Biomaterial encapsulation to improve cell therapy for Parkinson’s disease

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I, Rachael Evans, confirm that the work presented in this thesis is my own. Where information has been derived from other sources; I confirm that this has been indicated in the thesis.

Signed: ________________________

Date: _________________________
Acknowledgements

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Publications

Papers


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Conference proceedings


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Abstract

Current therapeutic approaches for Parkinson’s disease (PD), such as administration of levodopa and dopamine agonists are not ideal as they are only effective in the early stages of the disease. With PD being the second most common neurodegenerative disorder new therapeutic approaches are of great necessity. Over the past decade, cell therapy for CNS injury and disease has looked extremely promising. However, both pre-clinical and clinical evidence shows high rates of cell death after implantation and one cause of this is the host cellular response. The overall aim of this programme of work is to develop an advanced therapeutic for PD which overcomes the challenges of cell survival, focusing on protecting the cells from detrimental host glial cell and immune responses by synaptically isolating therapeutic cells in a hydrogel.

Human induced pluripotent stem cells (hiPSCs) were successfully expanded and differentiated to dopaminergic neurons (DA-hiPSCs). A new protocol for encapsulating cells in an alginate hydrogel was developed and optimised using SH-SY5Y cells. Alginate gels were then used to encapsulate DA-hiPSCs and the ability of encapsulation to protect cells was tested in vitro. Formulations of alginate hydrogels in microbeads and made using ThinCert™ 24-well plates were characterised for their physical and mechanical properties for use as a transplantable biomaterial for the CNS. Alginate hydrogels were further optimised to reduce the host response with the addition of poly-l-ornithine and hyaluronic acid. Local delivery of the immunosuppressant tacrolimus (FK506) was explored through the construction of drug-eluting nanoparticles using PLGA and PCL electrospraying.

Results indicated that encapsulation of DA-hiPSCs using alginate has the potential to protect therapeutic cells from detrimental host cell responses and that synaptic isolation from host neurons is a new paradigm for PD cell therapy to be explored further. Along with the combination of immunosuppressant FK506 for local delivery, the approach shows promise for improving cell survival in transplantation for PD.
Impact Statement

This thesis contributes to translational research in Parkinson’s disease (PD) through the development of a new tissue engineering technology. More than 10 million people worldwide are living with PD and this number is only increasing due to the aging population, with the estimated projection of people with PD expected to be 13 million by 2040. In the US alone the combined cost of treatment and social care is $52 billion per year. There is no current cure for PD and, the requirement for new and improved therapeutics is vital, as the current treatment levodopa has to be taken every day and is only beneficial in the early stages of the disease.

This project provides further validation for an established protocol for dopaminergic differentiation of pluripotent stem cells using a cell line suitable for clinical use. Furthermore, it develops new knowledge in the area of biomaterial encapsulation and local immunosuppression for delivery in the central nervous system. This work has led to the development of new alginate formulations for cell encapsulation and a new mechanical biomaterial analysis methodology, for material matching to host tissue.

Overall the impact of this research is the progression of knowledge for a one off therapeutic for PD. Potentially this therapy could reduce the overall cost burden of PD and increase the lives of thousands of patients. Furthermore, local immunosuppression has the potential to alleviate current off target toxic side effects seen with systemic immunosuppression.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>6-OHDA</td>
<td>6-Hydroxydopamine hydrobromide</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
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<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
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<tr>
<td>ATMP</td>
<td>Advanced therapy material product</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CAR-T</td>
<td>Chimeric antigen receptor T-cell</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAPT</td>
<td>N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine</td>
</tr>
<tr>
<td>DBS</td>
<td>Deep brain stimulation</td>
</tr>
<tr>
<td>DMA</td>
<td>Dynamic mechanical analysis</td>
</tr>
<tr>
<td>DNase I</td>
<td>Deoxyribonuclease I</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
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<tr>
<td>dSPN</td>
<td>Direct spiny projection neurons</td>
</tr>
<tr>
<td>EBs</td>
<td>Embryoid bodies</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGM</td>
<td>Endothelial cell growth media</td>
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<td>EHDA</td>
<td>Electrohydrodynamic atomisation</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>EMA</td>
<td>European Medical Agency</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagles minimum essential medium</td>
</tr>
<tr>
<td>ES</td>
<td>Electrospraying</td>
</tr>
<tr>
<td>ESCs</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>EVA</td>
<td>Ethylene vinyl acetate</td>
</tr>
<tr>
<td>F-DOPA</td>
<td>Fluoro-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S Food and Drug Administration</td>
</tr>
<tr>
<td>FK506</td>
<td>Tacrolimus</td>
</tr>
<tr>
<td>FOXA2</td>
<td>Forkhead box protein A2</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier-transform infrared spectroscopy</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GBA</td>
<td>Glucocerebrosidase</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial derived neurotrophic factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide 1</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>hfVM</td>
<td>Human foetal ventral mesencephalic</td>
</tr>
<tr>
<td>hiPSCs</td>
<td>Human induced pluripotent stem cells</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>iNOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>iPSCs</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>iSPN</td>
<td>Indirect spiny projection neurons</td>
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<tr>
<td>L-Dopa</td>
<td>Levodopa</td>
</tr>
<tr>
<td>Mpd</td>
<td>Mean pixel density</td>
</tr>
<tr>
<td>MPP+</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>NDM</td>
<td>Neural differentiation medium</td>
</tr>
<tr>
<td>NFL</td>
<td>Neurofilament derived peptide</td>
</tr>
<tr>
<td>NIM</td>
<td>Neural induction medium</td>
</tr>
<tr>
<td>NM</td>
<td>NovaMatrix</td>
</tr>
<tr>
<td>NP-hiPSCs</td>
<td>Neural progenitor hiPSCs</td>
</tr>
<tr>
<td>DA-hiPSCs</td>
<td>hiPSC-derived dopaminergic neurons</td>
</tr>
<tr>
<td>NPM</td>
<td>Neural proliferation medium</td>
</tr>
<tr>
<td>NSCs</td>
<td>Neural stem cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCL</td>
<td>Poly-ε-caprolactone</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDL</td>
<td>Poly-D-lysine</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly-ethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly-glycolic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>pHEMA</td>
<td>Poly-2-hydroxyethyl methacrylate</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly-lactic-acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-l-lysine</td>
</tr>
<tr>
<td>PLO</td>
<td>Poly-l-ornithine</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SAB</td>
<td>Salvianolic acid B</td>
</tr>
<tr>
<td>SBTI</td>
<td>Soybean trypsin inhibitor</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>SPNs</td>
<td>Spiny projection neurons</td>
</tr>
<tr>
<td>TFE</td>
<td>2,2,2-trifluoroethanol</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor -α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffraction</td>
</tr>
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Chapter 1: Introduction

1.1 Parkinson’s disease (PD)

1.1.1 Clinical overview
Parkinson’s disease (PD) is the second most common neurodegenerative disorder, first described by James Parkinson in 1817 (Parkinson, 1817). This devastating disorder affects approximately 6.3 million people worldwide, with this number only increasing with the aging population in Western countries (Wakeman et al., 2016). Neurological disorders are now believed to be the leading cause of disability in the world, with PD the fastest growing of those with numbers doubling from 1990 to 2015 (Feigin et al., 2017; Dorsey and Bloem, 2018). There is no current cure for PD and with the estimated projection of people with PD expected to be 13 million by 2040, the requirement for new and improved therapeutics is vital (Dorsey and Bloem, 2018).

1.1.2 Genetics and Pathology of PD
In PD the degeneration of dopaminergic neurons in the substantia nigra (SN) and the formation of Lewy bodies, which causes loss of innervation of neurons in the striatum (Figure 1.1) that leads to the well-known motor and behavioural impairments (Chung et al., 2001). Some of these symptoms include bradykinesia (slowness of movement), rigidity, tremor and postural instability but can also lead to dementia when systems other than dopamine are involved (Xiao et al., 2016). The motor symptoms of PD are thought to be predominantly caused by the death of dopaminergic neurons, the processes of which usually target the striatum in the midbrain, resulting in striatal dopamine levels being severely decreased (Caligiore et al., 2016). Clinical symptoms don’t manifest until 80% of dopaminergic neurons in the midbrain have degenerated. Lewy bodies are misfolded α-synuclein inclusions in the neuronal cell body and are now known to be found in multiple brain regions (Kalia and Lang, 2015; Przedborski, 2017). Braak et al. proposed a model for Lewy body pathology in 6 stages aligned with the clinical manifestation of the disease (shown in Figure 1.1.1) (Braak et al., 2003,
Furthermore, Lewy bodies are now believed to have a potentially instrumental part in the amplified degeneration and death of neurons (Halliday et al., 2008; Irwin et al., 2012). Due to the lack of knowledge on the pathogenesis and etiology of PD, developing therapeutics remains a challenge.

In comparison to sporadic PD, familial PD has an earlier average age of onset (~50) and has been linked to genes including ATP13A2, DJ-1, LRRK2, Parkin, PINK1 and SNCA (Klein and Westenberger, 2012). LRRK2 and SNCA are thought to be involved in both familial and sporadic PD (Redenšek, Trošt and Dolžan, 2017). Neuroinflammation is another area believed to be instrumental in the onset of PD, and could potentially lead to further degeneration following the start of dopaminergic neuron death (Mirza et al., 1999; Irwin et al., 2012; McGeer et al., 2012). Even though no current cure is known, PD is now believed to be a complicated interplay between genetic, environmental and other biological mechanisms.

Figure 1.1.1. The areas of the brain affected in PD and the progression of α-synuclein (Lewy body) pathology. The substantia nigra is where the majority of degeneration of dopaminergic neurons occurs, meaning loss of innervation to the striatum which is made up of the caudate nucleus and putamen (shown in the orange arrows as the dopamine pathway). The progression of Lewy body build up in PD is shown from a minimal amount in the lower brain stem (red) and cortex (yellow) at the beginning (Braak stage 1 and 2), increasing in the brain stem and advancing into the midbrain causing the characteristic motor symptoms of PD (Braak stage 3 and 4). Eventually this progression infiltrates the limbic system and surrounding cortical neurons (Braak stage 5 and 6). (Braak et al., 2004) (Figure edited from Poewe et al., 2017)
1.1.3 Current treatment

Current treatments for PD include precursors of dopamine, dopamine agonists and neuromodulation such as deep brain stimulation (DBS). Levodopa (L-Dopa), a dopamine precursor, is currently the gold standard of treatment for PD. The discovery of L-Dopa in the 1960s was ground-breaking for the field and patients and it continues to be the best symptomatic treatment today. One limitation is that it is more beneficial in the early stages of the disease and has some significant side effects such as dyskinesia (involuntary movement). Other drugs to stimulate dopamine production or dopamine receptors such as other dopamine agonists and monoamine oxidase type B inhibitors, as well as different formulations of L-Dopa have been explored, including slow-release and other administration methods. However, none of these have shown promise compared to the original L-Dopa design (Kalia and Lang, 2015; Przedborski, 2017). On the other hand, surgical intervention with DBS, where electrodes producing a high frequency current are introduced into the substantia nigra, shows improvements in the motor symptoms of patients (Antony et al., 2013; Stoker and Barker, 2016). DBS is a serious and invasive procedure and can have side effects such as intracranial haemorrhage and it does not stop the disease from progressing (Adil et al., 2017). PD drug treatments that involve replacing dopamine in the midbrain however have not been successful due to the requirement to reach the specific target area of the striatum and differing dose requirements depending on the patient (Stoker and Barker, 2016). No currently marketed therapy for PD prevents the degeneration of dopaminergic neurons or causes regeneration.

1.2 Regenerative medicine research in PD

There is a clear clinical need to improve upon the limited current therapies for PD. The main area of regenerative medicine under investigation for PD is cell transplantation, which will be discussed in detail in section 1.2.2, but before that some other options including the administration of drugs, neurotransmitters and gene therapy will be briefly discussed. Development of the next generation of
therapies for PD is likely to involve treating both the symptoms and the complicated neural pathology, as a combinational therapy.

1.2.1 Gene therapies, growth factors and small molecules

Gene therapy for PD aims to restore striatal dopamine levels, modulate the basal ganglia network and administer the delivery or overexpression of neurotrophic factors (Elkouzi et al., 2019). The indirect and direct pathway of dopamine transmission is altered in PD (discussed further in Chapter 3), this also regulates the output of GABAergic neurons in the basal ganglia, so targeting GABA has become another area of interest in regenerative medicine therapies for PD. Gene therapy for PD has targeted the synthesis of GABA and dopamine or overproduction of neurotrophic factors (Axelsen and Woldbye, 2018). Overexpression of glutamic acid decarboxylase (GAD) which increases GABA levels in the striatum has been shown to reduce Parkinson’s motor symptoms in both rodent and rhesus monkey models of PD (Luo et al., 2002; Emborg et al., 2007). This led to clinical trials in which Parkinson’s patients showed a reduction in clinical motor scores a year after injection and no adverse events (Niethammer et al., 2017). Adeno-associated virus (AAV) administration of GAD to enhance GABA transmission in the substantia nigra proved to be both safe and effective, showing alleviation of bradykinesia and motor symptoms in both Phase I and II trials (Kaplitt et al., 2007; LeWitt et al., 2011). This shows great promise as clinical benefits persisted after 12 months, however both sample sizes in the trials were <50 and there is currently no planned Phase III.

Another gene therapy which shows great promise for PD uses the lentiviral vector encoding enzymes which are essential for dopamine synthesis (tyrosine hydroxylase (TH) and cyclohydrodrolase 1 which both convert tyrosine to levodopa and aminoacid decarboxylase which coverts levodopa to dopamine). In previous studies it was shown that the combination of these enzymes (TH, cyclohydrodrolase 1 and aminoacid decarboxylase) expressed in non-dopaminergic neurons, is able to induce dopamine production (Azzouz et al., 2002). This therapy known as ProSavin® produced by
Oxford BioMedica has shown in 15 patients, in a Phase I/II studies in the UK and France (NCT00627588 and NCT01856439), improvements in motor symptoms in all patients with an average increase of 30% and one patient having 41% improved motor score at the 6 month time point (Palfi et al., 2014).

Glial Derived Neurotrophic Factor (GDNF), due to its neuroprotective and potential regeneration properties, has been of interest for the treatment of neurodegenerative diseases and specifically PD (Garbayo et al., 2009). Development of GDNF gene delivery to the putamen for PD has also been researched, with primate studies displaying improved motor symptoms and increased levels of GDNF in both the striatum and substantia nigra (Kells et al., 2010; Emborg et al., 2014). AAV delivery of GDNF is also currently under investigation in a Phase I clinical trial of 25 patients estimated to be completed in 2027, with no current data published (NCT01621581).

Direct GDNF infusions to the putamen for PD were looked at over a 40-week clinical trial following good safety and efficacy findings from earlier trials (Nutt et al., 2003; Lang et al., 2006; Whone et al., 2019). Although GDNF showed some improvement in patients, the findings showed no significance compared to the placebo and patients had variable results (Whone et al., 2019). Issues such as the amount of GDNF being delivered to the right location are concerns and this is now being considered by combinational therapy of GDNF in a biomaterial matrix (Garbayo et al., 2009; Whone et al., 2019). But if GDNF therapy is to be taken to the next stage in the clinic, trial design requires optimisation and drug dose and the length of the trial will need to be considered (Kirkeby and Barker, 2019).

Research into small molecules to prevent α- synuclein synthesis and aggregation is an exciting target for disease modification, however true progress can’t be made in this area until its physiological role in the disease is fully understood (Elkouzi et al.,
A link between PD and Gaucher disease has been shown, as patients who are homozygous for the glucocerebrosidase (GBA) gene are at increased risk of developing PD (Tayebi et al., 2003). Treatment with a chaperone for GBA to treat PD is currently in a Phase I clinical trial (AiM-PD NCT02941822), and a Phase II trial aiming to inhibit the enzyme which breaks down GBA glucosylceramide is planned to be completed in 2022 (MOVES-PD NCT02906020) (Alcalay et al., 2012; McNeill et al., 2014; Sardi et al., 2017). With neuroinflammation having a huge impact on PD pathology, NSAIDS are being analysed extensively for their protective properties (Emami et al., 2011).

In theory, the local delivery of dopamine to the striatum might be considered to be a logical approach to both treat the degeneration of cells and the motor symptoms caused by the loss of dopaminergic neurons. Depending on the amount of dopamine required, this could even be considered through implantation of an osmotic pump or dopamine-eluting biomaterial. From published data, a calculation was made as to how much dopamine is required in the striatum for a specific amount of time (Figure 1.2.1). The calculation illustrated that the minimal amount of dopamine required for a healthy individual for 1 year is 24g (Costa et al., 1975; Hardman et al., 2002). If this amount is required to treat a patient with PD then it is a substantial amount to be loaded into a material, plus the weight of the material would need to be considered in addition to this. Even if only 1g was loaded, this is 4% of the total amount required for a year, which is a third of the mass of an average human putamen (3.5g, Yin et al. 2009). This calculation is only an estimate, but it indicates that simply using a biomaterial to release stored dopamine over an extended period is not likely to be a suitable option for treating PD.
Minimal for 1 year

\[
18.8 \text{nmol/g/h} \times 8760 \text{ (hours in 1 year)} = 164688 \text{nmol/g or 0.000164mol/g}
\]
\[
\therefore 0.00016488 \text{mol/g} \times 153.18 \text{(MW)} = 0.0252 \text{g}
\]
0.0252 x 956 (rat to human) = **24.115g for 1 year**

Maximal for 1 year

\[
23.4 \text{nmol/g/h} \times 8760 =
\]
\[
204984 \text{nmol/g or 0.000204mol/g}
\]
\[
\therefore 0.000204 \text{mol/g} \times 153.18 = 0.0314 \text{g}
\]
0.0314 x 956 = **30.02g for 1 year**

Minimal for 3 year

\[
18.8 \text{nmol/g/h} \times 26280 \text{ (hours in 3 year)} = 494064 \text{nmol/g or 0.000494mol/g}
\]
\[
\therefore 0.000494 \text{mol/g} \times 153.18 \text{(MW)} = 0.0756 \text{g}
\]
0.0756 x 956 (rat to human) = **72.35g for 3 years**

Maximal for 3 year

\[
23.4 \text{nmol/g/h} \times 26280 =
\]
\[
614952 \text{nmol/g or 0.000614mol/g}
\]
\[
\therefore 0.000614 \text{mol/g} \times 153.18 = 0.0942 \text{g}
\]
0.0942 x 956 = **90.05g for 3 years**

Considering:

- In Parkinson’s Disease the expected dopamine level would not be more than 0.5 ng/mg in the caudate and 0.7 ng/mg in the putamen (Kish et al., 1992)
- Dopamine level of healthy 22 year old- 6.4ng/mg and 84 year old 2.5ng/mg (Kish et al., 1992).
- Average weight of normal human putamen is 3.5g (Yin et al., 2009)
- Total striatal tissue concentration of dopamine is 69 nmol/g wet wt (Patterson and Schenk, 1991).
- In the striatum, the concentration of dopamine peaks within ~200 ms and is cleared after several hundred milliseconds (Ford et al., 2010)
- DA turnover rate in rats: minimal- 18.8nmol/g/h, maximal- 23.4nmol/g/h (Costa et al., 1975)
- To calculate mass of dopamine required over time; 1 year =8760 hours, 3 years = 26280 hours
- Mass of rat brain is 1.4g (Hardman et al., 2002)
- Mass of human brain is 1339g (Human is 956x larger than Rat) (Hardman et al., 2002)
- Molecular weight of dopamine is 153.18g/mol or daltons (Sigma Aldrich)

**Figure 1.2.1** A calculation indicating how much dopamine would be therapeutically required to treat PD (Costa et al., 1975; Patterson and Schenk, 1991; Kish et al., 1992; Hardman et al., 2002; Yin et al., 2009; Ford et al., 2010).
A recent clinical trial in PD patients has shown alleviation of symptoms following treatment with Exenatide, a glucagon-like peptide 1 (GLP-1) agonist licensed for type 2 diabetes, with 32 patients treated over 48 weeks (Athauda et al., 2017). These results replicate previous in vivo data showing neuroprotective effects in the striatum and reduction in motor symptom scores in adult rats. However, the mechanism for this effect of Exenatide is uncertain and could possibly be through immunomodulatory effects or a direct impact on pathways associated with PD (Bertilsson et al., 2008; Foltynie and Aviles-Olmos, 2014).

Although, both small molecule and gene therapies show promising results for PD they require vehicles to either cross the blood brain barrier or for delivery to the target site. Furthermore, small molecules particularly would require multiple doses or injections or a long-term formulation. To overcome these issues cell therapy is a promising option for PD treatment allowing implanted cells to provide dopamine and other growth factors to the striatum and substantia nigra continuously, to replace the lost dopaminergic neurons.

1.2.2 Cell therapy

Therapeutic cells can be either cells from the patient, also known as autologous cells, or from a donor, known as allogeneic cells. Stem cells are capable of proliferation, self-renewal and differentiation into multiple cell types. Certain stem cells are multipotent, meaning they can only differentiate into particular cell lineages, and others are pluripotent meaning they can differentiate into any cell of the body. Stem cell therapy has shown promise in animal models for several neurological disorders which include stroke, Huntington’s disease, multiple sclerosis, Alzheimer’s disease and PD (Gögel, Gubernator and Minger, 2011; Lunn et al., 2011; Amemori et al., 2015). Some of these diseases involve specific cell loss, which is why such therapeutic cell replacement may be advantageous. The actual beneficial mechanism of action in many cases may however be due to modulation of the recipient tissue environment and immune response rather than direct cell replacement.
Since the 1970s, neural grafting has been investigated as a therapy for PD, with clinical trials involving surgical implantation of human foetal tissue into the striatum (Lindvall, Kokaia and Martinez-Serrano, 2004; Barker, Drouin-Ouellet and Parmar, 2015; Stoker and Barker, 2016). Although, these transplants have shown some serious side effects in patients, they have also highlighted great promise for cell therapy (Lindvall, Kokaia and Martinez-Serrano, 2004). Foetal grafting is still under investigation, with the ongoing TRANSEURO trial (NCT01898390) building on previous clinical trials by optimising cell delivery and trial design, and the results planned to be published in 2021 (Kirkeby, Parmar and Barker, 2017; Barker et al., 2019).

TRANSEURO started in 2009, a European Union funded trial using human foetal ventral mesencephalic (hfVM) tissue for transplantation of dopaminergic neurons to the striatum in PD patients. Although hfVM transplantation as a cell replacement therapy showed some efficacy, the approach is still limited by ethical issues of the requirement for numerous human foetal donors per patient and the associated difficulty in procuring a sufficient amount of tissue or cells. The TRANSEURO trial, unlike previous hfVM transplant clinical trials, was conducted to GMP standards and includes imaging outcome measures such as measuring changes in F-DOPA PET (discussed in Chapter 1.2.3). 11 patients received foetal implants and the results of the trial are set to be evaluated in 2021 (Kirkeby, Parmar and Barker, 2017; Barker et al., 2019). The biggest issue highlighted by foetal transplant studies is how to deliver a therapeutic dose of surviving cells to the striatum, hence the need for alternative cell types and improvements to cell survival, integration and function once transplanted. G-Force PD was set up in 2014 running in parallel to TRANSEURO, bringing together leading groups working on stem cell-derived dopaminergic cells for use in PD.
Stem cells are found in many tissues, but some are more appropriate than others as a source of therapeutic cell for transplantation into the CNS, due to their origin and capability to differentiate. Cells researched for CNS transplantation include embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), foetal or adult neural stem cells (NSCs) and induced pluripotent stem cells (iPSCs, Figure 1.2.2) (Gögel, Gubernator and Minger, 2011; B. Xiao et al., 2016).

![Figure 1.2.2](image_url)  
**Figure 1.2.2.** A schematic representation of the various cell sources and examples of their origins for use in therapeutics for CNS disease and injury. (Taken from Gögel, Gubernator and Minger, 2011).

### 1.2.2.1 MSCs

One potential population of cells are MSCs. These can be sourced from numerous adult tissues, with bone marrow MSCs being the most universally used, especially for the treatment of blood related diseases (Hass *et al.*, 2011; Ranganath *et al.*, 2012). MSC classification criteria to set out uniform and consistent data were published in 2006 (Dominici *et al.*, 2006). MSCs have been transplanted and show improvements in PD due to their secretome being neuroprotective, and this could lead to a novel PD therapy (Teixeira *et al.*, 2017). Furthermore undifferentiated MSCs pre-treated...
with hypoxic conditions increased endogenous dopaminergic neuron survival in rats but they haven’t been successfully differentiated into dopaminergic neurons for transplantation (Wang et al., 2013).

1.2.2.2 ESCs

ESCs are derived from blastocysts and provide a reliable cell source as they can be produced in high numbers readily and are pluripotent, therefore able to differentiate into all cell types of the human body (Gögel, Gubernator and Minger, 2011). However, ESCs continually proliferate so produce a high risk of cancerous cell formation after transplantation. To reduce the probability of tumorigenesis, appropriate quality control measures must be in place to ensure no pluripotent cells remain in the final product (R. A. Barker, Parmar, Studer, & Takahashi, 2017; Kirkeby et al., 2017) and in depth karyotyping of each cell line must be conducted routinely to ensure genomic stability (Barker et al., 2017; Kirkeby, Parmar and Barker, 2017; Natalwala and Kunath, 2017).

Human ESCs (hESCs) were first used in a human trial for spinal cord injury, and safety and efficacy has been demonstrated for the use of ESC-derived retinal epithelial cells for macular degeneration (Lebkowski, 2011; Da Cruz et al., 2018). ReNeuron’s CTX0E03 line is a conditionally immortalised NSC product which has been shown to be safe and well tolerated in a Phase II trial for stroke (PISCESII) with a Phase IIb trial ongoing (Kalladka et al., 2016). Conditional immortalisation allows consistent clonal expansion of cells by using an inducible transgene which when activated allows the cells to divide. This is controlled by an operator gene so that when a large enough yield of cells is reached the transgene is then switched off and cells return to a post-mitotic state (Wall, Toledo and Jat, 2016). Therefore, gene modified cells provide future promise for reducing safety concerns with specific cell lineages.
Differentiation of stem cells into dopaminergic neurons is well established for ESCs, NSCs and iPSCs, as highlighted in Figure 1.2.3 (Bjorklund et al., 2002; Barker, Drouin-Ouellet and Parmar, 2015). Recent developments in dopaminergic differentiation protocols have allowed research in this area to progress further. With the first protocols using ESCs (Perrier et al., 2004; Park et al., 2005; Kriks, J. W. Shim, et al., 2011), dopaminergic neurons did not express features such as forkhead box protein A2 (FOXA2). The change in protocol resulted from elucidation of the transitory developmental structure of the floor plate, which is key to midbrain dopamine neuron development. Therefore, the new protocol instead of transitioning the cells through the neuroepithelial intermediate stage now progresses through a floor plate stage. This means the cells from this protocol now show genetic and biochemical features of human dopaminergic midbrain neurons shown in Chapter 3 Figure 3.1.2 (Kirkeby et al., 2012; Tabar and Studer, 2014). Transplantation of mouse and human ESCs differentiated into dopaminergic neurons have demonstrated improved functional recovery in vivo (Bjorklund et al., 2002; Kim et al., 2002; Ben-Hur et al., 2004; Kriks, J.-W. Shim, et al., 2011). A major limitation of ESCs is the survival of only a small percentage of dopaminergic neurons after transplantation (Ben-Hur et al., 2004).
1.2.3 Multiple therapeutic options for treating PD. Human foetal VM tissue (hfVM) (A), Blastocyst into ESCs (B), Fibroblasts into iPSCs (C) which go through the developmental stages during differentiation including the neural plate (NP) and neuroepithelial cells (NEP), then through the midbrain floor plate (mFP) to midbrain dopaminergic progenitors (mDP), then postmitotic neuroblasts (mDNb) and finally midbrain dopaminergic neurons (mDA). This programming can also be done directly from fibroblasts (D). All lead to cell transplantation therapy for PD (E) but other strategies include gene therapy (F) and the use of these cells for in vitro disease modelling for drug discovery (G). (Arenas, Denham and Villaescusa, 2015).

1.2.3.3 iPSCs

iPSCs are cells reprogrammed to pluripotency from adult cells which, like ESCs, are able to differentiate into any other cell type in the body (B. Xiao et al., 2016). iPSCs were first generated by Dr Yamanaka’s team who showed that pluripotent cells could be created by reprogramming skin fibroblasts using just four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka, 2006). Since their discovery, iPSCs have been generated from multiple tissues and organs such as stomach cells, liver, neural stem cells and peripheral blood cells (Okano et al., 2013; Tan et al., 2014). iPSCs provide the exciting potential of patient specific cell therapy for treating neurological diseases without the ethical concerns around embryo destruction to obtain ESCs and NSCs.
iPSC research in PD is promising, with iPSCs differentiated into dopamine producing neurons resulting in reduction of rotational asymmetry following transplantation into a rat lesion model of PD (Wernig et al., 2008). Perhaps the greatest concern with iPSC-derived neurons is that their transplantation could lead to tumour formation, particularly if upon transplantation they spontaneously resort to their original pluripotent proliferative nature. Compared to ESCs, iPSCs avoid the use of ethically sensitive material and could be genetically or immunologically matched to a patient.

iPSCs are currently being tested in PD, spinal cord injury, diabetes and heart disease in Phase I and II clinical trials (Trounson and DeWitt, 2016; Martin, 2017; Natalwala and Kunath, 2017; Takahashi and Price-Evans, 2019). The potential effectiveness of using iPSCs to treat PD has been demonstrated through transplantation into a neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) primate model in Japan which showed improvement in a rating scale of Parkinson’s symptoms and neurite extension into the striatum (Kikuchi et al., 2017). With banks of iPSC lines being generated to allow human leukocyte antigen (HLA) matching across the world to prevent immunological rejection of cells once they are transplanted, this approach shows great promise for multiple diseases (Taylor et al., 2012).

Following the successful primate study of iPSC transplantation for PD, the first pluripotent cell therapy clinical trial in PD has begun (Kikuchi et al., 2017; Takahashi and Price-Evans, 2019). This hiPSC-derived dopaminergic neuron clinical trial began at the end of 2018, aiming to recruit 11 PD patients (Takahashi and Price-Evans, 2019). The trial is expected to last at least 2 years, with immunosuppressant FK506 being given, administered for 12-24 months post transplantation (Barker et al., 2017). The hiPSC line being used in the trial originates from peripheral blood cells from a healthy donor whose HLA haplotype is homozygous and matches 17% of the Japanese population. Studies in Europe are not far behind for both ESCs and iPSCs, with plans to begin recruitment for a cell therapy trial this year (Kirkeby, Parmar and Barker, 2017; Barker et al., 2019). Although these trials look promising for PD there are a number of outstanding issues with the cell therapies developed to date, which will be discussed in the next section.
1.2.3 Outstanding issues with current cell therapies

Current clinical trials for PD include cellular therapies using dopaminergic neuron progenitors to replace cells which are lost from the disease and therefore have the potential to restore some of the dopamine. Clinical trials of cell therapies for PD have shown variable results, with poor graft survival, development of dyskinesias and vulnerability of grafted cells to Lewy body pathology (Halliday et al., 2008; Li, Chen and Li, 2015; Zhu, Caldwell and Song, 2016). The limitations of cell transplantation currently include; difficulties in producing a high enough yield of cells, low cell survival during or post-transplantation, uncontrolled proliferation, unsuccessful integration, host immune response and high cost (shown in Figure 1.2.4). Prior to transplantation, generating ‘Good Manufacturing Practice’ (GMP) grade cells of a high enough yield for the cells to be therapeutically advantageous once injected, is a complex and time-consuming task. As talked about previously GForce-PD, the consortium of global organisations working on a cell therapy for PD, are now at a stage where they have multiple GMP protocols to derive functional midbrain dopaminergic neurons (Barker et al., 2017; Kirkeby, Parmar and Barker, 2017). Currently the protocols are small-scale in normal tissue-culture flasks, though due to the highly efficient yield this can produce sufficient cell numbers for Phase I/II trials and associated quality control and required preclinical safety and efficacy testing (Kirkeby et al, 2017). However, moving onto Phase III or further, scale-up and automation methodologies would need to be developed (Barker et al., 2017).

During transplantation, cells undergo multiple mechanical stressors such as compressive, tensile and shear forces from delivery (Amer et al., 2017). This can lead to many cells dying before even reaching the target area; under 0.1% survival of neural stem cell precursors has been reported in animal models of stroke (Bacigaluppi et al., 2009). Once the cells have been transplanted, they then have many biological hurdles to overcome such as recognition by host immune cells of non-self-antigens on the surface of transplanted cells which can elicit an unwanted immune response. Prior to this an inflammatory response from host glial cells to cell injection would further initiate an immune response. This is highlighted by removal
of immunosuppressants after transplantation leading to decline in the positive symptoms related to the treatment (Olanow et al., 2003). Therefore, cell survival at the target tissue is also an issue, as shown in clinical trials of foetal nigral transplants in 34 patients, with a low percentage of cells remaining at the end of the trial (Olanow et al., 2003). Previously, measuring cell survival clinically was difficult until post mortem examination. Imaging techniques, including fluoro-3,4-dihydroxyphenylalanine (F-DOPA) positron emission tomography (PET) scanning, allow assessment of the function of the dopamine system via radiotracers (Barker et al., 2019). F-DOPA PET has been successfully used to show recovery of the nigrostriatal system after neural transplantation in PD patients (Dunnett and Björklund, 1999). This technique is being used in the ongoing TRANSEURO clinical trial (described in 1.2.2). Another potential issue with cell therapies is proliferation of the transplanted cells and tumorigenesis (B. Xiao et al., 2016). Furthermore, the high cost of reagents used for pluripotent cell differentiation and therefore great cost for scale-up makes it an issue that must be addressed before the approach can become widely available.

Figure 1.2.4. Summary of the current issues with cell therapies.
As discussed, cell therapy is a promising approach to tackling the issues with current PD therapies. However, combining cell therapy with small molecules to enhance neurogenesis is also an exciting option. Furthermore, tissue engineering approaches such as the use of biomaterials can be considered to overcome many of the issues with cell therapies. Currently most neural cell therapies rely on synaptic integration for functional recovery. However for diseases such as PD where levels of neurotransmitters released from the degenerating neurons are reduced, a potential use of cell therapy could be to use biomaterials to synaptically isolate therapeutic cells which release these neurotransmitters such as dopamine in the areas of the brain where they are diminished. For example, encapsulation of therapeutic cells within a biomaterial could protect cells against mechanical damage during delivery, extend the survival of the cells in the host brain, or promote integration of the transplanted cells within the host tissue, tackling many of the concerns highlighted in this section. Furthermore, for PD, therapeutic cells protected within a material promoting cell survival and steadily releasing dopamine would also provide a long-term treatment for patients and repair motor and behavioural symptoms.

1.3 Biomaterials which could support CNS regeneration

1.3.1 Biomaterials for CNS repair and regeneration

Unlike many other tissues in the body, the central nervous system (CNS) has a poor capacity for intrinsic repair, regeneration and restoration of function after injury. Historically, the CNS was thought unable to regenerate at all, yet, over time, this assumption has become increasingly challenged. Indeed, given the appropriate biological, chemical and physical cues it is now widely accepted that the CNS at least has some capacity for regeneration.

One method to encourage CNS repair is to use biomaterial scaffolds to promote local neuroregeneration. Biomaterials can mimic the extracellular matrix (ECM) and provide support (Newland et al., 2013; Tam et al., 2014). Through injury or disease process CNS tissue undergoes liquefactive necrosis, where cells and the extracellular
matrix are digested by enzymes, sometimes leaving a fluid filled cavity (Lim and Spector, 2017). This type of cavity could potentially be filled with a biomaterial that could replace the lost tissue and provide an environment in which regeneration could be promoted. Due to the specific environment of the CNS, biomaterial selection for use in therapies is particularly important.

The range of biomaterials explored to promote CNS repair has been varied, yet soft hydrous materials have shown more promise and been preferred experimentally compared to stiff materials. This is due to their mechanical similarity to the brain and spinal cord tissue, and the reduced potential for adverse tissue reactions to occur (particularly from astrocytes) if there is a mismatch in mechanical properties. Biomaterials used for CNS repair and regeneration can be natural, synthetic, or a combination of both, to achieve the best desired properties. Materials that have been used in recently completed or ongoing clinical trials to treat CNS disorders are shown in Table 1.3.1.
<table>
<thead>
<tr>
<th>Type</th>
<th>Trial name</th>
<th>Stage</th>
<th>Location</th>
<th>Participants</th>
<th>Intervention</th>
<th>Primary outcome measure</th>
<th>Secondary outcome measures</th>
<th>Sponsor / funding</th>
<th>Start date</th>
<th>Status</th>
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<tr>
<td>Brain</td>
<td>The Safety and Efficacy Assessment of Injectable Collagen Scaffold™ Combined With Mesenchymal Stem Cell Transplantation in Patients With Brain Injury (NCT02767817)</td>
<td>Phase I (2-arm, randomised, double-blinded)</td>
<td>China</td>
<td>35-75 y/o intracranial haemorrhage (25-40ml), GCS 9-15, N = 30</td>
<td>Collagen scaffold combined with MSCs</td>
<td>Adverse events at 6 months</td>
<td>NIHSS, Brunnstrom stage, BI, VAS, EEG, SSEP &amp; Head CT Scan at 1, 3, 6, 12, 18 and 24 months</td>
<td>Chinese Academy of Sciences</td>
<td>Mar 2016</td>
<td>Completion scheduled Dec 2019</td>
</tr>
<tr>
<td>Spinal Cord</td>
<td>Functional Neural Regeneration Collagen Scaffold Transplantation in Acute Spinal Cord Injury Patients (NCT02510365)</td>
<td>Phase I (open-label)</td>
<td>China</td>
<td>18-65 y/o with thoracic ASIA A within last 21 days, N = 20</td>
<td>Collagen scaffold + comprehensive rehabilitation</td>
<td>Adverse events at 6 months</td>
<td>ASIA grade, SSEP and MEP at 12 months</td>
<td>Chinese Academy of Sciences</td>
<td>Apr 2015</td>
<td>Completion scheduled Dec 2019</td>
</tr>
<tr>
<td>Spinal Cord</td>
<td>The INSPIRE Study: Probable Benefit of the Neuro-Spinal Scaffold for Treatment of AIS A Thoracic Acute Spinal Cord Injury (NCT02138110)</td>
<td>Humanitarian device exemption (open-label)</td>
<td>USA / Canada</td>
<td>16-70 y/o with ASIA A graded thoracic or upper lumbar injury within past 96 hours, Non-penetrating injuries only, N = 25</td>
<td>Neuro-spinal scaffold</td>
<td>Improvements in ASIA grade of one or more levels</td>
<td>Motor, sensory, bowel, bladder, quality of life, independence, pain; adverse effects at 6 months</td>
<td>InVivo Therapeutics</td>
<td>Apr 2014</td>
<td>Final data collection for primary outcome scheduled Dec 2017, accompanying publication pending. Related first-in-man case study publication (Theodore et al., 2016)</td>
</tr>
<tr>
<td>Study Title</td>
<td>Phase</td>
<td>Region</td>
<td>Age Criteria</td>
<td>N</td>
<td>Treatment</td>
<td>Outcome Measures</td>
<td>Sponsor/Research Center</td>
<td>Completion Date</td>
<td>Final Data Collection Date</td>
<td>Notes</td>
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<tr>
<td>Pilot Study of the Neuro-Spinal Scaffold for the Treatment of AIS A Cervical Acute SCI (NCT03105882)</td>
<td></td>
<td>Canada</td>
<td>16-70 y/o with recent ASIA A graded cervical injury; non-penetrating injuries only</td>
<td>10</td>
<td>Neuro-spinal scaffold</td>
<td>Adverse effects at 6 months</td>
<td>Not specified</td>
<td>Aug 2017</td>
<td>Feb 2018</td>
<td>NCT03105882</td>
</tr>
<tr>
<td>NeuroRegen Scaffold™ With Bone Marrow Mononuclear Cell Transplantation vs. Intradural Decompression and Adhesiolysis in SCI (NCT02688062)</td>
<td>Phase I / II</td>
<td>China</td>
<td>18-60 y/o ASIA A with complete thoracic SCI as assessed by MRI and electrophysiology. Stable baseline after rehabilitation</td>
<td>22</td>
<td>Local scar resection, NeuroRegen™ scaffold plus either BMMCs and comprehensive rehabilitation, or surgical intradural decompression and adhesiolysis</td>
<td>ASIA grade, SSEP and MEP at 24 months</td>
<td>Chinese Academy of Sciences</td>
<td>Jan 2016</td>
<td>Completion scheduled Dec 2019</td>
<td>Relevant interim publications (Z. Xiao et al., 2016; Y. Zhao et al., 2017)</td>
</tr>
<tr>
<td>Neural Stem Cell Transplantation in Traumatic Spinal Cord Injury (NCT02326662)</td>
<td>Phase I / II</td>
<td>Russia</td>
<td>18-50 y/o with traumatic injury between 1 month to 5 years previously; any spinal level, stable ASIA A/B after rehabilitation</td>
<td>30</td>
<td>NSC transplant by intraspinal or intrathecal injection ± NWL Regeneration Matrix™ if large lesion cavity size; rehabilitation</td>
<td>Feasibility &amp; safety at 24 months</td>
<td>Federal Research Clinical Center of Federal Medical &amp; Biological Agency, Russia</td>
<td>Jul 2014</td>
<td>Final data collection for primary outcome scheduled Mar 2018, accompanying publication pending.</td>
<td></td>
</tr>
</tbody>
</table>
1.3.1.1 *Natural biomaterials*

Natural materials are derived from many different starting materials which include mammalian, plant-based tissues and crustaceans. Some examples of these include agarose, alginate, fibronectin, keratin, gelatin, fibrin, chitosan, gellan gum, collagen, elastin and hyaluronan (HA) (Orive, Anitua, *et al.*, 2009; Scarano, Carinci and Piattelli, 2009; Khaing *et al.*, 2014; Tam *et al.*, 2014; Mazzuca *et al.*, 2017). Natural extracellular matrix proteins such as laminin and fibronectin are involved in neural development, and cells are more likely to survive *in vivo* when transplanted within a matrix of these proteins (Tate *et al.*, 2009). Natural materials are typically degraded enzymatically which is beneficial for applications where material breakdown is necessary, such as for engraftment of transplanted cells within the host ECM or gene delivery to a target tissue. However, for materials used for drug release, enzymatic degradation of the material could result in the drug load being released too rapidly, or sporadic release kinetics. Many growth factors and small molecules such as neurotrophin-3, ionic cobalt, brain derived neurotrophic factor (BDNF) and GDNF and fibroblast growth factor have been successfully delivered and released using fibrin, collagen and chitosan hydrogels in rodent models of brain and spinal cord injury (Johnson, Parker and Sakiyama-Elbert, 2009; Wu *et al.*, 2012; Macaya *et al.*, 2013; Khaing *et al.*, 2016; Whone *et al.*, 2019). Hydrogels can also be used to provide an artificial extracellular environment for cases where it is lost due to disease degeneration and as a material for cell delivery. Matrigel™ is a hydrogel made from ECM proteins secreted by mouse sarcoma cells. It has been shown to improve mouse ESC derived neural precursor cell survival post transplantation in the mouse striatum (Uemura *et al.*, 2010). However, Matrigel™ is unlikely to be used clinically as it includes the presence of variable amounts of heterogenous constituents, including growth factors likely to promote proliferation of undifferentiated cells, leading to risk of tumorigenicity.
1.3.1.1 Alginate

Alginate is derived from brown algae (seaweed) and crosslinks with calcium chloride to form a hydrogel. It has been both European Medical Agency (EMA) and U.S Food and Drug Administration (FDA) approved for applications such as wound healing, and GMP grade alginates are available. Alginate is made up of mannuronic acid (M residues) and guluronic acid (G residues) which can be altered to change the ratio between them for specific medical applications (structure shown in Figure 1.3.1). For example, this can affect its degradability, and as it can be non-degradable this means it has great potential for long term implantation into the brain. Furthermore, the low immunogenicity of alginate is associated with its purity and endotoxin level but also the M:G ratio. High M alginates have shown increased cytokine production from macrophages such as IL-6 and tumor necrosis factor-α (TNF-α) in rats (Otterlei et al., 1991; De Vos et al., 2012). In comparison, high G alginate shows a more fibrotic reaction compared to high M and intermediate alginate, whereas an intermediate ratio of M:G shows decreased osmotic pressure implanted in the peritoneal cavity of both rat and mouse models (De Vos, De Haan and Van Schilfgaarde, 1997; Duvivier-Kali et al., 2001; Omer et al., 2005; Tam et al., 2011).

![Figure 1.3.1. Structure of alginate (Liang et al., 2015)](image-url)
Alginate has been widely used for both small molecule or growth factor delivery and cell encapsulation with the most successful application being for diabetes. *In vitro* models have used alginate to optimise 3D differentiation protocols for stem cells such as ESCs to dopaminergic neurons (Kim, Sachdev and Sidhu, 2013). Hyaluronic acid (HA) is found abundantly in the brain and is synthesised by neurons so therefore is a good material to consider for injection into the brain (Fowke *et al.*, 2017). The combination of HA and alginate has shown increased viability of mesenchymal cells *in vitro* compared to either material by itself (Cañibano-Hernández *et al.*, 2017). Extensive *in vivo* application of alginate has been used for spinal cord injury, cell delivery, BDNF release and diabetes therapy (summarised in Table 1.3.2). In diabetes, alginate has been a crucial biomaterial to encapsulate mouse and human ESCs differentiated into islet cells for insulin production with non-human primate studies leading to a clinical trial (De Vos, De Haan and Van Schilfgaarde, 1997; Duvivier-Kali *et al.*, 2001; G. *et al.*, 2011; Bochenek *et al.*, 2018).
<table>
<thead>
<tr>
<th>Application</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alginate encapsulated fibroblasts engineered to release BDNF</td>
<td>Significantly improved recovery compared to cells implanted alone</td>
<td>(Tobias et al., 2005)</td>
</tr>
<tr>
<td>transplanted in a rodent model of spinal cord injury</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biocompatibility of alginate- PLL microcapsules implanted in the</td>
<td>After 1 month, fibrosis was more severe in high G alginate</td>
<td>(De Vos, De Haan and Van Schilfgaarde, 1997;</td>
</tr>
<tr>
<td>peritoneal cavity of rats</td>
<td></td>
<td>De Vos et al., 2012)</td>
</tr>
<tr>
<td>Alginate microbeads containing adipose derived stem cells implanted</td>
<td>Both degradable and non-degradable alginate localised cells in the site</td>
<td>(Leslie et al., 2016)</td>
</tr>
<tr>
<td>intramuscularly in athymic nude mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alginate microbeads containing rabbit adipose derived stem cells implanted</td>
<td>12 weeks after transplantation microbeads with cells were still</td>
<td>(Leslie et al., 2018)</td>
</tr>
<tr>
<td>mid-cartilage in the ears of male white rabbits</td>
<td>present and caused cartilage regeneration</td>
<td></td>
</tr>
<tr>
<td>Porcine islet cells encapsulated in NovaMatrix SLM and SLG alginate</td>
<td>SLM alginate showed no inflammatory reaction and islets functioned for</td>
<td>(Vériter et al., 2010)</td>
</tr>
<tr>
<td>transplanted subcutaneously in rats for up to 12 weeks</td>
<td>60 days post transplantation, with no immunosuppression</td>
<td></td>
</tr>
<tr>
<td>RGD-coupled alginate encapsulating mouse myoblasts engineered to produce</td>
<td>Long term functionality of cells with sustained release of erythropoietin</td>
<td>(Orive, De Castro, et al., 2009)</td>
</tr>
<tr>
<td>erythropoietin implanted in the subcutaneous space of mice</td>
<td>over 300 days</td>
<td></td>
</tr>
<tr>
<td>PLO and PLL coated alginate of different M:G ratios transplanted in the</td>
<td>PLO and PLL coated alginites compared to alginate alone showed less</td>
<td>(Tam et al., 2011)</td>
</tr>
<tr>
<td>peritoneal cavity of mice</td>
<td>immune cell adhesion after 2 days</td>
<td></td>
</tr>
</tbody>
</table>
1.3.1.1.2 Hyaluronic acid (HA)

HA is a natural glycosaminoglycan found abundantly in the matrix of the brain. Neurons are able to autonomously produce HA throughout development (Fowke et al., 2017). Increasing research now highlights HA has a role in both peripheral and CNS cell signaling, migration, differentiation and proliferation (Toole, 2004; Preston and Sherman, 2011). There is substantial evidence for HA’s use in CNS neural tissue engineering as a matrix for cell encapsulation. HA hydrogels as a support matrix for NPC delivery into the brain show a significant decrease in the inflammatory response in a rat ischemia model (Hsieh, Tseng and Hsu, 2015). HA hydrogels have also been used as a delivery system, containing BDNF and Vascular Endothelial Growth Factor (VEGF) -loaded poly(lactic-co-glycolic acid) (PLGA) microspheres for controlled growth factor delivery, which have shown improved NSC survival and proliferation in vitro (Wang et al., 2011).

As a drug delivery system HA has been exploited in combination with other natural polymers as it provides an adaptable scaffold for encapsulation of both hydrophobic and hydrophilic molecules. One example is the inclusion of Exenatide in HA and alginate microspheres for improving delivery of the drug for patients with type 2 diabetes (Zhang et al., 2014). This study showed sustained release following oral delivery of the microspheres in mice (Zhang et al., 2014). Additionally, research has focussed on the variation of release kinetics from small molecules entrapped in nanoparticles which have been encapsulated within HA hydrogels compared to nanoparticles alone. The research highlighted the benefit of hydrogel encapsulation. Drug loaded nanoparticles in hydrogels can attain sustained drug release at remote tissue locations, where nanoparticles alone would diffuse away after long time periods (Kresna et al., 2013; Tajdaran et al., 2015).
1.3.1.2 Synthetic

Synthetic biomaterials are derived from inorganic compounds, including polymers such as PLGA/ Poly-lactic-acid (PLA)/ Poly-glycolic acid (PGA), Poly-ethylene glycol (PEG), Poly-2-hydroxyethyl methacrylate (pHEMA), Poly-ε-caprolactone (PCL) and Ethylene Vinyl Acetate (EVA) (Kalachandra, Dongming and Offenbacher, 2002; Orive, Anitua, et al., 2009; Newland et al., 2013; Tam et al., 2014). Due to the nature of the CNS and the desired soft composition of biomaterials, only a select few synthetic materials have been used clinically for brain repair (shown in Table 1.3.1). In contrast to many natural biomaterials, synthetic materials do not encourage cell attachment unless functionalised (Zhu and Marchant, 2011). An advantage of synthetic materials however is that they can be engineered to change parameters such as chemical composition or degradation rate (Zhu and Marchant, 2011).

1.3.1.2.1 Poly-lactic-co-glycolic acid (PLGA)

PLGA is an FDA approved (Figure 1.3.2), biodegradable, easily tuneable polymer (Makadia and Siegel, 2011). It has been significantly researched for the delivery of small molecules for extended release purposes. However this is also characterised by an initial burst release phase (Danlier et al., 2012; Han et al., 2016). The ratio of lactic and glycolic acid can be adjusted for the specific requirement, as well as modification of the surface to enhance cell attachment and interaction with other materials (Makadia and Siegel, 2011; Han et al., 2016). PLGA has been used for many nanoparticle formulations and has also been generated as microspheres incorporating dopamine for use in PD in vitro, however as suggested before (in Figure 1.2.1), this would require either a very large number of microspheres/ nanoparticles to be delivered or delivery in multiple applications (Kumari, Yadav and Yadav, 2010; Shin, Kim and Lee, 2014).

![Chemical structure of poly-lactic-co-glycolic acid (PLGA)](image)

Figure 1.3.2. Chemical structure of poly-lactic-co-glycolic acid (PLGA)
1.3.1.2 Poly-ε-caprolactone (PCL)

Poly-ε-caprolactone (Figure 1.3.3) is a synthetic aliphatic polymer utilised in drug delivery and tissue engineering. This is due to its biocompatibility and slow degradation, when compared to polylactide, polyglycolide and its copolymers (Kumari, Yadav and Yadav, 2010). The FDA-approved polymer is hydrophobic in nature, with its slow degradation determined by the hydrolysis of the ester linkage in physiological conditions, and produces a less acidic environment compared to PLGA (Woodruff and Hutmacher, 2010; Bock et al., 2011). The very slow degradation has been shown to vary from months to years (Malikmammadov et al., 2018). For drug delivery systems, PCL is compatible with a wide range of drugs due to high permeability to many active pharmaceutical ingredients (Woodruff and Hutmacher, 2010; Malikmammadov et al., 2018). These properties render PCL suitable for long-term implantable drug delivery systems (DDS).

![Chemical structure of poly-ε-caprolactone (PCL)](image)

Figure 1.3.3. Chemical structure of poly-ε-caprolactone (PCL)

1.3.1.3 Combinational

Hydrogels have water as a liquid phase and a polymer network which can be made up of either natural or synthetic materials or a combination of the two. Natural hydrogels, for example those made with collagen, are able to form during changes in conditions such as temperature or pH (Kretlow, Klouda and Mikos, 2007; Newland et al., 2013). Stimulus responsive hydrogels have also been made from synthetic materials which provide great benefit for CNS delivery as they can be prepared in vitro in solution and then form gels post-injection (Yu and Ding, 2008; Newland et al., 2013). NeuroGel™ is a non-degradable biomaterial hydrogel made from over 90% water and synthetic polymer solution which has been used for spinal cord repair and has also shown promise in regeneration of nerves in animal models (Woerly et al.,...
The presence of cell adhesion motifs in scaffolds is an important parameter, which is where natural materials are specifically advantageous. Research has investigated coating synthetic materials with adhesion proteins such as laminin, particularly due to its published ability to provide enhanced support for NSCs survival in scaffolds in vitro and in vivo (Flanagan et al. 2006; Nakaji-Hirabayashi et al. 2012; Tate et al. 2009).

1.3.2 Material property considerations for use in CNS tissue

1.3.2.1 Biocompatibility / non-toxicity

‘Biocompatibility’ in the conventional sense of chemical harmlessness to surrounding tissue needs to be considered at a higher level for the CNS. This is due to the sensitivity of neural cells and the serious permanent effects of causing additional damage. Additionally, the physically contained nature of the CNS (e.g. within the skull) means that swelling can be much more serious here than in other tissues. Sensitivity of cells to changes in stiffness and the very soft environment mean that toxic breakdown products could accumulate more easily than in other areas. Biocompatibility has no scale to which a material can be compared. The concept that biocompatibility is very complex and should always be considered in terms of both the material in question and the biological host was recently proposed (Williams, 2014). Whilst the need for biocompatibility in the sense of the material being non-toxic is a given for therapeutics, ensuring they do not stimulate a hazardous effect in the surrounding cells and tissue is also essential. This challenge then influences the considerations around selecting natural versus synthetic materials, where materials of natural origin are typically non-toxic but can elicit an immune response, and synthetic materials may be non-inflammatory but can include or be made with toxic chemical components. Not only does the biomaterial need to be non-toxic upon implantation but also its by-products (if it is degradable) need to be metabolised and cleared safely.
1.3.2.2 Physical/Mechanical properties

Mechanically, materials need to be soft and match stiffness of ECM brain tissue, have elasticity, be malleable and have tensile strength. This is discussed further in Chapter 4.1.2.1. Furthermore, physically solid materials need to be small enough to be injected into the CNS. Injectability of materials is essential to minimise damage to other areas in the brain following transplantation and hydrogels can be used to protect cells from shear stress during injection (Pakulski, Ballios and Shoichet, 2012; Amer et al., 2017). Mechanical matching of a biomaterial to the appropriate host tissue and cellular cargo is important for graft and cell survival (Moshayedi et al., 2014; Tam et al., 2014). The architecture of the scaffold has to complement that of host tissue for successful regeneration. This includes both the microarchitecture such as the size of the cells and also the macroarchitecture such as the size of the scaffold and the organisation of the molecules and material within the scaffold (Bartlett, Choi and Phillips, 2016). Hence, the challenges of getting the mechanical properties correct are vast and this is further heightened by the lack of effective methods for assessing bulk properties of soft materials, and also the variability of literature reporting CNS biomechanics.

1.3.2.3 Degradation

Degradation of a material is a key feature to be considered, especially the safety of the break-down products and how they are metabolised. This is specifically crucial in the CNS because any toxic waste products may diffuse into adjacent areas of the CNS and cause additional damage to previously healthy tissue. The degradation rate of a material can depend on which CNS area is targeted for therapy, how long the therapy is required and the formulation of the material. For PD, a non-degradable material is used for DBS electrodes in the subthalamic nucleus. This is connected to a pulse generator in the chest, which modulates an electrical current leading to improvement in motor symptoms (Ughratdar, Samuel and Ashkan, 2015). Issues with these electrodes have been caused by their unwanted degradation, leading to migration of the electrodes from the transplant site and infections. Such effects
highlight how crucial the potential degradation of a material can be (Rizzi et al., 2015).

Comparable to this, transplantation of cells within a biomaterial can require the material to have a quick degradation rate, if the material is there to provide mechanical support upon injection only. However, the material could also be required to last until the transplanted cells have interacted with host cells and created their own ECM. On the other hand, perhaps a non-degrading material would be desirable to permanently isolate cells. This has been shown to be advantageous specifically for the delivery of islet cells for diabetes as degradable alginate microbeads elicit an inflammatory response (Leslie et al., 2016). Both degradable and non-degradable alginate was injected intramuscularly in athymic nude mice where macrophage infiltration was only seen in the degradable alginate in tissue sections. After 6 weeks the non-degradable alginate was still present, but the degradable alginate was not seen and had caused an inflammatory response (Leslie et al., 2016).

1.3.2.4 Host immunological response

Immunoisolation of cells for implantation purposes is based on enveloping the cells in a biocompatible and selectively permeable membrane that enables the transport of essential nutrients and therapeutic agents but inhibits immune recognition by restricting interactions with T-lymphocytes, macrophages and other immune cells (Figure 1.3.4). Research has shown the importance of macrophage phenotype after injury or during the disease state. In the M1 state macrophages release reactive oxygen species (ROS) which initiates a pro-inflammatory response, slowing down tissue repair and regeneration. On the other hand, in the M2 state, macrophages release anti-inflammatory cytokines enabling the injury site to recover. In the CNS the resident macrophages are known as microglia, and the recruitment or transplantation of M2 state cells have shown promising results for CNS regeneration including in a mouse model of traumatic brain injury (Kigerl et al., 2009; Kumar et al., 2015).
Another cell type in the CNS which has been shown to be important in host response is astrocytes and they have become the targets for therapy in CNS injury and disease. Biomaterials used for CNS repair therefore need to prevent an inflammatory response to prevent further tissue damage at the diseased or injury site. Some biomaterials in the literature designed to promote the M2 state of macrophages use release or upregulation by gene therapy to deliver cytokines IL-4 and IL-10 known to promote this polarization (Mokarram et al., 2012; Boehler et al., 2014). Another technique is looking at hydrogels entrapping astrocytes to switch them from their pro-inflammatory ‘reactive’ state and this is further promising with new protocols from stem cell research showing successful differentiation into inflammation-response astrocytes (Macaya et al., 2013; Phillips, 2014; Santos et al., 2017).

In clinical trials for transplantation into the striatum of PD patients, immunological rejection of foetal allogeneic cells has been prevented using systemic immunosuppressive treatment. Clinical trials to date have shown the most improvements when patients received immunosuppression for a year after the transplant, with the least improvements observed where no immunosuppression was given (Sundberg et al., 2013). There are multiple side effects associated with repeated dosing required from systemic immunosuppression and off target effects. Therefore, local delivery could potentially reduce these issues and improve cell therapy for PD. Treatment with the immunosuppressant tacrolimus (FK506) (discussed further in Chapter 5.1.2.1) has been shown to elicit both neuroprotective and neurotrophic effects independent of its immunosuppressant ability and is being used systemically in the current PD hiPSC clinical trial (Konofaos, 2013; Barker et al., 2017).
1.3.2.5 Delivery mechanism

Delivery of biomaterials to the CNS can potentially involve a number of different routes. Direct surgical access is feasible for more accessible places such as the spinal cord or the outer parts of the brain, but in most cases for repair of deeper brain regions, injection is likely to be the most appropriate delivery method. Nanoscale biomaterials can also be engineered with surface markers which interact with specific cells or tissue such as lipoproteins and NSC targeting molecules like the neurofilament derived peptide NFL-TBS.40-63 which can carry small molecules across the BBB into the CNS (Béduneau et al., 2007; Mout et al., 2012; Lépinoux-Chambaud, Barreau and Eyer, 2016). Furthermore, successfully formulated nanoparticles made of chitosan delivered caspase 3 inhibitors to a mouse model of ischemic brain injury (Karatas et al., 2009; Carradori et al., 2017). Hydrogels are the preferred biomaterial for injection as they can be created in a liquid state and then upon delivery to the specific site, change to a semi solid gel due to the changes in microenvironmental conditions. Specifically, developments in cyrogels have shown promise as cancer therapeutics as they are able to retain a specific architecture post-injection and sustain their pore size (Kretlow, Klouda and Mikos, 2007; Bencherif et al., 2012, 2015; Pakulska, Ballios and Shoichet, 2012; Koshy et al., 2014).
1.4 Formulating biomaterials for use in the CNS

As described, the CNS is a complex environment for targeting both cell delivery and biomaterials, therefore the development of biomaterials is crucial. Materials can be formulated for release of small molecules from microparticles and nanoparticles. Dopaminergic neuron replacement has shown improved motor symptoms in PD using hVM and a further improvement could be to protect cells through encapsulation in a biomaterial including an immunomodulatory molecule. This would still allow dopamine secretion to potentially alleviate the motor symptoms in PD, while reducing the immune response to non-host cells.

1.4.1 Drug/Growth Factor/ Gene Delivery

Various bioactive molecules have been delivered to the CNS for tissue repair and regeneration. An example of this are GDNF loaded PLGA microspheres which have been shown to be successful for promoting axonal regrowth following spinal cord injury (Garbayo et al., 2009) and are being researched extensively for PD (Zhang et al., 2009; Li et al., 2015). In rodents, PLGA- GDNF nanoparticles improved rotational behaviour in 6-OHDA rats and showed continued GDNF release over 5 weeks (Garbayo et al., 2009).

Growth factor delivery in combination with cell delivery has also become increasingly researched. Collagen hydrogels encapsulating rat primary DA neurons and GDNF showed improved TH cell percentage survival and striatal innervation in a rat model of PD (delivery method shown in Figure 1.4.1) (Moriarty, Pandit and Dowd, 2017; Moriarty et al., 2019). Biomaterials can further act as nucleic acid carriers for gene therapy, through cell engineering, small interfering RNA (siRNA) and plasmid DNA. The stability of siRNA by itself is low therefore to escape engulfment by host cells and the immune response, siRNA is frequently transported via PEG to improve this stability (Smith et al., 2015; Liang et al., 2019). Lipid-based siRNA delivery to the liver in mouse and primate models of cancer showed high selectivity and efficacy for targeted disease treatment (Smith et al., 2015; Liang et al., 2019). Furthermore with
recent advances in engineered T-cell therapies such as chimeric antigen receptor T-cells (CAR-T), biomaterials could be used in the future to enhance the clinical outcome for multiple diseases (Uludag, Ubeda and Ansari, 2019), with development of PLGA nanoparticles being of utmost interest (Uludag, Ubeda and Ansari, 2019).

Figure 1.4.1. Biomaterials for delivery of cells and GDNF for PD (Moriarty and Dowd, 2018)

1.4.2 Nanoparticles
Polymeric nanoparticles made up of either natural or synthetic polymers such as PLA and PLGA are between 10-1000nm diameters. They can be designed to cross the blood brain barrier (BBB) following systemic injection, or directly injected, or incorporated into other materials. PLA-PEG nanoparticles for delivery of a neuroprotective peptide to the brain via systemic injection using transferrin like molecules (as transferrin receptors are located on the epithelia of the BBB and transport ligands across the BBB) resulted in a larger number of particles reaching the brain (Liu, Gao, et al., 2013; Liu, Jiang, et al., 2013). Nanomedicine-based technology can not only help deliver small molecules or growth factors to the CNS but can also be helpful in cell delivery through the use of nanostructured scaffolds to increase the viability of the transplanted cells with incorporation of growth factors or immunomodulatory molecules (Carradori et al., 2017).
1.4.3 Electrospraying

1.4.3.1 Overview

Electrospraying (ES) is a one-step, electrohydrodynamic atomisation (EHDA) technique where micro- and nanoscale drug delivery particles can be continuously synthesised by the atomisation of polymer solutions. The material properties of the particles, such as morphology, physicochemical properties and surface functionalisation, can be controlled by manipulating the experimental parameters (Williams, Raimi-Abraham and Luo, 2018). Conventional particle synthesising methods, including solvent evaporation, single and double emulsions, have drawbacks including low drug-loading efficiency, particle-size polydispersity and difficulty of incorporating hydrophilic drugs (Seeram Ramakrishna, Zamani and Molamma P Prabhakaran, 2013). EHDA is thought to overcome these issues in particular, as the fast drying of the solvent helps to remove the organic solvents in a rapid manner, leading to the formation of a solid product (D.-G. Yu et al., 2018). Electrospinning for the fabrication of fibres is the most popular EHDA. The working fluids for ES have lower viscoelasticity than for electrospinning with fewer physical entanglements, and a semi-dilute entangled regime of the polymer chains, which will lead to spherical and homogenous particles (Zhou et al., 2017; Williams, Raimi-Abraham and Luo, 2018; D.-G. Yu et al., 2018).

The amorphous form is of interest in drug delivery due to enhanced dissolution rate, especially in the formulation of poorly water-soluble active pharmaceutical ingredients (Lopez et al., 2014). In amorphous solid dispersions the components in the solutions are uniformly dispersed due to their rapid movements via Brownian motion and the faster the evaporation from liquid to solid, the better the homogeneity of the product. EHDA has extremely fast evaporation of the solvent, which can occur in the order of $10^{-2}$ seconds (Lopez et al., 2014; D.-G. Yu et al., 2018).
1.4.3.2 Method and principles

Figure 1.4.2. Schematic diagram of the electrospraying apparatus (co-axial spinneret with two solution feeding in from two syringes shown).

The EHDA apparatus is composed of four main parts shown in Figure 1.4.2, a high-voltage power supply, a precision syringe pump, a spinneret fitted to a syringe and a collector. The high-voltage power supply, in the kV range, is connected to the spinneret and the collector plate with opposite polarities. The syringe contains a liquid, often a polymer dissolved in a volatile solvent, with one or more active ingredients, which will extrude at a rate controlled by the syringe pump. A droplet will form at the nozzle of the spinneret and when the applied electric field is sufficient to overcome the surface tension of the liquid, a critical value is met and the spherical droplet will change shape to conical, the so-called ‘Taylor cone’, and ejection begins as a jet of particles. The charged droplets will travel towards the collector by the
attractive forces and the solvent will evaporate by Coulombic forces, and the particles are collected on the plate (Williams, Raimi-Abraham and Luo, 2018; D.-G. Yu et al., 2018).

The technique can be directly modified to fabricate particles with the desired target product characteristics. Factors which affect the electrospraying process can be divided into solution parameters, processing parameters and environmental parameters. Solution parameters include viscosity, polymer concentration, and the molecular weight of the polymer. Processing parameters include applied voltage, distance between spinneret and collector and solution flow rate. Environmental parameters include humidity and temperature (Williams, Raimi-Abraham and Luo, 2018).

The coaxial spinneret contains a needle within another needle in a concentric manner. The shell fluid is fed into the outer component and the inner component extrudes the core, allowing synthesis of core-shell particles with a more controlled release profile of drug dissolved within the core solution. Benefits include protection of the core from the outside environment, designing the structural composition, and preventing initial burst release by uniform distribution of the therapeutic molecule (Williams, Raimi-Abraham and Luo, 2018; D.-G. Yu et al., 2018).

Biomaterial formulation of molecules which modulate the immune response once transplanted could address some of the current issues with cell therapy for PD. This includes microspheres and nanoparticles which could be formulated using electrospraying or more traditional methods. The benefit of using electrospraying is being able to control the parameters to formulate a unique of core/shell particles to provide a controlled delivery profile over a sustained period.
1.5 Aims of the project

The aim of this project is to use tissue engineering and biomaterials to improve cell therapy for PD. Current therapeutic approaches such as administration of L-Dopa and dopamine agonists are not ideal as they are only effective in the early stages of the disease. Over the past 20 years evidence has emerged that local delivery of various neurotransmitters, growth factors and drugs, and in particular the administration of therapeutic cells, can improve motor function and other symptoms in animal models. Transplanted cells which integrate with host neurons have produced significant improvements in motor symptoms in primate models of PD. However, there is an issue with cell survival post injection and immune rejection.

A novel approach could be encapsulating therapeutic cells for dopamine release, without them forming synapses with host cells (Figure 1.5.1). Instead, a long-term encapsulation approach is hypothesised, in which the dopaminergic neurons won't integrate synaptically but they will supply the neurotransmitters and growth factors known to be beneficial and induce neurogenesis in PD. This has not yet been explored in the brain, but research with encapsulated islet cells has proved this to be successful for insulin production in diabetic patients. The overall aim of this project is to develop clinically competitive cell and biomaterial-based dopamine replacement therapy for PD which is ready to be taken into in vivo experiments.

This leads to 3 main aims:

1. Select and characterise an appropriate GMP grade dopamine releasing cell type for PD therapy through differentiation into midbrain dopaminergic neurons.

2. Develop a biomaterial formulation for encapsulation of therapeutic cells for the purpose of CNS delivery ensuring high cell viability and matching the mechanical properties to the brain.
3. Investigate the effect of encapsulation on dopamine and secreted factors from therapeutic cells and model the host response *in vitro*. Develop a biomaterial formulation for the local delivery of an immunosuppressant within the material encapsulating the therapeutic cells and investigate how this affects the host cell response *in vitro*.

*Figure 1.5.1.* Illustrative summary of the hypothesis for this project.
Chapter 2: Materials and methods

2.1 Materials

The following tables list the cells (Table 2.1.1), the consumables (Table 2.1.2) and the antibodies (Table 2.1.3) used within this research project.

Table 2.1.1. Cells used in this project

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Supplier</th>
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</tr>
</thead>
<tbody>
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<td>SH-SY5Y</td>
<td>Sigma Aldrich</td>
<td>94030304</td>
</tr>
<tr>
<td>HUVECs</td>
<td>PromoCell, Germany</td>
<td>C-12253</td>
</tr>
<tr>
<td>hiPSC (CGT-RCiB-10)</td>
<td>Cell and Gene Therapy</td>
<td>Kindly provided for research use under a Material Transfer Agreement.</td>
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</table>

Table 2.1.2. List of materials used in this project

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<th>Supplier</th>
<th>Catalogue Number (#)</th>
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<td>2,2,2-trifluoroethanol (TFE)</td>
<td>Alfa Aesar</td>
<td>A10788</td>
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<tr>
<td>24 Well Thincert. 1.0um pore</td>
<td>Greiner Bio-One</td>
<td>662610</td>
</tr>
<tr>
<td>diameter</td>
<td></td>
<td></td>
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<tr>
<td>6-HYDROXDOPAMINE HYDROBROMIDE,</td>
<td>Sigma Aldrich</td>
<td>162957</td>
</tr>
<tr>
<td>(6-OHDA) 95%</td>
<td></td>
<td></td>
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<tr>
<td>Accutase</td>
<td>Sigma Aldrich</td>
<td>A6964</td>
</tr>
<tr>
<td>Alginic acid sodium salt</td>
<td>Sigma Aldrich</td>
<td>180947-100G</td>
</tr>
<tr>
<td>AQIX® RS-I solution</td>
<td>Aquix</td>
<td>AQIX-RSI-P</td>
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<tr>
<td>B-27 without vitamin A</td>
<td>Thermo Fisher</td>
<td>12587010</td>
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<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma Aldrich</td>
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<tr>
<td>Calcium chloride</td>
<td>Fisher Scientific</td>
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<td>----------------------</td>
<td>--------------</td>
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<td>Cell activation cocktail</td>
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<td>CellTrace CFSE Cell proliferation kit</td>
<td>Thermo Fisher</td>
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<td>CryoStor CS10</td>
<td>Sigma Aldrich</td>
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<td>CT99021</td>
<td>Axon Med Chem</td>
<td>1386</td>
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<td>Cultrex® Antibiotic-Free Mouse Laminin I, PathClear®</td>
<td>Amsbio</td>
<td>3401-010-02</td>
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<td>Deoxyribonuclease I from bovine pancreas Type II, (DNase I)</td>
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<td>D4527</td>
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<td>Dopamine ELISA</td>
<td>Elabscience®</td>
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<td>DPBS</td>
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<td>Earls balanced salt solution</td>
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</tr>
<tr>
<td>Human Brain derived neurotrophic factor (BDNF)</td>
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<td>Human cytokine array</td>
<td>R&amp;D Systems</td>
<td>ARY005B</td>
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<td>Human Plasma Fibronectin</td>
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<td>FC010</td>
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<td>L-Ascorbic acid</td>
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<td>L-Glutamine</td>
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<td>Lymphoprep</td>
<td>Stem Cell technologies</td>
<td>07801</td>
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<td>MagCellect Rat CD4+ T cell Isolation kit</td>
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<td>MAGR302B</td>
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<td>Magnesium Sulphate (MgSO4)</td>
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<td>MEM Non-essential amino acid solution (100X)</td>
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<td>N2 (100X)</td>
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<td>Neurobasal Medium</td>
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<td>Noggin</td>
<td>R&amp;D systems</td>
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<td>Normal Horse Serum blocking solution</td>
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<td>Paraformaldehyde (PFA)</td>
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<td>Sigma Aldrich</td>
<td>P0781</td>
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<td>Phosphate buffered saline (PBS)</td>
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<td><strong>Propidium iodide (PI)</strong></td>
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<td>81845</td>
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<td><strong>Recombinant Human Glial derived neurotrophic factor (GDNF)</strong></td>
<td>R&amp;D systems</td>
<td>212-GD-010</td>
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<td><strong>ROCK Inhibitor Y27632</strong></td>
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<td><strong>SHH-C24II</strong></td>
<td>R&amp;D systems</td>
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<td><strong>Trypsin inhibitor from Glycine max (soybean) powder</strong></td>
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<td><strong>Vitronectin</strong></td>
<td>Life Technologies</td>
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Table 2.1.3. Primary and secondary antibodies used in this project.

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<th>Product code</th>
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<td>Santa Cruz</td>
<td>J0809</td>
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</tr>
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<td>Nestin</td>
<td>Mouse</td>
<td>Millipore</td>
<td>MAB5326</td>
<td>1:400</td>
</tr>
<tr>
<td>Beta-III-Tubulin</td>
<td>Mouse</td>
<td>Sigma</td>
<td>T8660-.2ml</td>
<td>1:400</td>
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<td>Beta-III-Tubulin</td>
<td>Rabbit</td>
<td>Sigma</td>
<td>T2200-200µl</td>
<td>1:400</td>
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<td>Tyrosine Hydroxylase</td>
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<td>Sigma</td>
<td>T2928-100µl</td>
<td>1:500</td>
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<td>GFAP</td>
<td>Rabbit</td>
<td>Dako</td>
<td>Z0334</td>
<td>1:300</td>
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<tr>
<td>FoxA2</td>
<td>Goat</td>
<td>R&amp;D systems</td>
<td>AF2400</td>
<td>1:500</td>
</tr>
<tr>
<td>LMX1A</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>Ab139726</td>
<td>1:400</td>
</tr>
</tbody>
</table>

Secondary Antibody

| Dylight 488 Horse Anti- Mouse IgG | Mouse | Vector Biolabs | DI-2488 | 1:400 |
| Dylight 549 Goat Anti- Rabbit IgG | Rabbit | Vector Biolabs | DI-1549 | 1:400 |
| Dylight 549 Horse Anti- Mouse IgG | Mouse | Vector Biolabs | DI-2549 | 1:400 |
| DyLight 594 Horse Anti-Goat IgG Antibody | Goat | Vector Biolabs | DI-3094 | 1:200 |
2.2 Methods

2.2.1 Cell Culture

2.2.1.1 SH-SY5Y culture

SH-SY5Y neuroblastoma cells were obtained from Sigma Aldrich and maintained in 1:1 Hams F12: Eagle’s Minimum Essential medium (EMEM) media supplemented with 1% essential amino acid solution, 2mM L-glutamine, 15% FBS and 1% penicillin/streptomycin (P/S). Cells were provided at passage 15 and used up to passage 50. The cells were passaged when 70-80% confluency was reached by trypsinization, centrifugation at 100 x g for 5 minutes and re-suspension in fresh media, and seeded at the appropriate density. Flasks were kept in a humidified incubator at 37 °C with 5% CO₂ in air.

2.2.1.2 Induced pluripotent stem cells

Human induced pluripotent stem cells (hiPSCs) generated from CD34+ peripheral blood cells from a donor in New Zealand were provided by The Cell and Gene Therapy Catapult as a ‘GMP-ready’ cell line (CGT-RCIB-10). This means that the cells were manufactured to an equivalent standard as the equivalent clinical grade cells. These cells were reprogrammed by episomal expression of Oct 4, Sox 2, Klf4, Myc and Lin28 genes. Cells were provided at passage 35 and used up to passage 50. Cells were expanded and maintained on vitronectin (0.5µg/cm²) coated 6 well plates or T25-75cm² flasks in Essential 8/Essential 8 flex media (Thermo Fisher), split every 4/5 days at a ratio of 1:4 and cryopreserved at 1x10⁶ cells/ml in CryoStor10 (Stem Cell Technologies). Flasks were kept in a humidified incubator at 37 °C with 5% CO₂ in air. Cells were seeded at the appropriate density required dependent on the experiment.

2.2.1.3 Human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell and were grown in Complete Endothelial Cell Growth Media (EGM) (PromoCell, Germany), supplemented with 10% FBS (FirstLink, UK) and 1% P/S (Gibco, UK). They were used between passage 4 and 9. The medium was replaced every 2 days.
2.2.1.4 Differentiation protocol

hiPSCs were differentiated into neural stem cells and then dopaminergic neurons following the protocol from Kirkeby et al (2013) shown in Figure 2.2.1. hiPSCs were split using 0.5mM EDTA and placed in Neural Induction Medium (NIM) (described in Table 2.2.1) + Y-27632 (10μM) + SB431542 (10μM) + Noggin (100ng/ml) + CHIR99021 (200ng/ml) + SHH-C24II (200ng/ml). On day 0 an appropriate plate or flask was coated with poly-l-ornithine (PLO) (15μg/ml) in H2O and incubated for 48h at 37 °C. After 48h coated wells/flasks were washed with H2O three times and a solution of fibronectin (0.5mg/ml) and laminin (5μg/ml) in PBS was added to the same plates/flasks for a further 48h at 37 °C. Cells formed embryoid bodies (EBs) between day 1-4. On day 4 cells were attached to plates coated with fibronectin and laminin and PLO and media was changed to Neural Proliferation Medium (NPM) (described in Table 2.2.1) + SB431542 (10μM) + Noggin (100ng/ml) + CHIR99021 (200ng/ml) + SHH-C24II (200ng/ml). On day 11 cells were plated in newly coated plates and media changed to Neural Differentiation Medium (NDM) (described in Table 2.2.1) + GDNF (10ng/ml) + BDNF (20ng/ml) + Ascorbic Acid (0.2mM). On day 14 medium was changed to NDM + BDNF + GDNF (10ng/ml) + Ascorbic Acid (0.2mM) + DAPT (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester) (1 μM) and maintained in this for any future use.

![Figure 2.2.1](image.png)

*Figure 2.2.1* Representative diagram of differentiation methodology from Kirkeby, Nelander and Parmar 2012.
<table>
<thead>
<tr>
<th>Neural Induction Media (NIM)</th>
<th>Neural proliferation media (NPM)</th>
<th>Neural differentiation media (NDM)</th>
</tr>
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<tbody>
<tr>
<td>DMEM/F-12: Neurobasal (1:1) 1× N2 (1:100)</td>
<td>DMEM/F-12: Neurobasal (1:1) 0.5× N2 (1:200) 0.5× B27 (1:100) 2 mM L-Glutamine (1:100)</td>
<td>Neurobasal 1× B27 (1:50) 2 mM L-Glutamine (1:100)</td>
</tr>
<tr>
<td>1× B27 (1:50) 2 mM L-Glutamine (1:100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2.1. Outline of media used for hiPSC differentiation into dopaminergic neurons (Kirkeby, Nelander and Parmar, 2013).

2.2.1.5 Primary rat T-cell isolation, purification and culture

T-cells were isolated from the spleens and lymph nodes of 5, 8 week male rats killed by CO₂. Collection and use of tissue from animals were conducted in accordance with the European Union Directive 2010/63/EU and S.I. No. 565 of 2012 and approved by the UCL Animal Welfare and Ethical Review Body. Briefly, the isolated tissue was teased apart in order to generate a single cell suspension which was then passed through a strainer to remove any cell clumps and/or debris. Cellular suspension was exposed to a red blood cell lysis buffer for 10 minutes on NH₄Cl basis (RBC buffer), which is designed for the preferential lysis of red blood cells, yielding intact T-cells for further applications. Isolation of CD4+ T-cells was performed via a negative selection principle using a commercially available kit (MagCellect Rat CD4+ T Cell Isolation Kit, R&D Systems). Briefly the cell suspension following red cell lysis was suspended in the MagCellect buffer provided and then centrifuged at 400 x g for 5 minutes. Following this the cells were re-suspended to 2 x10⁸ cells/ml and the biotinylated antibody added for 15 minutes at 2-8°C. Cells were washed by adding MagCellect Buffer and then centrifuged for 8 minutes at 300 x g. Streptavidin Ferrofluid was then added in MagCellect buffer for 15 minutes at 2-8°C and then the reaction volume increased to 1ml in each Eppendorf. The reaction tube was then placed on a MagCellect magnet and incubated for 10 minutes at room temperature (RT) to allow unwanted magnetically tagged cells to migrate towards the magnet. The CD4+ T cells were collected in the supernatant and then this step was repeated.
in a fresh Eppendorf to remove further unwanted cells. T-cells were cultured in complete RPMI-10 medium (1% P/S, 1% L-glutamine, 1% essential amino acids, 10% FBS) for up to 6 days in a humidified incubator at 37 °C with 5% CO₂.

2.2.1.6 Primary rat astrocyte and microglia culture

Cortical astrocytes were isolated and purified from 19 postnatal day 2 (P2) rat pups killed by decapitation. Collection and use of tissue from animals were conducted in accordance with the European Union Directive 2010/63/EU and S.I. No. 565 of 2012 and approved by the UCL Animal Welfare and Ethical Review Body. Following decapitation, cerebral cortices were dissected out and the meninges and associated blood vessels removed with fine forceps. The cortices were then finely chopped with a scalpel blade and placed in 250 µg/ml trypsin in 10ml disaggregation medium comprising 14mM glucose, 3mg/ml bovine serum albumin, 1.5 mM MgSO₄ in Ca²⁺ and Mg²⁺-free Earle’s balanced salt solution for 15 minutes at 37 °C. Subsequently, a dilute solution of soya bean trypsin inhibitor (SBTI) and deoxyribonuclease 1 (DNase) (21µg/ml and 6µg/ml respectively in disaggregation media) was added and the cell suspension was centrifuged for 2 minutes at 100 x g. The tissue pellet was re-suspended in 500µl of a concentrated solution of SBTI and DNase (133µg/ml and 40µg/ml respectively in disaggregation media) and triturated using a 1ml pipette to achieve a homogenous suspension. The suspension was then left to allow large tissue fragments to settle.

When the two phases were clearly separated, the top fraction (red) was removed to a separate 15ml tube. This procedure was repeated twice more, keeping the top fraction in each case and the bottom fraction discarded after the third trituration and separation step. The resulting cell suspension was free of debris and was then underlaid with 4% (w/v) bovine serum albumin (BSA) dissolved in disaggregation media and centrifuged at 400 x g for 5 minutes. The supernatant was carefully discarded, and the pellet re-suspended in DMEM-complete and plated on poly-D-lysine (PDL) coated 75cm³ flasks (50 µg/ml PDL coated for 1h at 37 °C, 5% CO₂) in a
ratio of 1 brain per flask with 10ml DMEM-complete medium. Flasks were then placed in a humidified incubator at 37 °C with 5% CO₂ in air.

Astrocytes were maintained in PDL-coated flasks and expanded in culture for at least 10 days before detaching by incubation with 0.25% trypsin-EDTA solution for 7-10 minutes at 37 °C, 5% CO₂. Cells were recovered by centrifugation at 400 x g for 5 minutes and then re-suspended in DMEM-complete medium for use in various applications. Microglia were shaken off flasks after 7 days in culture at 100rpm for 2 hours. Resulting cells were either stored at -150°C until required or plated on PDL coated flasks.

2.2.2 In vitro cell assays

2.2.2.1 Live/Dead assay
To assess cell viability, cultures were stained using the Syto 21/Propidium Iodide (PI) live/dead Double Cell Staining Kit (Thermo Fisher #04511) which allows for the simultaneous staining of viable and dead cells. In darkness (to prevent bleaching), the treatment medium was removed from the dishes, which were then washed three times with 1.0ml of media (37 °C). Subsequently, 500 μl of Syto 21/PI solution (1:1000 dilution) (37 °C) was added. The covered dishes were incubated for 15 min at 37 °C before removing Syto-21/PI solution. The dishes were then washed briefly with 1.0ml of corresponding culture media. Finally, an additional 1.0ml of culture media was added to each well prior to image acquisition.
2.2.2.2 Flow Cytometry

Following the T cell proliferation assay (2.2.2.8), cells were washed with 1ml PBS and centrifuged at 400 x g for 5 minutes. Cell pellet was re-suspended in PBS prior to analysis on the MACSQuant® Analyzer 10 flow cytometer. MACSQuant® Running buffer (Miltenyi Biotec, cat.no.: 130-092-74) was used for the analysis and MACSQuant® calibration beads (Miltenyi Biotec, cat.no.: 130-093-607) were used to calibrate the equipment.

2.2.2.3 Luminescent proliferation assay

Viability was also examined by measuring ATP as an indicator and generating a luminescent readout, using the CellTiter-Glo® 3D Assay (Promega). Based on the manufacturer’s protocol, a volume of reagent equal to that of the culture media was added to each experimental well and following a 30-min incubation at room temperature, 100μl assay solution were transferred to a microplate and luminescence was quantified on a plate reader (Flx800, BioTek or Synergy™ HTX Multi-Mode Microplate Reader). The number of viable cells was determined in culture by measuring the intensity of luminescence signals at several time points, 1, 7, and 14 days. This was determined based on the calibration curve shown in Figure 2.2.2.

![Calibration curve Celltiter Glo](image_url)

Figure 2.2.2. Celltiter Glo calibration curve
2.2.2.4  Cell preparation for cytokine array and dopamine ELISA

Conditioned media from undifferentiated hiPSCs, neural progenitor hiPSCs (NP-hiPSCs) at day 16, dopaminergic neurons (DA-hiPSCs) at day 35 of differentiation, and from encapsulated DA-hiPSCs at day 25, were collected and stored at -20°C (for <1 month) until required for use in the array or ELISA. All cells were plated at 1 x 10^6 cells/well in a 6 well plate for 48 h then media was collected. Encapsulated DA-hiPSCs were added to NovaMatrix alginate at a density of 1 x 10^6 cells/ml and 6 beads per well (500µl alginate solution equates to ~ 15 beads) were seeded and media was taken after 48h in culture.

2.2.2.4.1 Cytokine array

Human cytokine array (R&D Systems) was used according to the manufacturer’s instructions. Briefly, all media was brought to room temperature (RT) before starting and 2ml of array buffer, to serve as a blocking buffer, was added to each membrane and incubated for 1h on a rotating shaker at RT. 1ml of each media sample was added to 0.5ml of array buffer and 15µl of human cytokine array detection antibody cocktail, mixed thoroughly and incubated for 1h at RT. After blocking buffer was removed, sample/antibody mixture was added to each well of the 4-well multi dish with the membranes and incubated overnight at 2-8°C on a rocking platform. Each membrane was washed 3 x 10 minutes in 1x wash buffer then incubated in 2ml of streptavidin-HRP for 30 minutes at RT on a rocking platform shaker. The wash step was then repeated. 1ml of Chemi Reagent mix was added to each membrane and incubated for 1 minute before imaging using the SynGene GeneGnome Imaging system with exposure time of 45 seconds. Analyses and quantification were carried out using ImageJ software, where mean pixel density (mpd) was analysed for each cytokine or chemokine from the duplicate antibody spots (table of cytokines shown in Chapter 3, Table 3.2.1).
2.2.2.4.2  **Dopamine ELISA**

Dopamine ELISA (Elabscience®) was used as per manufacturer’s instructions. Briefly, all media was brought to RT before starting, then 50µl of standard or sample was added to each well. Immediately after that 50µl of biotinylated detection antibody was added to each well and incubated for 45 minutes at 37 °C. Wells were then washed 3x with 1x wash buffer (350 µl /well). 100 µl of HRP conjugate was then incubated in each well for 30 minutes at 37 °C. Wells were then washed 5x with 1x wash buffer (350 µl /well). 90µl of substrate reagent was then added for 15 minutes at 37 °C followed by 50µl of stop solution. Samples were read immediately at 450nm on a Synergy™ HTX Multi-Mode Microplate Reader. A standard curve was generated (Figure 2.2.3) using internal controls and the concentration of DA in the samples was then determined by comparing the OD of the samples to the standard curve.

![Dopamine ELISA standard curve](image)

**Figure 2.2.3.** Standard curve from Dopamine ELISA using internal dopamine controls fitted on a sigmoidal curve.

2.2.2.5  **Neurotoxin assay**

SH-SY5Y cells were seeded at a density of 20,000 cells/well in a 24 well plate and left for 48h to allow neurite extension in media described in 2.2.1.1. After 48h, 100µM MPP+ (1-methyl-4-phenylpyridinium) was added for 24h. Additionally some wells were pretreated with Salvianolic acid B (SAB) for 30 minutes before addition of neurotoxin. After 24h the neurotoxins were removed and the conditioned media from day 16-20 differentiated DA-hiPSCs alone was added to the wells for 48h. Cells were then fixed with 4% paraformaldehyde (PFA) for 24 hours and analysed following the monolayer immunofluorescence protocol in 2.2.3.1.
2.2.2.6 Neurite outgrowth assay

SH-SY5Y cells were seeded at a density of 30,000 cells/ml in a 48-well plate and allowed to attach prior to replacement of the culture medium with treatment solutions consisting of fresh culture medium (described in 2.2.1.1) and varying concentrations of tacrolimus (FK506) dissolved in 0.01% DMSO (1mg FK506 in 10ml DMSO). All treatment solutions were prepared and then allowed to temperature-equilibrate for a period of at least 20 min. After 48h, cells were fixed with 4% PFA in PBS for 24 hours and washed three times in PBS. Following fixation cells were quantified by immunocytochemistry and neurite length measured, method shown in section 2.2.3.1.

2.2.2.7 Microglia viability assay

Microglia were seeded at a density of 20,000 cells/ml in a 48-well plate and allowed to attach prior to replacement of the culture medium with treatment solutions consisting of fresh culture medium (described in 2.2.1.6) and varying concentrations of tacrolimus (FK506) dissolved in 0.01% DMSO (1mg FK506 in 10ml DMSO). All treatment solutions were prepared and then allowed to temperature-equilibrate for a period of at least 20 min. After 48h, viability of cells were asses by live/dead analysis (2.2.2.1).

2.2.2.8 T-cell proliferation assay

Purified rat CD4+ T-cells were stained with an intracellular fluorescent dye (CellTrace™ CFSE) at 5 µM in corresponding media and then plated at a density of $10^6$ cells/ml. Cells were then stimulated by an activation cocktail of PMA (phorbol 12-myristate-13-acetate), ionomycin, and protein transport inhibitor (Brefeldin A) (Cell activation cocktail, Biolegend) for 6 hours. Upon removal of the activation cocktail, cells were exposed to different biomaterial-based stimuli. T cell proliferation was determined 4 days later by flow cytometry analysis (described in 2.2.2.2) of CFSE fluorescence intensity.
2.2.2.9 Astrocyte reactivity assay using collagen gels

Primary rat astrocytes were cultured for 6 days on PDL coated flasks in complete-DMEM. Astrocytes were then detached by incubation with 0.25% trypsin-EDTA solution for 7-10 minutes at 37 °C. Cells were counted using a haemocytometer and then centrifuged at 400 x g for 5 minutes. Astrocytes were then re-suspended at a concentration of 1 x 10^6 cells/ml in complete DMEM. To prepare cellular collagen gels, the cell solution comprised 10% of the total volume so if 8ml total volume, cells were re-suspended in 800μl complete DMEM. The other 90% was, 10% (800μl) 10x MEM and 80% (6.4ml) rat tail collagen type 1. Collagen solution was kept on ice and neutralised with dropwise addition of 0.1M and then 0.01M sodium hydroxide based on phenol red pH indicator colour (the solution turned to a rusty red colour at neutral pH), whereupon cell solution was added, and the solution mixed by swirling. Gels were made in 24 well plates (500μl) and left to set for 20 minutes at 37 °C. Media was then added on top of the gels. After 24 hours 10ng/ml TGF-beta was added for 10 days to stimulate astrocyte reactivity as a positive control or hiPSC 16 day differentiated cells were seeded on top of the collagen gel at a density of 1 x 10^6 cells/ml with or without 100ng/ml FK506. To test the effect of encapsulation on astrocyte reactivity, alginate beads alone or containing 1 x 10^6 cells/ml NP-hiPSC day 16 were put on top of collagen gels (10 for 24 well-plate). After 10 days gels were fixed for 24 hours in 4% PFA at 4°C and then analysed by 3D immunofluorescence GFAP staining (2.2.3.2) and confocal microscopy (Zeiss Axio observer z1).

2.2.3 Immunofluorescence

2.2.3.1 Monolayer Immunofluorescence

Coverslips were fixed with 4% PFA in PBS at 4 °C overnight. Cells were permeabilised using 0.1% Triton-X-100 (Sigma Aldrich) in PBS and washed three times in PBS. Non-specific protein-protein interactions were blocked using 5% normal goat serum (NGS) or 5% normal horse serum (NHS) diluted in PBS (200μl of blocking solution on each coverslip) for 15 minutes in a humidified box at RT. The choice of blocking serum was matched to the species in which the secondary antibody was raised. Coverslips were washed in PBS as described above. Primary antibody was added for 1 hour 30
minutes at RT or overnight at 4 °C and then wash steps repeated. Coverslips were incubated with secondary antibody for 45 minutes at RT, then wash steps were repeated and coverslips mounted using VECTASHIELD® Hardset™ Antifade Mounting Medium with DAPI (Vector laboratories #H-1500) on glass slides. Coverslips were viewed using a fluorescence microscope (Zeiss- Axio Lab.A1). Cell counts were sampled using a x20 objective lens, standardised sampling regions were taken as shown in Figure 2.2.4, each region 625μm apart. The quantification % of cells immunoreactive for a specific marker, was conducted using ImageJ software.

![Figure 2.2.4. Representative image of sampling methodology for manual cell counting on monolayer cultures with each region 625μm apart.](Image)

### 2.2.3.2 3-dimensional immunofluorescence

Collagen hydrogels were fixed with 4% PFA in PBS overnight. Cells were permeabilised using 0.1% Triton-X-100 (Sigma Aldrich) and washed three times in PBS. Non-specific protein-protein interactions were blocked for 30 minutes in 5% normal goat serum (NGS) or 5% normal horse serum (NHS) diluted in PBS. The choice of blocking serum was matched to the species in which the secondary antibody was raised. Collagen hydrogels were then washed 3x in PBS. Primary antibody was added overnight at 4 °C and then wash steps repeated. This procedure was repeated for the secondary antibody and after 1 hour 30 minutes, wash steps were then repeated. Cell counts were sampled and imaged using a Zeiss Axio observer z1 using the x10 objective lens, z stack images were taken based on regions shown in Figure 2.2.5 and quantification carried out with ImageJ software, where a max projection was created from each stack and a threshold analysis taken for cells and area measured.
2.2.4 Biomaterials

2.2.4.1 Preparation of Alginate hydrogels in ThinCerts™

Sodium alginate (Sigma or Novamatrix) was dissolved in DMEM or Neurobasal media (according to cell type used), under sterile conditions, to form final concentrations of 1%, 1.5% or 2% (w/v), respectively. 100μl of alginate solution was placed in the inside of a ThinCerts™ in a 24-well plate (shown in Figure 2.2.6). 1ml of 102 mM calcium chloride (CaCl₂) was added into the well of the plate and this was then placed in a humidified incubator at 37 °C with 5% CO₂ in air for 30 minutes, 1h or 2 h. For cell encapsulation, cells at the desired density (5 x 10⁵-2 x10⁶) were dispersed in the alginate solution and placed inside a ThinCerts™ in a 24-well plate. Following the crosslinking step, the CaCl₂ solution was removed and hydrogels were washed twice with 0.9% (w/v) saline. After hydrogels were washed, they were placed in the appropriate media for the respective cells for use in experiments.

Figure 2.2.6. Representative diagram of alginate hydrogel (100μl) formation in a ThinCerts™ with 1μm porous PET membrane.
2.2.4.2 Preparation of Alginate microbeads.

Sodium alginate (Sigma or Novamatrix) was dissolved in DMEM or Neurobasal media, under sterile conditions, to form final concentrations of 1%, 1.5% or 2% (w/v), respectively. The mixture was extruded into a 102 mM calcium chloride (CaCl$_2$) solution in H$_2$O from a syringe equipped with needle (15-27 gauge size) and dropped at a flow rate of 25ml/h (shown in Figure 2.2.7). Formed beads were left in the CaCl$_2$ bath for 25min for polymerization to occur. For cell encapsulation, cells at the desired density (2.5 x 10$^5$-2.5 x 10$^6$) were dispersed in the alginate solution prior to extrusion into CaCl$_2$. Following the crosslinking step, the CaCl$_2$ solution was removed and the beads were washed twice with 0.9% (w/v) saline. Saline was then replaced with cell culture medium or coating solution. For the latter, beads were suspended in 0.1 % poly-L-ornithine (PLO) solution for 10 minutes, washed with saline and then 0.3% alginate or 0.3% alginate-0.3% hyaluronic acid (HA) solution to neutralize the residual positive charge (shown in Figure 2.2.7). After that, beads were re-suspended in the CaCl$_2$ solution to form the second layer. Finally, beads were allowed to crosslink for an additional 5 minutes, washed with saline to remove the excess Ca$^{2+}$, and suspended in the culture media. Freshly prepared beads were used for further analysis. The diameters of the beads under different conditions were measured using Image J software after images on a camera (Nikon 200) set up on a tripod were taken and then beads were measured compared to a reference standard. Cells in beads were analyzed using the Live/Dead assay described in 2.2.2.1 and also imaged using 3D immunofluorescence described in 2.2.3.2.
2.2.4.3 Swelling of Alginate

The swelling of alginate beads was determined as previously described (Stagnaro et al., 2018). Briefly, samples were immersed in physiological saline or culture media, then removed from the solution and weighed, until the maximum swelling equilibrium was attained. Hydrogel absorption properties were evaluated by estimating their swelling degree based on equation:

\[ SW = \frac{(W_{\text{swollen}} - W_{\text{dry}})}{W_{\text{dry}}} \]

Where, \( W_{\text{swollen}} \) is the weight of the completely swollen sample and \( W_{\text{dry}} \) is the weight of the dry sample.

2.2.4.4 Diffusion properties of alginate beads

In order to assess the diffusion properties of alginate beads, model molecules with different molecular weights were tested using FITC labelled dextran (Mw 44 and 150kDa). For encapsulation and release purposes, the molecule of interest was added to the final alginate solution (5mg/ml) before gelation. After formation, beads were washed twice with 2ml of Dulbecco’s phosphate-buffered saline (DPBS), and the solution was collected in order to measure the entrapment efficiency. The beads were immersed in fresh DPBS and aliquots of 2ml were collected to measure the fluorescence of released dextran. A calibration curve for each dextran \( \lambda_{\text{ex}}=490\text{nm}, \lambda_{\text{em}}=535\text{nm} \) was made. At 2, 4, 24, 48, 96 and 168h, aliquots were collected, and the amount of FITC-dextran released was determined by measuring the fluorescence intensity of the filtrate at a given time versus the amount of the dextran loaded in the microbeads at time 0.
2.2.4.5 Migration of cells in alginate

Sigma alginate was prepared in ThinCerts (2.2.4.1) and either 1x 10⁶ cells/ml SH-SY5Y, HUVECs or a co-culture of SH-SY5Ys and HUVECs at a ratio of 1:1 were seeded on the top of the alginate, inside the alginate or on the bottom of the well plate (Figure 2.2.8). Cells were then analysed after 48h by live/dead (2.2.2.1). The number of live/dead cells on either the bottom of the well plate, on the underside of the ThinCert membrane or inside the gel (shown in the diagram in Figure 2.2.8) were quantified to show percentage migration of total cells.

![Cells on top](Cells on top)

**Figure 2.2.8.** Representative diagram of where cells in migration assay were seeded and analysed.

2.2.5 Drug Loading (DL)

2.2.5.1 Coaxial-nozzle electrospraying of PCL microparticles and nanoparticles

The working solution was prepared by dissolving a fixed amount of polycaprolactone (Sigma Aldrich) pellets in 2,2,2-trifluoroethanol (TFE, Alfa Aesar) : deionized water (DI water) 9:1 v/v under stirring at room temperature to obtain a 10% or 5% w/v solution. Stirring was continued until the polymer was completely dissolved. The collector plate was at a fixed distance of either 15cm or 18cm from the tip of the spinneret (Figure 2.2.9). In order to improve the recoverability of the particles, aluminium foil was covered by a film of polyvinylpyrrolidone (PVP) dissolved in ethanol 10% w/v or 5% w/v, which dried overnight. The aluminium foil was sealed over a metal collector plate 14.7cm x 20cm. The working solution of PCL was carefully loaded into a 5ml disposable plastic syringe (Terumo, UK), ensuring there was no air bubble formation. The syringes were mounted separately onto two syringe pumps (a 78-9100 C syringe pump (Cole Parmer, UK) or a KDS-100-CE syringe pump (KD...
The flow rate for the core was set to 0.1ml/h and the shell 1ml/h for microparticles, and the core 0.01ml/h and the shell 0.1ml/h for nanoparticles and left to calibrate for an hour before the current was switched on, with the process performed under RT conditions (shown in Figure 2.2.9). The positive electrode was connected to the metal tip of the coaxial spinneret and the grounded negative electrode was attached to the metal collector plate. The current was set at 19kV and particles were collected between 1-3h.

Following spraying the PCL nanoparticles were collected by dissolving the PVP film in ultrapure H₂O for 15 minutes and pipetting to break the film apart. The PVP nanoparticle solution was then centrifuged at 400 x g for 10 minutes to collect the nanoparticles. To remove any residual PVP the solution was filtered through a 0.45 µm filter to ensure a homogenous solution and then freeze dried before characterisation or release studies. For the fabrication of PCL-FK506 particles, FK506 was dissolved in ethanol 0.1 % w/v. The solution was loaded into a 1ml disposable plastic syringe (Terumo, UK) and used for the core solution using the flow rates described above.

Figure 2.2.9. Representative diagram of the coaxial-nozzle electrospraying of PCL nanoparticles.
2.2.5.2 *Preparation of tacrolimus loaded PLGA nanoparticles*

PLGA nanoparticles were prepared by the o/w emulsion solvent evaporation method (Elkharraz et al., 2006). 1 mg of tacrolimus (FK506) and 5 mg of PLGA (PURASORB® PDLG, Corbion, Netherlands) were dissolved in 2ml of dichloromethane. This solution was slowly added to 3ml of 0.5 w/v% polyvinyl alcohol aqueous solution with 1000 rpm agitation by a vortexer. After 5 hours stirring to allow nanoparticles to harden, the solution was centrifuged twice (500 x g, 20 min); and the precipitate was then dispersed in deionized water. The aqueous dispersion was then filtered and the residue was freeze-dried. The control PLGA microspheres were prepared by the same procedure without the incorporation of the drug (Kojima et al., 2015).

2.2.5.3 *Determination of entrapment efficiency*

The percentage of FK506 incorporated into both types of polymeric nanoparticles was estimated by UV-Visible spectrophotometry at the characteristic wavelength of FK506 absorption (195 nm). 1mg of FK506-loaded NP powder was immersed into 10ml of acetonitrile solution. Following release of the drug into the solution samples were vortexed for 10 min and then centrifuged at 400 x g for 10 min. Aliquots of 2ml of reference stock solution and sample solution were transferred to a quartz cuvette and the absorbance was measured at 195 nm against a solvent blank. FK506 concentration was determined using a calibration curve (Figure 2.2.10) of purified FK506 concentrations in acetonitrile solution vs. absorbance values at 195 nm: 

\[ A = 0.0331x +0.111 \] (R² =0.9721), where A is the absorbance at 195 nm and C is the FK506 concentration (ng ml⁻¹). The entrapment efficiency (EE) was calculated using the following equation:

\[ \text{EE} (\%) = \frac{Q_i}{Q_t} \times 100, \]

where \( Q_i \) is the amount of FK506 incorporated into the polymeric matrix and \( Q_t \) is the amount of FK506 initially added to the starting solutions (1mg).
2.2.5.4 *In vitro FK506 release study*

FK506 release profiles of PLGA FK506-loaded NPs were performed as follows: 0.1 mg of NP powder was ground and immersed into 10ml of DPBS solution. The release medium temperature was set at 37 ± 1°C. Aliquots of 2ml were withdrawn with a syringe at fixed time intervals for analysis. Following removal of insoluble solid NPs by centrifugation (8000 x g, 5 min), the remaining clear solution was analysed by UV–Visible spectroscopy. The amount of FK506 released was determined by UV-Visible spectrophotometry at 195 nm, through the aid of a calibration curve (Figure 2.2.11) of four standard purified FK506 concentrations (0-200 ng ml\(^{-1}\)) (in DPBS buffer pH 7.4) vs. UV absorbance values: \(A = 0.007121x + 0.0079\) (\(R^2 = 0.9886\)), where \(A\) is the absorbance at 195 nm and \(C\) is the FK506 concentration (mg ml\(^{-1}\)).

Figure 2.2.10. Calibration curve for FK506 in acetonitrile for measuring particle entrapment efficiency for PLGA nanoparticles

Figure 2.2.11. Calibration curve used for FK506 in DPBS for PCL microparticles
Release study tests from PCL core shell nanoparticles were performed in triplicate in vials in an incubator set at 37 °C. 55 mg of particles were suspended in 1ml of deionised water by vortex for 10 minutes before the vials were placed in the incubator. The insoluble particles were removed by centrifugation (8000 x g, 5 minutes) and the remaining supernatant was stored at 4 °C for further analysis. The insoluble particles were re-suspended in preheated deionised water by vortexing and re-added to the vials to maintain a constant volume. The amount of FK506 released was determined by UV-Visible spectrophotometry at 195 nm, through the aid of the following calibration curve (Figure 2.2.12 and equation $y=0.01729x + 0.08868$)

![Calibration curve of FK506 in deionised water used for PCL nanoparticles.](image)

**2.2.6 Characterisation**

**2.2.6.1 Scanning Electron Microscopy (SEM)**

The morphology, size and size distribution of the particles were assessed with the use of a scanning electron microscope. Samples of approximately 1 mg of powder mounted onto aluminium SEM stubs and coated with a thin layer (20 nm) of gold using Quorum Q150T Sputter Coater (Quorum Technologies Ltd, UK) in an argon atmosphere. Subsequently, the materials were imaged with a field emission scanning electron microscope (FEI Quanta 200F, FEI, USA), at an accelerating voltage of 5 kV. The mean particle diameter was obtained by measuring 50 different particles in three SEM images using ImageJ software (National Institutes of Health, Maryland, USA).
2.2.7 Physicochemical characterisation

2.2.7.1 Differential scanning calorimetry (DSC)
DSC was used to explore whether solvent and the electrospraying process would alter the crystallisation of PCL, impacting the physiochemical characteristics of the particles. Samples (approximately 2 to 3 mg) were prepared in non-hermetic Tzero aluminium pans and sealed with pin-holed aluminium lids. The sample was heated from 0 °C to 200 °C using a Q2000 DSC instrument (TA instruments) at a rate of 10 °C /min under a nitrogen gas flow of 50ml/min. The resultant data were analysed using the TA Universal Analysis software version 4.5A (TA Instruments, USA).

2.2.7.2 Thermogravimetric analysis (TGA)
A Discovery TGA (TA Instruments) was employed and the samples heated from 40 °C to 300 °C at a rate of 10 °/min under a nitrogen gas flow of 25ml/min. Approximately 1mg of sample was heated in tared open aluminium pans using the Trios software version 3.3.0.4055.

2.2.7.3 Fourier transformation infrared spectroscopy (FTIR)
To examine the integration between the FK506 and the PCL NPs, infrared spectra were obtained using a Spectrum 100 spectrometer fitted with an attenuated total reflectance (ATR) sampling accessory (PerkinElmer, Waltham, MA, USA) in the range of 500-4000cm⁻¹ with a resolution of 1 cm⁻¹.

2.2.7.4 X-ray diffraction (XRD)
XRD was performed using a Miniflex 600 diffractometer (RigaKu, Tokyo, Japan) with Cu Kα radiation at 40 kV and 15 mA. The patterns were recorded from 3-60° at a scan speed of 5°/min.
2.2.7.5 Rheology

Rheological properties of alginate hydrogel samples were measured on a CVO Rheometry System (CVO, Bohlin Instruments, UK) using 40mm diameter plate geometry. The rheology test was performed at 37.5 °C in a constant-temperature environment maintained using an open-bath circulator with stainless steel bath (DC-10, Thermo Haake®, UK). To determine the linear viscoelastic region of hydrogels, separate strain sweep tests were made. Based on these results, a common strain value was chosen and later used to record viscoelastic properties during oscillatory experiments at a fixed strain of 0.01%, which was within the linear region, under constant frequency of 1Hz.

2.2.7.6 Dynamic Mechanical analysis (DMA)

Compressive DMA analysis of hydrogels was performed using an ElectroForce 3200 instrument (ElectroForce 3200, BOSE, US). Hydrogels of different alginate compositions were prepared using transwell inserts, equilibrated at 25 °C for 30 min, then mean height was calculated by an optical angle meter (Cam 200, KSV Instruments, Finland). Hydrogels or tissue were placed between two 10 mm metal platens. An initial ramp of 15% pre-strain was applied to ensure robust sample contact and a 1 Hz pre-conditioning cycle performed to minimise inter-sample variability. Then samples underwent a defined DMA cycle that consisted of an ascending 1 - 70 Hz frequency sweep with 2 % dynamic mechanical amplitude - these values were adapted from an unpublished company protocol developed for testing soft polyvinyl alcohol hydrogels (Bose, 2012). Once the sweep had been completed, a 1 Hz validation frequency was then repeated to assess for signs of mechanical destruction. Likewise, height measurements were repeated to check for evidence of geometric change at 25 °C.

2.2.7.6.1 Brain DMA

Male Sprague-Dawley rats weighing approximately 200g were culled using pentobarbital in accordance with Home Office Schedule 1 regulations. Collection and
use of tissue from animals were conducted in accordance with the European Union Directive 2010/63/EU and S.I. No. 565 of 2012 and approved by the UCL Animal Welfare and Ethical Review Body. For brain tissue, the head was decapitated, and skin removed. The whole brain was removed intact and placed in ice-cold AQIX® RS-I solution. The specimen was then transported to the laboratory and carefully dissected under a stereomicroscope. For cortex, dura and surface blood vessels were delicately removed using micro-forceps. For corpus callosum, a micro-spatula was gently placed in the longitudinal fissure to separate cortices and the tip of a No. 12D carbon steel scalpel blade (Swann-Morton) used to precisely dissect the corpus callosum from surrounding deep brain structures. Tissue was kept in ice-cold AQIX® RS-I solution during the dissection process and until the point of geometric measurement and mechanical testing. These were then geometrically assessed using a Canon EOS camera, height, length and width measurements were then extracted using ImageJ, and geometric values entered into the DMA WinTest 7 software to determine pre-strain and dynamic mechanical amplitude for each individual sample. After geometric dimensions had been determined, samples were transferred to a Bose Electroforce 3200 for compressive DMA as summarised above 2.2.6.6.

2.2.8 Data analysis
Normality was determined using Shapiro-Wilk test and if there was not a normal distribution then a non-parametric Mann-Whitney test was used. If normal distribution was shown then either a 1- or 2-way statistical analysis of variance (ANOVA) as appropriate with a post-hoc test by either a Dunnett’s, Tukey’s or Bonferroni multiple comparison test, was performed considering all group pair comparison. Degrees of significance were assessed by three different rating values: *p<0.05 (significant), **p<0.01 (very significant) ***p<0.001 (highly significant) and ****p<0.0001 (extremely significant) or non-significant (p>0.05). In the results the initial ANOVA analysis is stated in the main text and the post hoc analysis is indicated on the related figure (*) and explained in the figure legend.
Chapter 3: Differentiation of hiPSCs into dopaminergic neurons

3.1 Introduction

3.1.1 The function of dopamine in health and disease

Dopaminergic neurons in the midbrain are mainly located in the substantia nigra and innervate the basal ganglia, most specifically the striatum (Surmeier, Graves and Shen, 2014). As the largest nuclei in the basal ganglia, the dorsal striatum is known as the motor control centre, receiving actions primarily from the cortex and passing this to other areas in the basal ganglia nuclei (Borroto-Escuela et al., 2018; Zhai et al., 2019). Dopamine modulates this responsiveness mainly in GABAergic spiny projection neurons (SPNs), and therefore is believed to not only regulate action choices but also the memory of which choice was made and the result to initiate future decisions (Surmeier, Graves and Shen, 2014; Zhai et al., 2019). SPNs either project directly into the nuclei of the basal ganglia (dSPNs) expressing mainly D₁ dopamine receptors or indirectly project to the interface nuclei (iSPNs) and express mainly D₂ dopamine receptors (Surmeier, Graves and Shen, 2014). It is believed for movement control both dSPNs and iSPNs are activated and both compete for the basal ganglia outcome and harmonise with each other to both mutually encourage and prevent specific motor actions (Cui et al., 2013). Therefore, this pathway (shown in Figure 3.1.1) highlights dopamine’s crucial function in both control and choice in movement.
Figure 3.1.1: Schematic of the indirect (blue) and direct (red) pathways for a) normal physiological conditions and in b) Parkinson’s disease (PD). The substantia nigra (SN) made up of the pars compacta (SNpc) and substantia nigra pars reticulata (SNpr). The globus pallidus pars interna (GPI) receives projections of dSPNs representing the direct pathway. Activation of iSPNs, which project indirectly to the SNpr via the globus pallidus pars externa (GPe) and the subthalamic nucleus (STN), inhibiting neurons. In PD the degeneration of dopaminergic neurons leads to decrease in dopamine (DA) in the striatum causing an imbalance in both pathways and reduced motor activity (Edited from Calabresi et al., 2014).

Due to the degeneration of dopaminergic neurons and therefore reduction in dopamine levels in PD the innervation in the striatum is lost. This results in a loss of control over the output from the basal ganglia from the imbalance in selection between the iSPN and dSPN signals and consequently inhibition of movement (Cui et al., 2013; Surmeier, Graves and Shen, 2014). In PD this imbalance in the pathways causes inhibition of thalamic neurons which project to the cortex leading to reduced motor activity and locomotion (Figure 3.1.1) (Calabresi et al., 2014). The complexity of this interaction and pathway in PD is now highly characterised. However, it still requires significant research to understand the true interplay of all the cell types (Zhai et al., 2019). Because the loss of dopaminergic neurons is well established as a key aspect of PD, cell therapy to replace the degenerating or lost dopaminergic neurons is an important area of current research.
3.1.2 Cell therapy for PD

As mentioned previously, (Chapter 1.2.2) in the past two decades much research has surrounded transplantation of foetal tissue to treat PD, with clinical trials involving surgical implantation of hfVM tissue into the striatum (Olanow et al., 2003; Politis et al., 2010). After 30 years of hfVM tissue research, improved motor symptoms in patients and long-term cell survival have been observed (Clarkson, 2001; Kirkeby, Parmar and Barker, 2017). However some research has been contradictory with trials not always showing any improvements and also some grafts resulting in side effects such as dyskinesias (Barker, Drouin-Ouellet and Parmar, 2015; Astradsson and Aziz, 2016). Post mortem research and analysis of early hfVM transplants allowed trial optimisation, for the number of cells required and length of time to see clinical benefits, which created the basis for the TRANSEURO trial (Barker, Drouin-Ouellet and Parmar, 2015). The European funded trial, TRANSEURO, started in 2009 using hfVM tissue transplantations in PD patients discussed in detail in Chapter 1.2.2.

Although hfVM transplantation yielded promising results, the approach is limited by ethical and logistical issues around requiring the use of numerous human foetuses and this therefore makes it difficult to produce a scalable amount of tissue or cells (Kirkeby, Parmar and Barker, 2017). Consequently, there is a need for an alternative cell source to overcome these issues. GForce-PD was set up in 2014 to bring together leading researchers in Europe, USA and Japan working on stem cell-derived dopaminergic cells for transplantation in PD. Particular cell sources of interest are ESCs and iPSCs (described in detail in Chapter 1.2.1). Early work on undifferentiated mouse ESCs injected into the mid-brain of rats have improved functional recovery in animal models of PD and furthermore transplantation of ESCs differentiated into dopaminergic neurons also showed improved function (Bjorklund et al., 2002; Kim et al., 2002; Ben-Hur et al., 2004; Kriks, J.-W. Shim, et al., 2011; Kirkeby et al., 2012). A potential limitation highlighted in ESC studies was that only a small percentage of dopamine positive cells survived after transplantation in rat models (Ben-Hur et al., 2004). Despite this, with GMP grade cell sources now available and robust differentiation protocols (discussed in detail in 3.1.3), ESCs show huge potential.
Another cell source is iPSCs, with iPSCs differentiated to NSCs and then to dopaminergic neurons and transplanted into rats showing improvement in behaviour (Wernig et al., 2008). iPSCs are very similar to ESCs in that they respond to the same cues that lead to midbrain dopaminergic differentiation (Kriks et al., 2012; Doi et al., 2014; Kikuchi et al., 2017). Comparable to ESCs, an issue with iPSCs is that their transplantation could lead to tumour formation, particularly if upon transplantation they spontaneously resort to their pluripotent proliferative nature. However in vivo rat and primate models transplanting differentiated iPSCs have shown no tumour formation for nearly 2 years (Kikuchi et al., 2017). Unlike with ESCs, there is additional concern with iPSCs due to their method of reprogramming because this could potentially increase the chance of a reversion to a pluripotent state. Compared to ESCs, iPSCs can be genetically matched to a donor and are not associated with the ethical issues of using human embryos (Morizane et al., 2017).

3.1.3 Dopaminergic neuron differentiation protocols

Differentiation of stem cells into dopaminergic neurons is well established for ESCs and iPSCs (Kriks, J. W. Shim, et al., 2011; Kriks et al., 2012; Kirkeby, Nolander and Parmar, 2013). Recent developments in dopaminergic differentiation protocols have allowed research in this area to progress further. With the first protocols using ESCs (Perrier et al., 2004; Kriks, J. W. Shim, et al., 2011), dopaminergic neurons did not express features such as forkhead box protein A2 (FOXA2). The change in protocol resulted from elucidation of the transitory developmental structure of the floor plate, which is key to midbrain dopamine neuron development. Therefore, instead of transitioning the cells through the neuroepithelial intermediate stage, this protocol now progresses cells through a floor plate stage. This means the cells from this protocol now show genetic and biochemical features of human dopaminergic midbrain neurons (Kirkeby et al., 2012; Kirkeby, Nolander and Parmar, 2013; Tabar and Studer, 2014). For ESCs, using FGF-8 achieved a further optimised protocol for more controlled rostro-caudal patterning to allow GMP grade differentiation, shown in Figure 3.1.2 (Kirkeby et al., 2017; Nolbrant et al., 2017).
hiPSC protocols for midbrain dopaminergic neuron differentiation are very similar to ESCs, using the Wnt signalling pathway for midbrain specification (Kriks, J.-W. Shim, et al., 2011; Takahashi, 2017). By day 19 in differentiation no pluripotent cells are present and all cells express early neuronal markers (PAX6 and SOX1) (Katsukawa et al., 2016). Previous research suggests that cells positive for Nurr1, which is expressed in the middle stage of neuronal differentiation, result in the best survival of ESC-derived dopaminergic neurons following transplantation (Ganat et al., 2012). This indicates that progenitor cells and not mature dopaminergic neurons have the greatest chance of survival. Both ESC and iPSC protocols use ROCK Inhibitor (Y-27632) on the first day of differentiation, to improve survival following differentiation. It has also shown improvement in survival following cryopreservation of cells, but did not have any significant effect when administered directly to the striatum in a rat model of PD (Watanabe et al., 2007; Maldonado et al., 2016; Rodriguez-Pallares et al., 2016). To further prevent tumour formation by ensuring an enriched population of cells and to guarantee optimal survival of transplanted cells, the hiPSC protocol uses CORIN expression to sort the cells (Figure 3.1.3). CORIN is expressed particularly in dopaminergic progenitor cells in the floor plate, so using FACS for cell sorting of CORIN+ cells permits efficient isolation of hiPSC dopaminergic progenitors (Yan et al., 2000; Samata et al., 2016; Kikuchi et al., 2017). Successful transplantation of CORIN+
hiPSC dopaminergic neurons has been illustrated in both rat and primate models (Doi et al., 2014; Samata et al., 2016; Kikuchi et al., 2017).

![Figure 3.1.3: Schematic of the iPSC midbrain dopaminergic neuron differentiation protocol](Taken from Kikuchi et al., 2017).

### 3.1.4 Cell therapy outlook to the future

With the results of TRANSEURO imminent and GForce-PD leading clinical trials across the world in stem cell therapy for PD, there has clearly been significant progress with the translation of this approach. In Japan a clinical trial using dopamine progenitors differentiated from hiPSCs is already underway, (Takahashi and Price-Evans, 2019) and an ESC trial is due to start in Europe within the next year.

To reduce immune rejection in allogenic cell therapy, human leukocyte antigen (HLA) matched hiPSC lines are being generated (Taylor et al., 2012). In organ transplantation, HLA matching is shown to improve engraftment, therefore this could improve allogeneic cell therapy. This is because HLA is expressed on the transplanted cell surface and is recognised by host T cells so therefore plays a crucial role in the immune response. If the HLA isotypes expressed by the transplant and host are
mismatched the host lymphocytes will recognise the cell, tissue or organ as foreign, resulting in an immune response and rejection. Currently it is estimated that 50 HLA lines would cover 90% or 73% of the Japanese population and 150 lines would be required to match 93% of the UK population (Nakatsuji, Nakajima and Tokunaga, 2008; Okita et al., 2011; Taylor et al., 2012). The first HLA matched hiPSC-derived dopaminergic neuron clinical trial began at the end of 2018 on 11 PD patients (Takahashi and Price-Evans, 2019). The trial will be over 24 months and is delivering around 3 million dopaminergic progenitors to 12 sites in the striatum and will immunosuppress patients with FK506 for 12-24 months (Barker et al., 2017; Takahashi and Price-Evans, 2019).

Although current stem cell options for PD are promising there are still multiple issues surrounding their survival, with most ESC and iPSC therapies alone only having 10% survival rate of cells following transplantation over time. Regenerative medicine approaches such as a combination of cells and materials could overcome these problems. Furthermore, therapeutic cells protected within a material promoting cell survival and steadily releasing dopamine, could also provide a long-term treatment option for patients to relieve motor and behavioural symptoms.
Aims for this study

The work reported in this Chapter aimed to identify a specific stem cell type suitable for GMP production and relevant for clinical transplantation, then expand these cells and differentiate them into dopaminergic neurons. Having selected ‘GMP-ready’ hiPSCs provided by the UK Cell and Gene Therapy Catapult, these cells were expanded and characterised. The first aim was to optimise differentiation of these GMP hiPSCs to dopaminergic neurons, by adapting the Kirkeby et al 2013 ESC-differentiation protocol (Shown in Chapter 2.2.1.4) to hiPSCs for the first time. Differentiated cells were then characterised through immunocytochemical analysis for neuronal and dopaminergic markers. Finally, the level of dopamine release from differentiated cells was determined. The purpose of this work was to test the feasibility of using this particular source of hiPSCs to generate dopamine-releasing differentiated cells suitable for use as a new cell therapy option in PD, and to take forward for material characterisation studies in the next part of this thesis. This led to 3 specific aims:

- Establish an appropriate stem cell line relevant for clinical use
- Optimise a protocol for dopaminergic differentiation and characterise the cells for specific pluripotent, neural progenitor, midbrain and dopaminergic markers
- Explore the secretome of undifferentiated and differentiated cells, particularly concentrating on dopamine production
3.2 Results

After reviewing the current landscape in cell therapy, hiPSCs were chosen as the therapeutic cell type of choice because they provide the exciting potential of patient-specific cell therapy for treating neurological diseases without the ethical concerns around embryo destruction or use of foetal tissue associated with ESCs and NSCs. Furthermore, they are currently being used in both Phase I and II clinical trials for multiple disorders, including PD (Martin, 2017; Takahashi, 2017; Takahashi and Price-Evans, 2019). The first objective for this project was to determine whether the chosen hiPSC line could be differentiated into dopaminergic neurons and therefore is a relevant choice of therapeutic cell for use throughout the project. Initially the cells were characterised for pluripotency markers (Oct3/4, Nestin) and then further characterisation of neuronal and dopaminergic neuron markers (β-III-Tubulin, TH, LMX1A and FOXA2) and their secretome pre- and post-differentiation were analysed.

3.2.1 Expansion and Characterisation of hiPSCs

CGT-RCiB-10 hiPSCs were maintained in Essential 8 flex medium (refer to Materials and Methods Chapter 2.2.1.2). Cells were expanded for a month and morphology and characteristics were compared to those reported by the supplier (Figure 3.2.1). The resulting light microscopy images show the formation of small colonies on day 1 with similar hexagonal flat cell morphology as the images provided by the CGTC (Figure 3.2.1 and 3.2.2). By day 4/5 the cells then formed large colonies with little space in between colonies. They reached 80-90% confluency by day 5, confirming the cells were the expected morphology (Figure 3.2.1 and 3.2.2). The cells were successfully expanded and then characterised using pluripotency markers OCT3/4 and Nestin (Figure 3.2.4).
Figure 3.2.1. Phase Contrast Light Microscope images of cell expansion day 1 after split to day 4/5 before split. Images A and B on top were provided by Cell and Gene Therapy Catapult and the images C and D are the equivalent cells grown at UCL, (scale bars 200µm).

Figure 3.2.2. Phase Contrast Light Microscope images of hiPSCs at different magnification on day 4 after splitting. HiPSCs expanded from CGTC at 4x, 10x and 20x (left to right) confirm correct flat hexagonal morphology and cell colony formation (scale bars 100µm).

Characterisation by immunofluorescence shows the hiPSCs stained positively for OCT 3/4 and Nestin (day 0, Figure 3.2.4). The pluripotency marker OCT 3/4 showed 98 ± 1.6% of cells were positively marked, with 81 ± 3.8% of cells positive for Nestin (Figure 3.2.4). Staining controls with just secondary antibody were performed and no background or non-specific binding was observed (no Figure shown).
3.2.2 Differentiation of hiPSCs into dopaminergic neurons

The Kirkeby et al. 2013 protocol was chosen for this project as this has shown reliable differentiation from ESCs into functional dopaminergic neurons. However to the best of our knowledge it has not been used previously to differentiate CGTC hiPSCs (Kirkeby, Nelander and Parmar, 2013). It is also the shortest and appeared to be the most robust protocol published at the time of beginning this project. hiPSCs were successfully expanded and differentiated to a neural phenotype over 14 days. hiPSCs differentiated first into EBs between day 1-4 (shown with the blue arrow in Figure 3.2.3) and then once attached to the flask surface from day 14 they began to show neural morphology and neurites (orange arrows in Figure 3.2.3) can be seen from day 14 (Figure 3.2.3).

Figure 3.2.3. Phase contrast microscope images following Kirkeby et al. 2013 differentiation protocol. hiPSCs differentiated first into embryoid bodies (blue arrow) between day 1-4 and then attached to the flask surface from day 7. They begin to show neural morphology and extend neurites (orange arrows) from day 14.
Neuronal differentiation was characterised by immunocytochemical analysis (Figure 3.2.4-8). Oct 3/4, which is a marker of pluripotency, was present in 98 ± 1.6% of cells at day 0, this reduced to 1 ± 0.4% in the cells by day 14 (Figure 3.2.4; Group; $F_{(1,5)} = 19.2$, $p < 0.0001$). By 21 days Oct 3/4 quantification was 0 ± 0% positive in the cells. Nestin expression was used as a neural stem cell marker and this showed an increase over the period of differentiation from 80 ± 3.8% at time point 0 to 98 ± 1.3% positive cells per field at day 14 and 82 ± 7.0% at day 21 (Figure 3.2.4 and 3.2.6; Group; $F_{(1,7)} = 37.2$, $p < 0.0004$).
Figure 3.2.4. Immunofluorescence images following Kirekby et al (2013) differentiation protocol, comparing iPSCs at day 0 to day 14 and 21 stained with markers for OCT3/4 (red), Nestin (green) and DAPI (blue).
Figure 3.2.5. Immunofluorescence images following Kirkeby et al (2013) differentiation protocol, comparing hiPSCs at day 0 to day 14 and day 21 differentiated cells stained with markers for β-III-Tubulin (green) and DAPI (blue). Scale bars, 100µm.

Figure 3.2.6. Quantification of pluripotency and neuronal markers at different differentiation stages using immunocytochemical staining for OCT 3/4, Nestin and β-III-Tubulin. Data are expressed as mean ± SD, where the mean of 5 fields of view per coverslip were analysed, N=6 separate cultures of cells that have been expanded and characterised independently. One-way ANOVA with Tukey’s multiple comparison test where **p<0.01, ***p<0.001 and ****p<0.0001.
Following the Kirkeby et al (2013) protocol cells successfully differentiated into neuronal precursors (NP-hiPSCs) and showed positive expression of the neuron-specific cytoskeletal marker βIII-Tubulin (Figure 3.2.5). At day 0 cells showed no expression of βIII-Tubulin, this increased to 90 ± 5.1% by day 14 and then decreased to 68 ± 5.7% at day 21 (Figure 3.2.6 Group; \( F_{(5,10)} = 1.1, p < 0.0001 \)). Midbrain and floor-plate markers LMX1a and FOXA2, nuclear expressed transcription factors, were also investigated by immunocytochemistry (Figure 3.2.7). Comparing differentiated hiPSCs at day 25 stained for LMX1a alone and co-stained with FOXA2 revealed that LMX1A alone showed nuclear localisation (Figure 3.2.7). However, when co-stained with FOXA2, localisation appeared to be cytoskeletal for both LMX1A and FOXA2.

<table>
<thead>
<tr>
<th></th>
<th>DAPI</th>
<th>LMX1A</th>
<th>FOXA2</th>
<th>Merge</th>
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</table>

Figure 3.2.7. Immunocytochemistry for midbrain dopaminergic markers. Immunofluorescence detection of LMX1a (green) alone and with FOXA2 (red) in day 25 differentiated hiPSCs. Nuclei are stained using DAPI (blue).

Mature dopaminergic neurons are characterised by the expression of TH, which is the rate limiting enzyme converting tyrosine to L-DOPA and is only expressed in dopaminergic neurons. To investigate maturation of differentiated hiPSCs, cells were maintained for a longer period and TH expression assessed after 35 days to look at dopaminergic neuron expression (DA-hiPSC). At day 0 no cells expressed TH. This increased to 11 ± 3.8% by day 21, and then 30 ± 4.4% by day 35 (Figure 3.2.8; Group; \( F_{(5,10)} = 0.82, p < 0.0001 \)).
Figure 3.2.8. TH quantification for mature dopaminergic neurons following Kirkeby et al 2013 differentiation protocol. Graph shows quantification of TH staining as a proportion of total Hoechst-stained cells stained positive for TH, from day 0, 21 and 35. Immunocytochemistry shows 35 day differentiated hiPSCs stained with markers for β-III-Tubulin (green), TH (red) and DAPI (blue). Data are expressed as mean ± SD where the mean of 5 fields of view per coverslip were analysed, N=6 separate cultures of cells that have been expanded and characterised independently. One-way ANOVA with Tukey’s multiple comparison test **p<0.01, ****p<0.0001.
3.2.3 Secretome analysis of differentiated hiPSCs into dopaminergic neurons

To analyse and compare the secretome of hiPSCs before and after differentiation a cytokine array was used to assess media from hiPSCs alone, day 16 differentiated NP-hiPSCs and day 35 differentiated DA-hiPSCs (Figure 3.2.9). The human cytokine array contains antibodies for 36 cytokines (shown in Table 3.2.1) and media from each of the cell populations was collected and stored for <1 month prior to analysis (detailed in 2.2.2.4). Out of the 36 cytokines only 4 cytokines were detected on the array after the protocol was followed as per the manufacturer’s instructions. This is shown in both Table 3.2.1 and Figure 3.2.10. Mean pixel density (mpd) levels of CCL5 was the highest in hiPSCs, with CXCL12, IL-1β and CD40 also present. In comparison, after 16 days differentiation, CCL5 in DA-hiPSC media had decreased but CXCL12 had increased (Figure 3.2.9A). DA-hiPSC secretome at day 16 did not contain detectable levels of IL-1β, and the amount of CD40 was similar to that in hiPSCs alone. Day 35 DA -hiPSC media contained similar levels of both CCL5 and CXCL12, but both IL-1β and CD40 were not present.
<table>
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<th>DA-hiPSC</th>
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</tr>
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<td>x</td>
</tr>
<tr>
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<td>x</td>
<td>x</td>
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Table 3.2.1. Summary of the cytokines/chemokines the human cytokine array kit can detect (#ARY005B) and the ones detected (√) or not (x) after exposure to media from hiPSCs, NP-hiPSCs and DA-hiPSCs immerse.
Figure 3.2.9. Human cytokine array analysis. The secretome of hiPSCs before and after differentiation, media from hiPSCs alone (black), day 16 differentiated DA-hiPSC media (grey) and day 35 differentiated DA-hiPSC media (dark grey). A) graph showing mean pixel density (mpd) for each cytokine at each condition and B) a heat map for visual analysis of changes in expression through differentiation. Data are expressed as mean ± SD of the two spots on the array for each cytokine (n=1).
For the purpose of this project it was important to produce cells that not only displayed a dopaminergic neuron phenotype but were also capable of releasing a substantial amount of dopamine without additional stimulation. To quantify this an enzyme-linked immunosorbent assay (ELISA) (see Chapter 2.2.2.5), was carried out using the media from hiPSCs alone, day 16 differentiated DA-hiPSC, day 30 differentiated DA-hiPSC and day 35 differentiated DA-hiPSC (Figure 3.2.10). Media for each condition was collected after 48h contact with the cells. hiPSCs alone, day 16 differentiated DA-hiPSC, day 30 differentiated DA-hiPSC all showed less than 52 ± 3.2pg/ml DA. In comparison day 35 differentiated DA-hiPSC released 216 ± 31.7pg/ml DA (Figure 3.2.10; Group; $F_{(3,8)} = 17.18, p <0.0001$).

Figure 3.2.10. ELISA quantification of dopamine secreted by hiPSCs before and after differentiation and maturation in vitro. (A) Media was collected over 48h from hiPSCs before differentiation and at day 16, 30 and 35 of differentiation. (B) shows immunocytochemistry on the cell populations that were used in the secretome analysis after media collection. Cells were fixed with 4% PFA and immunostained to detect Oct 3/4 (red) β-III-Tubulin(green), TH (red) and DAPI (blue). Data are expressed as mean ± SD (n=3) One-way ANOVA with Dunnett’s multiple comparison test comparing differentiated cell groups to hiPSCs ****p<0.0001.
3.3 Discussion

3.3.1 hiPSC characterisation and differentiation

hiPSCs were provided for this project by Cell and Gene Therapy Catapult (CGT-RCiB-10) and were successfully transferred to UCL and expanded showing similar morphology to the images provided by CGTC, with a large nucleus to cytoplasm ratio. Unlike most commercially available hiPSC lines which are only used for research such as modelling disease, these cells have the potential to be used therapeutically at GMP grade and originated from enriched CD34+ cells from peripheral blood of a donor that was screened and consented with therapeutic use in mind. Previous analyses by Cell and Gene Therapy Catapult including whole genome karyotyping, gene expression by qRT-PCR and flow cytometry showed over 90% of the cells were positive for Oct4. Along with the promise that these cells could be clinical grade, there are many benefits of hiPSCs over other therapeutic cells such as their pluripotency, ability to differentiate easily, and their potential to be autologous. An issue with hiPSCs is they require highly intensive maintenance in culture, with the need for a media change every day which could be costly during clinical-grade manufacture. hiPSC also have the potential to revert to pluripotency due to reprogramming factors which can lead to tumour formation (Xu et al., 2016). hiPSCs can be engineered to be made safer using ex vivo gene therapy to counteract their ability to induce tumours, by editing tumour-progression genes in the pluripotent cells (Ben-David and Benvenisty, 2011).

Characterisation of hiPSCs by immunocytochemistry replicated key aspects of the flow cytometry data from CGTC. The marker Oct3/4 was present in 98% of cells, confirming their pluripotency. Oct3/4 maintains pluripotency but also plays a role in integrating responses to signals differentiating ESCs and loss of Oct3/4 deregulates WNT signalling (Simandi et al., 2016). The other marker used for characterisation was Nestin, a cytoplasmic intermediate filament protein which stabilises the cytoskeletal architecture of cells and is expressed in non-differentiated cells and has a role in cell regeneration (Dhingra et al., 2011; Ryuge et al., 2011). Nestin is expressed during
early stages of neuronal development and becomes downregulated as neurons mature. Immunocytochemistry characterisation of the hiPSCs showed ~88% were positive for Nestin, which is in line with what would be expected shown in a previous study which looked at neural progenitor cell Nestin expression by immunocytochemistry in brain slices from both rats and humans (Hendrickson et al., 2011).

For the differentiation of hiPSCs into dopaminergic neurons (DA-hiPSCs) the protocol established by Kirkeby et al (2013) was followed. This method is based on passing the cells through the floor plate stage during development, which is key to midbrain dopaminergic neuron development (Kirkeby, Nelander and Parmar, 2013; Grealish et al., 2014). Following the protocol, EBs could be visualised between day 1-4. Embryoid body differentiation could not be quantified reliably at this stage as the cells do not attach to plates until day 6-7 of the protocol. To analyse this further the cultures could potentially be set up in a 3D matrix so that the embryoid bodies could be immobilised to facilitate immunocytochemistry, or alternatively the cells could be analysed using flow cytometry. By day 14 the cells no longer expressed Oct 3/4, showed projections and had a neural morphology with what appeared to be neurites by light microscopy. This indicated that the cells were no longer stem cells as the pluripotency marker Oct 3/4 was no longer present. Nestin expression increased at day 14 and 21 from the ~88% in hiPSCs to 100%. This is consistent with the differentiation of the pluripotent stem cells to a neural lineage.

By day 14 of differentiation cells showed a positive expression of βIII-Tubulin, a cytoskeletal marker expressed in post-mitotic neurons (Roskams, Cai and Ronnett, 1998; Karki et al., 2013), indicating that by day 14 DA-hiPSCs are of neuronal phenotype. βIII-Tubulin increased to 90.6% by day 14 and then decreased to 68% at day 21 (Figure 3.2.7). The decrease could be due to the density of the neurons in these cultures, as to progress with differentiation cells have to be kept at a high density of 800,000 cells per cm² (Nolbrant et al., 2017) (which was avoided here to
facilitate immunocytochemistry manual counting). To overcome this issue, future studies could quantify for neuronal markers and also sort them using flow cytometry and fluorescence-activated cell sorting (FACS).

Midbrain dopaminergic neuronal progenitors have been defined previously as cells expressing both LMX1A and FOXA2 (Kirkeby et al., 2012; Kriks et al., 2012). The two proteins were present in the differentiated hiPSCs at 25 days as detected using immunofluorescence staining, which indicates that these cells exhibited a midbrain dopaminergic phenotype. LMX1A immunoreactivity was localised to the nuclei, which is consistent with its role as a nuclear transcription factor, but dual staining to detect both LMX1A and FOXA2 simultaneously resulted in more diffuse cytosolic staining. This phenomenon has been reported previously and a 2017 Nature Protocols paper suggested that LMX1A immunostaining could appear cytoplasmic if cells were not plated at a sufficiently high density on day 11 (Nolbrant et al., 2017). This was tested here by increasing the density, but the cells were then over-confluent and either died or had to be passaged. It is therefore likely that the dual immunofluorescence staining protocol used here disrupted the detection of the two markers, so refinement of the methodology or the use of alternative primary and secondary antibodies should be explored in future studies before this aspect of characterisation can be relied upon.

Tyrosine hydroxylase (TH) is a definitive marker for dopaminergic neurons and the result presented here indicated that 30.19% of cells were TH+ by day 35 of hiPSC differentiation. This is comparable with previous ESC differentiation which yielded between 10-30% TH+ cells by day 35-50, and hiPSC differentiation yielding 30-40% TH+ cells by day 42 (Doi et al., 2014; Kirkeby et al., 2017; Nolbrant et al., 2017). Cell sorting using the floorplate marker CORIN has been used in Japan to improve the percentage of dopaminergic specific progenitors in differentiated hiPSCs, increasing the TH+ cells from 20 to 40% (Doi et al., 2014; Kikuchi et al., 2017). This type of approach could be applied in future studies to the DA-hiPSCs obtained in this study,
further enriching the population. As it has been shown therapeutically that ~30% TH+ cells leads to successful transplantation of dopaminergic neurons in vivo, this study was able to successfully achieve this from GMP-ready hiPSCs. It also highlights that characterisation by staining is important and useful, but ultimately it is the functionality of the cell population that really matters, therefore subsequent analyses looked at key aspects of the secretome rather than conducting further characterisation using additional immunostaining, flow cytometry or qRT-PCR.

3.3.2 Secretome analysis from DA-hiPSCs

Release of CCL5, a pro-inflammatory chemokine, was relatively high in undifferentiated hiPSCs compared to differentiated cells, decreasing to similar levels in day 16 and 35 samples. This is consistent with previous work which showed that both ESCs and hiPSCs showed increased detection of CCL5 than in their differentiated neural precursors (Gao et al., 2016). CCL5 is important for immune cell recruitment and thought to be involved in neuronal glial cell interaction and can be both pro-inflammatory and protective against neurotoxins (Lanfranco et al., 2018). CCL5, also called RANTES, has been suggested as a biomarker for PD as serum levels are upregulated in PD patients aged 80+ (Tang et al., 2014). CCL5 is also upregulated in the substantia nigra of PD brains shown in post mortem studies compared to age matched healthy controls, and antibody blocking of RANTES reduced T cell infiltration into the substantia nigra in mice who were injected with MPTP (Chandra et al., 2016). Since chemokine attenuation could be an important therapeutic approach for PD it may be advantageous for levels of CCL5 to be relatively low in implanted cells.

CXCL12, or stromal derived factor-1 alpha (SDF-1α), has been shown to be involved in multiple CNS roles including promoting neural apoptosis and neuromodulation such as influencing the migration of GABAergic neurons (Shimoji et al., 2009; Réaux-Le Goazigo et al., 2013). Levels of CXCL12 in the secretome increased in differentiated cells compared with hiPSCs, with a greater increase apparent at day 16 than at day 35. Increased CXCL12 is seen in PD patients compared to controls in post mortem
brain analysis and it has been shown to be expressed widely in both the developing and adult nervous system (Banisadr et al., 2002; Stumm et al., 2007; Schönemeier et al., 2008). CXCL12 is considered to be homeostatic rather than inflammatory, modulating synaptic transmission. Thus increased levels are likely to be beneficial in therapeutic cells (Réaux-Le Goazigo et al., 2013; Janssens, Struyf and Proost, 2018).

Interestingly, IL-1β was detected in hiPSC media but not in the secretome of differentiated cells. This inflammatory cytokine is produced in the CNS in disease and injury and increased IL-1β in a rat model of PD was associated with neuroinflammation and degeneration of DA neurons (Srinivasan, 2004; Koprich et al., 2008). This result indicates that differentiation may reduce the potential for DA-hiPSCs to encourage neuroinflammation via IL-1β following transplantation.

CD40 is essential for T cell regulation and if concurrently blocked with the CD-28 pathway this increases acceptance of skin and cardiac allografts (Larsen et al., 1996). While hiPSCs and day 16 differentiated cells expressed CD40, day 35 DA-hiPSC cells did not. Upregulation of the CD-40 pathway by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 has been shown to have a pro inflammatory effect on dopaminergic neurons and could play a role in the neuroinflammatory side of PD (Okuno et al., 2005). As CD40 was not detected in the secretome of the mature DA-hiPSCs, there may be advantages in ensuring that transplantation takes place after the NPC stage, to reduce potential inflammatory effects.

The cytokine analysis data is useful in providing some indication about the differences in levels of key signalling molecules in the secretome released from hiPSCs and their differentiated counterparts. However, the results must be interpreted with caution since only one cytokine array was used and only 4 molecules were shown on the arrays, for comparison at only 2 differentiation stages. Furthermore, the cells were grown on a stiff 2D surface in vitro in an environment
that is mechanically and chemically very different to the situation they would encounter in vivo. So, while useful, these results might not predict the behaviour of transplanted cells.

DA-hiPSCs successfully produced picogram levels of dopamine quantified by ELISA, compared to very low levels in hiPSCs and NP-hiPSCs. This meant as a therapeutic cell the CGT-hiPSCs could be progressed to the next stage of the project. So that encapsulation and dopamine production could be explored further in Chapter 5.2.1.

3.4 Conclusion

hiPSCs were successfully differentiated using the Kirkeby et al (2013) protocol into TH+ dopaminergic neurons. This is the first reported differentiation of CGT-RCiB-10 hiPSCs into dopaminergic neurons and they successfully produced dopamine quantified by ELISA. Cytokine release was analysed and, among the molecules considered, the homeostatic CXCL12 was elevated in dopaminergic neurons compared to hiPSCs whereas the pro-inflammatory cytokines were present at lower levels or absent in the secretome of differentiated cells. Overall these cells have been shown to be suitable for use in the next stage of the project because they meet key requirements. These include being from a hiPSC source with potential for GMP manufacture and HLA-matched or autologous transplantation, being differentiated to a DA neuron phenotype via a robust protocol and generating a secretome enriched with dopamine and a potentially beneficial cytokine profile.

Dopaminergic cells implanted into the striatum could promote functional recovery in PD, by increasing local dopamine concentration where it has been lost by the disease. For this approach to work, the cells need to survive long term and remain in the target area, therefore encapsulation in a soft material to support this is explored in Chapter 4.
Chapter 4:  Developing materials for encapsulating therapeutic cells

4.1 Introduction

To achieve the optimal properties for CNS repair and regeneration biomaterials can be natural, synthetic, or a combination of both, described in detail in Chapter 1.3.1. Injectable biomaterials are often favoured for the CNS over those that require surgical implantation, particularly due to some of the deep brain targets required for treating specific injuries and diseases. For example, biomaterials provide the potential to deliver therapeutic cells in PD, but in order to access the striatum, where the dopamine is required, with minimal damage to the overlying cortex, an injectable therapeutic would be required.

4.1.1 Hydrogels for cell encapsulation

Hydrogels are networks of polymers that trap large amounts of water and are commonly used for delivery of cells. Advanced biomaterials technologies have the potential to address the challenges associated with cell transplantation, to protect cells during injection and to provide a supportive microenvironment for cells post-injection that improves retention, survival and integration. Despite considerable advances in therapeutic cell technologies for treating CNS conditions, many transplants still use injections of cells in suspension and accept that the majority of the cells will die, compensating for this through increased cell dose.

Studying hfVM transplants into the rat striatum has shown that up to 99% of cell death is associated with the transplant process itself, and most cell death is observed in the first 7 days post-transplantation (Boonman and Isacson, 1999). Previously, injection of rat primary neural progenitors within a collagen hydrogel improved...
survival in the rat striatum by up to 70% one day after transplantation. However, by 4.5 months only 9% of cells had survived (Moriarty, Pandit and Dowd, 2017).

Alginate hydrogels are commonly used as cell transplantation support matrices in vitro and alginate as a biomaterial is discussed in detail in Chapter 1 (1.3.1.1.1). For stem cells specifically, alginate has been used to guide neural differentiation of both human ESCs and NPCs (Li, Alexander E Davidovich, et al., 2011; Kim, Sachdev and Sidhu, 2013; Cizkova et al., 2015). In diabetes therapy, alginate has been a crucial biomaterial to encapsulate mouse ESCs differentiated into islet cells for insulin production (De Vos, De Haan and Van Schilfgaarde, 1997; Duvivier-Kali et al., 2001; Wang et al., 2009). For retinal cell delivery alginate hydrogels encapsulating both human ESCs and iPSCs significantly improved retinal differentiation over 45 days in vitro compared to HA hydrogels (Hunt et al., 2017). Alginate has also been shown to localise rat adipose-derived stem cells delivered subcutaneously in an in vivo rat model (Leslie et al., 2016). In these examples alginate was used alone, however, research in translational therapeutics is moving towards combinational biomaterials. This is particularly true for the CNS where more sophisticated approaches are likely to be required to overcome the complicated challenges associated with CNS cell transplantation (Mitrousis, Fokina and Shoichet, 2018).

4.1.2 Considerations for material use in the CNS
Biomaterials for therapy require multiple considerations such as biocompatibility, mechanical properties, immune response, release kinetics and degradation (discussed in detail in 1.4 and summarised in Figure 4.1). Issues around the host response and materials which can reduce the acute immune response are considered further in Chapter 5.1. This Chapter will look at establishing a biomaterial capable of encapsulated cell delivery, which does not degrade and therefore protects the cells over a prolonged time. Furthermore, for both cell delivery to the CNS and biomaterial acceptance by the host ECM, materials must be mechanically matched to the host tissue.
4.1.2.1 Mechanical properties

The range of biomaterials used to promote CNS repair experimentally has been diverse, yet soft hydrogels have shown increased potential and been favoured for both *in vitro* and *in vivo* studies (Tam *et al.*, 2014; Adil *et al.*, 2017; Lim and Spector, 2017). This is largely due to their mechanical resemblance to both the brain and spinal cord tissue, which means there is less chance of adverse host responses as the result of a mechanical mismatch.

Stiffness of the cellular environment has been shown to affect multiple biological processes and could consequently be crucial for defining the efficiency of advanced therapeutics. Astrocytes in the CNS are directly sensitive to mechanical cues such as local stiffness differences, therefore biomaterial tissue matching is important for CNS delivery (Moshayedi *et al.*, 2010, 2014; East *et al.*, 2013). Mechanical changes can
affect the behaviour and phenotype of cells, with stiffness being shown to influence differentiation, attachment and migration of stem cells (Engler et al., 2006; Clause, Liu and Tobita, 2010; Hadden et al., 2017; Haugh et al., 2018). The mechanical properties of biomaterial or host tissue rigidity also influence neuronal behaviour, including the time taken for neurones to extend neurites (Balgude et al., 2001), the route of outgrowth from axons (Koch et al., 2012) and amount of branching (Flanagan et al., 2006). Mechanical properties are thus an important concern for biomaterial-based therapy for the CNS.

Despite the significance of mechanical cues, and the desire to match mechanical properties to that of the native CNS, the required properties of biomaterials for successful CNS therapy remain poorly understood and associated protocols for successful characterisation have not yet been optimised. As CNS tissues are amongst the softest in the body compared to musculoskeletal tissues and most other organs, this means they are particularly difficult to test using conventional compressive and tensile material testing equipment (Bartlett, Choi and Phillips, 2016). Further complications arise from the experimental challenge of determining which tissue is a representative model for native CNS mechanical properties. Freshly harvested rodent brains or post mortem human tissues are confounded by the changes in stiffness associated with loss of perfusion and the effects of tissue decomposition. Furthermore, hydrogels and CNS tissue tend to exhibit viscoelastic behaviours (under strain a material exhibits characteristics of both a viscous liquid and an elastic solid), meaning that the stiffness changes according to the rate at which deformation is applied, so single values for tissue modulus obtained from conventional tensile and compressive testing may not be fully representative of the overall response. Techniques such as atomic force microscopy (AFM) provide excellent spatial resolution, yet they require substantial tissue processing and the resulting data involve highly localised mechanical properties on only the surface of the tissue. This makes it inappropriate for assessment of the complete bulk properties of a tissue or making guidelines for biomaterials used in CNS regenerative medicine. Rheometry is
another technique used for mechanical property analysis of viscous liquids and materials such as hydrogels, however it only provides shear modulus values.

4.1.3 Alginate and hyaluronic acid for CNS cell therapy

In order to develop biomaterials for encapsulating therapeutic cells to shield them from the host immune response and increase survival of the cells once transplanted, a combinations of alginate hydrogels with HA and poly-l-ornithine (PLO) were explored here. For this study, alginate was chosen due to it being a well-developed biomaterial option used in other applications, in particular its success in encapsulation of therapeutic cells to treat diabetes (Bochenek et al., 2018). Combbinations of alginate and HA have been used previously to improve the delivery of small molecules, for example Exenatide-loaded alginate-HA microspheres improved bioavailability after oral delivery in mice, overcoming the limitations of administering this drug via injection (Zhang et al., 2014). Mouse adipose derived stem cells encapsulated within the core of alginate material with a poly-l-lysine (PLL) layer were viable for 70 days in vitro (Bhujbal et al., 2014). Mixed results have been observed in studies reporting immune system responses to alginate, although it has been established that the mannnuronic acid (M residues) are responsible for stimulation of human monocyte produced cytokines such as TNF-α (Otterlei et al., 1991). In a rat in vivo model, coating alginate with PLL to prevent stimulation of a lymphocyte mediated cytokine response by concealing the M residues, decreased the inflammatory response compared to alginate alone (De Vos et al., 2012). Alginate beads coated with PLO compared to PLL showed greater resistance to swelling and degradation under osmotic stress in vivo and also lower immunogenicity after 2 days in mice (Tam et al., 2011).

HA is a component of brain ECM available at GMP grade and widely used in plastic surgery (described in more detail in Chapter 1.3.1.1.2). Previously, primary neural progenitors from rats within a collagen hydrogel improved survival in the rat striatum up to 70% one day after transplantation. However, by 4.5 months only 9% of cells
had survived (Moriarty, Pandit and Dowd, 2017). Therefore, the proposed work is to improve this by using a combination of alginate with a HA/PLO layer to reduce the host immune response and reflect the host tissue matrix. It is important when transplanting biomaterials to match them to the surrounding tissue to both reduce inflammation and rejection. HA is found abundantly in the brain and is synthesised by neurons so therefore it is reasonable to suggest that it might be a good material for injection into the brain (Fowke et al., 2017).

Alginate is a good candidate material for encapsulating cells for PD because it can be made into microbeads that would be injectable, therefore protecting cells during injection and once in situ its stiffness modulus would be similar to brain tissue (Drury, Dennis and Mooney, 2004; Banerjee et al., 2009). Furthermore, using a non-degradable alginate could provide long-term protection to cells, shielding them from rejection whilst permitting exchange of nutrients and O2 and allowing dopamine and growth factors through (Vériter et al., 2010; Gasperini, Mano and Reis, 2014; Ramos et al., 2018).
4.1.4 Aims of this study

The aim of this Chapter was to develop and test biomaterial formulations for the encapsulation of therapeutic cells, with a view to injection into the CNS to treat PD. Multiple formulations of alginate including a GMP grade version were analysed in vitro to look at cell viability, cell migration, physical and mechanical characterisation. To enable the stiffness of the material to be matched to the host tissue environment, detailed characterisation of the mechanical properties of brain tissue was undertaken. Furthermore, combination biomaterials were studied for the addition of small molecule release, or to improve host immune reaction. This was investigated by the incorporation of an outer layer using either PLO or HA to coat the alginate. Specific objectives to do this include:

- Develop formulations and protocols for generating stable cellular microbeads
- Characterise cell viability changes in response to seeding density and chemical environment in vitro
- Explore the migration of cells and macromolecules within alginate hydrogels
- Establish target mechanical properties based on characterisation of brain tissue stiffness modulus
4.2 Results

Prior to construction of microbeads, alginate formulations were developed initially using ThinCerts™ to form cylindrical gels to act as an in vitro screening tool to optimise cell viability and study cell migration and material mechanical properties. Two alginate sources were chosen to be explored in this work, Sigma alginate for its low cost and ease of use to set up conditions and then SLM NovaMatrix alginate, a biomaterial manufactured under GMP guidelines with <1% endotoxins. Sodium alginate (Sigma or NovaMatrix) was made to a final concentration of 1%, 1.5% or 2% (w/v) and 100μl of alginate gel was cast in the inside of a ThinCert™ in a 24-well plate. Alginate microbeads were formulated using a syringe pump and manual synthesis into a beaker containing heated and stirring CaCl₂. The microbeads were created to look at a potential method to encapsulate cells, which could be taken in vivo and translated to humans. PLO and HA were also incorporated into the microbeads to reduce immunoreactivity and reflect the host tissue extracellular matrix, respectively.

4.2.1 Cell survival in different formulations of alginate hydrogels

To investigate the effects of cell survival in alginate, SH-SY5Y cells were seeded in 2% alginate (Sigma) at different seeding densities (0.1 x 10⁶ cells/ml – 1 x 10⁶ cells/ml) and altering amounts of time in CaCl₂. Viability was quantified by using PI for dead cells and Syto 21 for live cells, and the percentage of live and dead cells was calculated from the total cell number. To study whether osmotic shock impacts cell viability when synthesising the alginate hydrogels, viability was tested in CaCl₂ made both in DMEM media and in H₂O. Neither cell seeding density, CaCl₂ solution conditions nor length of time in CaCl₂ had an effect on the viability of the cells with all conditions showing over 90% total live cells (Figure 4.2.1).
Figure 4.2.1. SH-SY5Y cell viability is not affected by either cell density or time in CaCl\(_2\). Alginate hydrogels (2% Sigma) with 0.1, 0.5 and 1 x 10\(^6\) cells/ml were synthesised in ThinCerts. All showed over 90% cell viability in both CaCl\(_2\) made in either H\(_2\)O or DMEM media (A). Alginate hydrogels (2% Sigma) made with 1 x 10\(^6\) cells/ml left in CaCl\(_2\) for 30min, 1h or 2h showed no significant difference in cell viability with all conditions being over 90% total live cells. N=3 Means ± SD. Two-way ANOVA with Tukey’s multiple comparison test non-significant (p>0.05).

The Sigma alginate is non-sterile and may contain endotoxins, therefore a potential GMP grade alginate (NovaMatrix) was looked at as a comparison, which manufactured under GMP guidelines. 1 x10\(^6\) cells/ml in 1h of CaCl\(_2\) was chosen as the cell density and time for crosslinking for future experiments. For each alginate formulation, SH-SY5Y viability was quantified at 3 time points over 168h using live/dead quantification (Figure 4.2.2). There was a significant decrease in cell viability following culture in both alginates at 96 and 168h, but both showed over 50% viability at 168h (Figure 4.2.2 A; Group live cells; \(F_{(2,7)} = 9.2, p <0.0001\), Figure 4.2.2 B; Group live cells; \(F_{(3,8)} = 8.4, p <0.0001\)).
Figure 4.2.2. SH-SY5Y cell viability over time in Sigma and NovaMatrix alginate. Alginate hydrogels (2%) with 1 x 10^6 cells/ml were synthesised in ThinCerts and cell viability was calculated using PI and Syto 21 at 72, 96 and 168h, in either Sigma (A) or NovaMatrix (B) alginate. Both alginates showed significant decrease in live cells after both 96 and 168h compared to 24h. N=6 Means ± SD, two-way ANOVA with Dunnett’s multiple comparison test where live cell percentage is compared to 24h for each where ***p<0.001 and ****p<0.0001.

Alginate microbeads were created using syringes with needles to create drops that then cross-linked in CaCl₂ solution. Syringes were controlled manually (manual synthesis) (example shown in Figure 4.2.4) or using a syringe pump at a flow rate of 25ml/h (Figure 4.2.3). Acellular alginate hydrogels (1.5 & 2%) were synthesised using different size needles (15G-27G). The two different alginate concentrations were explored to assess how this compared in terms of mechanical properties. The largest needle (15G) produced the biggest beads at 4mm and the smallest (27G) produced 1.5-2mm beads with significant difference between gauge size for each condition (Figure 4.2.3A-D). There was a significant difference in bead size between syringe pump and manual synthesis, with syringe pump synthesis creating smaller bead sizes with the exception of 21G at 1.5% alginate (Figure 4.2.3 E; Row factor x time; F (4,25) = 7.9, p <0.0003; Figure 4.2.3 F; Row factor x time; F (4,25) = 2.4, p =0.0769).
Figure 4.2.3. Sigma alginate bead size changes with needle gauge size. Acellular alginate microbeads (2 & 1.5%) were produced by syringe pump (A,B) or manual (C,D) synthesis using different size needles (15G-27G), with the largest (15G) producing the biggest beads at 4mm (A) A digital image of the beads was taken 30 minutes after the samples were synthesised (Nikon D200, Nikon, Japan) for determination of the sample diameter. Comparison between synthesis method for 2% (E) and 1.5% (F) N=4 samples per repeat, Means ± SD, one-way ANOVA with Bonferroni’s multiple comparison test for A-D (Black compared to 15G, red compared to 21G, blue compared to 23G and green compared to 25G) and two-way ANOVA with Bonferroni’s multiple comparison test (E-F) where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001
Figure 4.2.4. Alginate bead made using manual synthesis of Sigma alginate bead by 21G needle.

Figure 4.2.5. Swelling rate of Sigma alginate measured by the percentage weight change over time. Analysed in media (A, C) and saline (B, D) for beads of 2% (A,B) and 1.5% (C,D) alginate solution, synthesized using needle gauges (15G-27G). Samples were incubated at 37.5 °C, and their weight was recorded at 0, 2, 4, 24 and 48h. Data expressed as Means ± SD, N=3, 4 samples per repeat.
Stability of alginate microbeads made using a range of 5 different needle gauges was assessed by allowing them to swell in saline or in media over 48h and monitoring the change in their weight (Figure 4.2.5). The swelling rate described in Chapter 2.2.4.3, was higher for beads immersed in saline solution than those in media, with the 1.5% alginate showing a larger effect (Figure 4.2.5). In all cases there was a rapid increase in weight over the first 4 hours then the rate of change decreased, levelling off between 24 and 48 hours in most cases. The microbeads immersed in saline solution exhibited greater swelling than those in media, and in general the 1.5% alginate microbeads swelled more than the 2% material. In all conditions the 25G needle exhibited the greatest percentage weight change and the 21G showed the least.

The 21G needle was chosen as the size to move forward with, so in subsequent experiments all beads were made with 21G (Figure 4.2.4). To investigate if cell density impacts cell viability in the alginate microbeads, cell viability at densities 2.5 x 10^5 cells/ml - 2.5 x 10^6 cells/ml in either 1.5 % (Figure 4.2.6 A; Group; F (3,8) = 3.6, p =0.0641) or 2% alginate (Figure 4.2.6 B; Group; F (3,32) = 1.5, p =0.225) using 21G needle synthesis was measured after 24 hours by quantifying live/dead cells. This showed that at both 1.5% and 2% alginate high densities of cells (2.5 or 1 x 10^6 cells/ml) had a significantly higher viability compared to the microbeads with <1 x 10^6 cells/ml. Microbeads encapsulating 1 x 10^6 cells/ml were synthesised using a 21G needle with 2% alginate, alginate with PLO coating and alginate with PLO and HA coating (described in methods 2.2.4.2). Cell viability was quantified over 14 days, showing no significance between alginate alone, alginate with PLO and alginate with PLO and hyaluronic acid (Figure 4.2.6 C; Time; F (2,25) = 6.9, p =0.053).
Figure 4.2.6: SH-SY5Y cell viability is consistent in different cell densities and over time. Alginate microbeads (21G) alone or with PLO with $2.5 \times 10^5$ cells/ml- $2.5 \times 10^6$ cells/ml in either 1.5% (A) or 2% (B) were synthesised using a syringe pump and cell viability after 24 hours using the live/dead method. (C) Cell viability ($1 \times 10^6$ cells/ml) over 14 days in alginate microbeads alone, with PLO or with PLO/HA using 21G needle was analysed by the same method. N=4 samples per repeat, Means ± SD, two-way ANOVA with Bonferroni’s multiple comparison test between alginate, alginate + PLO or alginate +PLO/HA where *p<0.05 and non-significant (p>0.05).
The viability of SH-SY5Y cells in the microbeads was further analysed by measuring their metabolic activity over time using the CellTiter Glo assay, which looks at luminescence levels relative to ATP concentrations produced. CellTiter Glo was used to measure metabolic activity of the cell populations over 14 days at cell densities from $2.5 \times 10^5$ cells/ml- $2.5 \times 10^6$ cells/ml in microbeads made with alginate alone, alginate with PLO coating, and alginate with PLO and Hyaluronic acid (HA) compared to a SH-SY5Y cell monolayer control (Figure 4.2.7). Similar to the live/dead results (Figure 4.2.6), $2.5 \times 10^5$ cells/ml showed the lowest levels of metabolic activity with a decrease after 14 days in all formulations. As expected, $2.5 \times 10^6$ showed the highest relative luminescence values in all formulations over 14 days with a significant increase over time. Compared to alginate, alginate + PLO and alginate +PLO/HA show no decrease after 14 days in $2.5 \times 10^6$ cells/ml, but similar to alginate all conditions show consistently low metabolic activity at both $2.5$ and $5 \times 10^5$ cells/ml (Figure 4.2.7 alginate; Group; $F_{(12,75)} = 5.9$, $p < 0.0001$; Figure 4.2.7 alginate + PLO; Group; $F_{(12,75)} = 5.7$, $p < 0.0001$; Figure 4.2.7 alginate + PLO/HA; Group; $F_{(8,30)} = 9.3$, $p < 0.0001$).
Figure 4.2.7: Cell metabolic activity over 14 days in 2% alginate microbeads and is consistent in different formulations. Cell proliferation from the measured luminescence values from 2% Sigma alginate using the CellTiter-Glo® assay after 24h, 7 and 14 days in alginate alone (A), PLO coated alginate (B) or alginate coated with PLO/HA (C). N=3, (3 beads per N). Means ± SD, two-way ANOVA with Dunnett’s multiple comparison test where each condition was compared to the respective monolayer control *p<0.05, **p<0.01 or non-significant p>0.05.
Following use of the SH-SY5Y cell line for *in vitro* optimisation of alginate microbeads, the viability of stem cells in alginate was investigated. Undifferentiated hiPSCs were encapsulated in alginate and viability assessed using the live/dead assay. Initial experiments found that hiPSCs did not survive in Sigma alginate (data not shown). This was presumed to be due to endotoxin levels of Sigma alginate. Therefore, NovaMatrix alginate was used for all subsequent hiPSC encapsulation experiments at a concentration of 1.5% for all further cell experiments and then both 1.5% and 1% for mechanical testing. 2% NovaMatrix is too viscous to use and therefore could not be continued with. Alginate was synthesised in either DMEM or Essential 8, however at all densities viability of the cells was less than 50% after 24 hours in both formulations (Figure 4.2.8 A; Group; $F_{(4,12)} = 20, p < 0.0001$ and Figure 4.2.8 B; Group; $F_{(3,11)} = 3.6, p = 0.048$). The addition of different concentrations of ROCK Inhibitor (Y-27632), known to improve survival of hiPSCs (discussed in Chapter 3.1.3), was investigated. However, with the addition of ROCK Inhibitor (1-100µM) in Essential 8 or DMEM, viability remained at less than 50% after 24h (Figure 4.2.9 B; Group; $F_{(2,12)} = 31.7, p < 0.0001$). To investigate this further, cell densities of 0.5 x 10⁶ cells/ml- 2 x 10⁶ cells/ml in alginate hydrogels treated with vitronectin (0.25µg/ml), Y-27632 (500µM) and fibronectin (5µg/ml) were analysed for viability. All showed no significant change compared to the control apart from 500µM Y-27632 at both 0.5 x 10⁶ and 1 x 10⁶ having a significant increase compared to the control after 24 hours (Figure 4.2.9A; Group; $F_{(9,16)} = 7.2, p < 0.0001$).
Figure 4.2.8: Viability of hiPSC increases at higher cell densities. Alginate hydrogels (1.5% Novamatrix) with 0.1 x 10^6 cells/ml - 4 x 10^6 cells/ml after 24h. Alginate was made in either DMEM (A) or Essential 8 media (B) N=6 Means ± SD. One-way ANOVA with Tukey’s multiple comparison test between live cell groups where ***p<0.001 and ****p<0.0001.

Figure 4.2.9. Improving hiPSC viability with different compounds. Alginate hydrogels (1.5% Novamatrix) with 0.5 x 10^6 cells/ml- 2 x 10^6 cells/ml with addition of Vitronectin, Y-27632 and Fibronectin (A) all showed less than 60% viability after 24h. Alginate with 1 x 10^6 cells/ml with 1-100µM Y-27632 (B) N=6, Means ± SD, two-way ANOVA with Dunnett’s multiple comparison test for A, where each concentration is compared to the respective control and Tukey’s multiple comparison test for B, where each concentration compared for both media formulations, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 or non-significant (p>0.05).
As previously described (Chapter 3) hiPSCs were differentiated into dopaminergic neurons (DA-hiPSCs). To measure the viability of the day 16 progenitors (NP-hiPSCs) in NovaMatrix alginate, ThinCerts were prepared with 2% alginate and NP-hiPSCs at densities of $1 \times 10^6$ cells/ml, $2 \times 10^6$ cells/ml and $3 \times 10^6$ cells/ml, then analysed after 24h for live/dead (Figure 4.2.10A; Group; $F_{(2,9)} = 84$, $p < 0.0001$). Both $1 \times 10^6$ cells/ml and $2 \times 10^6$ cells/ml gels contained significantly more viable cells than $3 \times 10^6$ cells/ml gels. Viability of cells encapsulated within microbeads was then measured to see whether viability was affected by formulation. NP-hiPSCs were encapsulated at a density of $1 \times 10^6$ cells/ml in NovaMatrix alginate alone, PLO coated, and PLO and HA coated alginate beads. After 24h and 7 days, viability was quantified used live/dead (Figure 4.2.10B; Group; $F_{(5,17)} = 9.4$, $p = 0.0002$). Encapsulation of cells in alginate alone did not affect viability over 7 days, with 24h and cells after 7 days being...
equivalent. Cells within PLO coated alginate had a significant reduction in viability after 7 days. In comparison cells in HA and PLO coated alginate had a significantly lower viability at 24h, however this stayed constant up to 7 days (Figure 4.2.10B).

NovaMatrix alginate beads containing NP-hiPSCs at 1x 10^6 cells/ml for 24h, 7, 14, 21 and 28 days were analysed by live/dead cell viability quantification (Figure 4.2.10C; Group; F_{(4,23)} = 14, p < 0.0001). No significant decrease was found from 24 hours to 7 or 14 days, however, after 21- and 28days viability reduced significantly. Overall a 14% decrease in % live cells over 28 days in NovaMatrix was seen, only significantly lower than 24h at 28 days (Figure 4.2.10C).
4.2.2 Characterisation of the physical properties of alginate

Figure 4.2.11. Cell migration through alginate. SH-SY5Y, HUVECs and a co-culture of both (1:1) SH-SY5Y and HUVECs both at a density of 1x 10^6 cells/ml were seeded on top of (A), or on the bottom of the plate (B), or inside of (C) Sigma alginate ThinCert hydrogels. Cell viability was quantified by live/dead after 48h. N=4, Means ± SD, multiple t-test, comparing between each individual location (inside, membrane or bottom) where ****p<0.0001.
To analyse whether alginate is physically an appropriate material for CNS therapeutics, properties such as cell migration, dextran diffusion and the degradation of alginate were quantified. To look at cell migration, Sigma alginate was prepared in ThinCerts and either SH-SY5Y, Human Umbilical Vein Endothelial cells (HUVECs) or a co-culture of SH-SY5Ys and HUVECs were seeded on the top of the alginate (Figure 4.2.11 A; Group; $F_{(4,18)} = 82, p < 0.0001$), inside the alginate (Figure 4.2.11 C; Group; $F_{(4,18)} = 187, p < 0.0001$) or on the bottom of the well plate (Figure 4.2.11 B; Group; $F_{(4,18)} = 58, p < 0.0001$). The number of live/dead cells on either the bottom of the well plate, on the underside of the ThinCert membrane or inside the gel (shown in the diagram in Figure 4.2.11) were quantified after 48h and each population presented as a percentage of total cells. HUVECs were chosen as the second cell type because vascularisation will be key to implanted cell survival, and it will be useful to understand whether endothelial cells would be able to penetrate a transplanted cellular alginate gel or would be limited to surrounding the outside.

Overall SH-SY5Y cells tended not to migrate, with all or most of them remaining in the place they started (Figure 4.2.11). Conversely, a significant proportion of the HUVECs which started out on or in the gel migrated to other locations (Figure 4.2.11). The migration pattern in the co-cultures reflected that in the HUVEC-only group, with significant migration onto the membrane from the gel or the bottom of the plate.

On transplantation of cells encapsulated within materials it is essential that nutrients and oxygen can diffuse through the alginate for the survival of the therapeutic cells. Furthermore, in the context of this project it is important that the dopamine released from the DA-hiPSCs can diffuse out of the material and into the host tissue. To analyse diffusion, release of 40kDa and 150kDa FITC-dextrans from within alginate gels into media was quantified using a fluorescence plate reader (Figure 4.2.12). Diffusion from both 1.5% and 2% Sigma alginate alone, with PLO coating and with PLO/HA coating were analysed, and for 40kDa dextran 100% release was observed by 48h for all conditions (Figure 4.2.12A). 1.5% alginate alone showed the fastest
release with 84% at 3h, the 2% PLO and PLO/HA coated was the slowest at 71% at 24h (Figure 4.2.12A). In contrast, 150kDa dextran only reached between 30-50% for each condition after 168h, however as with the 40kDa dextran the 1.5% alginate alone showed the fastest release with 46% release after 168h compared to only 19% release from the PLO/HA coated 2% alginate (Figure 4.2.12B).

Figure 4.2.12. Release of different size dextrans from both 1.5% and 2% alginate. FITC labelled dextran (Mw 44 and 150kDa) was added to the final alginate solution (5mg/ml) in both 1.5 and 2% alginate. After formation, beads were washed twice with 2ml of Dulbecco’s phosphate-buffered saline (DPBS), fluorescence of released dextran was measured at 2, 4, 24, 48, 96 and 168h, the amount of FITC-dextran released was determined by measuring the fluorescence intensity of the filtrate at a given time versus the amount of the dextran loaded in the microbeads at time 0. Release of 40kDa dextran from alginate alone, PLO coated, and PLO and HA coated for both 1.5% and 2 % alginate (A). Release of 150kDa dextran from alginate alone, PLO coated, and PLO and HA coated for both 1.5% and 2 % alginate (B). Means ± SD, N=3.
4.2.3 Mechanical properties of alginate and tuning this for CNS therapeutics

4.2.3.1 Evaluation of the time-dependent viscoelastic shear behaviour of alginate

Rheometry was performed to understand the gelation behavior of alginate. The tests were completed at 37 °C using 1hz as a constant frequency which is within the linear viscoelastic region of alginate (Appendix, Figure 7.1).

Following the addition of CaCl₂, gelation or crosslinking started after ~ 100 seconds and was complete at 43 minutes for 2% Sigma alginate and 51 minutes for 1.5% Sigma alginate (Figure 4.2.13). The same was shown for 1.5% NovaMatrix and 2% NovaMatrix was too viscous to be used to form gels in the same way, with gelation at 56 minutes (Figure 4.2.14). This was determined as the time when three sequential measurements did not change by > 0.5% (indicated by the arrows on the graph).

![Graph showing viscoelastic behavior of alginate](image)

<table>
<thead>
<tr>
<th>Shear force analysis (Rheometry)</th>
<th>2% w/v alginate</th>
<th>1.5% w/v alginate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Storage modulus</td>
<td>0.536 ± 0.017 kPa</td>
<td>0.232 ± 0.019 kPa</td>
</tr>
<tr>
<td>Loss modulus</td>
<td>0.054 ± 0.019 kPa</td>
<td>0.024 ± 0.010 kPa</td>
</tr>
<tr>
<td>Gelation time</td>
<td>43 min</td>
<td>51 min</td>
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**Figure 4.2.13. Rheology for Sigma Alginate.** 100μl of un-crosslinked polymer solution and CaCl₂ were added to a parallel plate layout and subjected to single frequency (1Hz) oscillatory tests at 37.5 °C and 0.01% strain. Table shows a summary of the mean modulus values obtained by rheological measurements in the graph. Arrows highlight gelation time. Data expressed as Means ± SD, N=3.
100μl of un-crosslinked polymer solution and CaCl$_2$ were added to a parallel plate layout and subjected to single frequency (1Hz) oscillatory tests at 37.5 °C and 0.01% strain. Arrow highlights gelation time of 56 mins. Data expressed as Means ± SD, N=3

4.2.3.2 Diversity in mechanical properties of specific brain regions

To test the mechanical properties of bulk brain regions and biomaterials independently, the technique dynamic mechanical analysis (DMA) was chosen. For the present study all analyses were conducted using rat brains due to feasibility of procurement. Although the area of interest for tissue matching would be the striatum, the rat striatum would be too small to conduct accurate measurements, therefore the cortex and corpus callosum were chosen, due to cortex being predominantly grey matter and corpus callosum predominantly white matter. Since the striatum contains a mixture of both white and grey matter, it made sense to test areas as an example of each tissue type. Sections were dissected from rat brains as described in 2.2.6.6.1, then at 40 minutes post-mortem DMA was used to assess the compound, storage and loss moduli of the fresh tissue samples (Figure 4.2.15 A, B). Brain regions showed significant differences in tissue stiffness, with compound modulus for cortex being 4.83 kPa and for corpus callosum 32.15 kPa at a frequency of 1Hz (Figure 4.2.15C; Group; $F_{(10,55)} = 2, p = 0.0394$). Both the white and grey matter areas became stiffer as test frequency increased, shown by compound modulus values of 10.21 and 63.92 kPa at 70 Hz. The tan delta between cortex and corpus callosum (Figure 4.2.15D; Group; $F_{(10,55)} = 1, p = 0.3902$) showed at higher frequencies the corpus callosum displayed a more elastic behaviour than cortex which displayed a relatively more viscous behaviour.
4.2.3.3 Alginate can be adjusted to match the stiffness properties of specific brain regions

The same DMA protocol (described in 2.2.6.6.1) was successfully applied to measure the mechanical properties of both Sigma alginate and clinical-grade NovaMatrix alginate using the ThinCert formulation. 2% alginate had higher compressive and shear modulus values at 1-70hz, which indicates higher material stiffness, as expected by the concentration.

All alginites, 1.5% and 2% Sigma and 1% and 1.5% NovaMatrix had a storage modulus which was close to 10 times that of their respective loss modulus, demonstrating that the materials display elastic behavior (Figure 4.2.16 A - D). Alginate hydrogels show a plateaued tan delta response, indicating a relative inability to stiffen in response to
increasing strain rate and suggesting almost exclusively elastic behaviour (Figure. 4.2.16F). When comparing the compound modulus (E*) of each alginate, 2% Sigma alginate exhibited the highest value across all frequencies. Interestingly, both 1.5% Sigma and NovaMatrix alginate gels exhibited very similar compound modulus values and 1% NovaMatrix had the lowest (Figure. 4.2.16E; Group; \( F_{(2,112)} = 114, p < 0.0001 \) and Figure 4.2.16F; Group; \( F_{(2,90)} = 61, p < 0.0001 \)).

To determine whether alginate is a suitable material for neural transplantation, values were then compared to fresh tissue. The cortex was chosen for comparison to
biomaterials as cortex it is likely to represent the soft end of the brain mechanics scale, therefore, this was the most relevant stiffness range to be trying to match. (Figure 4.2.17). Stiffness (Compound modulus) values for 1% NovaMatrix alginate and cortex were very similar with 1.5% NovaMatrix being stiffer (Figure 4.2.17A; Group; F (20,110) = 2.4, p = 0.98). However, the viscoelastic response (Tan delta) to deformation at higher frequencies was notably different for both 1% and 1.5% NovaMatrix and cortex (4.2.17B; Group; F (20,110) = 17, p < 0.0001).

![Figure 4.2.17](image)

Figure 4.2.17. Matching the mechanical properties of 1% NovaMatrix alginate to specific brain regions to be used for regenerative medicine. (A) the similarity in compound modulus for 1% NovaMatrix and cortex and difference in 1.5% NovaMatrix; (B) the difference in the tan delta for both 1% and 1.5% NovaMatrix and cortex. All samples tested at 40 minutes post-mortem. Means ± SEM, N=6.
4.3 Discussion

4.3.1 Cell viability in alginate

Alginate was chosen as the main biomaterial component for cell encapsulation in this project because it is a natural material which crosslinks on contact with calcium chloride to form a hydrogel. It has been FDA approved for applications such as wound healing, and GMP grade materials are available. It can also be non-degradable which is relevant for the present application to enable long term implantation into the brain. For many of the initial experiments, Sigma alginate which contains a 1:4 G:M ratio and it was used because it could be obtained at low cost. For the further development of a more clinically relevant formulation, NovaMatrix alginate (PRONOVA SLM100) was used, which has a 60:40, G:M ratio, and can be supplied at GMP grade. As discussed previously (Chapter 1.3.1.1.1); high M ratio can lead to an increased macrophage response, therefore this alginate was chosen due to having lower M concentration. Initially, the ThinCert approach to generating alginate gels was used to create a simple in vitro model to look at cell survival and migration, based on previous literature (Gut et al., 2018; Nagaraju et al., 2019). SHSY5Y cells (a human neuroblastoma cell line) were used for material characterisation as a representative neuronal population of cells which are quick and easy to culture.

SH-SY5Y cells survived well in alginate gels regardless of the source of material or the formulation methods used. This is consistent with previous reports where SH-SY5Ys remained viable in bioprinted alginate gels in vitro for 5 days (Fantini et al., 2019). However, cells in the report by Fantini et al had a lower viability (<60%) than shown in this research which could be due to the bioprinting process used in their method. To the best of our knowledge this is the first reported data of extended viability (up to 14 days) of SH-SY5Y’s in alginate in vitro, although it has been used for ESC differentiation over 24 days (Li, Alexander E. Davidovich, et al., 2011).

Microbeads were then created following similar methodology to a previously published protocol (Hoesli et al., 2017). For microbead synthesis, alginate solution
can be either dropped in to CaCl\textsubscript{2} from a manually operated syringe where the flow rate is not controlled, or a syringe pump can be used to control the flow of liquid. For this project, analysis of gauge (G) size was first investigated with manual vs syringe pump synthesis, showing a significant difference in bead size produced depending on the method used (Figure 4.2.3A). The range in bead size was between 1.3-2.4mm from the differences in gauge needle sizes. Using the syringe pump produced more consistent smaller beads with less variability between experiments. For all further in vitro experiments 21G was taken forward as a reliable method for generating cellular microbeads, although these would be too large for injection (~2-2.5mm depending on alginate concentration) so the dimensions would need to be scaled down for future in vivo experiments. It has been shown that the ideal needle size for cell injection is >26G with 32G producing too high sheer stress on the cells in rodent models (Wahlberg et al., 2018). Clinically, 20G needles or 50µl Hamilton syringes (Mendez et al., 2005; Amer et al., 2017) are commonly used for intracerebral cell delivery which would correspond to a diameter of 0.6mm and 1.03mm respectively. Smaller needles are one future option for generating smaller microbeads for cell encapsulation which could be explored, but if that failed due to physical limitations then another approach might be to use thermosetting alginate. Thermoresponsive alginate could be injected as a cell suspension liquid that then sets in situ and forms hydrogel microbeads, therefore this could be explored for future translation of this as a therapy for PD (Liu et al., 2017).

The swelling rate of hydrogels is important to be assessed for transplantation in vivo, because this can increase degradation rate or cause bursting of the hydrogel and therefore tissue damage in the host. A litre of 0.9% saline (physiological) contains 154mM sodium ions compared to DMEM media which contains 109mM. Most increase in swelling is caused by osmosis and fluctuations in ion levels, which is why a greater increase was observed in all formulations in a saline solution compared to media. Previous research suggests hydrogels which promote neural survival are more likely to exhibit biomaterial swelling, which could be a reflection on the extracellular ion environment that is favourable to neurons (Kuo and Ma, 2008; Matyash et al., 2017).
2012). Alginate showed good potential as a biomaterial for CNS delivery as it exhibited some swelling but this was limited and gels remained stable.

To investigate methods to improve cell viability and survival post transplantation, PLO with or without the addition of HA as coating for the alginate microbeads was assessed. HA hydrogel-based models have been used for in vitro hiPSC-derived model tissues in previous literature such as studies of 3D migration and differentiation of NSCs (Zhang et al., 2016; Simò et al., 2017; Wu et al., 2017). PLO was chosen as a coating for the alginate beads for stabilisation and it has showed greater resistance to degradation and reduced immune response compared to PLL in an in vivo mouse model (Tam et al., 2011). Immune responses to coated and uncoated alginate materials will be explored further in the next Chapter. An additional benefit of coating the alginate is that previous studies have found a reduction in surface roughness of alginate microbeads when coated with PLL which increased mechanical stability (Bhujbal et al., 2014). Viability measured by live/dead cell quantification in alginate beads with and without coating was consistent, with higher cell densities (1 or 2.5 x 10^6 cells/ml) surviving significantly better than lower densities. Although, in all formulations and across all cell densities, viability was above 70%. This finding is in agreement with Dvir-Ginzberg et al., 2003, who also showed better survival in alginate gels when hepatocyte cells were encapsulated at a higher density. Further processing of materials can also improve their uses for regenerative medicine. For example, linking poly (N-isopropylacrylamide) (PNIPAM) on alginate makes it thermoresponsive, which could improve injectable delivery for cell therapy (Liu et al., 2017; Ciocoiu, Staikos and Vasile, 2018). Method of delivery is just one of the considerations for use of biomaterial delivery to the CNS, multiple factors have to be addressed for transplantation to such a complex tissue environment.

Having used SH-SY5Y cells to develop alginate encapsulation methodologies, the feasibility of encapsulating hiPSC-derived therapeutic cells was tested. Viability of hiPSCs in both formulations of alginate (Sigma and NovaMatrix) was quantified in the
first instance, however viability did not reach higher than 50% in either. To improve this, the addition of cell survival and attachment molecules to the media such as vitronectin, fibronectin and the rock inhibitor Y-27632 were tested. 500µM Y-27632 at cell densities of both 0.5 and 1 x 10⁶ cells/ml showed a significant improvement in survival compared to controls. This is supported by literature using up to 50µM Y-27632 for 12-36h improving survival and proliferation compared to controls. Furthermore, that work highlights that pre-treatment with Y-27632 reduces expression of pluripotency markers OCT4 and NANOG (Maldonado et al., 2016). hiPSCs have been shown to require RGD motifs for cell attachment, and these are not present in alginate which could be a reason why cell survival is so low (Adil et al., 2017; Hunt et al., 2017). However, to the best of our knowledge there is no reported viability data for the encapsulation of either pluripotent ESCs or iPSCs in alginate, with all previous studies exploring survival quantification post differentiation. In order to overcome the problem of poor viability when undifferentiated hiPSCs were encapsulated, the differentiated DA neural progenitors (day 16 NP-hiPSCs) were tested. This option was also based on 16 days into differentiation being the time-point for transplantation/freezing of progenitors specified in the published protocol (Nolbrant et al., 2017). NP-hiPSCs at day 16 encapsulated in alginate of both formulations, in ThinCert gels and microbeads with and without coatings all showed much better viability than the undifferentiated hiPSCs. This is in agreement with previous iPSC and ESC studies where alginate is used as a platform for differentiation for over 20 days in vitro and fibroblasts delivered in 2% alginate were shown to be viable for 150 days (Banerjee et al., 2009; Li, Alexander E. Davidovich, et al., 2011; Hunt et al., 2013; Kim, Sachdev and Sidhu, 2013; Gu et al., 2017).

4.3.2 Physical properties of alginate

For material transplantation it is essential that nutrients and oxygen can diffuse through the material to the encapsulated cells to ensure their survival. For the purpose of this project the aim is for the therapeutic cells to stay within the biomaterial, but blood vessels surrounding the material being able to provide required molecules is a necessity. To look at this these aspects, the migration of
neurons and endothelial cells through alginate gels was investigated, along with the permeability of alginate gels to diffusion of molecules of different molecular weights.

The cell migration assay, modified from Cell and Boyden, 2005, was performed using the neural cell line SH-SY5Y and the human endothelial cell line HUVECs. SH-SY5Ys showed no migration when seeded on the top of the gel or the bottom of the plate and <5% when seeded inside the gel, compared to HUVECs which showed over 30% migration in all conditions. A co-culture of HUVECs and SH-SY5Y was looked at to see how whether different cell interaction increased migration. For all conditions the co-culture showed an increase in migration compared to just either cell type alone. This highlights that the DA-hiPSCs are not expected to migrate outside of the alginate gel, but that endothelial cells and therefore blood vessels might have some capacity to penetrate. Previous studies with islet cell implants for diabetes therapy, contradict these results as the vasculature has been shown to surround the material and not penetrate alginate (Omer et al., 2005; Vériter et al., 2010; Farina et al., 2018). Although, these studies support the preferential exchange of soluble factors and no islet cell movement out of the material. This has not been explored for the combination of biomaterials and therefore, it would be interesting to investigate whether the PLO and PLO + HA coatings might alter this behaviour. Furthermore, this is a 3D model, so it would be of interest to look at this in vivo, to explore how vasculature formation occurs in both coated alginate and alginate alone.

Migration into an implanted cellular material of some cell types, especially host immune cells such as B and T cells, could be detrimental to the survival of allogeneic therapeutic cells. Furthermore, nutrient and oxygen diffusion would be essential for survival of the implanted cells, and diffusion out of dopamine and beneficial growth factors is a key functionality. Therefore, an important consideration in the development of this cell encapsulation technology is the permeability of the material, which should ideally permit free diffusion of soluble factors, nutrients, dissolved gases, growth factors and neurotransmitters, but block invasion of cells and host antibodies.
Permeability was explored through a FITC-dextran release assay. For both the 1.5% and 2% alginate 100% of the low molecular weight (40kDa) dextran was able to diffuse through the alginate hydrogels, including the ones coated with PLO and PLO/HA. In comparison the larger dextran (150kDa) showed <50% release over 168h for all conditions, with the 1.5% alginate alone showing the fastest release and the PLO/HA 2% alginate showing the slowest release. This highlights that as the concentration of polymer increases the diffusion of molecules decreases. This is supported by Wang et al 2009 who looked at insulin release from encapsulated ESC derived islet cells in alginate, where increase in alginate concentration decreased insulin secretion. The low molecular weight dextran did show slower release than that represented in previous studies, but this could be because in this study the gels were not mechanically agitated (Tate et al., 2009; Wang et al., 2009). Dopamine is 153 g/mol (153Da), so therefore is small enough to diffuse out of the alginate (PubChem). The 150kDa dextran was looked at because it is a similar size to many antibodies (Vidarsson, Dekkers and Rispens, 2014), and therefore this restrictive release in all alginate formulations indicates that antibodies are unlikely to be able to readily diffuse through them.

4.3.3 Mechanical properties of alginate

4.3.3.1 Alginate shows decreased gelation with increased concentration
To assess how the different alginate concentrations and formulations gel, rheometry was used under constant sheer stress of 1hz. For Sigma alginate, the higher concentration (2%) resulted in quicker gelation time by 8 minutes compared to 1.5% Sigma alginate. In comparison, NovaMatrix at 1.5% was 5 minutes slower than the equivalent Sigma concentration. 2% NovaMatrix is too viscous to use and 1% could not appropriately set to be measured using the rheometer. This is a useful technique for measuring gelation of hydrogels, and gelation time is similar in the literature for 2% alginate (~50 minutes) but 1.5% alginate in the literature shows ~20 minute gelation time (Kuo and Ma, 2001).
4.3.3.2 Specific brain regions show distinctive stiffness differences

DMA was used to measure the mechanical properties of cortical tissue for predominantly grey matter, and corpus callosum tissue for white matter (Büchel et al., 2004; Fitsiori et al., 2011). Previously brain tissue has been thought to be particularly soft with literature showing it has an elastic modulus of 0.5-1 kPa using rheometry with a strain rate of 2% (Gefen and Margulies, 2004; Georges et al., 2006). Neural cells in particular are sensitive to changes in mechanical stiffness, which influences their survival and that is why biomaterial matching for these properties is particularly important for the CNS (Hynes et al., 2008). Interestingly, the corpus callosum (white matter tissue) exhibited a stiffness modulus that increased more steeply in proportion to strain rate. Furthermore, corpus callosum tissue exhibited a more elastic response at strain rates >50hz. Bulk mechanical properties of brain tissue are hard to compare within the literature due to variances in species, time delay post mortem and tissue storage medium prior to analysis. Previous reports have found a 34% difference in stiffness between white and grey matter (Budday et al., 2015). Furthermore, due to differences in methodologies, this highlights discrepancies in measurements such as how using AFM shows higher stiffness values for grey matter (Koser et al., 2015). AFM is a well-used technique for mechanical analysis, but it just measures the tissue surface and not the total bulk properties, therefore here a technique was developed using DMA to analyse brain stiffness.

4.3.3.3 Alginate shows viscoelastic behaviour and increased concentration increases stiffness

The same compressive DMA protocol used for brain analysis was then used to quantify the different alginate hydrogels. As expected, the highest concentration (2%) Sigma alginate showed the top stiffness values with a compound modulus between 30-40kPa. Interestingly 1.5% for both NovaMatrix and Sigma had very similar stiffness values. The tan delta values for each highlight alginate has almost solely elastic behaviour. Tensile analyses from previous literature showed high-G alginates had sturdier properties compared to high-M alginates (Drury, Dennis and Mooney, 2004). The compressive analysis was done using a Bionix 100 MTS and was
similar to the 2% Sigma alginate described here. This is the first time DMA has been used to show bulk alginate material mechanical analysis under multiple frequencies. The DMA protocol was conducted at 23°C and not physiological 37 °C, and previous literature has shown this could increase the stiffness values by 10% (Ganachaud et al., 2013).

4.3.3.4 Mechanical matching of biomaterials to brain tissue to improve regenerative medicine

Mechanical matching of a biomaterial to the appropriate host tissue and cellular cargo is important for graft and cell survival (Moshayedi et al., 2014; Tam et al., 2014). Mechanical matching is likely to be important for the outcome of biomaterial tissue transplantation. From quantifying the bulk mechanical properties of white vs grey matter and comparing different formulations of alginate it is possible to compare both the stiffness and viscoelastic response of each biomaterial to CNS tissue. The only specific match was in the stiffness values (Compound modulus) of cortex and 1% NovaMatrix. Highlighting that although 1% alginate displayed comparable stiffness values to brain cortical tissue, the Tan Delta and therefore viscoelastic response to increasing frequency was very different. However, these frequencies where the materials deviate from the tissue are above normal physiological strain rate, so it is an interesting observation but may not be a problem in terms of stiffness matching.
4.4 Conclusion

In this Chapter, formulations and protocols were developed for generating alginate biomaterials that could be used to encapsulate therapeutic cells. This included the use of a GMP-grade material, in which therapeutically relevant DA-hiPSCs remained viable for an extended period in vitro. The influence on encapsulated cells of seeding density and chemical modification of the environment and coating of the alginate materials was characterised. Microbeads were generated and characterised in terms of size and stability, and the migration of therapeutic cells and endothelial cells, and of large and small molecules, was investigated. The final formulation resulted in using 1.5% NM alginate and explored the use of a PLO/HA coating. Finally, the stiffness environment of fresh rodent brain tissue was measured using DMA for the first time, and the same technique was applied to characterise the viscoelastic mechanical properties of alginate materials across a comprehensive range of strain rates. In conclusion, alginate microbead encapsulation technology has been advanced towards applications in CNS cell transplantation, along with increased understanding about host tissue biomechanics.
Chapter 5: Encapsulation and local drug release: effects on host cell responses

5.1 Introduction

5.1.1 Refining current cell therapies for PD

Cell encapsulation is not only about improving transplanted cell survival, but also allowing local delivery of growth factors and molecules produced by the therapeutic cells to the target area. This is particularly relevant for PD although most previous research has focussed on using transplanted cells to form synapses with host neurons. The success of hfVM grafts is supported by in vivo rodent model research, highlighting that synaptic connections between host and graft cells led to motor improvements (Björklund and Stenevi, 1979; Clarke et al., 1988). More recently studies using ESCs and iPSCs have indicated the benefit of host engraftment and integration in rodent and non-human primate PD models, respectively (Grealish et al., 2014; Kikuchi et al., 2017). The central hallmark of PD is the degeneration of dopamine producing cells, and it is this loss of dopamine which leads to the clinical motor symptoms. Therefore, if the requirement for PD is restoring local dopamine levels, encapsulating cells which produce dopamine could improve their survival post transplantation allowing them to act as a local dopamine source. Improving survival has been explored with the release of trophic factors to further protect therapeutic cells, such as GDNF, increasing cell survival in a rat model of PD (Wang et al., 2016; Moriarty et al., 2019). Collagen hydrogels encapsulating primary neural progenitors from rats and GDNF showed improved TH+ cell percentage survival and striatal innervation in a rat model of PD (Moriarty, Pandit and Dowd, 2017; Moriarty et al., 2019).

Over the past decade research into the cell secretome as a therapeutic for multiple diseases has had much interest for regenerative medicine. The secretome is defined as factors or molecules such as proteins, nucleic acids, growth factors, chemokines,
cytokines, lipids and extracellular vesicles released into the extracellular space from cells (Drago et al., 2013; Vizoso et al., 2017; Marques et al., 2018). Most literature has explored the secretome of MSCs with a view to development of a therapy where the cells are not required (Ranganath et al., 2012; Vizoso et al., 2017). For PD the secretome of MSCs was injected into a 6-OHDA rat model which increased the amount of dopaminergic neurons and improved motor symptoms (Teixeira et al., 2017). Therefore therapeutic cell secretome rather than just host cell connectivity and synapse formation shows promise for the CNS and neurodegenerative disease treatment (Drago et al., 2013; Tran and Damaser, 2015).

Biomaterials used to protect or support therapeutic cell delivery have been used across multiple diseases and medical disorders including retinal repair, cardiovascular implants, neurodegenerative diseases, targeting tumors in cancer, replacing islet cells in diabetes, encouraging wound regeneration, and implants for kidney failure (summarised in Figure 5.1.1). Arguably the most advanced of these is diabetes, with 8 current clinical trials in Phase I or II encapsulating islet cells for type I diabetes (Farina et al., 2018). For neurodegenerative diseases, NsGene have developed clinical implants for both PD and Alzheimer’s disease (AD), encapsulating therapeutic cells (Wahlberg et al., 2012; Farina et al., 2018). An implantable polymer made of poly (acrylonitrile-co-vinyl-chloride) for AD releasing NGF from a human cell line showed a reduction in the progression of the disease in patients (Wahlberg et al., 2012; Eyjolfsdottir et al., 2016). For PD a polysulfone implant which protects GDNF producing baby hamster kidney cells was shown to be promising over a year after transplantation into the putamen of rodents and using Human ARPE-19 cells is now in Phase II trials sponsored by the Michael J Fox foundation (Lindvall and Wahlberg, 2008; Emerich et al., 2014, 2019; Farina et al., 2018). Overall biomaterials encapsulating therapeutic cells show great promise for CNS diseases.
Figure 5.1.1. Schematic highlighting the benefit of encapsulating cells for multiple disorders, diseases and replacement of whole tissues or organs (Farina et al., 2018).
5.1.2 Immunosuppression for cell therapy

Another benefit of encapsulating therapeutic cells is protecting them from host immune cells. Currently cell therapies in the clinic use systemic immunosuppression for a minimum of 6 months following cell transplantation (Barker et al., 2017). Clinical trials in PD cell therapy have ranged from using combinations of immunosuppressants such as Ciclosporin A (CicsA), Azathioprine, Basiliximab and Tacrolimus (FK506) systemically (Barker et al., 2017, 2019). However systemic immunosuppression has multiple issues and, in some cases, serious side effects including hypertension, nausea, severe diarrhoea, constipation, diabetes and neurological problems like migraines and tremors (Bamoulid et al., 2015). Furthermore, in clinical trials for transplantation into the striatum of PD patients, immunological rejection of hfVM grafts has been prevented using systemic immunosuppressive treatment. Clinical trials to date have revealed the greatest benefits are from when patients received immunosuppression for up to a year following transplantation, with the smallest clinical improvement seen where no immunosuppression was provided (Sundberg et al., 2013).

5.1.2.1 Tacrolimus (FK506)

The fungus Streptomyces tsukubaensis produces FK506 (Figure 5.1.2) which is a FDA and EMA approved macrolide lactone used as an immunosuppressant, utilised in clinical practice for prevention of allograft rejection in solid organ, kidney and liver, transplantation (Shin et al., 2010). FK506 binds to immunophilin FK506 binding protein (FKBP-12) which creates a FK506-FKBP-12 complex which then selectively inhibits calcineurin phosphatase which subsequently blocks the activation of T lymphocytes. T cells are essential to the upregulation of the expression of cytokines involved in the immune response such as; the interleukins (IL-2, IL-3, IL-4), tumour necrosis factor alpha (TNF-α) and interferon-γ (De Oliveira et al., 2018). FK506 causes the prevention of the proliferation, maturation and differentiation of T cells (Rahman, Zidan and Khan, 2013). FK506 is a Class II drug in the Biopharmaceutical Classification System being lipophilic, insoluble in water and highly soluble in organic
solvents such as ethanol and DMSO, which can then represent a challenge in formulation strategies (Rahman, Zidan and Khan, 2013).

Figure 5.1.2. Chemical structure of tacrolimus (FK506).

FK506 is used topically for inflammatory skin disease, and given orally and by intravenous infusion to prevent graft rejection (Rahman, Zidan and Khan, 2013; Gabriel et al., 2016). Although effective as an immunosuppressant and widely used still today, FK506 is associated with multiple side effects such as toxicity in certain tissues and also patient to patient variability (Hesselink et al., 2014; Sikma et al., 2015). In the CNS, neurotoxic complications have included hallucinations, postural tremor and speech disorders (Konofaos and Terzis, 2013). However, these complications are all associated with systemic delivery of FK506 due to high dosage and its ability to bind multiple receptors in humans in different organs and tissues which can lead to off-target toxicity. The lipophilicity of tacrolimus means that accumulation in fatty tissues throughout the body also occurs and this can lead to neurotoxicity, nephrotoxicity, hyperkalaemia, hypertension and myocardial hypertrophy and subtherapeutic levels could lead to immune rejection (Van der Merwe et al., 2017).
5.1.2.2 Overcoming issues of toxicity

To improve allogeneic cell delivery and ensure engraftment is long-term, immune evasion by immunosuppression or other means is essential. One option for this is to provide local immunosuppression, due to many of the toxic effects being caused by high systemic dose. In the CNS, FK506 has been shown to elicit both neuroprotective and neurotrophic effects independent of its immunosuppressant ability, enhancing nerve regeneration through calcium modulation (Konofaos and Terzis, 2013; Van der Merwe et al., 2017). Furthermore, FK506 is shown to reduce neutrophil and macrophage activation and glial cell activation (Hailer, 2008; Van der Merwe et al., 2017). Therefore, local delivery of FK506 should not only deliver a dose specifically to the target site but also potentially provide neuroprotective benefits. To provide local immunosuppression, biomaterials can be used to encapsulate and release the drug controllably in the specific area. This has already been looked at for CicsA in a rat model of stroke, with a hyaluronan and methylcellulose hydrogel releasing CicsA for local immunosuppression, increasing endogenous neural stem cells and significantly reducing stroke injury volume (Tuladhar, Morshead and Shoichet, 2015).

5.1.3 Local delivery of small molecules for the CNS

Delivering small molecules to the CNS is a challenge but one which is significantly researched due to many oral and intravenous therapeutics not being able to cross the blood-brain-barrier (BBB). Materials which are implanted generally need to provide appropriate release kinetics to release their therapeutic molecule over time into the target tissue. A successful example of this which is used clinically is GLIADEL® used for metastatic brain cancer (Westphal et al. 2003; Bregy et al. 2013; Perry 2007). GLIADEL® wafers are implanted directly to the resection cavity to prevent brain tumours metastasising (Perry, 2007; Martinez-Ramos et al., 2012). They are made of polyanhydride a biodegradable polymer loaded with carmustine and deliver locally (each wafer containing 7.7mg carmustine) for up to 30 days after surgery to help reduce the risk of tumour reoccurrence (Martinez-Ramos et al., 2012). Small molecules and growth factors have also shown sustained release from multiple hydrogel formulations (Carballo-Molina and Velasco, 2015). For sustained release,
zero-order controlled release is more preferable for predictable release kinetics of therapeutic molecules, this is defined as where the drug release rate is constant over a period of time (Y. N. Zhao et al., 2017). Previous work has shown zero-order drug release following incorporation of bevacizumab in core/shell electrospun poly-ε-caprolactone (PCL) nanofibers for delivery in the eye (Angkawinitwong et al., 2017). An in vitro model of PLGA microparticles releasing platelet derived growth factor show that burst then slow release stimulated cell survival and proliferation of oligodendrocyte precursors (Pinezich et al., 2018). For spinal cord injury, a combinational hydrogel of PLGA-HA and methylcellulose showed sustained release of BDNF in a rodent model (Khaing et al., 2016). Furthermore a promising strategy for PD uses decellularised brain tissue to create a hydrogel for the release of basic fibroblast growth factor, which demonstrated sustained neuroprotection in rats (Lin et al., 2017). Due to the nature and complexity of delivering small molecules directly into the CNS, it is logical to assume that the smaller the delivery vehicle, the lower the risk of damage due to physical disruption, therefore nanoparticles are of particular interest for CNS drug delivery.

5.1.3.1 Nanoparticles for drug delivery to the CNS

Nanoparticles are promising vehicles for CNS drug delivery due to their ability to cross the BBB and also when locally administered they are able to reach and interact with neuronal cells. PLA, PLGA, PCL, chitosan and gelatin are the most extensively used polymeric nanoparticles in drug delivery systems (Kumari, Yadav and Yadav, 2010; Seeram Ramakrishna, Zamani and Molamma P Prabhakaran, 2013). Nanoparticles made of PLGA used to deliver nicotine to the CNS of PD mice show improved neuroprotection and increased TH mRNA levels (Tiwari et al., 2013). PLGA is the most commonly used polymer in electrospraying, where a high current is used to disperse a polymer solution to create micro- or nano-sized particles (technique described in more detail in Chapter 1.6) (Bock et al., 2011). PCL nanoparticles have been used for intranasal delivery of NAPVSIPQ (NAP) a neuroprotective drug for AD, illustrating transfer to the rat cerebrum resulted in memory improvements (Liu, Jiang, et al., 2013). The size of nanoparticles is key for therapeutic purposes, particles
smaller than 100nm can cross the BBB but if they are less than 5nm they are secreted by cells and size also effects cellular uptake (Chithrani, Ghazani and Chan, 2006; Kim et al., 2009; Duan and Li, 2013; Jo et al., 2015).
5.1.4 Aims for this study

Cell therapy has looked increasingly promising for PD, however there is an issue of low cell survival and one of the factors which causes this is the host immune response to foreign cells. Dopamine releasing cells have been successfully generated and encapsulated in hydrogels in the previous Chapters and an outstanding issue is the immune response to allogeneic cells, therefore the aim for this work is to address this in a way that avoids systemic immunosuppression. Current research has looked at encapsulating cells in a biomaterial to create a barrier between the implanted cells and the host immune cells (Moriarty and Dowd, 2018). In Chapter 3, the original hypothesis, was that the implanted cells needed to release soluble factors, including DA, which would have a beneficial effect on host cells. Therefore, one of the aims here is to build on this previous work from Chapter 3, which looked at cytokine and DA release. In order to understand whether the secretome can exert neuroprotective effects, and also whether DA is still released when cells are encapsulated in alginate.

After ESC and hiPSC transplantation, immunosuppression is required for a minimum of 6 months to see a clinical benefit and ensure no tissue rejection (Barker et al., 2017). However, systemic immunosuppression can lead to toxic and unwanted side effects for patients. This study looks at improving current encapsulation techniques by using alginate as a hydrogel to encapsulate the cells and then a second layer of HA/PLO to release the immunosuppressant FK506 in a sustained and controlled manner. This led to 4 individual aims, shown below.

Specific aims:

- Investigate functional effects of the secretome of DA-hiPSCs on neurons subjected to toxic insult
- Measure DA release from encapsulated DA-hiPSCs
- Characterise the effects of FK506 on relevant host cells (neurons, glia, T-cells)
- Develop and characterise local FK506 delivery systems using synthetic polymer particles
5.2 Results

5.2.1 Functional effects of DA-hiPSC secretome

To look at whether the secretome of the DA-hiPSCs could promote regeneration or reduce neurodegeneration, an MPP+ (1-methyl-4-phenylpyridinium) neurotoxicity assay was set up using SH-SY5Y cells. Salvianolic acid B (SAB) has previously shown to be neuroprotective in cell culture neurotoxicity studies and so was used as a positive control (O’Rourke et al., 2017). Cells were cultured in 24 well plates (full description in Chapter 2.2.2.5) for 48h, to allow neurite extension, then either SAB was added for 30 minutes before addition of MPP+, or MPP+ was added alone. After 24h MPP+ was removed and either fresh SH-SY5Y media or DA-hiPSC conditioned media was added for 48h. Cells were fixed after 48h and neurite outgrowth quantified by immunofluorescence (Figure 5.2.1A), where neurite length was measured using Image J software. Data illustrate that MPP+ causes significant degeneration, decreasing mean neurite length by ~ 57% compared to the control (Figure 5.2.1B; Group; $F_{(5,30)} = 31, p < 0.0001$). This is prevented by the neuroprotective agent SAB prior to treatment with MPP+. Significantly, DA-hiPSC conditioned media increased neurite length by 15% compared to control and then, following MPP+ treatment, increased neurite length to $64.7 \pm 5.3 \mu m$ compared to $25.9 \pm 2.4 \mu m$ with MPP+ alone.

A key aim of encapsulating DA-hiPSCs and transplanting them for PD is to provide dopamine to the striatum to help counteract the loss of dopaminergic neurons caused by the disease. To look at DA levels released from alginate-encapsulated 35 day differentiated DA-hiPSCs, 1ml media was collected after 48 hours exposure to 6 cellular beads with $1 \times 10^6$ cells/ml. DA concentration was measured using ELISA and the result is shown alongside the DA release data obtained from the monolayer cell cultures (shown previously in Chapter 3, Figure 3.2.11). Encapsulated cells showed a significantly lower release of dopamine compared to non-encapsulated ($143.3 \pm 7.3 pg/ml$ and $216.7 \pm 31.7 pg/ml$, respectively), with equivalent cell number in each condition (Figure 5.2.2; Group; $F_{(4,10)} = 69, p < 0.0001$).
Figure 5.2.1. Neuroprotective effects of conditioned media from DA-hiPSCs on SH-SY5Y cells. SH-SY5Y cells extended neurites for 48h and were then treated with MPP+ to induce toxicity, DA-hiPSC conditioned media, or control media for 24h. In one group, SAB, a known neuroprotective agent, was included prior to and during the MPP+ treatment as a positive control. After 24h media was replaced with control SH-SY5Y media or day 35 differentiated DA-hiPSC-conditioned media for a further 48h then immunostained with β-III-Tubulin (green) and DAPI (blue) (A) and neurite growth quantified (B). Mean neurite length per neuron was measured using ImageJ. N=6, Means ± SD, One-way ANOVA with Dunnett’s multiple comparison test where *p<0.05, **p<0.01, ***p<0.001 compared to untreated control. Scale bars are 100µm.
5.2.2 The local effect of FK506 on neurons, glial cells and T cells.

To investigate local immunosuppression FK506 was chosen due to its use in current PD clinical trials (Barker et al., 2017; Takahashi, 2017). First an in vitro dose response of FK506 was investigated on SH-SY5Y neuronal cells. Concentrations between 10ng/ml and 10,000ng/ml were added to the culture media for 48h followed by fixation in 4% PFA and stained for β-III-Tubulin to measure neurite growth (Figure 5.2.3; Group; $F(7,16) = 8, p = 0.0003$). No concentration had a negative effect on neurite growth and, interestingly, every concentration significantly improved neurite length compared to the control (0ng/ml FK506).

The effect of 72h exposure to FK506 was then explored for primary rat microglial cells. Microglia were plated on poly-D-lysine (PDL) plates and allowed to attach for 24h before addition of FK506. Only $\sim 38\%$ of the control cells were shown to be live after the 72h assay period (Figure 5.2.4; Group; $F_{(1,4)} = 19, p = 0.0114$). However, the addition of FK506 increased cell viability at all concentrations tested, with 1000ng/ml having the largest effect increasing the percentage of live cells by $\sim 46\%$.
Figure 5.2.3. Dose response of the effect of FK506 on neurite length in SH-SY5Y cells. SH-SY5Ys were allowed to attach before treatment with 0, 10, 30, 100, 300, 1000, 3000 or 10,000 ng/ml FK506 for 48h. Quantification of neurite length was carried out by immunofluorescence and neurite length was measured using Image J software. Data are expressed as mean ± SD (n=3). One-way ANOVA with Dunnett’s multiple comparison test where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared to 0ng/ml control, line of best fit is a non-linear fit using Richard’s five parameter dose response curve (Giraldo et al., 2002).

Figure 5.2.4. A dose response of FK506 increases survival of primary rat microglia. Primary rat microglia were allowed to attach for 24h before treatment with 0, 10, 30, 100, 300, 1000, 3000 or 10,000ng/ml FK506 for 72h. Quantification of the percentage of live cells was done using syto-21 compared to total cells counted by inverted florescence microscope. Data are expressed as mean ± SD (n=3), One-way ANOVA with Dunnett’s multiple comparison test where *p<0.05 and **p<0.01, compared to 0ng/ml control, line of best fit is a non-linear fit using Richard’s five parameter dose response curve (Giraldo et al., 2002).
To investigate the local effect of FK506 on astrocytes, a 3D assay was set up according to a previously established protocol (East et al., 2013). Astrocyte reactivity can’t be accurately quantified in 2D cultures due to the stiffness of tissue culture plastic causing them to become reactive. Therefore, primary rat astrocytes were cultured in 500µl collagen gels. After 24 hours, 10ng/ml TGF-β was added for 10 days to stimulate astrocyte reactivity as a positive control (Figure 5.2.5 and Figure 5.2.6A).

**Figure 5.2.5. Astrocyte reactivity assay in collagen gels.** Primary rat astrocytes were cultured in 500µl collagen gels after 24 hours 10ng/ml TGF-β was added for 10 days to stimulate astrocyte reactivity. After 10 days gels were fixed and stained for GFAP (green) and DAPI (pink) then analysed by 3D confocal microscopy (Zeiss Axio observer 11). The edge effect of GFAP+ astrocytes in the control is shown by the orange arrows. Micrographs of TGF- β treated cells show characteristic hypertrophied ramified GFAP+ reactive astrocytes (red arrows) compared to control (non-reactive shown in blue arrows).
Figure 5.2.6: Astrocyte response to alginate with or without cells or FK506. Primary rat astrocytes were cultured in 500µl collagen gels. After 24 hours, 10ng/ml TGF-β was added for 10 days to stimulate astrocyte reactivity as a positive control (A). To test the effect of biomaterials on astrocyte reactivity, alginate beads alone (sigma and NovaMatrix (NM) or coated with HA/PLO) were placed on top of astrocyte collagen gels (B) or hiPSC 16 day differentiated cells were seeded on top of the collagen gel at a density of 1 x 10^6 cells/ml with or without 100ng/ml FK506 and encapsulated alginate (NM and HLA/PLO NM) containing 1 x 10^6 cells/ml DA-hiPSC day 16 were put on top of collagen gels (10 for 24 well-plate) (C). After 10 days gels were fixed for 24 hours in 4% PFA at 4°C and stained for GFAP (green) and DAPI (pink) then analysed by 3D confocal microscopy (Zeiss Axio observer z1). N=3-6, with 3 z stacks per condition, Means ± SD, Two-tailed T-test comparing control to TGF-β and One-way ANOVA with Tukey’s multiple comparison test comparing everything for B and comparing to DA-hiPSC for C, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.
To see how astrocytes responded to NP-hiPSC, 16 day differentiated cells were seeded on top of the collagen gel at a density of $1 \times 10^6$ cells/ml. Then to see if immunomodulation could reduce any astrocyte response, $100\text{ng/ml FK506}$ with NP-hiPSC day 16 cells was added. To test the effect of encapsulation on astrocyte reactivity, Sigma and NovaMatrix (NM) alginate beads containing $1 \times 10^6$ cells/ml NP-hiPSC day 16 with or without HA/PLO coating were cultured on top of collagen gels (10 beads per 500ul collagen gel). After 10 days, gels were fixed for 24 hours in 4% PFA at 4°C and immunostained for GFAP and DAPI then analysed by 3D confocal microscopy (Zeiss Axio Observer Z1) (Figure 5.2.6).

Addition of TGF-$\beta$ significantly increased GFAP+ area (Figure 5.2.6A; Group; $F_{(1,5)} = 123$, $p < 0.0001$). Sigma alginate also significantly increased the percentage of reactive astrocytes compared to the control GFAP cells, by 5-fold (Figure 5.2.6 B; Group; $F_{(3,12)} = 15.82$, $p = 0.0002$). Sigma alginate caused a significantly more reactive astrocyte response than NM alginate and HA/PLO coated NM alginate, by 2-fold and 3-fold respectively. NM alginate and HA/PLO coated alginate had no significant difference between them in terms of their effect on astrocyte reactivity according to GFAP+ area.

DA-hiPSCs alone seeded on top of the collagen gels, increased astrocyte reactivity by 4.5-fold compared to control (Figure 5.2.6 C; Group; $F_{(3,8)} = 19$, $p = 0.0005$). The addition of $100\text{ng/ml FK506}$, reduced GFAP+ area compared to DA-hiPSCs alone by 3-fold, to a level close to that of the control. Furthermore, encapsulation of DA-hiPSC inside NM alginate reduced the percentage area GFAP+ cells compared to DA-hiPSCs alone by 1.8-fold, showing that encapsulated cells produce less of an astrocyte response than non-encapsulated cells. Incorporation of the PLO/HA coating on NM alginate also reduced astrocyte reactivity in terms of the percentage area occupied by GFAP+ cells by 2.7-fold compared to DA-hiPSCs alone. So, encapsulation and FK506 both reduce the response astrocytes have to DA-hiPSCs.
To look at the effect of alginate on primary rat T cell proliferation with and without the addition of FK506 an assay was established using flow cytometry. 1x 10^6 cells/ml were stained with the intracellular fluorescent dye CellTrace™ carboxyfluorescein diacetate succinimidyl ester (CFSE). Then NM alginate beads, PLO/HA coated alginate beads, 100ng/ml FK506 in the media or 100ng/ml FK506 encapsulated in alginate were added to wells containing T cells, or cells were activated with phorbol 12-myristate 13-acetate (PMA)/Ionomycin. Flow cytometry for CFSE staining showed that alginate beads alone resulted in 100% T cell proliferation compared to the non-proliferating control. T-cell proliferation in response to alginate beads in vitro was reduced by PLO coating (39.7 ± 1.7% vs control 34.1 ± 2.9%) (Figure 5.2.7; Group; F (1,20) = 3, p = 0.0010). The addition of FK506 either in free form in the culture media or encapsulated in the coating of the beads limited T-cell proliferation more efficiently (13.3 ± 1.2% and 20.4 ± 3.1 % respectively).

**Figure 5.2.7. T cell proliferation assay for release of FK506 from alginate beads.** Primary rat CD4+ T cells were isolated from the spleen and lymph nodes, then stained with CellTrace™ CFSE and plated at 1 x 10^6 cells/ml. Cells were then activated with activation antibody (phorbol 12-myristate 13-acetate (PMA)/Ionomycin) for 6 hours then media (activated control), alginate beads alone, PLO coated alginate, 100ng/ml FK506 in the media or FK506 encapsulated in alginate were added to the wells containing T cells. After 4 days proliferation was analysed by Flow Cytometry and the percentage of proliferation compared to the non-proliferating control was measured. N=3, with 3 repeats per condition, Means ± SD, One-way ANOVA with Dunnett’s multiple comparison test where **p<0.01, all conditions compared to the activated control.
5.2.3 Encapsulating immunosuppressant FK506 in PLGA nanoparticles

As the data in this Chapter show, the addition of FK506 in vitro increases neurite outgrowth of SH-SY5Ys and can and can potentially modulate the response of host glial cells and T-cells in a positive manner. Therefore, a method was developed for local delivery by creating FK506 nanoparticles to suspend in the alginate hydrogel containing therapeutic cells (DA-hiPSCs). PLGA was chosen as the first polymer to explore for nanoparticle formation using an oil to water emulsion solvent evaporation method (Elkharraz et al., 2006). A ratio of 1:5 w:w, FK506 to PLGA was used and SEM was used to look at particle size and morphology (Figure 5.2.8). The mean particle size was 485 ± 190nm with a range of 301-966nm. Encapsulation efficiency of the NPs was 5.3% and total amount of tacrolimus loaded was 1.75 μg/mg of nanoparticles.

Figure 5.2.8. SEM images of PLGA nanoparticles. PLGA nanoparticles (A, B) and FK506 encapsulated PLGA nanoparticles (C). The frequency distribution of particle size (D) taken from images B and C (25 nanoparticles), quantified using Image J.
To measure release of FK506 from nanoparticles, DPBS was used as the release medium and the temperature was set at 37 °C. Following removal of insoluble solid NPs by centrifugation (8000 x g, 5 min), the remaining clear solution was analysed by UV–Visible spectroscopy at 195 nm. Release from NPs alone and from NPs encapsulated within the outside PLO/HA layer of alginate beads was measured over 21 days (Figure 5.2.9). NPs alone displayed a faster release profile, with nearly 100% release by 21 days compared to those encapsulated in microbeads (<40% in the same time period).

Figure 5.2.9. Release of FK506 from PLGA nanoparticles and nanoparticles encapsulated in alginate beads. FK506 release from encapsulated PLGA nanoparticles alone or embedded in the outside layer of alginate HA/PLO beads quantified by UV spectrophotometry as cumulative release over 23 days. Data are expressed as mean ± SD (n=3) with 3 repeats per condition.

5.2.4 Encapsulating immunosuppressant FK506 in PCL electrospayed microparticles

Following FK506 nanoparticle formulation using PLGA, a method to increase encapsulation efficiency and prolong release was explored. Electrospaying using 45kDa PCL was chosen for optimisation, as previous data has shown optimised first order drug release with PCL electrospinning (Angkawinitwong et al., 2017). The principles of coaxial electrospraying are discussed in detail in Chapter 1.6.2. For initial experiments, the apparatus was set up as shown in Figure 5.2.10, where a 10% w/v PCL solution was used for the shell and the core contained 1mg/ml FK506 in
ethanol with a flow rate of 1ml/h: 0/1ml/h, respectively. The voltage was kept constant at 20kV, with the collector at a 15cm distance from the Taylor cone and particles were sprayed onto a 10% w/v PVP film on foil and collected by scraping particles. These parameters were used as the standard set up, and initial experiments showed the mean diameter of the microparticles are 0.5-9μm (Figure 5.2.11). The microparticles were smooth in morphology, but significant aggregation and polydispersity of particle size is seen which is reflected in the SEM images (Figure 5.2.11).

Figure 5.2.10. Schematic diagram showing the electrospaying process. PCL core-shell microparticles were sprayed at a flow rate of 1ml/h:0.1ml/h, Shell:Core. The voltage was kept constant at 20kV, with the collector at a 15cm distance from the Taylor cone. Microparticles were sprayed onto a 10% w/v PVP film on foil and collected by scraping particles.
Batch-to-batch reproducibility of the microparticles was assessed as this can affect the potential to up-scale and translate into the clinic. This yielded a range of different size microparticles as shown in histograms in Figure 5.2.11. Particle morphology is important as it can affect the degradation properties of the polymer matrix, determining the release kinetics of the loaded therapeutic molecule component (Bock et al., 2011). To test whether aggregation of the microparticles could be altered after spraying they were sonicated for 10 minutes at 37 °C. The morphology of the microparticles after sonication was investigated using SEM. Open pores are observed in the shell of the sonicated microparticles (Figure 5.2.12).

Figure 5.2.11. SEM images to determine the size range of two batches of PCL-FK506 loaded core-shell microparticles. A) Batch 1 SEM of FK506 microparticles and (B) a histogram showing the size range of batch 1 (C), Batch 2 SEM of FK506 microparticles and (D) a histogram showing the size range of batch 2.
To characterise the physical properties of the FK506 PCL microparticles, thermogravimetric (TGA), X-ray powder diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) analysis were performed. The TGA analyses of raw PCL and FK506 and electrosprayed PCL-blank and PCL-FK506 core shell batch 1 & 2 microparticles showed high thermal stability (Figure 5.2.13A). FK506 is more sensitive to heat, with the TGA trace showing rapid weight loss of 87.4% from 225°C to 400 °C. The raw PCL and PCL-blank microparticles show a weight loss of 21.7% from 328°C to 400°C and 43.4% from 215°C to 400°C respectively. The lower degradation temperature of the ES PCL-blank microparticles compared with raw PCL could suggest the influence of TFE solvent on the PCL polymer matrix and the electrospraying process. The batch 1 & 2 PCL-FK506 microparticles present a similar weight loss pattern to the electrosprayed blank PCL. Batch 1 shows a weight loss of 42.9% from 239°C to 400°C and batch 2 shows a weight loss of 45.4% from 207°C to 400°C.
Figure 5.2.13. Characterisation of PCL-FK506 loaded core-shell microparticles. A) TGA thermograms of PCL and FK506 alone and blank electrosprayed microparticles (PCL-blank) and the PCL-FK506 core-shell microparticles. B) XRD patterns of PCL and FK506 alone, PCL-blank and the PCL-FK506 core-shell microparticles. C) FTIR spectra of PCL and FK506 alone, PCL-blank and the PCL-FK506 core-shell microparticles. D) DSC thermogram of PCL-blank and the two batches of PCL-FK506 core shell microparticles.

Figure 5.2.14. Characterisation of FK506 alone by DSC.
X-ray diffraction (XRD) was used to characterise the physical forms of raw FK506 and electrosprayed PCL-blank and PCL-FK506 batch 1 & 2, with the results shown in Figure 5.2.13B. FK506 is a crystalline material and the XRD pattern revealed characteristic peaks of the drug, the Bragg reflections, most prominent are the peaks between 10-20°. This indicates the high crystalline nature of the drug as reported in the literature (Pathak et al., 2016). The XRD pattern of electrosprayed PCL shows Bragg reflection peaks at 21° and 24° as reported in the literature, characteristic of the semi-crystalline nature of PCL (Cai et al., 2014). The PCL-FK506 microparticles batch 1 and 2 also showed higher in intensity peaks at 21° and 24° when compared to the electrosprayed PCL. The electrosprayed formulations of PCL-FK506 microparticles batch 1 and 2 showed no diffraction peaks from FK506 highlighting that FK506 inclusion does not affect the crystalline structure of PCL.

FTIR was used to examine the complex molecular structure interspersed within the microparticles (Figure 5.2.13C). Raw FK506 shows peaks at 1194cm⁻¹ and 1180cm⁻¹ showing the esteric C-O stretch, then at 1637cm⁻¹ showing the C=C stretch and finally at 3450cm⁻¹ showing O-H broad medium intensity stretch. The absorbance bands at 1738, 1728 and 1698 show resonating amidic C=O and ketonic C=O stretching vibrations and etheric C-O-C stretches are present at 1171 and 1084 cm⁻¹ (Pathak et al., 2016; Zidan, 2017). The FTIR spectra of electrosprayed PCL-blank microparticles shows typical absorption bands of PCL reported previously (Liao et al., 2015) with absorption bands at 2945 and 2867 showing CH₂ stretch, 1726 showing C=O stretch, 1238 and 1166 showing the C-O-C stretch. The PCL-FK506 microparticles do not show the typical absorption bands of FK506 but show the same spectra as PCL.

The DSC thermograms of raw materials, electrosprayed PCL-blank and PCL-FK506 microparticles are shown in Figure 5.2.13D & 5.2.14. The thermogram for FK506 alone (Figure 5.2.14) shows a clear broad endothermic peak 128°C which corresponds to the melting point of the drug and indicates that the drug is present in crystalline form as reported in the literature (Pathak et al., 2016). In contrast, PCL
shows a high endothermic peak at 57˚C (Figure 5.2.13D) which corresponds to its low melting point (Cai et al., 2014). The PCL-FK506 microparticle thermogram shows only characteristic peaks of PCL polymer at 57˚C and no endothermic transition due to tacrolimus. This could suggest the drug was dispersed or was present in the amorphous form within the microparticles. It appears from the DSC thermograms (Figure 5.2.13D) and XRD data (Figure 5.2.13B) that FK506 and PCL are compatible as shell and core working fluids, and FK506 is amorphously dispersed.

The drug release profile followed biphasic kinetics from the in vitro cumulative release plot shown in Figure 5.2.15A. In the first phase, after 24 hours, 25μg/ml is released showing burst release. In the second phase, a slower pseudo-steady-state with sustained release kinetics was observed. The microparticles were sonicated in a water bath prior to the in vitro drug release study being conducted because of the aggregated nature of the microparticles.

A linear fit of the tacrolimus cumulative release was constructed from 24 hours to 180 hours (Figure 5.2.15B) and the equation of the line was found to be $y=0.06425x + 22.06 \ (R^2=0.97814)$, where $y$ is the cumulative drug release and $x$ is the time in hours.
5.2.5 Encapsulating immunosuppressant FK506 in PCL electrosprayed nanoparticles

To optimise the particle size from the previously sprayed microparticles in section 5.2.4, parameters from the process were altered (Figure 5.2.16). These included the PCL solution, 45kDa PCL was still used but as a 5% w/v solution compared to 10% previously. The flow rate was also reduced as it was believed the lower flow would reduce particle size, the core was reduced to 0.01ml/h and the shell to 0.1ml/h. During optimisation when the voltage increased above 18kV, the stability of the Taylor cone was altered and therefore the kV was reduced from 20kV to 18kV for the following experiments. Due to previous aggregation of microparticles and difficulties in collecting the particles, the method was adapted so the nanoparticles were sprayed onto a 5% w/v PVP film on foil and collected by pipetting distilled H2O onto the foil membrane to wash the nanoparticles off.
Figure 5.2.16. Schematic diagram showing optimisation of the electrospraying process to make nanoparticles. PCL core-shell nanoparticles were sprayed at a flow rate of 0.1ml/h:0.01ml/h, Shell:Core. The voltage was kept constant at 18kV, with the collector at a distance of 18cm from the Taylor cone. Nanoparticles were sprayed onto a 5% w/v PVP film on foil and collected by pipetting distilled H2O on the foil membrane to wash the particles off. Nanoarticles were then freeze dried over 2 days.

Figure 5.2.17. SEM images of the electrosprayed PCL FK506 nanoparticles.
SEM images of initial nanoparticles can be seen in Figure 5.2.17, which either resulted in minimal particle formation (5.2.17A) or layers of nanoparticles forming due to length of time spraying being too long (1h+). To prevent this layering of nanoparticles but also ensure a large enough yield for release studies, spraying on each PVP film was kept to less than 30 minutes. Collected nanoparticles were then pooled together in solution and freeze dried over 2 days resulting in less aggregation and nanoparticles with a mean size of 82 ±39.98 nm (Figure 5.2.18).

Figure 5.2.18. FK506 core-shell nanoparticle SEM after freeze-drying and quantification of size range. SEM images with different magnification (A-C), histogram showing the size range from 3 images and 150 nanoparticles (D).
The drug release profile for the nanoparticles followed biphasic kinetics the same as the microparticles from the *in vitro* cumulative release plot shown in Figure 5.2.19A. In the first phase, after 24 hours, over 60% of FK506 is released showing burst release (Figure 5.2.19B). A linear fit of the tacrolimus cumulative release was constructed from 96 hours to 1500 hours (Figure 5.2.19C) and the equation of the line was found to be $y=0.01777x + 77.13$ ($R^2 = 0.9096$), where $y$ is the cumulative drug release and $x$ is the time in hours.

![Graphs showing drug release profile](image)

*Figure 5.2.19. FK506 core-shell nanoparticle release over 2 months.* Total cumulative release of FK506 from PCL nanoparticles over 2 months (A), 24-hour release (B) and linear release (red line) from 96h to 1344h (2 months). Data expressed as mean ± SD (error bars too small to see in some points) (n=2).
5.3 Discussion

5.3.1 Functional effects of DA-hiPSC secretome

The purpose of encapsulating the DA-hiPSCs is to act as a therapeutic for PD, by not only replacing the lost dopamine but also potentially providing regenerative properties to degenerating dopaminergic neurons. Therefore, the observation that DA-hiPSC conditioned media was both able to restore MPP+ treated neurite length to that of the untreated control, highlights its potential capacity to counteract neurodegeneration. SAB was used as the positive control due to its previously reported neuroprotective effects (Tian *et al.*, 2009; O’Rourke *et al.*, 2017). The DA-hiPSC media had a greater effect on both neurite length and number of neurites per field compared to SAB. Previous research using the secretome of MSCs injected into the striatum in a rat model of PD showed reversal of motor deficits back to that similar to the control and also improved degenerating neurons (Teixeira *et al.*, 2017).

In addition to the functional benefit of hiPSC secretome in the neurotoxicity assay, the ELISA confirmed that encapsulated day 35 DA-hiPSCs released dopamine, highlighting the potential for alginate-encapsulated differentiated hiPSCs to act as a dopamine depot option for PD. More exploration into the cell secretome of both NP-hiPSCs and DA-hiPSCs by proteomic analysis is required to fully understand which small molecules could be having a beneficial effect for PD. This also highlights that for cell therapy to be effective it is not always essential that the transplanted cells form synapses and connections with the host cells and that the secretome alone may be beneficial (Marques *et al.*, 2018). DA release from encapsulated DA-hiPSCs was measured and showed 357.5pg release from 6 beads in 48h. Considering 24g for 1 year minimal was calculated in Figure 1.2. One year with 6 beads is only 68.53ng, say 20 beads were transplanted this would be 0.00415mg, which is still significantly less than 24g. However clinical symptoms are not shown in PD until >80% of dopaminergic neuron cell death, therefore any improvement in dopamine production could reduce this. Furthermore, future studies could look at increasing cell density and whether this increases dopamine production in a linear way. In
primate studies using hiPSC for PD $4.8 \times 10^6$ cells per animal were used and saw survival of $4,300 \sim 13,000$ TH+ cells with dopamine influx calculated by $[^{18}\text{F}]$DOPA-PET recovering to 48% of the levels of normal primates (Kikuchi et al., 2017). To put this in terms of humans, around 16,000 TH+ cells in primates relates to 100,000 TH+ cells in humans (Kikuchi et al., 2017). Therefore, when 500µl of alginate with $1 \times 10^6$ DA-hiPSCs/ml corresponds to ~15 beads, this should relate to 500,000 TH+ cells, where 5x less has shown a significant clinical improvement in non-human primate PD models.

5.3.2 Effect of FK506 on CNS cells and the immune response in vitro

The results showed that FK506 is not toxic to neurons and microglia and actually seemed to benefit their survival and growth in vitro. Additionally, the presence of FK506 reduced astrocyte reactivity and T cell proliferation. FK506 increased neurite length in SH-SY5Y neurons at all concentrations, with a generally dose-dependent trend. Even the lowest dose tested (10ng/ml) had a significant effect, which was consistent with previous studies showing PC12 and dorsal root ganglia neuronal growth increase with 1pM to 1µM (Lyons et al., 1994). FK506 treatment of SH-SY5Y cells has previously been reported to prevent reactive oxygen species (ROS) production, but FK506 was shown to have no beneficial effect on motor symptoms in a rat 6-OHDA lesion model (Maňáková et al., 2005). Contrary to this, another study showed that pre-treatment of nigral cells with FK506 increased survival of grafted dopaminergic neurons in a rat (Castilho, Hansson and Brundin, 2000). Therefore, although this study looked at the effect of FK506 on a neuronal cell line it would be of interest in future studies to look at its effects on both NP-hiPSCs and DA-hiPSCs for neuroprotective properties.

The main objective for the next experiment was to test whether FK506 might have been toxic to microglia, but instead the data suggests that microglial viability was increased by treatment with FK506 concentration between 10ng/ml and 10,000ng/ml. 1000ng/ml had the largest effect, increasing the percentage of live cells by ~50%. Previously in vitro, FK506 has been shown to prevent stimulation and
cytokine expression caused by lipopolysaccharide (LPS) in microglia (Zawadzka and Kaminska, 2005), although no reports could be found of previous studies in which microglial viability following FK506 treatment was assessed directly. In a rat model of stroke, FK506 was able to block the expression of multiple genes associated with inflammation caused by microglia following ischemia (Zawadzka et al., 2012). Hence local administration of FK506 in both cell therapy and PD could not only reduce graft rejection but also potentially reduce inflammation resulting from both PD and transplantation.

In addition to microglia, the predominant glial cell type in the brain which mediates the host response to implanted cells and materials is the astrocytes. It was important to investigate whether there were any detrimental effects on astrocytes of the cell, material and drug components of the therapy under development here. Astrocytes respond to damage and disease by undergoing reactive gliosis, where they proliferate and adopt a ‘reactive’ phenotype with increased expression of GFAP and characteristic morphological changes (hypertrophy and ramification). Reactive gliosis is difficult to study using stiff monolayer cultures since that in vitro environment results in a highly reactive baseline. Therefore this study used a soft hydrogel 3D culture assay which avoids this problem (East et al., 2013), to look at astrocyte responses to both material and FK506. TGF-β1 has been used previously to induce astrocyte reactivity in vitro (Moreels et al., 2008; East et al., 2013) so was used as the positive control which significantly increased GFAP+ percentage area by 5-fold. Adding a sample of Sigma alginate to the top surface of the astrocyte gels also significantly increased the % area of gel occupied by reactive and therefore proliferative astrocytes compared to the control and this could be due to the endotoxin levels in the alginate. This is consistent with the smaller GFAP+ response following culture with NM alginate than Sigma, which contains <1% endotoxins and has previously been shown to be less immunoreactive than other alginates (Lan, Safiejko-Mroczka and Starly, 2010; Vériter et al., 2010; Fahmy-Garcia et al., 2018). DA-hiPSCs alone seeded on top of the collagen gel containing astrocytes increased GFAP+ cell percentage by 4.5-fold. In PD, reactive astrocytes and microglia both
contribute to an inflammatory response which is detrimental to dopaminergic neuron survival (McGeer and McGeer, 2008). Therefore, upon transplantation of DA-hiPSCs or encapsulated DA-hiPSCs, less activation of glial cells is highly advantageous. The reduced GFAP+ response following addition of 100ng/ml FK506 with the DA-hiPSCs highlights its potential for the reduction of inflammation. Furthermore, DA-hiPSC encapsulated in NM alginate did not increase GFAP+ cell percentage compared to the NM alginate alone. PLO/HA coating (with and without DA-hiPSCs) lowered the percentage area GFAP+ cells to levels similar to that of the control. So, encapsulation in NM alginate and FK506 both reduced the astrocyte response to DA-hiPSCs and coating encapsulated cellular microbeads with PLO/HA resulted in a response similar to that of the control. In a rat model injected with FK506 in the cortex there was a significant decrease of cytokines IL-1β, IL-6, and TNF-α and in vitro it reduced the amount of mRNA in astrocytes corresponding to TNF-α, and IL-1β (Zawadzka and Kaminska, 2005). For future studies it would be interesting to explore how FK506 and encapsulation change cytokine expression in glial cells both in vitro and in vivo.

An important immune cell involved in rejection of transplanted cells is the T lymphocyte. As calcineurin is believed to play a key role in activation of T cells, FK506 is an ideal choice of drug because it binds calcineurin, altering its activity and therefore suppressing T cell activation (Liu et al., 1991; Wallemacq and Reding, 1993; Miller and Ericson, 2007). The T-cell proliferation assay showed that PLO-HA coating reduces the T cell response compared to alginate alone, therefore highlighting this combinational biomaterial approach as a viable option for cell encapsulation and transplantation. Previously PLO coated alginate microcapsules with anti-TNF-α bound to the surface have shown reduction of TNF-α in vitro (Leung et al., 2008). However, no literature could be found on the use of PLO with HA and alginate together. FK506 in the media alone and encapsulated in alginate microbeads also successfully reduced T cell proliferation to significantly less than or similar to the activated control, respectively. This therefore suggests that a combinational biomaterial plus FK506 encapsulation should be effective in reducing T cell activation following cell transplantation. The cells used here were all rat derived, so future
studies would need to look at human cells to further validate these findings. Furthermore, the activation antibody used in this assay contains PMA/Ionomycin, which are a protein kinase C (PKC) activator and calcium binding protein, respectively. Future studies could extend this investigation by testing the therapeutic approaches in additional assays where the T-cells are activated by mismatched allogeneic stimulator cells in order to be more similar to the transplant situation (Juvet et al., 2017).

The Japanese hiPSC PD clinical trial (and other hiPSC approaches for other conditions) are starting to reduce the risk of immune rejection by matching HLA haplotype and generating cell banks, but there are limitations due to genetic diversity specifically in countries such as the USA with such genetic heterogeneity (Garreta et al., 2018). However, in the Japan DA-hiPSC PD trial they are still using immunosuppression of FK506 for a minimum of 6 months in patients even with HLA matching (Barker et al., 2019). So, although HLA matching could be the future of allogeneic therapy, biomaterial encapsulation and local immunosuppression also show great promise in improving the current progress of cell therapy.

5.3.3 Encapsulating FK506 in nanoparticles

5.3.3.1 PLGA

Considering the potential for FK506 to be used in local immunosuppression in combination with encapsulation in alginate for therapeutic cell delivery to the CNS, the next stage was to determine how to deliver FK506. Delivery could just be within the hydrogel itself however this would not provide sustained long-term drug release, therefore a system to encapsulate FK506 with a sustained release profile was explored. FK506 nanoparticles have previously been reported in the literature for liver transplantation, cancer therapy and skin disorder with sustained and long term delivery (Gao et al., 2012; Xu, Ling and Zhang, 2014; K. Yu et al., 2018). Consequently, we aimed to create nanoparticles containing FK506 which could be incorporated in the outside PLO/HA layer of the alginate hydrogels to deliver FK506 locally once the encapsulated cells had been transplanted.
For initial nanoparticles, PLGA was used as the polymer to encapsulate FK506 as this has been previously reported successfully (Li et al., 2018; Wu et al., 2019). FK506 PLGA nanoparticles showed a mean size of 484 ± 189nm, which is larger than previously reported (82.9± 1.3nm, Wu et al., 2019). Although encapsulation efficiency was low (5.3%), it is not far off the 8% described by Wu et al 2019 and often PLGA is reported in the literature as having <1% efficiency (Danhier et al., 2012). Most interestingly, the release was sustained over 21 days. In the nanoparticles alone the drug was all released during that time period however, when encapsulated in alginate, the release was slower such that only <40% had been released in 21 days. Consequently, PLGA FK506 nanoparticles look promising for local delivery, although encapsulation efficiency and release profile could be improved. The target product profile for this project would be to achieve first order drug release kinetics and the higher the encapsulation efficiency the better as that means maximum drug incorporated in the polymer.

5.3.3.2 Electrospraying PCL
To create a novel method for the generation of FK506 nanoparticles with a more prolonged release than shown from the PLGA particles described above, the technique of electrospraying was explored. This is where a high current is used to disperse a polymer solution to create micro or nano-sized particles. PLGA is the most predominantly used polymer in electrospraying (Bock et al., 2011), but PCL nanoparticles have been used for intravenous delivery to the brain (Liu, Jiang, et al., 2013) and PCL core-shell nanofibers have shown first order release properties in electrospinning (Angkawinitwong et al., 2017). PCL was chosen to encapsulate FK506, however initial experiments resulted in micro sized particles rather than nanoscale.

The TGA analysis of these PCL-FK506 microparticles have no FK506 shown in the formulation and this could be attributed to the low concentration of tacrolimus loading in the particles (1mg/ml) (Blaine and Rose, 2004). The TGA traces do show that FK506, PCL and the PCL-FK506 particles are likely to be stable at body
temperature of 37 °C. Furthermore, the raw materials and all formulations with and without FK506 were stable to be heated to 200°C during DSC analysis. The XRD analysis of the microparticles indicated that FK506 existed in an amorphous state within the PCL solution. Throughout the method of electrospraying, the drying rate is quick, and the dispersed state of drug and polymer will be fixed in place as solid particles. Hence, the movement of FK506 molecules is greatly limited, which stops the drug from enduring nucleation and crystal growth (Wang et al., 2018). The FTIR spectra for the core shell FK506-PCL microparticles show the same spectra as PCL with no FK506, this implies low drug loading of FK506 in the core-shell microparticles, or that the drug core is deep inside the shell beyond the surface-penetration limit of FTIR detection. A limitation of FTIR is that the probe can penetrate a depth of only 100nm, which may explain that the absorbance bands of tacrolimus in the PCL-FK506 microparticles cannot be visualised as the microparticles are on average 5µm in size (Wang et al., 2018).

The release study of the microparticles suggests that a promising drug delivery system for CNS transplantation can be fabricated by electrospraying with the local sustained release of appropriate concentrations of FK506. Therapeutically, this is advantageous as local delivery of FK506 for a prolonged time, at a clinically relevant concentration, currently 200ng/ml used systemically with a therapeutic window of 0.5-2µg/L in plasma (Wallemacq and Reding, 1993). This could potentially prevent off-target toxicity and reduce toxic side effects, whilst still having immunosuppressive properties at the host site. From the literature, PCL degradation is presented to be months to years, therefore this formulation could be optimized to prevent burst release (Malikmammadov et al., 2018). The initial phase of burst release could be interpreted as high drug loading close to the surface of the polymer, with drug diffusion through the superficial layers of the polymeric particle. For future studies, the cumulative release values can be fitted to the Korsmeyer-Peppas equation \( \frac{M_t}{M_\infty} = k \cdot t^n \) to investigate the release model, where \( M_t \) represents the fraction of drug released at time \( t \), \( M_\infty \) is the complete measure of drug, \( k \) is the constant of apparent release and \( n \) the diffusion exponent (Korsmeyer et al., 1983).
The initial burst release of tacrolimus is inconsistent with the previous study fabricating PLGA-FK506 microparticles (Pathak et al., 2016), however the subsequent sustained release of tacrolimus from the PCL matrix is consistent with the use of PCL core-shell nanofibers as a drug delivery system (Angkawinitwong et al., 2017) and shows promise for the application proposed here.

The microparticles developed here are likely to be too large for use in the alginate hydrogels with DA-hiPSCs for CNS delivery. The optimisation of nanoparticles using electrospraying yielded a more appropriate size profile (82.23± 40nm), with smaller SD than both the PCL microparticles and PLGA nanoparticles and they also fit the therapeutic profile for the CNS of <100nm (Newland et al., 2013; Jo et al., 2015). Nevertheless, electrospraying proved difficult to get high yields without causing layering of nanoparticles. Also, physical characterisation (TGA, XRD, DSC and FTIR) of PCL nanoparticles is required as for microparticles. The next step would be to add nanoparticles to hydrogels with cells to check for toxicity and explore how the release profile affects neuronal cells and immune cells such as T cells, then following this moving in vivo to look at toxicity and prolonged release and efficacy in a suitable PD model.
5.4 Conclusion

This part of the project aimed to explore the secretome of DA-hiPSCs and develop a delivery method for local immunomodulation through sustained release of FK506. DA-hiPSC secretome was shown to have neuroprotective properties and when encapsulated dopamine release was analysed to be in picograms. Consequently, not only can encapsulated DA-hiPSCs potentially provide some of the lost dopamine in PD, they can also potentially have a neuroprotective effect, although proteomic analysis of the DA-hiPSC secretome both alone and encapsulated would be required to understand the mechanism. The functional effects of the secretome of DA-hiPSCs on neurons subjected to neurotoxin exposure were investigated and showed that DA-hiPSC media recovered neurite length. DA release from encapsulated DA-hiPSCs was measured and found to be slightly less than that released from the equivalent cells in monolayer culture. FK506 had no toxic effect on relevant host cells *in vitro* and actually showed increased neurite length, survival of microglia and reduced astrocyte reactivity. Multiple local FK506 delivery systems using synthetic polymer particles were developed, using PLGA and PCL. PCL nanoparticles were synthesised using electrospraying and initial characterisation indicates that such PCL-FK506 nanoparticles could be further optimised for use as a potential drug delivery system for immunomodulation in the CNS to accompany cell delivery for PD.
Chapter 6: Overall conclusions and future directions

6.1 Conclusions

The main objective of this work was to formulate a translational encapsulation device for therapeutic cells, to isolate them from the host immune response. This included the aim of identifying a dopamine releasing cell which, when encapsulated, is able to replace the dopamine lost in PD. Furthermore, another aim was to establish a delivery system for the local release of an immunosuppressant, to further protect the implanted cells. In Chapter 3, the objective of finding an appropriate GMP-ready cell for PD therapy was successfully completed (CGT-RCiB-10 hiPSCs). These cells were differentiated into midbrain dopaminergic neurons and most importantly produced dopamine. This means they could potentially be used therapeutically for dopamine replacement, allowing the project to continue to the next stage of cell encapsulation. In Chapter 4, a GMP grade alginate microbead formulation was developed and mechanically matched to cortical brain tissue (rat) showing appropriate stiffness matching for neural transplantation. Subsequently, in Chapter 5, the secretome from encapsulated DA-hiPSCs, was shown to successfully recover the health of neurotoxin-treated neurones and encapsulated cells could still release dopamine. Electrosprayed FK506 nanoparticles were also optimised in Chapter 5, and this immunosuppressant delivery approach showed potential to positively modulate host cell responses in vitro. Overall encapsulation of DA-hiPSCs in alginate and the incorporation of FK506 nanoparticles could offer an advanced cell transplantation therapy for PD.

The rationale here was that injection of dopamine-releasing cells into the striatum could promote functional recovery in PD, by increasing local dopamine concentration where it has been lost by the disease. This is the first reported differentiation of CGT-RCiB-10 hiPSCs into dopaminergic neurons and the requirement for these cells to release dopamine was successfully demonstrated within this project. The differentiation protocol chosen here (Kirkeby, Nelander and Parmar, 2013) has been
applied to other cell types previously (Kirkeby, Parmar and Barker, 2017), so these results validate the utility of the protocol and demonstrate its effectiveness using an additional previously untested stem cell type.

For this technology to work, the cells need to survive long-term and remain in the striatum, therefore cell encapsulation in alginate was investigated. Formulations and protocols were developed which included the use of a GMP-grade alginate (SLM NovaMatrix), in which therapeutically relevant DA-hiPSCs remained viable for 28 days in vitro. While detectable amounts of DA were released from encapsulated DA-hiPSCs, it would be interesting to further explore ways to maximise the amount of DA released, for example with extended differentiation time or genetic modification. Ultimately, in vivo studies would be required to determine the optimal release rate and amount of DA from encapsulated cells, by evaluating motor symptom and cognitive outputs as well as measuring changes in F-DOPA PET.

Importantly, previous literature has suggested that less than 10% of transplanted cells survive, yet this still shows clinical motor symptom improvements (Ben-Hur et al., 2004). Whilst this study was limited to in vitro environments, encapsulation of DA-hiPSCs in Chapter 3, for up to 1 month in alginate showed around 60% survival of NP-hiPSCs. Previously, injection of primary rat neural progenitor cells within a collagen hydrogel was shown to improve cell survival in the rat striatum up to 70% one day after transplantation. However, by 4.5 months only 9% of cells had survived (Moriarty, Pandit and Dowd, 2017). Therefore, if encapsulation in alginate increases the survival of cells in vivo, proportionally fewer cells would be required to achieve the same therapeutic benefit. This reaffirms that cell encapsulation could help to address the challenges associated with poor cell survival in cell therapy for PD.

A new protocol using DMA was created to mechanically match biomaterials to tissue stiffness, which involved generating novel data about brain tissue biomechanical properties, in this case in 2 brain areas in order to capture both white and grey matter. Using cortex for grey matter, DMA was able to show that 1% NovaMatrix had very similar stiffness (compound modulus) to cortex. It is important when
transplanting a material to ensure mechanical properties are equivalent to host ECM to reduce any risks associated with inflammatory responses and rejection. The striatum (the proposed delivery site) is a mixture of both white and grey matter and in order to match future biomaterials as closely as possible it would be interesting to investigate the local stiffness of striatal tissue, ideally in human brain tissue. Practically this might be challenging however, since human brain tissue is particularly hard to acquire, particularly in a fresh state where mechanical testing would be relevant. Alginate above 1% has been described as a shear-thinning material and this property is useful for this application, because such materials can flow under shear during injection and then form gels which are stiffer at the transplantation site (Qin, 2018). Ideally, the flow rate and concentration of the material would be controlled so the therapeutic cells are well protected during injection and then once set, the stiffness would match with the host tissue. To optimise these parameters would require knowledge of the material and host tissue stiffness under various strain rates and the work in this project establishes a new paradigm for mechanical testing where these properties can be explored in more detail and potentially progressed to human brain tissue.

Another factor which can affect the survival of transplanted cells in PD is the host immune response to the foreign transplanted cells following injection. The potential for local delivery of the immunosuppressant FK506 to the CNS alongside cell transplantation was explored here. Functional assays to investigate the effect of FK506 in vitro on host immune cells (microglia, astrocytes and T-cells) revealed no toxic effect and actually showed increased neurite length, improved survival of microglia and reduced activation of astrocytes. Multiple local FK506 delivery systems using synthetic polymer particles were developed, using PLGA and PCL. To the best of our knowledge no other studies have reported this type of local immunosuppression approach with FK506 for long-term cell transplantation in PD. The long-term dose that is required for a single application for PD is likely to require more sustained drug-delivery biomaterial systems than those that have gone before, which is why core-shell electrospraying is a promising technology to test in this situation. The mean size of the PCL nanoparticles was <100nm which has been shown
to be a clinically acceptable size (not big enough to be destructive or small enough to be cleared) (Chithrani, Ghazani and Chan, 2006; Kim et al., 2009; Duan and Li, 2013; Jo et al., 2015). The incorporation of the nanoparticles in the alginate microbeads with the cells, will affect the total stiffness and this could be explored further in future studies. Furthermore, how the nanoparticles will affect the DA-hiPSCs and potentially change the phenotype should also be investigated. Local delivery of FK506 has not been explored in detail so any long-term accumulation and side effects are not known and therefore further long-term in vivo toxicity studies would be required. Here the effect of FK506 on glial cells and T-cells was shown to be protective and FK506 released from PCL nanoparticles may have a similar effect, thus suggesting they are worth further investigation in vivo. Initial release studies from PCL nanoparticles alone show ~450µg/ml release over 60 days, this needs to be explored further to see if its relatable to the current clinical systemic dose of FK506 (0.2-5 mg/kg twice a day) (Udina et al., 2002; Tajdaran et al., 2019).

![Figure 6.1.1](image)

**Figure 6.1.1** Diagram showing the final proposed therapeutic device with encapsulated DA-hiPSCs in Alginate surrounded by a layer of FK506 nanoparticles in a alginate and PLO/HA layer.

Overall the work presented here has displayed promising new biomaterial formulations to protect therapeutic cells and positively modulate host responses in PD cell transplantation treatment. The final potential device from this project includes the DA-hiPSCs encapsulated in 1.5% NovaMatrix alginate, surrounded by a layer of PCL core shell nanoparticles combining FK506 entrapped in NovaMatrix alginate and PLO/HA (Figure 6.1.1). This approach could be translated across multiple disease areas where cell therapy is an option but, for the CNS specifically, the option
of synaptically isolated but chemically integrated cells in biomaterials is an exciting emerging research area. Furthermore, this has highlighted the benefit of the DA-hiPSC secretome, encapsulated DA-hiPSCs and local delivery of FK506 for suppression of host cell response in the CNS. With further in vivo validation, this combinational advanced therapy shows exciting translation for a PD therapeutic.

6.2 Future translation
Although there are many exciting conclusions from this area of work, there are also limitations and opportunities for improvements. Firstly, with regards to the DA-hiPSCs, ESC and hiPSC differentiation protocols show robust dopaminergic neuron maturation can take 50+ days (Kikuchi et al., 2017; Nolbrant et al., 2017). Therefore, it would be beneficial to continue to study the maturation of differentiated CTG DA-hiPSCs for additional time. Furthermore, it would also be relevant to conduct RT-qPCR on the NP-hiPSC and DA-hiPSC to confirm the presence of specific dopaminergic markers after differentiation, and to explore the effect of encapsulation on differentiation in order to optimise the timing for future manufacturing and implantation. Although the CTG hiPSCs are a suitable exemplar hiPSC cell choice, there are improvements that could be potentially made to cells such as these. For example, ex vivo gene therapy could be used to modify candidate hiPSC-derived DA neurons, for example to increase DA production, improve safety (e.g. through conditional immortalisation) (Wall, Toledo and Jat, 2016), improve efficacy (e.g. through overexpression of other factors like GDNF) (Hoban et al., 2013), or reduce immunogenicity (e.g. modify expression of cell surface antigens) (Lanza, Russell and Nagy, 2019).

As time was limited the DA-hiPSCs and FK506 nanoparticles were never combined and analysed experimentally. For further in vitro validation of the device proposed in this project (shown in figure 6.1.1), it would be interesting to look at the response of multiple CNS derived cells (e.g neurons and glial cells) both in a ‘healthy’ state and with the combination of a neurotoxin such as MPP+ (shown in figure 6.2.1). Analysis
from this in vitro experiment could include immunocytochemistry to see how the morphology of the cell types change with the presence of the device and to see whether after MPP+ treatment the device is able to promote recovery of any other cell types. Furthermore secretome analysis from all the cell types to look at cytokine release with and without the device present to look at pro-inflammatory markers would be useful for taking the device into *in vivo* experimentation.

**Figure 6.2.1** Diagram showing a proposed *in vitro* study incorporating the final therapeutic device of encapsulated DA-hiPSCs in alginate surrounded by a layer of FK506 nanoparticles in an alginate and PLO/HA layer. Using 4 combinations of cell types SH-SY5Y neurons, primary rat microglia, astrocytes and macrophages alone and combined in a 3D collagen matrix. Multiple (~6) microbeads are then placed on the collagen gels containing the cells, with our without the neurotoxin MPP+ and cultured for 3-7 days and the cell morphology, survival and inflammatory marker release would be analysed.
Alginate was chosen as the biomaterial for encapsulation in this project and its potential for successful long-term cell viability was confirmed. However, using a 27G needle produced microbeads which were still larger than 1mm. Ideally for PD therapy smaller beads would be required so they can be drawn up via a syringe, 20G needles or 50µl Hamilton syringes (Mendez et al., 2005; Amer et al., 2017) are commonly used for intracerebral delivery in clinical trials for cell therapy, which would correspond to a diameter of 0.6mm and 1.03mm respectively. Consequently, using needles smaller than 27G could be explored for alginate bead manufacture, or techniques such as microfluidics or electrospraying, in order to generate smaller structures. Nevertheless, there is a trade-off between making small microbeads so they can be injected with minimal disruption but also creating them big enough to encapsulate a sufficient number of cells. An alternative option might be to use in situ gelling using thermoresponsive alginate, rather than manufacturing structures for injection in their final form. Microfluidics to create micron sized alginate beads has proven to be an exciting technique with the potential to encapsulate cells in beads ~200µm (Workman et al., 2007, 2008; Hidalgo San Jose et al., 2018). For manufacturing they show great promise as they are highly reproducible and manufactured under GMP conditions. Consequently the company Dolomite microfluidics now produces commercially available GMP produced relatively low cost micron size alginate beads with proven application for drug delivery and cell encapsulation (Dolomite microfluidics, 2019).

This research has shown a combinational approach of using a biomaterial to protect cells for transplantation, and local immunosuppression to reduce the host immune response further. Another strategy for evading the host immune system after allogeneic cell therapy is by engineering cells so that they are not detected by the host immune system. One technique aims to manipulate genes involved in host recognition of transplanted cells, of specific interest are HLA class I and II proteins (Morizane et al., 2017; Lanza, Russell and Nagy, 2019). Alternatively, generation of homozygous HLA haplotyped hiPSC banks which could match a large percentage of the population could provide donor cells for transplantation which may be tolerated by the host immune system (Taylor et al., 2012). In regard to this work it would be
interesting to look at the difference between the effectiveness of cell encapsulation and local immunosuppression vs HLA-matching or systemic immunosuppression in in vitro and in vivo models. This would allow comparison of this novel approach, against the current best options. Following the successful in vitro development of cell encapsulation and local immunosuppression methods using novel biomaterial formulations, future work in vivo is necessary to further validate this approach. This could be carried out using a rat unilateral 6-OHDA lesion model to assess transplantation of allogeneic rat cell transplants to the striatum, in comparison to alginate encapsulated cells with local FK506 delivery. A humanized mouse model could also be used to assess the performance of the hiPSCs in this research.

Demonstrating GMP compliance is essential for the translation of any clinical product, and therefore suitability of materials and methods for GMP processes should not be viewed as an afterthought. This process should be considered at the pre-clinical/ in vivo stage or earlier to justify the translation into the clinical setting, as done in this project for both cells and materials. The location of GMP facilities is also paramount, particularly for time-critical formulations where prolonged transportation times could adversely affect the viability of the end clinical product, potentially leading to failure of release criteria and unacceptable financial risk. As this therapy includes both cells, materials and drugs it would be classed as an Advanced Therapy Medicinal Product (ATMP). All of the biomaterials used for CNS repair clinically are relatively simple and yet they still face considerable challenges before reaching fully-fledged, licensed therapies as medical devices. Conversely, where new material formulations and complicated combinations of components such as ATMPs are believed to be required, it will be important to justify why additional complexity is advantageous or indeed necessary. Simply increasing the complexity of biomaterial formulations in the quest for ‘novelty’ will not in itself lead to new biomaterial-based therapeutics for CNS disorders. Breaching the gap for complex ATMPs between in vivo and the clinic is of great importance for diseases of the CNS, where the gold standard treatment is not good enough or there is no current treatment. Therefore, this un-met need can’t just be looked at in animal models this needs to be translated into first in human trials where safety and efficacy can actually be determined.
This project has established a multidisciplinary cell encapsulation biomaterial approach for PD, as well as exploring the advantage of local immunosuppression in the CNS. This could underpin further future development of cell therapies in the CNS and comes at a particularly exciting time where regenerative medicine approaches are starting to become a clinical reality, with suitable manufacturing processes available and cell therapies being developed at the clinical trial stage in diseases such as Stroke, Diabetes and PD. This work further progresses the practicalities of translating encapsulated cell therapies for cell delivery and long-term survival and provides an exciting additional contribution to the future of advanced therapeutics for PD.


Basta, G. et al. (2011) ‘Long-term metabolic and immunological follow-up of nonimmunosuppressed patients with type 1 diabetes treated with microencapsulated islet allografts: Four cases’, *Diabetes Care*.


Stumm, R. et al. (2007) ‘Patterns of SDF-1α and SDF-1γ mRNAs, migration pathways, and phenotypes of CXCR4-expressing neurons in the developing rat telencephalon’, *Journal of Comparative Neurology*. doi: 10.1002/cne.21336.


Figure 7.1: Amplitude sweep tests of 1.5% w/v (A) and 2% w/v (B) to determine their linear viscoelastic region.