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A Biomaterials Approach for an Ex-Vivo Multiple Sclerosis Model of Inflammatory Demyelination

A thesis submitted to the National University of Ireland for the degree of Doctor of Philosophy

By

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August, 2013

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ABBREVIATIONS

6-OHDA - 6-hydroxydopamine

AAV- Adeno associated virus

AD – Alzheimer’s disease

ADSCs- Adipose tissue derived stem cells

AF- Annulus fibrosus

APCs- Antigen-presenting cells

APP- Amyloid precursor protein

AT-EAE- Adoptive transfer experimental autoimmune encephalomyelitis

BBB- Blood brain barrier

CFA- Complete Freund’s adjuvant

CNP- 2',3'-cyclic nucleotide 3’ phosphodiesterase

CNS- Central nervous system

CSF- Cerebrospinal fluid

DA- Dark agouti

DE-ATRP- Deactivation enhanced atom transfer radical polymerisation

DMAEMA - 2-dimethylaminoethyl methacrylate

DMEM – Dulbecco’s modified eagles medium

DMF- Dimethyl formamide

DOPE - Dioleoylphosphatidyl ethanolamine

DPI- Days post injection

FBS- Fetal bovine serum

EAE- Experimental autoimmune encephalomyelitis
EB - Ethidium bromide

EBiB - Ethyl α-bromoisobutyrate

EGDMA – Ethylene glycol dimethacrylate

GFAP- Glial fibrillary acid protein

GFP - Green fluorescent protein

GPC - Gel permeation chromatography

HBSS – Hank’s balanced salt solution

$^1$H NMR- Proton nuclear magnetic resonance

IFNγ- Interferon gamma

IL- Interleukin

LPC- Lysophosphatidylcholine or lysolecithin

LPS- Lipopolysaccharide

MAG- Myelin-associated glycoprotein

MBP- Myelin basic protein

MHV- Mouse hepatitis virus

MOG- Myelin oligodendrocyte glycoprotein

MNCs- Mononuclear cells

MRI- Magnetic resonance imaging

MS - Multiple sclerosis

NAWM- Normal appearing white matter

NHS- Normal horse serum

NO- Nitric oxide

NP- Nucleus pulposus
OSC- Organotypic slice cultures
PAMAM – Poly(amido amine)
PBS- Phosphate buffered saline
PD - Parkinson’s disease
PEG – Polyethylene glycol
PEGMEMA – Poly(ethylene glycol) methyl ether methacrylate
PEI – Poly(ethylene imine)
PLL – Poly-L-lysine
PLP- Proteolipid protein
PMDETA - N,N,N′,N′′,N″-pentamethyldiethylenetriamine
PP-MS - Primary progressive multiple sclerosis
PR-MS - Progressive relapsing multiple sclerosis
PS - Penicillin streptomycin
RR-MS - Relapsing remitting multiple sclerosis
SFV - Semliki forest virus
SP-MS - Secondary progressive multiple sclerosis
TEM - Transmission electron microscopy
TGF – Transforming growth factor
THF - Tetrahydrofuran
TMEV - Theiler’s murine encephalomyelitis virus
TNF - Tumor necrosis factor
ABSTRACT

Multiple sclerosis (MS) is characterised by the presence of inflammatory demyelinating foci throughout the brain and spinal cord, accompanied by axonal and neuronal damage. Although inflammatory processes are thought to underlie the pathological changes, the individual mediators of this damage are unclear. In order to study the role of pro-inflammatory cytokines in demyelination in the central nervous system, in this thesis, a novel non-viral gene transfection vector was utilized to establish an inflammatory demyelinating model of MS in an ex-vivo environment.

At first, a unique hyperbranched polymeric system with a linear poly 2-dimethylaminoethyl methacrylate (pDMAEMA) block and a hyperbranched polyethylene glycol methyl ether methacrylate (PEGMEMA) and ethylene dimethacrylate (EGDMA) block was designed and synthesized via deactivation enhanced atom transfer radical polymerisation (DE-ATRP) for efficient gene delivery. Using this unique structure, with a linear pDMAEMA block, which efficiently binds to plasmid DNA (pDNA) and hyperbranched polyethylene glycol (PEG) based block as a protective shell, efficient in-vitro and ex-vivo transfection was achieved with minimal cytotoxicity. Organotypic brain slices were then successfully transfected with the TNFα or IFNγ genes. TNFα and IFNγ expression and release in cerebellar slices via non-viral gene delivery approach resulted in inflammation mediated myelin loss, thus making it a promising ex-vivo approach for studying the underlying mechanisms of demyelination in myelin-related diseases such as MS.
Chapter 1

Introduction
1.1 Multiple Sclerosis

Multiple Sclerosis (MS) is a chronic inflammatory disease which invades the central nervous system (CNS). MS is the leading cause of neurological disability in young adults. MS affects about 2.5 million individuals throughout the world. Around 200 people are diagnosed with MS in Ireland every year. This disease usually affects those between the age of 20 and 40, with women being affected twice as often as men. According to Compston et al [1], MS, in the view of a pathologist, is a disorder of the central nervous system with acute focal inflammation and demyelination of axons with limited remyelination affecting multifocal sclerotic plaques. For a neurologist, it is a slow and continuous neurological disorder of young adults which is clinically diagnosed with at least two demyelinating lesions affecting brain and spinal cord. For a clinical scientist, MS is an inflammatory autoimmune disease of the central nervous system and for a patient; it is a threatening disease with certain recurring themes with an unpredictable course [1]. Despite the different definitions, multiple sclerosis still remains a difficult disease for which solutions are within reach, yet remain elusive.

1.1.1 Clinical Manifestation

Due to the broad range and subtleties of symptoms, MS is not diagnosed by a single clinical feature or by a single diagnostic test. An early attempt to ascertain the diagnostic criteria was made by Charcot in 1968. He outlined nystagmus, intention tremor and scanning speech as indicators of MS. However, this combination was clearly non-specific and is associated with almost any cerebellar lesion [2]. Currently, MS is diagnosed according to the fulfilment of diagnostic criteria, as described by McDonald et al in 2005 [3]. This criteria predominantly aims at the identification of disease dissemination in space and time as typically seen in MS. Diagnostic criteria for MS mainly involves a combination of clinical and paraclinical studies [2, 4-5]. Magnetic resonance imaging (MRI) is a very sensitive technique for the detection of MS lesions and is usually the preferred imaging procedure. Newer Magnetic resonance techniques, such as measurements of brain atrophy, magnetic resonance spectroscopy (MRS), magnetization transfer, and diffusion tensor imaging promise further progress in assessment and understanding of MS through imaging. In addition, increasingly sensitive methods for the study of
cerebrospinal fluid (CSF) have led to the recognition that most MS patients have evidence of abnormal immuno reactivity that can be demonstrated by CSF analysis [6]. Abnormalities include elevated immuno reactivity [7-8] as detected by elevated immunoglobulin IgG levels [9-13], increased IgG index [14-15], increased IgG synthesis rate [16-17], and the presence of oligoclonal bands [18-22].

Relapsing remitting MS (RR-MS), secondary progressive MS (SP-MS), primary progressive MS (PP-MS) and progressive relapsing MS (PR-MS) are the four clinical classifications of MS. Of these RR-MS and SP-MS are most common. About 80-90% patients with MS develop relapsing remitting phase at early stages, in which episodes of acute relapse are followed by partial or full recovery and separated by periods of remissions [23]. Most RR-MS patients later develop SP-MS [24]. During this stage, an ever-worsening neurological deficit accumulates even without relapses, though these may still occur. MS does not affect daily activities in about quarter of MS patients. However, around 15% of patients become severely disabled within a short time [1]. In 10-20% patients the disease is progressive from onset without relapses and slowly progresses resulting in the worsening of neurological deficits (PP-MS). PP-MS affects spinal cord and less frequently the optic nerve, cerebellum and cerebrum. PR-MS is the least common among the four disease course. It affects only 5% of people with MS. Similar to PP-MS, the patients with PR-MS also experience disease progression from the onset of the disease [25]. Relapses in PR-MS may occur more often and disability may accumulate more rapidly in PR-MS than in PP-MS. The suppressed phases between the first manifestation of MS and further relapses can last for several years, and intervals between relapses vary within patients and within disease course.

1.1.2 Pathology

MS is generally characterized by inflammation, demyelination and axonal degeneration. Active demyelination followed by remyelination and focal inflammation are the hallmark pathology of acute and relapsing MS. Cortical demyelination, global inflammation and axonal degeneration corresponds to the progressive stage [26]. Until recently, it was assumed that demyelination and the consequent failure of efficient axonal conduction were the primary causes of disability during the early stages of MS. However, results emerging from clinical and
laboratory studies over the past few years, shows the incidence of axonal damage, and inflammation in the very early stages of MS [27-29].

1.1.3 Demyelination

Demyelination occurs on individual fibers or on small groups of fibers (Figure 2). These demyelinating lesions (plagues) appear on the central myelin and the cells that form central myelin (oligodendrocytes). MS generally do not affect peripheral myelin; however, there are few cases that report peripheral demyelination [30-32].

Demyelination plaques are present in both white matter and grey matter region [33-34]. Until recently, grey matter lesions were less obvious and were undetected on MRI [35], because of the relatively small amount of myelin and reduced inflammation [36]. Recent studies with high field (9.4 Tesla) MRI has enabled the detection of grey matter lesions [37].

Mechanisms of Demyelination: Recent studies report four different patterns (I-IV) of demyelination in active MS lesions [38-40]. Pattern I and pattern II share similar features. Pattern I is seen in RR-MS patients with active demyelination and is associated with T-lymphocyte and macrophage dominated inflammation without significant amounts of antibody or complement deposition [41]. Pattern II, also seen in RR-MS patients on the other hand is associated with active demyelination with T-lymphocyte and macrophage dominated inflammation with extensive antibody deposition (mainly IgG and complement C9neo antigen) in the tissue and in astrocyte cytoplasm [42]. Both these patterns are perivenular in location and are sharply demarcated from the surrounding periplaque white matter. Loss of myelin proteins from damaged myelin sheaths occurs simultaneously.

Oligodendrocytes, which are lost during active demyelination, tend to reappear in high densities in inactive sites ensuring effective remyelination. Axonal density is reduced in active demyelination sites resulting in acute axonal injury [43]. Pattern III is also seen in RR-MS patients and is associated with active demyelination with an infiltrate of T-lymphocytes and activated macrophages and microglia.
Figure 1.1 Schematic representation of neuron, healthy nerve and myelin ruptured/damaged nerve.
In contrast to pattern I and pattern II, demyelination is found around small inflamed vessel and is frequently seen within the plaques and is surrounded by a strip of preserved myelin. The borders of active lesions are ill-defined, showing diffuse spread into the surrounding white matter. Deposition of Ig and complement is also absent in this pattern [44].

Another prominent feature is the significant loss of myelin-associated glycoprotein (MAG) relative to other myelin proteins such as myelin basic protein (MBP), proteolipid protein (PLP) and 2',3'-cyclic nucleotide 3' phosphodiesterase (CNP) [45]. These changes in myelin protein expression are associated with nuclear condensation and fragmentation of oligodendrocytes, closely resembling the structural changes of apoptosis. All these changes indicate that in these pattern III lesions, oligodendrocytes are the primary target in the demyelinating process.

Pattern IV is primarily seen in PP-MS patients. As with pattern III, pattern IV also closely resembles a disease effecting oligodendrocytes. Demyelination is associated with oligodendrocyte death in a small rim of periplaque white matter, adjacent to the zone of active demyelination [42]. Pattern IV resembles pattern I and pattern II in perivenous distribution of the lesions, and in the sharp separation of the plaques and the association of macrophages with injured myelin. Deposition of activated compliments are absent in this pattern. Myelin proteins such as MBP, PLP and CNP revealed no difference in staining patterns [42].

1.1.4 Inflammation

Demyelinated areas in MS patients are characterised by inflammatory infiltrates that contain blood-derived myelin specific T cells, B cells [46-47]. Activated T cells and B cells are normal constituents of the immune system. Once activated, T cells cross the blood brain barrier (BBB), proliferate and secrete pro-inflammatory cytokines, which in turn stimulates microglia, macrophage, astrocytes and recruit B cells. This process results in the damage of myelin, oligodendrocytes and axons [48]. This pathogenetic description has led to the conclusion that MS is a chronic, inflammatory, autoimmune, demyelinating disease of the CNS [49-51]. The density of inflammatory infiltrates in PP-MS patients is significantly higher in inactive plaques and in the normal appearing white matter of patients with progressive
disease compared to that in the white matter of age matched controls [33]. In the progressive stage of multiple sclerosis, inflammation is trapped behind a closed or repaired blood brain barrier suggesting that inflammation is an essential force driving tissue injury in multiple sclerosis. Involvement of different components of the immune system may, however, vary between disease stages and between individual patients [52].

Macrophages and activated microglia cells dominate the inflammatory reaction in all MS lesions and have the potential to induce tissue injury by secreting a variety of cytotoxic molecules [53-56]. Macrophages and microglia cells can be activated in MS lesions by cytokines produced by activated T-lymphocytes. Once the process is initiated, resting/inactive parenchymal microglia exit from their resting/inactive state and secretes the Th1 promoting cytokines interleukin (IL): IL-12 and IL-13 and become phagocytic dendritic cells [57-58]. These microglia secreted cytokines, in turn, promote encephalitogenic Th1 cell differentiation and myelin damage by secreting other myelin toxic agents such as nitric oxide (NO) and glutamate [59-60].

Focal, acute and recurrent inflammatory lesions of MS can be visualized in-vivo and on post mortem samples by MRI scans obtained after the intravenous administration of a paramagnetic contrast agent, such as gadolinium [61-62]. In-vivo studies have shown that gadolinium enhancement is related to the number of inflammatory cells within the lesions and mainly represents macrophage activation [63-64]. Gd-enhanced MRI does not provide information about the extent of inflammation, the presence of low-grade or short-lasting inflammation, the nature of the cellular components of the immune system trafficking across the damaged BBB or the degree and nature of the associated tissue destruction [65]. The addition of a magnetization transfer (MT) pulse to enhanced T1-weighted scans increases the sensitivity for the detection of enhancing MS lesions by reducing the signal of the background tissue making low grade inflammation detectable [66]. Cellular and metabolic features of MS inflammation are imaged by more sophisticated tools such as MRS and cellular imaging of lymphocytes labelled with a super paramagnetic iron oxide contrast agent [67-70].
1.1.5 Axonal Degeneration

MR studies from RR-MS patients have shown that in addition to a prominent inflammatory demyelinating component, axon degeneration also occurs from the onset of clinical disease [71-72]. However, studies have shown that axon damage is mild during the onset of early MS in patients, is extensive in the destructive regions of acute MS, and is associated with secondary tract degeneration, brain atrophy and ventricular dilatation in late chronic patients [73]. Damaged axons are mostly found in, or around, the borders of plaques and also in the normal appearing white matter (NAWM), to a lesser degree [74-75]. Damaged axons are easily detected by the reduced mean diameter of axons when compared to healthy axons [27]. Axonal degeneration is also characterized by the accumulation of mitochondria and other organelles with axonal swelling in active lesions [76]. It was seen that amyloid precursor protein (APP) was negligibly present in normal myelinated axons but was abundant in demyelinated lesions [77]. Accumulated APP inhibits fast anterograde transport and results in axonal damage [78]. Accumulation of pore-forming subunit of N-type calcium channels and metabotropic glutamate receptors also results in axonal damage [79-80].

Myelin is essential for axonal structure and functioning. Hence, destruction of myelin sheaths will indirectly result in axonal damage. Reports show that there is significant axonal loss within the plaques with degenerated axons not surrounded by myelin, suggesting demyelination prior to axonal damage [43, 81]. The effects of demyelination on both the microtubular and neurofilament components of the axonal cytoskeleton combined with the failure of tubulin transport also results in axonal degeneration associated with demyelination [82]. The pathology of the damaged axons also correlates with inflammation [27, 77]. Cytokines, enzymes, matrix metalloproteases, oxidative products and free radicals etc. secreted by activated immune cells also cause axonal damage [83-84]. Another possible mechanism of axonal degeneration in MS is a specific immunologic attack on the axon, suggested by the strong correlation between inflammation and axonal transection [85-87]. CD8+ T cell are active mediators of inflammation and axonal degeneration [88-89]. Macrophages and activated microglia have also been reported to be in close contact with degenerating axons [90]. Axonal damage can also be
caused by the direct attack of antibody mediated immune system [91]. While new inflammatory demyelinating lesions contribute to disability in RR-MS, the majority of SP-MS patients continue to decline neurologically without evidence of new inflammatory demyelinating lesions. This is due to the continuous process of axonal degeneration.

Measurements of brain atrophy present an adjunctive MRI marker reflecting the more disabling aspects of MS pathology [92]. Current methods to determine whole-brain atrophy in MS rely mostly on the measurement of brain volumes [93]. Brain volume is significantly decreased in the secondary progressive stage when compared to the relapse-remitting phase (Figure 1). However, this method does not provide a good representation for neuronal degeneration. Suppressive silver methods were used as a reliable tool for measuring neuronal degeneration [94-95]. Axonal degeneration is also measured by diffusion tensor MRI [96] and by MRS [97].

1.1.6 Role of Cytokines in MS

Cytokines play an important role in the pathogeneses of MS. They are contributors of oligodendrocytes cell death [98], inflammation [99-100] and axonal degeneration [101] which are the key features of MS. Experimental autoimmune encephalomyelitis (EAE) is an animal model that mimics many aspects of MS (discussed in section 1.1.7.1). EAE has provided significant information on the role of cytokines in MS [102-103]. Table 1.1 provides a list of cytokines and their role in either augmenting or suppressing the disease in MS positive patients and in EAE.

1.1.6.1 Role of TNFα in MS

TNFα is a pro-inflammatory cytokine and a member of the TNFα superfamily of ligands, many of which promote inflammatory signalling [104]. TNFα is synthesized as a monomeric type 2 transmembrane precursor protein (tmTNF) with a molecular weight of 26 kDa. It is mainly secreted by macrophages and monocytes, and additionally by B and T lymphocytes, natural killer (NK) cells, astrocytes, microglia, fibroblasts, adipocytes, and many other cells from immune and non-immune lineages. TNFα stimulates production of IL-12 which in turn induces IFNγ production [105].
Table 1.1 List of cytokines and their role in MS positive patients and in EAE.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>MS positive patients</th>
<th>EAE</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>↑</td>
<td>↑</td>
<td>[106-107]</td>
</tr>
<tr>
<td>IL-3</td>
<td>↑</td>
<td>↑</td>
<td>[108-109]</td>
</tr>
<tr>
<td>IL-4</td>
<td>ND</td>
<td>↓</td>
<td>[110]</td>
</tr>
<tr>
<td>IL-10</td>
<td>↓</td>
<td>↑ or ↓</td>
<td>[111-113]</td>
</tr>
<tr>
<td>IL-12</td>
<td>ND</td>
<td>↑</td>
<td>[111, 114]</td>
</tr>
<tr>
<td>IL-17</td>
<td>↑</td>
<td>↑</td>
<td>[115-116]</td>
</tr>
<tr>
<td>IL-18</td>
<td>↑</td>
<td>↑</td>
<td>[117-118]</td>
</tr>
<tr>
<td>TNFα</td>
<td>↑</td>
<td>↑ or ↓</td>
<td>[119-123]</td>
</tr>
<tr>
<td>IFNβ</td>
<td>↓</td>
<td>↓</td>
<td>[124-125]</td>
</tr>
<tr>
<td>IFNγ</td>
<td>↑</td>
<td>↑ or ↓</td>
<td>[111, 126-128]</td>
</tr>
<tr>
<td>TGFβ</td>
<td>↓</td>
<td>↑ or ↓</td>
<td>[128-130]</td>
</tr>
<tr>
<td>osteopontin</td>
<td>↑</td>
<td>ND</td>
<td>[131]</td>
</tr>
</tbody>
</table>

↑ - Aggravation, ↓ - Suppression or prevention, ND - Not determined, IL - Interleukin, TNFα - Tumor necrosis factor alpha, IFNγ - Interferon gamma, TGFβ – Transforming growth factor
TNFα is considered to be detrimental in autoimmune diseases such as MS due to its potent pro-inflammatory properties. A number of studies have reported elevation of TNFα in blood cells [132], CSF [121] and in serum [133] of patients with MS. Inflammatory responses are the primary mechanism leading to myelin destruction and oligodendrocyte cell death.

In MS, over expression of TNFα results in myelin and oligodendrocyte deterioration leading to myelin rupture in animal model [134], lymphocyte infiltration [135], astrocyte activation [136] and the up-regulation of major histocompatibility complex (MHC) I and II molecules [137] on CNS resident cells, thereby triggering T cell responses. Binding of TNFα to its receptors induces the caspase cascade which promotes cell apoptosis, and also the transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activation of Jun N-terminal kinase (JNK) pathway, which on the other hand, inhibits apoptosis [138]. Intracellular and extracellular regulatory mechanisms exist to control the relative dominance of the apoptotic and non-apoptotic pathways, which determine cell survival or death. It was seen that TNFR1 signalling mediated nerve demyelination whereas TNFR2 signalling appeared to be crucial for remyelination in the cuprizone mouse model of MS [139]. These findings were consistent with divergent roles for the individual TNF receptors in CNS autoimmunity, with TNFR1 playing roles in CNS inflammation (Figure 1.2) and demyelination and TNFR2, on the other hand, limited the pathology by eliminating auto reactive CD4+ T cells and macrophages and/or by playing roles in remyelination.

Several animal studies have reported the role of TNFα in demyelination and axonal damage. Systemic administration of TNFα increases the severity of EAE, prolongs its duration and induces relapses [119]. Direct injection of TNFα has been shown to cause demyelination of the optic nerve in mice [122] and in myelin oligodendrocyte glycoprotein (MOG) primed rats [140]. Transgenic mice have been developed to over express TNFα or its receptors in specific cell lines in the CNS. Glutamate excitotoxicity is recognized as a mechanism for oligodendrocyte and axonal damage in MS models. TNFα modulates the accumulation and release of glutamate from astrocytes and hence indirectly causes oligodendrocytes and neurons damage [141].
Primary demyelination involves the phagocytosis of myelin by activated microglia and recruited blood monocytes. This phenomenon proceeds with little damage to the myelin-forming oligodendrocyte. Elevated local TNFα expression induces oligodendrocyte apoptosis and myelin vacuolation, as well as primary inflammatory demyelination, which may be initiated by the pro-inflammatory effects of TNFα on local CNS glia and endothelial cells. The pattern of demyelination observed in TNFα transgene closely resembles that observed in a subgroup of multiple sclerosis patients whose lesions are characterized by primary oligodendropathy (image adapted from Probert et al [142]).
In EAE models, TNFα expression from astrocytes is induced by lipopolysaccharide (LPS), IFNγ and IL-1β. However, differential TNFα gene expression in response to LPS and IFNγ is strain and cell specific, and reflects both transcriptional and post-transcriptional control mechanisms. The capacity for TNFα production by Lewis astrocytes, especially in response to disease related cytokines such as IFNγ and IL-1β, has also contributed to disease susceptibility and to the inflammation and demyelination associated with EAE [123].

1.1.6.2 Role of IFNγ in MS

IFNγ is a pro-inflammatory cytokine which also plays a key role in modulating MS. IFNγ is produced by CD4+ T helper cell type 1 (Th1) lymphocytes, CD8+ cytotoxic lymphocytes, NK cells, B cells, NKT cells, and antigen presenting cells (APCs). The level of IFNγ in MS patients has shown conflicting results. Some studies have reported on the elevated IFNγ-mRNA in blood mononuclear cells (MNCs)[143] while some other studies report no change [144]. Similarly, some studies report an elevated IFNγ in serum of MS patients [145], while other studies report no change compared to controls [146]. In addition, other studies have also suggested that IFNγ may actually have a beneficial role in EAE and other demyelinating animal models. Hence, the exact role of IFNγ in MS is still not understood.

IFNγ secretion by NK cells and APCs results in early host defence against infection, whereas T lymphocytes become the major source of IFNγ in the adaptive immune response. IFNγ also activates microglia, which acts as an effector cell that damages CNS cells via phagocytosis and by the release of cytotoxic factors such as glutamate, nitric oxide, superoxide, and pro-inflammatory cytokines [147], suggesting that IFNγ and microglia play an important role in the initiation of MS. Hideyuki et al reported that IFNγ induces microglial apoptosis as a result of activation-induced cell death. This microglial apoptosis is associated with the up-regulation of pro-apoptosis proteins, especially Bcl-2–associated X protein (Bax). Microglial apoptosis was also observed in peak EAE mice, but not in early EAE mice, suggesting that IFNγ acts on microglia as part of a self-limiting negative feedback system. The authors suggested that the activation and subsequent death of microglia induced by IFNγ might have played pivotal roles in the pathogenesis of MS [148].
The role of IFN\(\gamma\) in demyelination is also controversial. In a study with EAE mice, Willenborg et al reported that IFN\(\gamma\) plays an important role in down-regulating EAE at both the effector and induction phase of disease [127]. However, Toufic et al reported IFN\(\gamma\) as playing a role in chronic demyelination and long-term disability following the induction of demyelinating disease. When myelinating oligodendrocytes of transgenic mice were expressed with IFN\(\gamma\), an up-regulation of MHC 1 and iNOS was observed with no evidence of CNS inflammation or demyelination. In contrast to control mice, which remit from EAE with resolution of glial reactivity and leukocytic infiltration, transgenics showed chronic neurological deficits. Activated microglia/macrophages persisted in demyelinating lesions for over 100 days, CD4\(^+\) T lymphocytes were no longer present in CNS, indicating the role of IFN\(\gamma\) in chronic demyelination [126]. Hence, IFN\(\gamma\) has an activating role in sustaining inflammation in EAE and MS, although it may have regulatory functions that become apparent in its complete absence.

1.1.7 *In-Vivo Models of MS*

Animal models for MS can be divided into four types:

- models that originate from an EAE-related protocol
- models that involve virus-induced CNS disease
- models that involve toxin induced mutations in the CNS
- models that involve physical injury.

1.1.7.1 *Experimental Autoimmune Encephalomyelitis (EAE)*

EAE is the most widely accepted and well characterised animal model of MS. EAE resembles MS in many aspects and has thus has become a useful tool in MS research. Similar to MS, EAE is an inflammatory, demyelinating disease of the CNS whose development and course varies due to several genetic and environmental determinants.

Active EAE and adoptive transfer EAE (AT-EAE) are the most common forms of EAE. Active EAE is induced by subcutaneous injection of an encephalitogenic peptide, mostly MOG [149] or PLP [150], which is emulsified in complete Freund’s adjuvant (CFA) containing mineral oil and Mycobacterium tuberculosis strain
H37RA, followed by intraperitoneal injection of pertussis toxin [151]. AT-EAE is induced by intravenous injection of myelin-reactive CD4\(^+\) Th1 lymphocytes into naïve animals [152]. In 1946, Kabat et al published a preliminary report of the induction of EAE in monkeys using three-weekly injections of rabbit brain emulsion in CFA [153]. This was followed immediately by more definitive reports of similar results in monkeys, rabbits, and guinea pigs [154-156].

For the past 50 years, rats and mice have proven extremely useful for investigating the pathogenesis of EAE. Initially most of the studies were largely restricted to SJL(H-2\(^s\)) and PL/J (H-2\(^u\)) mice induced by MBP antigen as this was easy to purify [157-158]. Later with the development of PLP antigens, EAE was induced in other strains [159]. SJL (H-2\(^s\)) mice had a high incidence of both clinical and histological markers of disease characterized by early onset of clinical signs when treated with PLP. In contrast, BALB/c (H-2\(^d\)), DBA/1 (H-2\(^q\)), C57BL/6 (H-2\(^b\)), AKR (H-2\(^k\)), CBA (H-2\(^k\)), C3H (H-2\(^k\)), B10.BR (H-2\(^k\)), and C57BR (H-2\(^k\)) mice showed a later onset of clinical signs and typically a lower disease incidence [159]. Subsequently it was shown that immunization of young male SJL mice, in contrast to female SJL, with PLP 139–151 overrides a defect in antigen presentation responsible for the resistance to EAE, and that natural processing and presentation of neuro antigens during the course of acute EAE induces Th2 cells that prevent the relapse of disease [160]. The discovery of CNS specific protein MOG was also encephalitogenic in SJL, AB/H(H-2dq1) mice [161], C57BL/6(H-2b) [162].

Rat models of EAE are also widely used. Acute, non relapsing EAE can be actively induced in Lewis rats using MBP, CFA, and M. tuberculosis [163]. However, chronic relapsing experimental allergic encephalomyelitis (CR-EAE) can be induced in rats with a higher dose of an emulsion of guinea-pig spinal cord tissue (GPSC) in CFA enriched with mycobacterium tuberculosis H37RA (Tbc) [164]. Lewis rats offer the advantage of predictable time of onset and uniform susceptibility to low (5-25μg) doses of MBP [165]. The disease is acute, and most animals spontaneously recover. However, they become resistant to further attempts at re-inducing EAE with MBP /CFA [166]. Cytokines that induce EAE is tabulated in Table 1.1. In summary cytokines such as TNF\(\alpha\) [167], IFN\(\gamma\) [168], IL-12, IL-23 [169], IL-10 and [170]
induces EAE in mice and rats. A list of agents that induce EAE in rodents is provided in Table 1.2.

Age and gender of species have influence on disease susceptibility, severity and course of EAE. Young male SJL mice immunised with PLP are relatively resistant to EAE whereas older males and female SJL mice of any age are susceptible. Young C57BL/6 mice and Wistar rats develop acute EAE and remission whereas middle-age males developed severe chronic EAE [171]. When MBP-specific T-lymphocytes derived from female mice were transferred, the female recipients developed more severe EAE and expressed higher levels of iNOS and NO than did male recipients [172].

However, there are also arguments that EAE might be a misleading model of MS. In a review article by Subramaniam et al, it is suggested that EAE is more of a model of acute central nervous system inflammation than the counterpart of MS [173]. One argument is that EAE differs immunologically and pathologically depending on the type of antigen used to induce it and the species used. Hence, none of the EAE models represent MS and they therefore are imprecise methods to elucidate either the pathogenesis or to develop therapeutic strategies in MS. The inability to apply the therapeutic successes from the EAE model to the human condition is another argument against the autoimmune hypothesis for the pathogenesis of MS.

1.1.7.2 Viral Models

Virus induced demyelination in CNS has shown similarities to the MS pathology. Viral infections induce autoimmunity via the following four mechanisms: molecular mimicry, epitope spreading, bystander activation and cryptic antigens [174]. Molecular mimicry, is the process by which cross-reactive T cells are generated during virus infection that recognizes both viral and self epitopes and epitopes of foreign pathogens, resulting in autoimmune disease following infection with the corresponding virus [175].
### Table 1.2 List of agents inducing EAE

<table>
<thead>
<tr>
<th>Inducing factor</th>
<th>Animal species</th>
<th>Disease type</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFA</td>
<td>rhesus monkey</td>
<td>acute disseminated encephalomyelitis</td>
<td>[154]</td>
</tr>
<tr>
<td>MBP</td>
<td>SJL mice</td>
<td>relapse remitting</td>
<td>[176]</td>
</tr>
<tr>
<td>MBP</td>
<td>SJL × B10.PL F1 mice</td>
<td>chronic</td>
<td>[177]</td>
</tr>
<tr>
<td>PLP</td>
<td>SJL mice (female)</td>
<td>relapsing EAE</td>
<td>[160]</td>
</tr>
<tr>
<td>PLP</td>
<td>(SJL/J × BALB/c) F1 mice</td>
<td>chronic relapsing</td>
<td>[178]</td>
</tr>
<tr>
<td>PLP</td>
<td>BALB/c mice</td>
<td>acute and relapsing</td>
<td>[179]</td>
</tr>
<tr>
<td>PLP</td>
<td>lewis rat</td>
<td>demyelinating</td>
<td>[180]</td>
</tr>
<tr>
<td>MOG</td>
<td>C57BL/6 mice</td>
<td>chronic</td>
<td>[181]</td>
</tr>
<tr>
<td>MOG</td>
<td>biozzi ABH mice, DA rats</td>
<td>chronic relapsing</td>
<td>[182-183]</td>
</tr>
<tr>
<td>MOG</td>
<td>NOD mice</td>
<td>secondary progressive</td>
<td>[184]</td>
</tr>
<tr>
<td>TNFα+IFNγ</td>
<td>lewis rat</td>
<td>relapse remitting</td>
<td>[140]</td>
</tr>
<tr>
<td>IL-12</td>
<td>(PL/J × SJL/J)F1 mice</td>
<td>spontaneous relapsing</td>
<td>[185]</td>
</tr>
<tr>
<td>IL-23</td>
<td>SJL mice</td>
<td>chronic</td>
<td>[169]</td>
</tr>
</tbody>
</table>

CFA - Complete Freund’s adjuvant, MBP - Myelin basic protein, PLP - Proteolipid protein, EAE - Experimental autoimmune encephalomyelitis MOG - Myelin oligodendrocyte glycoprotein, IL - Interleukin, TNFα - Tumor necrosis factor alpha, IFNγ - Interferon gamma
Briefly, activation of the cross-reactive T cells results in the release of cytokines and chemokines that recruit and activate resident and peripheral monocyte/macrophage cells that can mediate self-tissue damage. The subsequent release of self-tissue antigens and their uptake by APCs perpetuates the autoimmune disease (Figure 1.3A).

Epitope spreading is the development of immune responses to endogenous epitopes secondary to the release of self antigens during a chronic autoimmune or inflammatory response [186]. In the epitope spread model, persistent viral infection results in the activation of virus-specific Th1 cells, which mediate self-tissue damage and results in the release of self-peptides, which are engulfed by APCs and presented to self-reactive T helper cells (sTh1). Continual damage and release of self-peptides results in the spread of the self-reactive immune response to multiple self epitopes (Figure 1.3B).

Bystander activation is a non-specific mechanism for virus induced autoimmunity which occurs within the inflammatory context produced by chronic viral infection that leads activated lymphocytes to secrete inflammatory mediators that mediate tissue damage [187]. Briefly, activation of virus-specific Th1 cells and the up-regulation of immune functions throughout the tissue results in the increased infiltration of T cells to the site of infection and the activation of self-reactive Th1 cells by T-cell receptor (TCR)–dependent and TCR-independent mechanisms. Self-reactive T cells activated in this manner can then mediate self-tissue damage and further perpetuate the autoimmune response (Figure 1.3C).

The cryptic antigen model describes the initiation of the autoimmune response by differential processing of self-peptides. After viral infection interferons are secreted both by activated virus-specific Th1 cells and virus-infected cells. This up-regulates the immune functions of APCs and can lead to APC engulfing self-peptides. Cytokine activation of APC can induce increased protease production and different processing of captured self-epitopes resulting in “cryptic” epitopes. The presentation of these “cryptic” epitopes activates self-reactive Th1 cells and leads to self-tissue destruction (Figure 1.3D).


**Figure 1.3** Mechanisms of autoimmune disease induction after a viral infection (A) Molecular mimicry (B) Epitope spreading (c) Bystander activation and (D) Cryptic antigens. Image adapted from Olson *et al* [174].
A number of viruses, including Theiler’s Murine Encephalomyelitis Virus (TMEV), Semliki Forest Virus (SFV), and Mouse Hepatitis Virus (MHV) have been found to induce some aspects of disease course of MS. Table 1.3 summarises the list of virus induced in-vivo models of MS.

**Theiler’s Murine Encephalomyelitis Virus (TMEV):** TMEV, is an endogenous mouse pathogen that normally establishes a lifelong persistent CNS infection and leads to the development of a progressive CD4+ T cell–mediated demyelination and axonal injury that shares pathologic and immunologic similarities with human MS [188]. TMEV has also notably been used to demonstrate mechanisms by which autoimmunity may develop following a viral infection. It persists in varying degrees in different cell types including monocyte/macrophages, microglia, oligodendrocytes and astrocytes [189-190]. TMEV can be divided into two main groups: the highly neurovirulent viruses (strains GDVII) [191] that induce fatal encephalitis and results in death within one or two weeks post administration, and the low-neurovirulent viruses including Daniels (DA) [192] and BeAn strains [193] that promote a biphasic disease in which the chronic progressive phase is associated with demyelination in the white matter of the spinal cord. The cell types infected and the pathology of DA strains differs from BeAn strains although they share some similarities. DA strain results in greater demyelination, more viral RNA and more antigen specific cells in the spinal cord than BeAn strains [194].

**Semliki Forest Virus (SFV):** SFV induces an acute demyelinating model of MS. This virus is largely cleared out from CNS six days after post infection (dpi), with maximum demyelination at 14 dpi [195]. In this model, CD8+ T cells are required for inflammation mediated demyelination [196]. Another study on the mice model suggests that brain infiltrating B cells and anti-myelin antibodies contribute to myelin injury in SFV encephalomyelitis [197]. The neurovirulent L10 strain of SFV causes extensive neuronal damage in the CNS of infected rats, and is probably the cause of death [198]. SFV infection does not lead to severe axonal damage and hence it cannot lead to clinical disease. However, SFV infection is a good model to examine the impact of immunosuppressive therapies on CNS infections and demyelination.
### Table 1.3 List of virus induced *in-vivo* models of MS.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Animal species tested</th>
<th>Observations</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMEV (strains: DA, BeAn)</td>
<td>mouse</td>
<td>developed a chronic, progressive, T cell-mediated inflammatory demyelinating disease (DA strain). initiated organ specific T-cell mediated auto immunity (BeAn strain)</td>
<td>[174, 192]</td>
</tr>
<tr>
<td>TMEV (GDVII strain)</td>
<td>mouse</td>
<td>acute fatal grey matter encephalomyelitis kills the host within two weeks</td>
<td>[191]</td>
</tr>
<tr>
<td>SFV</td>
<td>mouse, rats</td>
<td>CD8⁺ mediated acute inflammatory demyelination in spinal cord and brain</td>
<td>[195-196, 198]</td>
</tr>
<tr>
<td>MHV</td>
<td>mouse, rats</td>
<td>demyelination due to specific immune attack</td>
<td>[199-200]</td>
</tr>
</tbody>
</table>

TMEV - Theiler’s Murine Encephalomyelitis Virus, DA – Dark agouti, SFV - Semliki Forest Virus, MHV - Mouse Hepatitis Virus
**Mouse Hepatitis Virus (MHV):** MHV is a natural mouse pathogen that infects all cells types within the CNS [201] and is dependent on the viral strain, mouse host strain and inoculation route. For example MHV-A59, a mouse hepatitis virus (MHV) strain causes brain and spinal cord inflammation and demyelination, while, MHV-2, is a non demyelinating strain [202]. MHV-A59 strain generally establishes a persistent CNS infection, leading to chronic neuro inflammation and demyelination [203]. In MHV demyelination begins about one week post-infection, showing its maximum level at around three to four week after dpi. Although virus infection contributes to the myelin damage by inducing oligodendrocyte apoptosis, the main effectors of demyelination are thought to be T cells and macrophages. Lesion repair and remyelination often follows. Axonal damage and immune mediated attack are also pathological features of MHV attack [204].

### 1.1.7.3 Toxin Models

Toxin models are generally used for studying demyelination in MS. Demyelination is induced by either focal application or systemic administration of the toxin. Lysophosphatidylcholine or lysolecithin (LPC) [205], ethidium bromide (EtBr) [206] and 6-aminonicotinamide [207] are the agents used for inducing focal demyelination. Cuprizone models utilize systemic administration. Timeline for demyelination and remyelination for various toxin models is compared in Figure 1.4.

Extent of demyelination and lesion size are mostly toxin and concentration dependent. EtBr is a DNA intercalating agent [208]. Hence it attacks all nucleated cell types. Astrocytes are damaged within the EtBr lesion area, therefore, Schwann cells are able to migrate and remyelinate before oligodendrocytes repopulate the area [209]. In EtBr models, spontaneous remyelination occurs in young animals from the outside of the lesion towards the centre. However, with increasing age (>12months) remyelination is limited [210]. LPC (a soluble detergent like agent) also induces spontaneous demyelination in primates [211] and rodents [212] within seven days and is usually followed by remyelination. The extent of demyelination with LPC is age dependent, with young rats showing remyelination within one month [205] and older rats still showing extensive demyelination even after one month post injection [213]. A major difference in the pathology induced by LPC and EtBr is the extent of astrocyte loss and the dynamics of myelin breakdown.
Figure 1.4 A comparison of timeline for demyelination and remyelination for various toxin models (Neurofilament heavy chains (NFH) (axons) in green and MBP (myelin) in red).
In a comparative study between different toxins it was seen that EtBr induced lesions remyelinated more slowly than those induced by LPC as the astrocyte damage incurred within the demyelinated area was greatest for EtBr induced demyelination [209]. It was also seen that EtBr induced larger lesions than LPC under the same volume and concentration (1 or 2% solution) [214]. A failure of remyelination in the old rats following LPC induced demyelination was also observed similar to EtBr. This may be related to sluggish responses of astrocytes and/or macrophages to demyelination [215]. In another study it was seen that in pregnant mice remyelination was enhanced in white matter lesions after LPC induced demyelination suggesting that maternal white matter plasticity imparts a striking ability to repair demyelination and identifies prolactin as a potential therapeutic agent [216]. See Table 1.4 for the list of demyelination models induced by focal administration of toxins.

Feeding animals with the copper-chelator cuprizone is another method to induce demyelination. It has been reported that wistar weanling male rats treated with 0.5-2% cuprizone showed oligodendrocyte perturbation and intramyelinic edema of the cerebral white matter, but do not develop demyelination [217]. In contrast to rats, mice challenged with cuprizone showed significant demyelination. A six weeks and longer diet of 0.2% cuprizone on adult mice have shown demyelination of the superior cerebellar peduncle [218] and Swiss or ICI mice fed 0.5 or 0.6% cuprizone have shown acute oligodendrocyte degeneration [219]. However, extensive remyelination did not occur if the animals were kept on cuprizone diet [220]. Acute demyelination is followed by spontaneous remyelination during subsequent weeks when mice are fed normally.

In another study C57BL/6 male mice were fed with 0.2% cuprizone for 12 weeks followed by 12 weeks of recovery on normal chow resulted in significant demyelination of the corpus callosum after 6 – 12 weeks of cuprizone ingestion followed by partial normalization during the remyelination phase [221-222]. This suggests that prolonged administration of cuprizone limits remyelination leading to chronic demyelination. Unlike EtBr or LPC models, in cuprizone models, age was not a factor which limited extensive remyelination in mice [223].
Table 1.4 List of demyelination models induced by focal administration of toxins.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Animal species</th>
<th>Concentration</th>
<th>Administration location</th>
<th>Demyelination</th>
<th>Remyelination</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-aminonicotinamide</td>
<td>rat (400gms)</td>
<td>2-10µl of 0.01 or 0.05 M solution</td>
<td>spinal cord</td>
<td>4-7 dpi</td>
<td>11-13 dpi</td>
<td>[207]</td>
</tr>
<tr>
<td>ethidium bromide (EtBr)</td>
<td>monkey (adult)</td>
<td>0.5µl of 0.03% solution</td>
<td>spinal cord</td>
<td></td>
<td>no remyelination up to 3 weeks post injection</td>
<td>[224]</td>
</tr>
<tr>
<td></td>
<td>rat (adult)</td>
<td>1µl of 0.1% solution</td>
<td>sciatic nerve</td>
<td>1-15 dpi</td>
<td>15-38 dpi</td>
<td>[225]</td>
</tr>
<tr>
<td></td>
<td>rat (1-1.5 months)</td>
<td>20µl of 1.5% solution</td>
<td>ventricle</td>
<td>1-10 dpi</td>
<td>12-40 dpi</td>
<td>[226-227]</td>
</tr>
<tr>
<td></td>
<td>rat (2 months)</td>
<td>4µl of 0.01% solution</td>
<td>CCP</td>
<td></td>
<td>4 weeks</td>
<td>[228]</td>
</tr>
<tr>
<td></td>
<td>rat (9-12 months)</td>
<td>4µl of 0.01% solution</td>
<td>CCP</td>
<td></td>
<td>no remyelination up to 9 weeks dpi</td>
<td>[228]</td>
</tr>
<tr>
<td></td>
<td>cat</td>
<td>0.01-1%</td>
<td>spinal cord</td>
<td>2 dpi (around injection site) 8-14 dpi (deep layers)</td>
<td></td>
<td>[229]</td>
</tr>
</tbody>
</table>

Introduction
Table 1.4 (Cont’d) List of demyelination models induced by focal administration of toxins.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Animal species</th>
<th>Concentration</th>
<th>Administration location</th>
<th>Demyelination</th>
<th>Remyelination</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>lysophosphatidylcholine (LPC)</td>
<td>rat (18 months)</td>
<td>1μl of 1% solution</td>
<td>spinal cord</td>
<td>---------</td>
<td>8 weeks</td>
<td>[228]</td>
</tr>
<tr>
<td></td>
<td>rat (100-122gms)</td>
<td>1μl of 1% solution</td>
<td>spinal cord</td>
<td>1-10 dpi</td>
<td>10-21 dpi</td>
<td>[230]</td>
</tr>
<tr>
<td></td>
<td>mouse (adult)</td>
<td>2μl of 1% solution</td>
<td>spinal cord</td>
<td>1-7 dpi</td>
<td>7-23 dpi</td>
<td>[212]</td>
</tr>
<tr>
<td></td>
<td>rat (2 months)</td>
<td>1μl of 1% solution</td>
<td>spinal cord</td>
<td>---------</td>
<td>complete remyelination in one month</td>
<td>[215]</td>
</tr>
<tr>
<td></td>
<td>rat (5 months)</td>
<td></td>
<td></td>
<td></td>
<td>partial remyelination in one month</td>
<td></td>
</tr>
<tr>
<td></td>
<td>rat (12 months)</td>
<td></td>
<td></td>
<td></td>
<td>no remyelination observed within one month</td>
<td></td>
</tr>
<tr>
<td></td>
<td>virgin mouse (6-8 weeks)</td>
<td>1.5μl of 1% solution</td>
<td>spinal cord</td>
<td>---------</td>
<td>pregnant mice showed 37% increased remyelination after 7 dpi and 52% increased remyelination after 11 dpi</td>
<td>[216]</td>
</tr>
<tr>
<td></td>
<td>pregnant mouse (6-8 weeks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A strain dependent susceptibility to cuprizone has been reported with SJL mice showing a unique pattern of demyelination different from C57BL/6 mice. SJL mice do not readily demyelinate at the midline within the corpus callosum as in C57BL/6 mice [231] but show greater demyelination directly lateral to midline [232]. Clinical and experimental observations describe sexual dimorphisms in MS with a higher prevalence in females of childbearing age and different clinical courses in the female and male population. However, when treated with 0.2% cuprizone female mice were partially resistant to demyelination, whereas male mice were more severely demyelinated [232].

Dose also plays an important role in cuprizone models. It was seen that mice on a diet of 0.3% or greater exhibited over toxic effects of cuprizone indicated by diminished weight, lethargy and lack of grooming while very little demyelination was induced by 0.1% cuprizone, with no significant difference from untreated group [232]. In another study cuprizone intoxication induced a severe demyelination of distinct cortical deep grey matter sub regions which is also a key feature of clinical multiple sclerosis. Striosomes, located within the caudate-putamen and the ventral part of the caudate nucleus displayed intense demyelination, whereas those within the globus pallidus and the head of the caudate nucleus were not affected [233]. See Table 1.5 for the list of various cuprizone models.

1.1.7.4 Physical Injury Models

Cognitive impairment [234] and motor dysfunction [235] are also associated with multiple sclerosis apart from inflammation and demyelination. In experimental and human spinal cord injury there is evidence of demyelination in white matter as seen in multiple sclerosis [236] and motor impairment [237]. Hence, a new method was developed for the clinical assessment of motor function and demyelination in rats after experimental spinal cord injury. Spinal cord lesions can be created by weight drop [238], aneurysm clip compression [239] calibrated forceps compression [240] contusion [241] and hemisection [242]. Behaviour and electrophysiology assessments on adult rat models evaluate the sensorimotor function rather than sensory or motor functions individually [243-244].
Table 1.5 List of various cuprizone models.

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Concentration</th>
<th>Administration time period</th>
<th>Demyelination</th>
<th>Remyelination</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>swiss mouse</td>
<td>0.5%</td>
<td>1-5 weeks</td>
<td>1-5 weeks</td>
<td>immediate remyelination</td>
<td>[219]</td>
</tr>
<tr>
<td>swiss mouse (8 weeks)</td>
<td>0.2%</td>
<td>12 weeks</td>
<td>6-12 weeks</td>
<td>slow remyelination 12-24 weeks</td>
<td>[221-222]</td>
</tr>
<tr>
<td>SJL mouse (8 weeks male and female)</td>
<td>0.1-0.5%</td>
<td>3-10 weeks</td>
<td>3-13 weeks with maximum at 7-10 (male) and 9-10 (female)</td>
<td>no significant remyelination</td>
<td>[232]</td>
</tr>
<tr>
<td>C57BL/6 mouse (8 week)</td>
<td>0.2 or 0.4%</td>
<td>1-5 weeks</td>
<td>1-5 weeks with less extent of demyelination in older rats</td>
<td>immediate remyelination</td>
<td>[233]</td>
</tr>
<tr>
<td>C57BL/6 mouse (6 month)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
After contusive spinal cord injury in adult rats it was seen that the overall number of demyelinated axons peaked at one day post injury which declined by 7–14 days post injury, and then progressively increased up to 450 days post injury. Oligodendrocyte and Schwann cell remyelinated axons appeared by 14 days to 450 days post injury with complete remyelination making it a model for chronic progressive demyelination [245]. Ischemic insult models produced by 90 minutes middle cerebral occlusion (MCAO) are also studied for evaluating white matter injury and demyelination [246-247].

1.1.8 *In-Vitro* Models of MS

Animal studies have significantly contributed to understanding MS. However, the relative expense of EAE models makes *in-vitro* models an attractive alternative for studying disease mechanisms and testing efficacy. *In-vitro* approaches are crucial to study the role of CNS cells, allowing manipulations that cannot easily be performed *in-vivo*.

1.1.8.1 Astrocytes

Astrocytes are star shaped glial cells in the brain and spinal cord communicating with neurons and other glial cells. Their processes extend to neuronal synapses, nodes of Ranvier, and to the BBB, and they are interconnected via gap junctions to each other, and to oligodendrocytes via heterotypic gap junctions [248]. They play an active role during demyelination by controlling chemokine expression and local inflammation in the CNS [249-252], provoking damage of oligodendrocytes and axons by secreting inflammatory cytokines [253], glial scarring [254] and also in remyelination [255]. Primary astrocyte cultures are mainly obtained from neonatal brain tissue from mice [256] or rats [257]. Primary astrocytes are also cultured from human tissues [258]. Astrocytes exposed to bacterial LPS has resulted in the appearance of nitric oxide synthase (NOS) activity [259], monocyte chemo attractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1a, MIP-1b mRNA expression [260], IL-12 [261], IL-1, IL-6 [262] and TNFα [263] which are the key cytokines that induce demyelination and axonal degeneration. Effect of cytokines such as TNFα, IFNγ, TGFβ1, IL-10, IL-1β in astrocytes has also been studied in *in-vitro* systems. Infection of primary astrocytes with neurotropic virus [262], human
herpesvirus 6 (HHV-6) [264] and Theiler's virus [265] has also provided a better understanding of the role of astrocytes in demyelination and inflammation. IFNγ activated mouse astrocytes express B7 co-stimulatory molecules necessary for efficient activation of naive T cells and MHC-II molecules which can process and present MBP and PLP epitopes to T cells [266]. Some in-vitro model used to gain an understanding the role of astrocytes and inducing factors are listed in Table 1.6

1.1.8.2 Microglia

Microglia are a type of glial cells that are resident macrophages of the brain and spinal cord. They are sensitive to brain injury and disease and alter their morphology and phenotype to adopt an activated state in response to pathophysiological brain insults. Microglia plays a controversial role in neuro degeneration by either being protective [267] or harmful [268], depending on the mode of activation. Phagocytic properties of microglia are well studied in in-vitro cultures [269]. Microglia can be isolated from mice [270], rats [271], rhesus monkeys [272] and human fetal brain or post-mortem tissue [273]. In-vitro studies highlight the two-sided nature of microglial cells. In-vitro studies on microglia cultures show that microglia activated to an inflammatory phenotype via LPS became neurotoxic by secreting TNFα, IL-1, IL-6 [273], nitric oxide (NO) [274]. However, microglia pre-treated with IL-4 became neuroprotective and induced neurogenesis and oligodendrogenesis in-vitro with IL-4-activated microglia showing a bias towards oligodendrogenesis and IFNγ activated microglia showing a bias towards neurogenesis [275]. Infection of microglial cells with human T-cell leukemia virus type I (HTLV-I) has resulted in the enhanced secretion of pro inflammatory cytokines such as TNFα and IL-6 which play a key role in inflammatory demyelination and gliosis [276]. Microglia isolated from rhesus monkeys infected with simian immunodeficiency virus (SIV) produced excess of IL-8 and TNFα than the uninfected cultures and displayed an increased capacity to secrete TNFα upon stimulation with LPS [277]. Female sex steroids estriol, β-estradiol and progesterone have been shown to inhibit LPS induction of NO production by primary rat microglia and by the mouse N9 microglial cell line [278]. Lists of agents that induce activation of microglial cells in-vitro are described in Table 1.7.
Table 1.6 List of inducing factors and *in-vitro* models used to understand the role of astrocytes.

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Inducing factor</th>
<th>Observations</th>
<th>Relevance to MS</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat (P1)</td>
<td>LPS (500mg/ml)</td>
<td>NOS activity</td>
<td>inducible NOS contribute to the neuronal damage associated demyelinating diseases</td>
<td>[259]</td>
</tr>
<tr>
<td>rat (adult)</td>
<td>LPS (0-10µg/ml)</td>
<td>LPS, IFNγ and TNFα induced β chemokine mRNA expression.</td>
<td>β chemokines contribute to the development of inflammatory demyelination</td>
<td>[260]</td>
</tr>
<tr>
<td></td>
<td>IFNγ (1–100U/ml)</td>
<td>TGFβ1 and IL-10 down regulated induced β chemokine mRNA expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TNFα (1–100U/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGFβ1 (10–1000ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-10 (10ng/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse (P1-2)</td>
<td>LPS (10µg/ml)</td>
<td>IL-12 expression</td>
<td>IL-12 regulates inflammatory immune response and plays a key role in the mechanism of multiple sclerosis</td>
<td>[261]</td>
</tr>
<tr>
<td>rat (P1-2)</td>
<td>LPS (10µg/ml)</td>
<td>LPS induced TNFα, IL-1 and IL-6</td>
<td>TNFα, IL-1, lymphotoxin, IL-6, IFNα and IFNβ play a significant role in the pathogenesis of immunologically and/or virally mediated CNS disease, in CNS intercellular communication, and in the interactions between the nervous and immune systems</td>
<td>[262]</td>
</tr>
<tr>
<td></td>
<td>neurotropic paramyxovirus</td>
<td>Virus induced TNFα, lymphotoxin, IL-6, IFNα and IFNβ</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.6 (cont’d) List of inducing factors and *in-vitro* models used to understand the role of astrocytes

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Inducing factor</th>
<th>Observations</th>
<th>Relevance to MS</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat (P1-2)</td>
<td>LPS (0-10µg/ml) IFNγ 0 to 1000 U/ml rIL-1β (0 to 1000 U/ml) LPS+ IFNγ</td>
<td>IFNγ and IL-1β alone do not induce TNFα production. combined treatment of IFNγ and IL-1β and IFNγ and LPS induce TNFα production</td>
<td>astrocyte produced TNFα have a pivotal role in augmenting intracerebral immune responses and inflammatory demyelination due to its diverse functional effects on glial cells such as oligodendrocytes and astrocyte themselves</td>
<td>[263]</td>
</tr>
<tr>
<td>human (fetal)</td>
<td>(HHV-6)</td>
<td>infected cells showed cytopathic effects, forming giant syncytia</td>
<td>MS patients show active, ongoing HHV-6 infections. This study suggest that infection of primary human astrocytes may play a role in the neuropathogenesis of HHV-6</td>
<td>[264]</td>
</tr>
<tr>
<td>mouse (P1-3)</td>
<td>theiler's virus</td>
<td>induces IL-12p40, IL-1, IL-6, TNFα, and IFNβ via NF-κB activation</td>
<td>IL-12p40, IL-1, IL-6, TNFα, and IFNβ are involved in the initiation and amplification of inflammatory responses in the CNS known to be critical for the development of immune-mediated demyelination</td>
<td>[265]</td>
</tr>
<tr>
<td>mouse</td>
<td>IFNγ (100U/ml)</td>
<td>express B7 costimulatory molecules and murine MHC-II molecules</td>
<td>MHC-II molecules process and present MBP and PLP epitopes to T cells</td>
<td>[266]</td>
</tr>
</tbody>
</table>

LPS - Lipopolysaccharide, IL - Interleukin, TNF - Tumor necrosis factor, IFN- Interferon, HHV- Human herpesvirus 6, NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells, MBP - Myelin basic protein, PLP - Proteolipid protein, MHC - Major histocompatibility complex
Table 1.7 Lists of agents that induce activation of microglial cells *in-vitro*.

<table>
<thead>
<tr>
<th>Inducing factor</th>
<th>Animal species</th>
<th>Observations</th>
<th>Relevance to MS</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS (0-10µg/ml)</td>
<td>human (16-24 week fetal brain)</td>
<td>LPS induced TNFα, IL-1β, and IL-6</td>
<td>TNFα, IL-1β, and IL-6 plays an important role in regulating MS hence the understanding of role of cytokines is crucial</td>
<td>[273]</td>
</tr>
<tr>
<td>TNFα (200 U/ml), IL-1 β (200 U/ml)</td>
<td></td>
<td>TNFα induced IL-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IL-1 β induced TNFα , IL-1 and IL-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS (10pg/ml)</td>
<td>rat (P2)</td>
<td>LPS and IFNγ induces metabolite nitrate (NO2)</td>
<td>ameboid microglial cell killing of oligodendrocytes and NO2-production suggests that NO may be a mechanism of death of the oligodendrocyte and possibly play a role in lesion formation in MS</td>
<td>[279]</td>
</tr>
<tr>
<td>rIFNγ(10 U/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα (100 U/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HTLV-I</td>
<td>human (patients undergoing partial temporal lobe resection)</td>
<td>HTLV-I enhanced the secretion of IL-6 and TNFα</td>
<td>both TNFα and IL-6 have been implicated in inflammatory demyelination and gliosis</td>
<td>[276]</td>
</tr>
</tbody>
</table>

LPS - Lipopolysaccharide, IL - Interleukin, TNF - Tumor necrosis factor, IFN- Interferon, TGF – Transforming growth factor, HTLV - Human T-cell leukemia virus
1.1.8.3 Oligodendrocytes

Oligodendrocytes are a type of glial cell and their main function is to provide support and to insulate the axons by creating myelin sheath. Myelin is about 40% water with the dry mass about 70 - 85% lipids and about 15 - 30% proteins. Oligodendrocytes play a major role in multiple sclerosis. There is evidence of myelin damage and oligodendrocyte cell death in MS lesions as a result of excitotoxicity [280] or by the production of NO or heat shock protein-65 [274, 281].

In MS the maintenance of myelin and remyelination following damage depends on manipulating oligodendrocytes in-vitro. Oligodendrocytes are obtained by gently shaking a flask of isolated rat cerebral tissue where the free-floating oligodendrocytes separated from the adherent microglia and astrocytes [282] or from differentiated rodent stem cells [283]. HOG, MO3.13, KG-1C, OLP-6, OLN-93, Oli-neu, CG4 are some examples of oligodendrocyte cell lines. OLN 93 and the Oli-neu cell lines are commonly used. In-vitro models are absolutely crucial to study the mechanisms of oligodendrocyte damage such as apoptosis and oxidative stress. The human transformed oligodendrocyte cell line, MO3.13 cells induced neuronal nitric oxide synthase (nNOS) which directly mediates oligodendrocyte injury and reduces cell viability leading to demyelination, following stimulation with LPS [284].

A combinational treatment of LPS and IFNγ reduced proliferation and viability of oligodendroglial cells and also resulted in the enhanced expression of inducible nitric oxide synthase (iNOS) and released micro molar concentrations of NO. However, treatment with IL-4 or IL-10 inhibited the expression of iNOS with IL-10 being more effective than IL-4 in suppressing iNOS expression. IL-10 conferred protection against oligodendroglial death caused by LPS/IFNγ [285]. Treatment of IFNγ alone also induced oligodendrocyte cell death suggesting that IFNγ plays a role in the pathogenesis of MS by activating apoptosis in oligodendrocytes [286]. The rate of proliferation, death and maturation of oligodendrocytes is controlled by multiple factors, including the extent of TNFα expression. Cultured oligodendrocytes exposed to various concentration of TNFα result in differing modes of proliferation and cell death. It was seen that maturation to the MBP positive stage was inhibited by about 36% by 1000–2000 U/ml of TNFα, while numbers of O4+/MBP− precursors were
Introduction

unaffected. In contrast, at 5000 U/ml TNFα, the specific effect on maturation was overtaken by cytotoxicity [287].

Lymphotoxin (LT) also caused injury to oligodendrocytes in a time and dose-dependent fashion. LT showed a far more potent cytotoxicity than TNFα towards oligodendrocytes with early retraction of cell processes, depolymerisation of F-actin and subsequent nuclear degeneration, suggesting its potent cytotoxic activity against oligodendrocytes and the major mechanism involved in this process of DNA fragmentation [288]. The mechanism of induction of TGFβ1 in brain and the identity of cells expressing TGFβ1 is better understood in an in-vitro culture of oligodendrocytes. It was seen that TGFβ1 was induced by IL-1 by both autocrine and paracrine mechanisms [289]. Stellate shaped or elongated NG2-positive oligodendrocyte progenitor cells were detected in chronic MS lesions [290]. In-vitro studies on oligodendrocyte cells have reported an activation of the TrkA NGF receptor in oligodendrocytes after introducing TrkA receptor into oligodendrocyte cell cultures which negates cell death by the p75 receptor. Activation of TrkA in oligodendrocytes resulted in suppression of c-jun kinase activity initiated by p75 suggesting that TrkA-mediated rescue involves not only activation of survival signals but also simultaneous suppression of a death signal by p75 [291]. In the immune system, T-lymphocyte proliferation depends on IL-2 interaction with specific receptors. In an in-vitro study on neonatal rat oligodendrocyte progenitor cells it was seen that IL-2 inhibited oligodendrocyte proliferation via Tac-positive receptors [292-293].

S-nitroso-N-acetyl-DL-penicillamine (SNAP), 3-morpholinosydnonimine-HCl (SIN-1) and pyrogallol can also cause significant mitochondrial enzyme damage in oligodendrocytes by inducing NO production. SNAP causes less than 25% cell death at 0.5mM while SIN-1 and pyrogallol causes 100% death and mitochondrial damage at 2mM after 18 h in-vitro [294]. While all the agents discussed above cause cell death and affect cell proliferation and maturation, FTY720 [295], platelet derived growth factor (PDGF), basic fibroblast derived growth factor (bFGF) [296], neurotrophin 3 (NT3) [297-298], glial growth factor 2 (GGF-2)[299] , insulin like growth factor (IGF-1) [300], Ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) [301] are examples of some agents that promotes cell
survival, proliferation migration and myelin induction to oligodendrocytes. A detailed list of agents causing and inhibiting oligodendrocyte survival is provided in Table 1.8.

1.1.8.4 Co-Culture Systems

Co-culture models are useful to study the impact of a certain cell type to oligodendrocyte injury and remyelination. Co-culture of dorsal root ganglia cultured together with Schwann cells is one of the most frequently used model developed by Ratner et al [302] or Schwann cell-like cells derived from adult rat bone marrow [303]. Other neuronal cell types are also used such as primary cultured embryonic cortical neurons [304]. Myelinating cell types other than Schwann cells in two oligodendrocyte cell lines [305] are also used. In mixed co-cultures in-vitro, oligodendrocyte progenitors express functional adenosine receptors and detect action potentials from axons with large intracellular calcium fluxes. These adenosine receptors inhibit oligodendrocyte progenitor proliferation, promotes its interaction with axons, and drive myelination over the course of 14 days in-vitro [306].

The action of LPS is better studied in a mixed culture system. It was seen that LPS selectively killed oligodendrocyte precursors (preOLs) by activating them in a non-cell autonomous fashion through the induction of TNFα in mixed glial cultures. Whereas, astrocytes and microglia, were not activated. Moreover, astrocytes promoted TNF-mediated preOL death through a cell contact-dependent mechanism, while microglia were the sole source for TNFα production in LPS treated mixed glial cultures [307]. This LPS induced toxicity can be inhibited by endogenous fractalkine. In a mixed microglial culture, effects of LPS on TNFα secretion were partially blocked (30%) by fractalkine by acting as an anti-inflammatory chemokine in cerebral tissue through its ability to control and suppress certain aspects of microglial activation [308]. The effect of peroxisome proliferator-activated receptor (PPAR) was better studied in cortical neuron glial co-cultures. PPARγ agonists such as 15d-PGJ$_2$, ciglitazone and troglitazone prevented LPS induced neuronal death and eliminated LPS induced NO, iNOS, COX-2 and PGE$_2$ release. It was seen that PPAR-α agonists such as clofibrate and WY14, 643 did not produce similar results [309].
Table 1.8 List of agents causing and inhibiting oligodendrocyte survival.

<table>
<thead>
<tr>
<th>Cell model</th>
<th>Cell death/demyelination inducing agent</th>
<th>Survival, proliferation, migration and myelin inducing agent</th>
<th>Observations</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO3.13 (cell line)</td>
<td>LPS (5µg/ml)</td>
<td></td>
<td>LPS induces nNOS</td>
<td>[284]</td>
</tr>
<tr>
<td>primary rat (P2)</td>
<td>LPS (1µg/ml) IFNγ (100U/ml)</td>
<td>IL-4 (ng/ml) IL-10 (ng/ml)</td>
<td>LPS/IFNγ induces iNOS treatment of infected cells with IL-4 or IL-10 suppressed iNOS</td>
<td>[285]</td>
</tr>
<tr>
<td>rat oligodendrocyte progenitors (P1)</td>
<td>IFNγ (50U/ml)</td>
<td></td>
<td>IFNγ induced oligodendrocyte cell death</td>
<td>[286]</td>
</tr>
<tr>
<td>primary rat (P2)</td>
<td>TNFα (1000-5000U/ml)</td>
<td></td>
<td>concentration dependant response of oligodendrocytes (1000–2000 U/ml of TNFα inhibiting maturation and 5000U/ml inducing cytotoxicity)</td>
<td>[287]</td>
</tr>
<tr>
<td>rat (P1)</td>
<td>NGF (100ng/ml)</td>
<td>TrkA retrovirus (0.5–1 pfu/cell)</td>
<td>activation of the TrkA NGF receptor in oligodendrocytes reduces cell death induced by NGF.</td>
<td>[291]</td>
</tr>
<tr>
<td>Cell model</td>
<td>Cell death/ demyelination inducing agent</td>
<td>Survival, proliferation, migration and myelin inducing agent</td>
<td>Observations</td>
<td>Ref</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------</td>
<td>----------------------------------------------------------</td>
<td>--------------</td>
<td>-----</td>
</tr>
<tr>
<td>primary calf (fresh calf white matter)</td>
<td>LT (100 or 500 U/ml) TNFα (100 or 500 U/ml)</td>
<td></td>
<td>LT showed a much more cytotoxicity than TNFα. LT-mediated effects were associated with early retraction of cell processes, depolymerisation of F-actin and subsequent nuclear degeneration</td>
<td>[288]</td>
</tr>
<tr>
<td>rat oligodendrocyte progenitor cells (P1)</td>
<td>IL-2 (5U/ml)</td>
<td></td>
<td>IL specifically inhibits oligodendrocyte proliferation via Tac-positive receptors</td>
<td>[292]</td>
</tr>
<tr>
<td>primary rat (P2)</td>
<td>SNAP (0-2mM) SIN-1 (0-2mM) Pyrogallol (0-2mM)</td>
<td></td>
<td>SNAP causes less than 25% cell death at 0.5mM. SIN-1 and pyrogallol causes 100% death and mitochondrial damage at 2mM</td>
<td>[294]</td>
</tr>
<tr>
<td>human fetal oligodendrocyte progenitor cells (19-23 week embryos)</td>
<td></td>
<td>FTY720 (10nM-1µM)</td>
<td>FTY720 induces time-dependent modulation of S1P receptors on human OPCs with consequent functional responses that are directly relevant for the remyelination process</td>
<td>[295]</td>
</tr>
</tbody>
</table>
Table 1.8 (cont’d) List of agents causing and inhibiting oligodendrocyte survival.

<table>
<thead>
<tr>
<th>Cell model</th>
<th>Cell death / demyelination inducing agent</th>
<th>Survival, proliferation, migration and myelin inducing agent</th>
<th>Observations</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>primary rat (P2)</td>
<td>PDGF-AA (10ng/ml) bFGF (5 ng/ml)</td>
<td>PDGF induces a motile state in progenitor cells growing in the presence of bFGF</td>
<td>[296]</td>
<td></td>
</tr>
<tr>
<td>primary rat (P2)</td>
<td>NT3 (10ng/ml)</td>
<td>NT3 promotes progenitor OL cell commitment to enter into S-phase of cell cycle to initiate DNA synthesis, in a similar manner to PDGF-AA</td>
<td>[298]</td>
<td></td>
</tr>
<tr>
<td>rat oligodendrocyte progenitor cells (P2)</td>
<td>GGF (200ng/ml)</td>
<td>high levels of GGF reversibly inhibit the differentiation and lineage commitment of oligodendrocyte progenitors and, in differentiated cultures, result in loss of O1 and myelin basic protein expression</td>
<td>[299]</td>
<td></td>
</tr>
<tr>
<td>human fetal oligodendrocyte progenitor cells (19-24 week)</td>
<td>IGF-1 (10ng/ml)</td>
<td>IGF-1 exerted a maturational effect</td>
<td>[300]</td>
<td></td>
</tr>
</tbody>
</table>

Oligodendrocyte precursors co-cultured with rat embryonic motor neurons on non-biological substrate (diethylenetriamine trimethoxy-silylpropyldiethylenetriamine), were induced to differentiate into mature oligodendrocytes that express myelin basic protein [310]. This reproducible model of *in-vitro* myelination is a valuable tool for the development of treatments for demyelinating diseases such as multiple sclerosis.

In an *in-vitro* co-culture system with dorsal root ganglion neurons and oligodendrocytes addition of soluble neuregulin (Nrg1) (a mitogen for oligodendroglial cells), provided an axonal signal implicated in oligodendrocyte survival and increased myelination, while the oligodendrocyte progenitor cells (OPCs) mitogens FGF-2 and PDGF-AA inhibited myelination, which was reversed by inhibiting PDGF for remyelination [311]. A reproducible myelination culture method using dissociated neuron-oligodendrocyte co-cultures from either the embryonic day 16 (E16) rat spinal cord or cerebral cortex by Pang *et al* [312]. It was seen that the spinal cord derived OPCs developed quickly into MBP⁺ mature oligodendrocytes and started to myelinate axons around 17 days *in-vitro*, with maximum myelination around six weeks. Myelination in these cultures systems is quantified by counting of MBP-stained cells [311] or the number of internodes/myelin segments, by toluidine blue staining [313] and by validation of compact myelin using electron microscopy [314] or time lapse confocal microscopy [315]. Examples of some agents causing and inhibiting myelination are listed in Table 1.9.

### 1.1.8.5 Organotypic Brain Slice Culture

Brain slice culture systems are more complex *ex-vivo* models for deeper understanding of cell-cell interactions and are advantageous over other *in-vitro* platforms as they can replicate many aspects of the *in-vivo* context. These slices largely preserve the tissue architecture of the brain regions including hippocampus [316], striatum [317], cortex [318], spinal cord [319], brain stem [320] and cerebellum [321]. Acute slices and organotypic slices are currently in use in neuroscience research. Acute slices are short lived and are used for short experiments that range for a few hours. Whereas organotypic slices, by contrast are designed for long term studies.
Table 1.9 Examples of agents that promote or inhibit myelin formation in mixed cultures.

<table>
<thead>
<tr>
<th>Co-culture model</th>
<th>Cell death/demyelination inducing agent</th>
<th>Cell survival and myelin inducing agent</th>
<th>Observation</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>mixed glial culture (rat P1-P2)</td>
<td>LPS (1µg/ml)</td>
<td></td>
<td>astrocytes promote TNFα mediated preOL death through a cell contact-dependent mechanism via LPS induction</td>
<td>[307]</td>
</tr>
<tr>
<td>mixed glial culture (rat P1-P2)</td>
<td>LPS (10ng/ml)</td>
<td>recombinant human fractalkine (100nM)</td>
<td>effects of LPS on TNFα secretion were partially blocked (30%) by fractalkine</td>
<td>[308]</td>
</tr>
<tr>
<td>neuron-oligodendrocyte coculture (rat P0)</td>
<td>FGF 2 (10ng/ml)</td>
<td>Nrg1(0.1-50ng/ml)</td>
<td>Nrg 1 promotes myelination FGF 2 and PDGF-AA inhibits myelination</td>
<td>[311]</td>
</tr>
<tr>
<td>neuron-oligodendrocyte coculture (mice E16)</td>
<td>TNFα (10ng/ml)</td>
<td>IL-1β (10ng/ml)</td>
<td>LPC (100µg/ml)</td>
<td>AntiMOG (10µg/ml)</td>
</tr>
</tbody>
</table>

Acute slices are mainly used as an alternative to electrophysiological studies *in-vivo* [322] and has enabled detailed studies to carry out on neuronal membrane properties [323], cellular actions of neurotransmitters (reviewed in [324]), synaptic mechanisms [325] and hence enabled a better understanding of cell death mechanisms [326-327]. Organotypic brain slices serve as an alternative to neurobiological studies and is a good model system for studying neurodegenerative disease [328] such as Alzheimer’s disease [329], Parkinson’s disease [330] Huntington’s disease [331], amyotrophic lateral sclerosis [332] and multiple sclerosis [333].

Organotypic brain slices are prepared using several methods. In early times, roller tubes or Maximov-type chambers were used. In roller-tube cultures, the tissue is embedded in either a plasma clot or in a collagen matrix on glass cover slips and then undergoes continuous slow rotation, ensuring oxygenation of these slices, because the slow rotation results in a continuous changing of the liquid gas interface [334]. The roller-tube technique offers the advantage that the slice cultures flatten to a monolayer, which results in optimal optical conditions and access to single neurons. Therefore, this technique is preferably used for electrophysiological recordings. However, this method is limited as it is difficult to prepare and is not reproducible due to the thinning of tissues to non physiological monolayer of cells. After a few weeks *in-vitro*, hippocampal slices cultured by means of the roller-tube technique thin down from an initial 400 mm to about 50 mm. Slices can be grown in culture dishes. The tissue is placed either directly on plastic dishes coated with collagen or in petriPERM® dishes that contain a gas-permeable bottom and may be embedded in three-dimensional collagen gels [335].

Since no special measures are taken to facilitate oxygenation the tissues grown in culture dishes are not ideal for long-term studies as they are typically used after a few days *in-vitro*, which limits it application. Membrane culture system developed by Stoppini *et al* is the most efficient and widely used method [336]. In membrane cultures, the slices are placed on a transwell [337] or millicell [338] semi porous membrane (for an air–medium interface) and is kept stationary during the entire culturing process. These slices obtain oxygen from above and medium from below. The medium below the membrane provides adequate nutrition to tissues via capillary action. All three techniques yield cultures that retain the characteristic
cytoarchitecture of the tissue of origin to a large degree and that display excellent cellular differentiation. The major difference between the various techniques is the final thickness of the slices and the time that they survive in culture. After a few weeks in-vitro, hippocampal slices cultured by means of semi porous membrane thin down from an initial 400 mm to about 100–150 mm. Brain slices are cut using a tissue chopper [316] or vibratome [339].

Organotypic slice cultures (OSC) are prepared from brains of adult [340], neonatal [341] or embryonic [342] tissue. Brain tissues from young animals are mostly used as they show a high degree of plasticity and resistance to mechanical trauma during the slice preparation. The cellular and molecular substrates that direct axon regeneration in adult white matter is better studied on organotypic brain slices. It was seen that adult cortical neurons were able to elaborate very short, highly branched, dendritic-like processes when seeded onto organotypic slice cultures of postnatal day 35 (P35) rat brain containing the corpus callosum. Adult dorsal root ganglion (DRG) neurons were able to regenerate lengthy axons within the reactive glial environment of this degenerating white matter tract. Fibronectin was found in abundance in callosum and played an important role in axon regeneration in white matter suggesting that the critical interaction between regrowing axons and astroglial associated fibronectin in white matter may be an additional factor to consider when trying to understand regeneration failure and when devising strategies to promote regeneration [343]. The nature of infiltrating T-cells has an impact on the expression of the B7 molecules on microglia, the resident APCs of the brain, was also illustrated in a mice hippocampus OSC (P11). It was seen that T-cells modulate B7 expression on microglial cells in the brain independent of antigen presentation through TCR/MHC-II ligation but presumably by soluble mediators [344].

Sagitally cut cerebellar organotypic slice cultures are mostly used to examine the effects of agents on myelination. Myelination was first reported in longer term cerebellar slices in 1957 [345]. In organotypic cerebellar slices, progesterone (PROG) (neuro steroid) [346], estrogen (neuro-steroid) [347], adenosine (neuro-glial transmitter) [306], N-cadherin (calcium-dependent cell adhesion molecule) [348] and glucose [349] are factors that promote formation of myelin. Demyelination was first reported in 1959 [350], by adding serum from animals with EAE. In 1981,
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Raine *et al.* exposed myelinated cultures of mouse spinal cord to sera raised in rabbits against whole white matter (anti-WM), myelin basic protein (anti-MBP) and galactocerebroside (anti-GC) to determine which factor in CNS tissue *in-vitro* is the target of serum demyelinating and myelin swelling antibodies. It was seen that demyelination and myelin swelling *in-vitro* are caused by antibodies against GC and not against MBP [351].

In 1986 Shahar *et al.* developed a demyelinating *ex-vivo* mouse spinal cord model by infecting spinal cord slices with Theiler’s Meningoencephalitis virus (GDVII) or WW virus. GDVII virus demyelinated about 50% of myelinated axon, whereas, infection with WW virus showed a more uniform and severe demyelination of about 90% of the myelinated axons [352]. In 1995, Roth *et al.* described a demyelinating and remyelinating model in an organotypic nerve tissue. Treatment with IGF-1 and anti-white matter antiserum with complement to the normal nutrient medium (NNM) produced demyelination. However, demyelination induced by IGF-1 was concentration dependent. Treatment of lower concentrations of IGF (0.01-0.1g/ml) showed remyelination after 14 days of treatment [353].

In 2004, Birgbauer *et al.*, developed a demyelinating rat cerebellar model by treating the slices with LPC otherwise called lysolecithin [354]. Treatment of cultures with LPC for 15-17 hours produced marked demyelination within 24 hours and showed its maximum extent at day two and remyelinated completely within eight days post treatment. However, remyelinated fibres had thinner myelin sheaths and shorter internodes [333]. This is still the most widely used model to study *ex-vivo* demyelination and remyelination. Demyelination was also induced in OSC via treatment with anti-MOG [355] and simvastain [356]. A list of agents that induce demyelination in OSC is provided in Table 1.10. Recent studies have shown that addition of fingolimod (FTY720) [357], olesoxime [358], Sphingosine-1-phosphate receptors (S1PRs) [359] or progesterone [360] inhibit demyelination by modulating cytokine expression and promotes remyelination in organotypic brain slice cultures.
<table>
<thead>
<tr>
<th>Slice region</th>
<th>Inducing factor</th>
<th>Animal species</th>
<th>Demyelination</th>
<th>Remyelination</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>spinal cord</td>
<td>anti-GC serum</td>
<td>mouse (embryos)</td>
<td>0-2 days post treatment</td>
<td>----</td>
<td>[351]</td>
</tr>
<tr>
<td>spinal cord</td>
<td>WW and GDVII virus</td>
<td>mouse (P17)</td>
<td>15-17 hours post treatment GD VII virus demyelinated of about 50% and WW virus about 90%</td>
<td>----</td>
<td>[352]</td>
</tr>
<tr>
<td>spinal cord</td>
<td>anti white matter serum IGF-1 (0.01-.5g/ml)</td>
<td>mouse (embryos)</td>
<td>0-15 days post treatment Anti white matter serum produced total demyelination 50%, demyelination for IGF-I between 0.1 and 0.5 g/ml concentration</td>
<td>remyelination was observed at day 14 after removal of IGF-1 (0.01-0.1g/ml)</td>
<td>[353]</td>
</tr>
</tbody>
</table>
Table 1.10 (cont’d) List of agents that induce demyelination and subsequent remyelination in OSC

<table>
<thead>
<tr>
<th>Slice region</th>
<th>Inducing factor</th>
<th>Animal species</th>
<th>Demyelination</th>
<th>Remyelination</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>cerebellum</td>
<td>LPC (0.5mg/ml)</td>
<td>SD rat postnatal (P10)</td>
<td>0-4 days post treatment</td>
<td>2-8 days post treatment</td>
<td>[354]</td>
</tr>
<tr>
<td>cerebellum, brain stem, spinal cord</td>
<td>LPC (0.5mg/ml)</td>
<td>mouse postnatal (P1-P2)</td>
<td>0-1 days post treatment</td>
<td>remyelination verified after 14 treatment remyelinated fibres have thinner myelin sheaths and shorter internodes</td>
<td>[333]</td>
</tr>
<tr>
<td>cerebellum</td>
<td>anti-MOG (60µg/ml)</td>
<td>mouse postnatal (P8-P10)</td>
<td>0-43 hours post treatment</td>
<td>53-77 hours post treatment</td>
<td>[355]</td>
</tr>
</tbody>
</table>

GC serum – Galactocerebroside serum, IGF – Insulin like growth factor, LPC – Lysophosphatidylcholine, MOG – Myelin oligodendrocyte glycoprotein
1.2 Gene Delivery Systems

Genes are the basic physical and functional units of heredity. They have specific sequences of bases that encode the amino acids of proteins. When genes are altered, the encoded proteins are often unable to carry out their normal functions, which may result in a disease phenotype.

Gene therapy is a technique that treats human disease by inserting a functioning gene into the cells of a patient to correct a genetic error or to introduce a new function to the cell. Gene therapy was first attempted by delivering naked DNA into the cells. Although this method is the safest approach in gene delivery, low levels of transfection efficiency limit its application. In order to increase transfection efficiency viral and non-viral vector were introduced in the later years. The success of gene therapy is largely dependent on the development of vector systems. Viral vectors show high transfection efficiency. These vectors made from viruses by removing their own genes and inserting therapeutic genes. Hence, their application is limited by their toxicity, difficulty in pharmaceutical processing and scale-up as well as the possibility of the reversion of an engineered virus to a wild type [361]. Non-viral vectors are generally synthetic molecules that are modified to carry DNA. Hence, they have gained their importance in scientific research because of their safety in handling and ease of application compared with viral vectors. Non-viral gene delivery can be classified into two general groups: (1) naked DNA delivery by a physical method, such as electroporation and gene gun and (2) delivery mediated by a chemical carrier such as a cationic polymer or a lipid.

Here, the focus is mainly on gene delivery in multiple sclerosis disease in-vivo and ex-vivo. Delivery of anti-inflammatory cytokines using a gene delivery vector will represent a possible therapeutic target in MS, which has yet not attempted on MS patient. Delivery of anti-inflammatory cytokines as gene therapy in MS patients has not yet been attempted. However, some experiments have been performed, using the animal models of MS (discussed in section 1.2.1 and 1.2.2). To date no reports have been published which use gene delivery approaches to induce MS model either in-vitro or in-vivo.
1.2.1 Viral Vectors

While there may be other drawbacks associated with their use, viral vectors are the most efficient means of gene transfer. They can strongly bind to a cell for ease of uptake, avoid intracellular degradation and are capable of entering the nucleus of a cell, making them the most effective transfection vectors. Retrovirus vector is widely chosen as it readily transduces dividing cells. Encephalitogenic T cells transduced with a retroviral gene complexed with IL-4, TNFα or MOG-Ig gene was able to delay the onset and reduce the severity of EAE when adoptively transferred to myelin basic protein immunized mice [362-364]. Retrovirus mediated gene transfer of the self antigen MBP into the bone marrow of mice was found to alter resistance to experimental autoimmune encephalomyelitis [365]. Injection of recombinant adeno associated virus containing the human gene for catalase to the right optic nerve heads of SJL/J mice suppressed EAE [366]. Transduction of a gene coding for IL-4 within the CNS of mice EAE, using non-replicative Herpes Simplex Virus type 1- derived vectors resulted in delayed disease onset [367-368]. All these studies suggest that gene delivery by using viral vectors may be a good therapeutic strategy for suppression of MS.

1.2.2 Non-Viral Vectors

Various methods of non-viral gene delivery been developed that involve delivery of naked DNA, delivery of genes using hydrodynamic technique, ultrasound, electroporation, gene gun, liposomes and by cationic polymers. The BBB protects the CNS from potentially harmful xenobiotics and endogenous molecules. Hence, it is very difficult for non-viral vectors to cross the BBB and transf ect efficiently. The relative numbers of non-viral vectors used in gene transfection to CNS are summarized in Figure 1.5. It was seen that mammalian expression plasmid for green fluorescent protein (GFP) gene injected into the lateral ventricle of rat embryos via electroporation expressed intensely in neural progenitor cells at one day in-vitro (DIV) which later differentiate into neurons and glia at 21 DIV [369]. Since then, the CNS has been frequently targeted for in-vivo and ex-vivo electroporation of prenatal, postnatal and adult rodents. Studies reported on in-vivo and ex-vivo electroporation to CNS are described in Table 1.11.
**Figure 1.5** Percentage distribution of non-viral vectors used in CNS gene delivery
**Table 1.11** List of previous studies reported on *in-vivo* and *ex-vivo* electroporation to CNS

<table>
<thead>
<tr>
<th>Animal species</th>
<th>In-vivo</th>
<th>Ex-vivo</th>
<th>Plasmid dose</th>
<th>Target region</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>wister rat</td>
<td>✓</td>
<td></td>
<td>0.2μl of (10 μg/μl),</td>
<td>hippocampal CA1 region</td>
<td>[370]</td>
</tr>
<tr>
<td>C57BL/6 mouse</td>
<td>✓</td>
<td></td>
<td>8.3μl of (1.0–2.5mg/ml)</td>
<td>cerebellum</td>
<td>[371]</td>
</tr>
<tr>
<td>rat</td>
<td>✓</td>
<td></td>
<td>0.25–2.0mg/ml</td>
<td>hippocampus</td>
<td>[372]</td>
</tr>
<tr>
<td>sprague–dawley rat and C57Bl/6J mouse</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5μl plasmid (1μg/μl), 0.05μl/minute</td>
<td>anterior cingulated cortex</td>
<td>[373]</td>
</tr>
<tr>
<td>long-Evans rat</td>
<td>✓</td>
<td></td>
<td>3–4μl plasmid</td>
<td>cerebellum</td>
<td>[374]</td>
</tr>
<tr>
<td>rat</td>
<td>✓</td>
<td></td>
<td>10μl of 1μg/μl</td>
<td>hippocampus</td>
<td>[375]</td>
</tr>
<tr>
<td>mouse</td>
<td>✓</td>
<td></td>
<td>10μl of 1μg/μl</td>
<td>cortex</td>
<td></td>
</tr>
<tr>
<td>sprague–dawley rat and C57Bl/6J mouse</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1μg/μl</td>
<td>cortex</td>
<td>[376]</td>
</tr>
<tr>
<td>sprague–dawley rat; SVE129 and OMP-GFP mouse</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1–2μl of (1–5μg/μl)</td>
<td>lateral ventricles</td>
<td>[377]</td>
</tr>
<tr>
<td>C57Bl/6J mouse</td>
<td>✓</td>
<td></td>
<td>0.05–0.5μg/μl</td>
<td>cortical layer</td>
<td>[378]</td>
</tr>
<tr>
<td>C57Bl/6, Z/EG and R26R mouse</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2μl</td>
<td>lateral ventricles</td>
<td>[379]</td>
</tr>
<tr>
<td>CD1 mouse</td>
<td>✓</td>
<td></td>
<td>2μl of (5mg/ml)</td>
<td>fore brain</td>
<td>[380]</td>
</tr>
<tr>
<td>C57Bl/6 mouse</td>
<td>✓</td>
<td></td>
<td>0.5μl of (5μg/μl)</td>
<td>mesopallium</td>
<td>[381]</td>
</tr>
</tbody>
</table>
Electroporation mediated gene therapy has been reported in *in-vivo* studies in MS models. In EAE models IFNβ gene delivery using an electroporation technology resulted in significant inhibition of disease progression and a significant reduction of EAE relapses compared to untreated mice or null vector treated mice [382].

Cationic lipids with positively charged functional groups that have an affinity for negatively charged DNA and form bilayered vesicles are termed as liposomes or lipoplexes. Liposomes are spherical in shape and have a diameter in the range of 50–1000nm that have proven useful as convenient delivery vehicles for biologically active compounds. Liposomes are widely used for transfecting brain cells. Lipotransfection has been used successfully to transfer genes to monolayer cultures, such as primary rat neuronal cells [383], neuroblastoma cell lines [384] and also in organotypic brain slice cultures [371, 385]. CNS primary tissue transfected using a liposomal transfection reagent, caused cell membrane damage within 24 hours after transfection. It was seen that nestin-positive target cells, which were used as morphological correlate, were severely diminished in some areas of the cultures after liposomal transfection [386].

Reports of CNS gene transfer using cationic liposomes began to appear in the literature from the early 1990s [390]. DNA-liposome complexes (lipoplexes) were successfully delivered to the brain by a single injection to the cerebral cortex. A single injection of lipoplexes showed a therapeutically significant decrease in the tumor volume while continuous intracerebral delivery of lipoplexes using an osmotic mini pump led to complete tumor regression in 36.4% of the treated animals [391]. Hecker *et al* in 2001 reported a successful delivery and expression of heat shock protein Hsp70 and reporter gene enzymes in the CNS of the rat after injection into the lateral ventricle via cationic lipid based gene delivery approach [392].

The delivery of *in-vitro* transcribed mRNA vectors encoding for Hsp70 and luciferase to the lateral ventricle of the rat via cationic liposomes demonstrated that the distribution, uptake, and expression of reporter sequences using lipid-mediated mRNA vector delivery is extensive in coronal sections throughout the rat brain, confirming the potential for lipid-mediated mRNA delivery to the CNS [387]. However, when injected into the parenchyma of the rat brain, enzyme activity is not widely distributed after injection of the vector into brain parenchyma, emphasizing
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the importance of CSF delivery to achieve widespread vector distribution [388]. The luciferase activity in the CNS of rats after transfection with cationic lipids was widespread and the transfection peaked at 72 hours after injection and was detected *in-vivo* for at least 7–10 days after peak expression. Cationic lipid/ liposomes have been modified for more efficient delivery. For example *trans*-activating transcriptional activator peptide (TATp)-modified liposomes have been used to enhance the delivery of the model gene, plasmid encoding for the green fluorescent protein (pEGFP-N1), to human brain tumor U-87 MG cells *in-vitro* and in an intracranial model in nude mice [389]. Neutral/anionic liposomes containing the polymer polyethylene glycol (PEG) showed that the genetic material of interest is actually encapsulated within the lipid core (as opposed to surface attachment for cationic liposomes) and the presence of PEG prevented the capture by the reticulo-endothelial system of the lipoplex [390]. Table 1.12 summarises the different gene delivery vectors used for *in-vivo* and *ex-vivo* transfection of CNS.

Cationic liposomes have also been used for treating neurodegenerative disease in *in-vivo* models of Parkinson’s disease model [391-392] and EAE [393-394]. For example EAE symptoms were inhibited by a single injection of therapeutic cytokine (IL-4, IFNβ, and TGFβ) lipoplexes directly into the central nervous system. Table 1.13 summarises gene delivery vectors used in MS.

Cationic polymers also exhibit the DNA condensing properties similar to cationic lipids. Poly ethyleneimine (PEI), poly-L-lysine (PLL), poly (β-amino esters), poly 2-dimethylaminoethyl methacrylate (pDMAEMA) and poly amido amine (PAMAM) are widely used as non-viral vectors because of their ability to condense DNA and to form complexes (polyplex) for more efficient uptake through endocytosis. However, it is difficult for the cationic polymers to enter the brain tissue by crossing BBB. A list of cationic polymer enabling *in-vivo* and *ex-vivo* transfection to CNS is provided in Table 1.12.

Polyamidoamine (PAMAM) dendrimers have emerged as a new class of nanoscopic, spherical polymers that have captured the interest of researchers in various scientific disciplines over the past few years. Arginine-grafted PAMAM dendrimer have shown high transfection efficiencies and low cytotoxicity in primary cortical cells including neurons, glial cells, astrocytes, microglia, and oligodendrocytes [395].
Table 1.12 Summary of gene delivery vectors used for \textit{in-vivo} and \textit{ex-vivo} transfection of CNS.

<table>
<thead>
<tr>
<th>Gene delivery vector</th>
<th>Animal species</th>
<th>\textit{In-vivo}</th>
<th>\textit{Ex-vivo}</th>
<th>Time points</th>
<th>Target region</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>cationic lipid</td>
<td>rat</td>
<td>✓</td>
<td></td>
<td>24 or 44 hours</td>
<td>lateral ventricle</td>
<td>[396]</td>
</tr>
<tr>
<td>cationic lipid</td>
<td>rat</td>
<td>✓</td>
<td></td>
<td>0-8 days</td>
<td>CSF and parenchyma</td>
<td>[388]</td>
</tr>
<tr>
<td>cationic liposome</td>
<td>rat</td>
<td>✓</td>
<td></td>
<td>24 hours</td>
<td>right cerebrum</td>
<td>[397]</td>
</tr>
<tr>
<td>cationic liposome</td>
<td>mouse</td>
<td>✓</td>
<td></td>
<td>24 or 48 hours</td>
<td>striatal parenchyma and in the paraventricular area</td>
<td>[398]</td>
</tr>
<tr>
<td>TATp-modified liposomes</td>
<td>mouse</td>
<td>✓</td>
<td></td>
<td>24 or 48 hours</td>
<td>intracranial brain tumors</td>
<td>[389]</td>
</tr>
<tr>
<td>anionic liposome</td>
<td>rat</td>
<td>✓</td>
<td></td>
<td>48 hours</td>
<td>neurons, choroid plexus epithelium, and the brain microvasculature</td>
<td>[390]</td>
</tr>
<tr>
<td>TransFast\textsuperscript{®}</td>
<td>mouse</td>
<td>✓</td>
<td></td>
<td>24 hours</td>
<td>cerebellum</td>
<td>[371]</td>
</tr>
<tr>
<td>cationic liposome</td>
<td>rat</td>
<td>✓</td>
<td></td>
<td>48 hours</td>
<td>ventral mesencephalon</td>
<td>[385]</td>
</tr>
<tr>
<td>cationic liposome</td>
<td>human</td>
<td>✓</td>
<td></td>
<td>two or four days</td>
<td>ventral mesencephalon</td>
<td>[399]</td>
</tr>
<tr>
<td>Lipofectamine\textsuperscript{®}</td>
<td>rat</td>
<td>✓</td>
<td></td>
<td>two or four days</td>
<td>ventral mesencephalon</td>
<td>[386]</td>
</tr>
<tr>
<td>Effectene\textsuperscript{®}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg-PAMAM</td>
<td>mouse</td>
<td>✓</td>
<td></td>
<td>48 hours</td>
<td>mixed neuronal-glia</td>
<td>[395]</td>
</tr>
</tbody>
</table>
Table 1.12 (cont’d) Summary of gene delivery vectors used for *in-vivo* and *ex-vivo* transfection of CNS.

<table>
<thead>
<tr>
<th>Gene delivery vector</th>
<th>Animal species</th>
<th>In-vivo</th>
<th>Ex-vivo</th>
<th>Time points</th>
<th>Target region</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>branched PEI</td>
<td>mouse</td>
<td>✔️</td>
<td></td>
<td>Three months</td>
<td>cerebral cortex, hippocampus, and hypothalamus</td>
<td>[388]</td>
</tr>
<tr>
<td>linear PEI</td>
<td>mouse</td>
<td>✔️</td>
<td></td>
<td>24 hours</td>
<td>neurons and glia adjacent to ventricular spaces</td>
<td>[389]</td>
</tr>
<tr>
<td>BPEI-SS-PEG-RVG</td>
<td>mouse</td>
<td>✔️</td>
<td></td>
<td>24 hours</td>
<td>brain</td>
<td>[390]</td>
</tr>
<tr>
<td>PEG-PDMAEM A</td>
<td>mouse</td>
<td>✔️</td>
<td></td>
<td>two days</td>
<td>third ventricle, lateral ventricle, hippocampus, substantia nigra, triangular nucleus, hypothalamus and cortical layer</td>
<td>[400]</td>
</tr>
<tr>
<td>PAMAM</td>
<td>mouse</td>
<td>✔️</td>
<td></td>
<td>two days</td>
<td>brain</td>
<td>[401]</td>
</tr>
<tr>
<td>PAMAM–PEG–Angiopep</td>
<td>mouse</td>
<td>✔️</td>
<td></td>
<td>two days</td>
<td>cortical layer, caudate putamen, hippocampus and substantia nigra</td>
<td>[402]</td>
</tr>
<tr>
<td>PAMAM–PEG–Lf</td>
<td>mouse</td>
<td>✔️</td>
<td></td>
<td>two days</td>
<td>brain</td>
<td>[403]</td>
</tr>
<tr>
<td>Tet1-PEG-PEI</td>
<td>mouse</td>
<td>✔️</td>
<td></td>
<td>two days</td>
<td>lateral ventricle</td>
<td>[404]</td>
</tr>
</tbody>
</table>
Table 1.13 Gene delivery vectors used in *in-vivo* models of MS

<table>
<thead>
<tr>
<th>Gene delivery vector</th>
<th>Animal species</th>
<th>Plasmids</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>cationic liposome</td>
<td>mouse</td>
<td>IL-4, IFNβ, and TGFβ</td>
<td>[393]</td>
</tr>
<tr>
<td>cationic liposome</td>
<td>mouse</td>
<td>IFNβ</td>
<td>[394]</td>
</tr>
<tr>
<td>naked DNA</td>
<td>rat</td>
<td>IL-4, IL-10, GM-CSF, and TNFα</td>
<td>[405]</td>
</tr>
<tr>
<td>naked DNA</td>
<td>mouse</td>
<td>TGFβ1, IL-4-IgG1</td>
<td>[406]</td>
</tr>
<tr>
<td>naked DNA</td>
<td>mouse</td>
<td>IL-4</td>
<td>[407]</td>
</tr>
</tbody>
</table>

IL - Interleukin, IFN - Interferon, TNF - Tumor necrosis factor, TGF – Transforming growth factor, GM-CSF – Granulocyte macrophage colony stimulating factor, IgG – Immunoglobulin G
PEI has also been shown to be a useful agent for the stable expression of plasmid-encoded genes in neuronal cultures [408]. Efficient *in-vivo* gene delivery in the brain using PEI was first demonstrated by Boussif *et al* in 1995 [409]. Later Abdallah *et al*, transfected brain using complexed plasmid DNA with branched PEI (25 kDa) at an electrostatic charge close to neutral [410]. Transgene expression was found more than three months after injection in cortical neurons. Subsequently, linear PEI (22-kDa) was used to condense DNA. Linear PEI-DNA complexes were reported to be highly stable and diffusible in biological fluids, and intra ventricular injection resulted in diffusion of the complex from the injection site to the entire brain ventricular space [411]. Later, ‘Jet PEI®’ (linear polyethylene imine derivative, free of components of animal origin) was used for efficient *in-vivo* gene transfection to the brain [412]. PEI modified with RVG peptide has been used for efficient gene delivery to brain to transport across BBB [413]. In another study, PEG modified PEI was linked to a neuronal targeting ligand, Tet1, a 12-amino acid peptide. Tet1-PEG-PEI complexes mediated increased transfection in brain tissue when compared to unmodified PEI-PEG complexes [404]. It has been reported that patients with PD have a decrease in fibroblast growth factor-2 (FGF-2) in the dopaminergic neurons of the substantia nigra before cell degeneration. It was seen that following PEI-mediated delivery of a dominant negative FGFR1 mutant, a decrease in number of Th-positive neurons in the SN on the FGFR1 (TK−) was observed around the injected side, as opposed to control side. This efficiency of PEI was found to be similar to that of helper-free herpes simplex virus-1 (HSV-1) amplicon as a FGFR1 (TK−) DNA delivery vector [414].

Recently Qian *et al* reported phage-displayed TGN peptide-decorated polymeric micelle-like polyplexes based on PEGylated poly(2-(dimethylamino) ethyl methacrylate) (PEG-PDMAEMA) as an efficient brain targeted gene delivery vector [400]. Tissue expression experiments indicated the widespread expression of an exogenous gene in mouse brain after i.v. administration with a PAMAM/DNA weight ratio of 10:1, the brain gene expression of the PAMAM-PEG-Tf/DNA complex was ~2-fold higher than that of the PAMAM/DNA and PAMAM-PEG/DNA complexes [401]. Later Angiopep-conjugated, PEG-modified PAMAM and lactoferrin modified PAMAM (PAMAM-PEG-Lf) was used for better efficiency [402]. PAMAM–PEG–Lf can be exploited as a potential non-viral gene vector
targeting to the brain via non-invasive administration and Lf as a promising ligand for the design of gene delivery systems targeting to the brain [403].

1.3 Hypothesis and Objectives

This introduction has thus far discussed different areas that are currently under investigation in the scientific community: pathology of MS, relevant organotypic brain slice system as models of multiple sclerosis and gene therapy. The project described build on these investigations. As described in section 1.1.8.5 current *ex-vivo* models of MS induces demyelination within one day of LPC or MOG antibody treatment and remyelinates within a week, making it an acute model. Since MS is a chronic disease, an ideal model would have a delayed remyelinating response. It was hypothesised that the controlled delivery of inflammatory TNFα and IFNγ genes into an organotypic brain slice system using a gene delivery approach would delay the remyelination process compared to current chemical or antibody induced methods. Hence, the ultimate aim of the project was to establish a MS *ex-vivo* model, showing a delayed remyelinating response with the use of a gene delivery approach. Since viral systems are associated with health risks, high production costs and a variety of other drawbacks, non-viral gene delivery vector was selected for the delivery of TNFα and IFNγ genes.

This research project had three phases. The first was to develop a non-viral gene delivery vector for efficient gene transfection. The second phase of the study was to optimize the transfection conditions for efficient non-viral gene delivery in an organotypic brain slice system. The final phase was to deliver inflammatory genes via the non-viral gene delivery vector developed in an organotypic brain slice system and to establish an *ex-vivo* model of MS. The specific objectives and hypothesis of each phase is described below.

1.3.1 Phase One (Chapter two and three)

**Overall aim:** To synthesize a pDMAEMA based hyperbranched polymer as a non-viral gene delivery vector for efficient gene transfection with minimal toxicity.
Hypothesis: Hyperbranched block copolymer with linear pDMAEMA and PEGMEMA/EGDMA can efficiently bind pDNA and can be used as a transfecting agent with minimal cytotoxicity.

Specific objectives:

- To synthesise a hyperbranched polymer with a linear pDMAEMA unit and a hyperbranched polyethylene glycol methyl ether methacrylate (PEGMEMA)/ethylene glycol dimethacrylate (EGDMA) unit.
- To characterise the hyperbranched polymer via gel permeation chromatography (GPC), nuclear magnetic resonance (NMR), size and charge analysis.
- To investigate the binding efficiency of the polymer with pDNA.
- To investigate the effect of molecular weight upon PEGylation on transfection and cellular metabolic activity.
- To investigate the effect of protonation on transfection and cellular metabolic activity.
- To investigate the effect of serum on transfection and cellular metabolic activity.
- To investigate the transfection ability and cytotoxicity of the polymer on various cell types.
- To optimize the correlation between transfection and viability as a function of pDNA concentration.
- To compare the transfection ability of the hyperbranched polymer with commercial transfecting agents.

1.3.2 Phase Two (Chapter four)

Overall aim: To optimize the transfection ability of the pDMAEMA based hyperbranched polymer in an organotypic brain slice system.

Hypothesis: Parameters such as brain slice thickness, brain regions, application of polyplexes, concentration of polyplexes, volume of polyplexes and ratio of polyplexes might have an influence in the transfection efficiency and cytotoxicity of non-viral vectors when used in an organotypic brain slice system.
Specific objectives:

- To investigate the effect of organotypic brain slice thickness (250µm and 400µm) on transfection ability and cytotoxicity.
- To investigate the effect of brain regions (cerebral and cerebellar slices) on transfection ability and cytotoxicity.
- To investigate the effect polyplex application procedure on transfection of brain slices.
- To investigate the transfection ability and cytotoxicity of the polyplex on brain slices by varying pDNA concentration (1µg, 2.5µg and 10µg).
- To investigate the transfection ability and cytotoxicity of the polyplex on brain slices by varying polyplex volume (30µl and 100µl).
- To investigate the transfection ability and cytotoxicity of polyplexes on brain slices on varying polyplex ratio (2:1, 8:1 and 15:1).
- To compare the transfection ability and cytotoxicity of the hyperbranched polymer with commercial transfecting agents and electroporation method in an organotypic brain slice system.

1.3.3 Phase Three (Chapter four)

Overall aim: To establish an ex-vivo model of multiple sclerosis via non-viral gene delivery approach.

Hypothesis: Delivery of inflammatory TNFα and IFNγ genes into an organotypic brain slice system using a hyperbranched polymer will delay demyelination compared to conventional chemically induced methods.

Specific objectives:

- To investigate the effect of TNFα and IFNγ upon transfection via non-viral gene transfection on myelin.
- To investigate the release of TNFα and IFNγ over a 21 day period.
- To compare the effect of demyelination by TNFα and IFNγ mediated non-viral gene transfection method with a chemically induced method.
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Project Overview:

Ex-vivo MS model via non-viral gene delivery

System development

Ex-vivo optimisation

Ex-vivo characterisation

Synthesis of pDMAEMA-based hyperbranched polymer

Analytical characterisation

In-vitro characterisation

- Brain regions
- Brain slice thickness
- Polyplex treatment
- Polyplex concentration
- Polyplex volume
- Polyplex ratio

TNFα and IFNγ expression and release

Demyelination
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System Development

The majority of this chapter has been previously published in:

2.1 Introduction

Gene therapy introduced in the early 1970’s [1-3], is a promising approach for the treatment of genetic disorders, mutation and intractable diseases, and its success relies on the transfection capabilities of its vectors. Three main types of gene delivery systems have been described so far: viral and non-viral vectors and the direct injection of genetic materials into the tissues using “gene guns” [4-9]. Viral vectors show high transfection efficiency. However, their application is limited by their toxicity, difficulty in pharmaceutical processing and scale-up as well as the possibility of the reversion of an engineered virus to a wild type [6]. Even though the idea of using non-viral cationic vectors as transfection agents was introduced in 1973 [10], this area was not fully explored until the mid to late 1990’s [11-12]. Non-viral vectors have increasingly drawn attention due to their large scale manufacture, transport, storage, reduced immunogenic response and the possibility of modifications and their capacity to carry large inserts [13]. In this regard, cationic polymers such as poly ethyleneimine (PEI), poly-L-lysine (PLL), poly (β-amino esters), poly 2-dimethylaminoethyl methacrylate (pDMAEMA) etc have been utilized largely because of their ability to condense DNA and form complexes (polyplex) for more efficient uptake through endocytosis.

pDMAEMA is widely used as non-viral gene delivery vector. At physiological pH pDMAEMA is partially protonated and behaves as a proton sponge and can transfect cells efficiently [14]. However, there are limits to its application due to the cationic property of these polyplexes, which leads to high levels of toxicity. Several researchers have attempted to reduce this toxicity while maintaining high transfection efficiency [15]. Poly-(ethylene glycol) (PEG) modified polymers generally exhibit good solubility and also provide a steric shield to the polyplexes from interaction with blood components [16]. These characteristics enable PEG modified polymers to circulate in the blood without aggregation for an extended period of time in the body [17]. PEG modified polymers also show reduced toxicity levels. However, maximum dosage of the polypelex which maintains high transfection with minimum toxicity is still an issue.
The synthesis of polymers with well-defined composition, architecture and functionality is absolutely crucial in determining the efficiency of the polymer as a gene delivery vector. Lack of control over the polymerisation to synthesize a well defined polymer is a major limitation of the conventional radical polymerisation [18]. With the recent progress in polymerization methods, it has become possible to design and prepare well-defined polymers by controlled/living polymerizations [19]. Atom transfer radical polymerisation (ATRP) has been widely employed for the preparation of well-defined biomaterials [20]. Wang et al introduced the deactivation enhanced atom transfer radical polymerisation (DE-ATRP) approach for the synthesis of hyperbranched copolymers [21]. In this approach a halogen–Cu$^I$/halogen–Cu$^{II}$ mixture was used to enhance the deactivation of the polymerization process which leads to a slow polymer growth. This method is advantageous for synthesis of soluble hyperbranched polymer instead of gelation.

Herein a new hyperbranched polymeric system with a linear pDMAEMA block and a hyperbranched polyethylene glycol methyl ether methacrylate (PEGMEMMA) and ethylene glycol dimethacrylate (EGDMA) block was synthesized via DE-ATRP technique and analysed for transfection capability and cytotoxicity over a range of plasmid DNA (pDNA) dosage. The rationale behind the synthesis and use of this new polymer is that the polymer structure with a linear pDMAEMA unit can efficiently bind pDNA whereas the PEG based hyperbranched unit will form the micelle-like structure in the water solution because of its amphipillicity to protect the polyplex and enhance its viability thereby reducing the cytotoxicity while maintaining its transfection efficiency at high dosages.

To prove this concept, two hyperbranched polymers one with a high PEG content (termed lpD-b-P/E 1) and another with low PEG content (termed lpD-b-P/E 2) were synthesised and compared with commercially available transfection vectors such as branched PEI (25KDa) (gold standard for non-viral gene delivery vector), partially degraded poly (amido amine) dendrimer (dPAMAM; commercial name: SuperFect®), and also with linear pDMAEMA. These polymers were tested on fibroblast cells which are generally easy to transfect and also on a primary cell line: adipose tissue derived stem cells (ADSCs) which are generally considered as “difficult to transfect cells” [22-23].
2.2 Materials and Methods

2.2.1 Chemicals

All reagents including HPLC and analytical solvents, synthesis and cell culture reagents were obtained from Sigma-Aldrich Chemical Co. (Ireland) unless otherwise stated. All reagents were used with a purity of more than 98%, and were used without further purification. dPAMAM was purchased from Qiagen, UK. A comprehensive list of materials has been supplied in Appendix A, Table A.1.

2.2.2 Synthesis of pDMAEMA

pDMAEMA was synthesized via ATRP as follows: DMAEMA (15g, 9.54x10⁻² moles), ethyl-α-bromoisobutyrate (EBiB) (0.146g, 7.51x10⁻⁴ moles), N, N, N’, N” pentamethyldiethylenetetramine (PMDETA) (0.0649g, 3.75x10⁻⁴ moles) and tetrahydrofuran (THF) (15ml) was added to a two-necked round bottom flask. After sealing the reaction flask, the mixture was purged with nitrogen for 20 minutes. Copper chloride (I) (0.0371g, 3.75x10⁻⁴ moles) was immediately added. The reaction flask was then sealed and immersed in an oil bath at 50°C. The reaction was stopped after 2.5 hours at a monomer conversion of 78%. The polymer was precipitated in hexane and was then dried under vacuum. For detailed description see Appendix (B1).

2.2.3 Synthesis of pDMAEMA - b - PEGMEMA/EGDMA

pDMAEMA -b- PEGMEMA/EGDMA was synthesized via deactivation enhanced atom transfer radical polymerization, with feed ratio 1: 90: 10. pDMAEMA (7.51x10⁻⁴ moles) was dissolved in THF (40ml) in a two-necked round bottom flask. PEGMEMA (28.538g, 6.008x10² moles), EGDMA (2.9779g, 1.502x10⁻² moles), PMDETA (0.0324g, 1.87x10⁻⁴ moles) were added thereafter. After sealing the reaction flask, the mixture was purged with nitrogen for 20 minutes. Ascorbic acid (0.0066g, 3.75x10⁻⁵ moles) was immediately added. The reaction flask was then sealed and immersed in an oil bath at 50°C. The reaction was stopped at 60% conversion for lpD-b-P/E 1 and 28% conversion for lpD-b-P/E 2. The polymer was precipitated in hexane and was dried under vacuum. Copolymer was purified using membrane dialysis (MWCO 8000; Spectra/Por®, Netherland) for three days against
distilled water and was then freeze dried and stored at -20 °C until use. For detailed description see Appendix (B2).

2.2.4 Gel Permeation Chromatography (GPC) Characterisation

Number average molecular weight ($M_n$), weight average molecular weight ($M_w$) and polydispersity ($M_w/M_n$) were obtained by Gel Permeation Chromatography (Varian 920-LC) with a RI detector. The columns (30 cm Polargel M, two in series) were eluted by dimethyl formamide (DMF) and calibrated with poly methyl methacrylate standards. All calibrations and analyses were performed at 40°C at a flow rate of 1 ml/minute. All samples were dissolved in DMF, and passed through 0.2µm filter before injection to demonstrate the absence of gelation. For detailed description of sample preparation see Appendix (C).

2.2.5 Proton Nuclear Magnetic Resonance ($^1$H NMR)

$^1$H NMR was carried out on a 300 MHz Bruker DPX300 and was analysed using MestRec™ processing software. The chemical shifts were referenced to the lock deuterated chloroform (CDCl$_3$). The final polymer composition was calculated by the integral values provided by the $^1$H NMR. For detailed description of sample preparation see Appendix (D).

2.2.6 Plasmid Propagation and Isolation

*E. coli* (Stratagene, Agilent, Germany) competent cells were transformed and selected twice in antibiotic containing LB broth and on LB agar plates. Plasmid expansion was performed as recommended in the Giga-Prep (Qiagen, UK) protocol and isolated using that kit. Plasmid purity was confirmed by UV spectroscopy (NanoDrop™ ND1000 Spectrophotometer, Thermo Scientific, UK) and gel electrophoresis. Gaussia Princeps Luciferase (GLuc) plasmid was obtained from New England Biolabs, USA and assayed with a kit from the same manufacturer. For detailed description see Appendix (E).
2.2.7 Agarose Gel Electrophoresis

pDNA binding ability of the hyperbranched polymers was examined by agarose gel electrophoresis. The polymer/pDNA complexes containing luciferase plasmid were prepared at varying polymer to pDNA weight ratios (1:1 to 15:1). 10µg of pDNA (50µl) was added to the polymer solutions (in phosphate buffered saline (PBS)) of different concentration (final volume 100µl). The mixtures were then allowed to incubate at room temperature for 30 minutes. Thereafter, the complexes were loaded into individual wells of 0.7% agarose x TAE gel containing 10µl SYBR® safe (Invitrogen, Ireland) and were electrophoresed at 100V for 90 minutes. Naked pDNA was used as the control. The gel was then visualized on a UV transilluminator (Syngene, UK). For detailed description see Appendix (F).

2.2.8 Polyplex Stability

To investigate the ability of the polymer to protect pDNA from endonucleases, Deoxyribonuclease I (DNase I) protection assay was performed. For this, the polymer/pDNA complexes containing luciferase plasmid were prepared at various polymer to pDNA weight ratios (1:1 to 15:1) as described previously. These polyplexes were subjected to 20 IU of DNaseI (2 IU to 1µg of DNA) in DNase I buffer (400mM Tris HCL (PH 8), 100mM MgSO₄ and 10mM CaCl₂) for 30 minutes at 37 ºC. The reaction was terminated with 4µl of 0.5M EDTA (pH8) and then placed on ice for 10 minutes. The samples were then analyzed by 0.7% agarose gel electrophoresis. To determine polyplex stability over time, the polymer/pDNA complexes containing luciferase plasmid were prepared at various polymer to pDNA weight ratios (1:1 to 15:1) described previously. The samples were stored in PBS at 4ºC and analyzed by 0.7% agarose gel electrophoresis after 1, 7 and 14 days.

2.2.9 Particle Size and Zeta Potential

Polymer/pDNA complexes were prepared as described previously, with weight ratios (1:1 to 15:1). After 30 minutes of incubation, the complex solutions were diluted eleven times in PBS (1X). Prior to analysis, the diluted complex solutions were allowed to stabilize for 30 minutes. The particle size and charge of the polymer/pDNA complexes were measured using Zetasizer Nano-ZS90 (Malvern Instrument Ltd., UK). The particle size and zeta potential measurements were
repeated for five runs for each sample, and the data were reported as the average of five readings.

2.2.10 Transmission Electron Microscopy (TEM)

TEM measurements were performed using a Hitachi H-7500 microscope. The TEM samples were prepared by depositing a diluted the polymer/pDNA complexes on a carbon-coated copper grid, followed by air drying.

2.2.11 Cell Culture

The transfection ability and effect of polymers on cellular metabolic activity of the synthesised polymer was evaluated on two different cell types. Human fetal foreskin fibroblast cells were purchased from Centre of Applied Microbiology and Research (CAMR, UK). ADSCs were extracted from rabbit adipose tissue as follows. Adipose tissue was digested by collagenase type I at 0.025% for one hour under agitation at 37°C. The enzymatic reaction was stopped by addition of complete medium (Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), 1% Penicillin Streptomycin (P/S)). The stromal fraction was collected by centrifugation five minutes at 1,200 rpm, re-suspended and filtered on a cell strainer 70 mm (Fisher, Ireland). After 24 hour incubation at 37°C in a humidified atmosphere of 5% CO₂, cells were washed to eliminate the contaminant cells (blood cells and adipocytes). Medium was changed every two to three days and cells were maintained sub-confluent. Adipogenic, chondrogenic and osteogenic differentiation assays were conducted to confirm the nature of the extracted cells. For detailed description see Appendix (G).

2.2.12 Cell Metabolism: alamarBlue™ Assay

Cellular metabolic activity was assessed by the alamarBlue™ assay, wherein dye reduction was considered proportional to the metabolic activity of the cells. Cells were seeded with a cell density of 6,500 cells/well for fibroblasts, 6,000 cells/well for ADSC cells in a six well tissue culture plate and grown overnight. Cells were washed and polymers with different concentrations (5, 25, 50, 100 µg/ml) were loaded on the cell and incubated for 48 hours in medium containing serum. alamarBlue™ assay was conducted according to the manufacturer’s instructions.
After three-hour incubation, absorbance at 550 and 595 nm was determined on a microplate reader (Varioskan™ Flash - 4.00.53, Thermo Scientific, Finland) and the percentage reduction of the dye was calculated. alamarBlue™ assay was also performed on fibroblasts (6000 cells/well) and ADSCs (6500 cells/well) after transfection. For detailed description of alamarBlue™ assay see Appendix (H).

2.2.13 Cell Proliferation: PicoGreen™ Assay

PicoGreen™ dsDNA quantitation kit (Invitrogen, Ireland) was used to measure cell proliferation by quantifying the amount of double-stranded DNA (dsDNA) in solution. Cells were washed twice with Hanks Balanced Salt Solution (HBSS) after alamarBlue™ assay, 110μl of DNAs free water was added and stored at -80°C until use. Cells were later freeze thawed at least three times. Samples were then aliquoted into a 9six well plate in triplicate and incubated with 100μl of PicoGreen™ working solution, 1:200, for five minutes at room temperature, protected from light. Fluorescence of the whole sample mixture was measured at excitation and emission wavelengths of 485 and 518 nm, respectively, on a micro plate reader (Varioskan™ Flash - 4.00.53, Thermo Scientific, Finland). For detailed description of PicoGreen™ assay see Appendix (I).

2.2.14 In-Vitro Gene Transfection

The in-vitro gene transfection ability of the polymer/pDNA complexes was assessed via G-luciferase activity. Under usual cell culture sterile conditions, cells were seeded in a 96 well plate with a cell density of 6,500 cells/well for fibroblasts and 6,000 cells/ well for ADSCs for 24 hours before the transfection experiment. pDNA (1μg per well) was mixed with 50μl polymer solutions with different weight ratios from 1:1 to 20:1. The mixture was then incubated for 30 minutes at room temperature to allow the formation of polymer/pDNA complexes, and 150μl of serum free medium was added to the polyplex solution. The cells were washed with HBSS. Complexes were then added into the well and incubated for four hours. The media was then removed and replaced with fresh medium with serum and was then incubated for 48 hours at 37°C. After 48 hours at 37°C, the cells were analysed for G luciferase activity. For detailed description of G luciferase Assay see Appendix (J).
2.2.15 Statistics

Results are expressed as mean ± standard deviation. Statistical significance was assessed using the analysis of variance (ANOVA). \( p \) values of <0.05 were considered statistically significant. In all studies, the minimum sample size was three.

2.3 Results and Discussion

2.3.1 Synthesis of pDMAEMA)-b-PEGMEMA/EGDMA

Linear pDMAEMA was synthesised via normal ATRP (Figure 2.1a). Then, this linear pDMAEMA was used as a macro initiator for DE-ATRP of PEGMEMA and EGDMA to produce a PEG based hybrid polymer with a hyperbranched structure (Figure 2.1b). Two hyperbranched polymers, one with a high PEG content (lpD-b-P/E 1), and another with low PEG content (lpD-b-P/E 2), were synthesized. A small amount of reducing agent was added, thus being different from DE-ATRP technique reported by Wang et al [21]. This was to facilitate a controlled polymer growth without gelation over a period of time [24]. Ascorbic acid was used as a reducing agent for the production of active \( \text{Cu}^1 \) species, while \( \text{Cu}^{11} \) remaining in the system controls growth of the polymer chain.

2.3.2 Gel Permeation Chromatography (GPC)

The molecular weights of the polymers were characterised by GPC. GPC clearly demonstrates the controlled polymer growth of lpD-b-P/E 1 over a period of time (Figure 2.2). Molecular weight of linear pDMAEMA was restricted to 8000 Da. The molecular weight lpD-b-P/E 1 was around 38000 Da and that of lpD-b-P/E 2 was around 14000 Da. Polydispersity index (PDI) of lpD-b-P/E 1 and lpD-b-P/E 2 were 1.17 and 1.19 respectively. Low PDI in both cases demonstrates the controlled growth of the polymer chain (Table 2.1).
Figure 2.1 Schematic representation of deactivation enhanced – ATRP showing the activated and deactivated routes, controlling the formation of a hyperbranched structure (a and b) and polyplex formation (c).
Figure 2.2 GPC trace showing the controlled growth of lpD-b-P/E 1 over time (2, 3, 6, 10 hours).
Table 2.1 Weight average molecular weight (Mw), number average molecular weight (Mn) and PDI of pDMAEMA and hyperbranched polymer (lpD-b-P/E 1 and lpD-b-P/E 2) determined by GPC.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mn(Da)</th>
<th>Mw (Da)</th>
<th>PDI</th>
<th>DMAEMA: PEGMEMA:EGDMA (molar ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>block 1 (pDMAEMA)</td>
<td>7500</td>
<td>8000</td>
<td>1.07</td>
<td>1:0:0</td>
</tr>
<tr>
<td>block 2 (lpD-b-P/E 1)</td>
<td>37900</td>
<td>44300</td>
<td>1.17</td>
<td>1:12.215:1.104</td>
</tr>
<tr>
<td>block 2 (lpD-b-P/E 2)</td>
<td>13700</td>
<td>16300</td>
<td>1.19</td>
<td>1:0.879:1.12</td>
</tr>
</tbody>
</table>

Mn – Number average molecular weight, Mw – Weight average molecular weight, DMAEMA - 2-dimethyl-aminoethylmethacrylate, pDMAEMA – Poly (2-dimethylaminoethylmethacrylate), PEGMEMA - Poly ethylene glycol methyl ether methacrylate, EGDMA – Ethylene glycol dimethacrylate, lpD-b-P/E - Poly (2-dimethyl-aminoethylmethacrylate)-block-poly ethylene glycol methyl ether methacrylate/ethylene dimethacrylate
2.3.3 Proton Nuclear Magnetic Resonance (\textsuperscript{1}H NMR)

The chemical structure of final hyperbranched polymer was determined using \textsuperscript{1}H NMR (Figure 2.3). The characteristic peaks at chemical shifts of 6.1 and 5.6 ppm are attributed to the vinyl functional groups in the hyperbranched polymer. The degree of branching within the polymer structure was analysed via the following equations (Table 2.1):

- DMAEMA = integrals of \( j/6 \)
- PEGMEMA = integrals of \( l/30 \)
- Linear EGDMA = integrals of \( d \) or \( e \)
- Branched EGDMA = \( \{[\text{integrals of (c+h+k)]} - [2^* \text{integrals of (j/6)}] - [2^* \text{integrals of (l/30)}] - [4^*\text{integrals of d or e}]/4 \)

The molar ratio of pDMAEMA to PEGMEMA to EGDMA for lpD-b-P/E 1 was found to be 1:12.215:1.104 with 1.054\% of branched EGDMA (Figure 2.3a) and 1:0.879:1.12 with 33.3\% of branched EGDMA for lpD-b-P/E 2 (Figure 2.3b). At the early stage of the reaction, EGDMA adds up to the polymer block leading to high branching ratio.

2.3.4 Agarose Gel Electrophoresis

Both lpD-b-P/E 1 and lpD-b-P/E 2 are able to form complexes with pDNA via electrostatic interactions (Figure 2.1c). G luciferase (GLuc) pDNA binding ability of the hyperbranched polymers was examined by agarose gel electrophoresis and was compared with controls at their optimum weight ratios, e.g. PEI and dPAMAM to pDNA at 2:1 and 8:1 (w/w ratio) respectively. It was seen that lpD-b-P/E 1 complexed with GLuc pDNA at 10:1 (w/w ratio), whereas lpD-b-P/E 2 complexed at 2:1 (w/w ratio) (Figure 2.4) and linear pDMAEMA as early as 1:1 (w/w ratio) (Figure 2.5c). lpD-b-P/E 1 has a larger PEG based unit than lpD-b-P/E 2. Therefore at a particular w/w ratio, the number of free cationic groups present in lpD-b-P/E 1 is less than lpD-b-P/E 2, which might be the reason why both the polymers complex at different w/w ratios. Since linear pDMAEMA has no PEG unit attached to it, the number of free cations present at a particular weight ratio is higher than lpD-b-P/E 1 and lpD-b-P/E 2.
Figure 2.3 $^1$HNMR spectra of (a) lpD-b-P/E 1 and (b) lpD-b-P/E 2. The characteristic peaks at chemical shifts of 6.1 and 5.6 ppm are attributed to the vinyl functional groups in the hyperbranched polymer.
Figure 2.4 pDNA binding ability of the hyperbranched polymers with w/w ratios (1:1 to 15:1) compared to PEI (2:1) and dPAMAM (8:1) at their optimum weight ratios (a) lpD-b-P/E 1 complexed at 10:1 w/w ratio (b) lpD-b-P/E 2 complexed as early as 1:1 w/w ratio.
**Figure 2.5** Stability of against DNase I degradation (a) lpD-b-P/E 1 (b) lpD-b-P/E 2 (c) linear pDMAEMA. Naked pDNA was used as the control. Results indicate that lpD-b-P/E 1 was stable from 8:1 w/w ratio, lpD-b-P/E 2 as early as from 1:1 w/w ratio and linear pDMAEMA from 2:1 w/w ratio against degradation by endonucleases. In contrast, the naked pDNA (control) was fully degraded by DNase I in 30 minutes.
This is the reason why linear pDMAEMA complexes as early as 1:1 w/w ratio. It should be noted that polymer/plasmid weight ratios were used in this study for increased accuracy over the commonly used N/P quotation because of differences arising in $M_n$ values across varying GPC instruments/calibration. Moreover, w/w ratio is more relevant to this particular study as the maximum safe polyplex loading was investigated so that the final administration weight of the polyplex can be assessed.

2.3.5 Polyplex Stability

The DNase I protection assay showed the capability of lpD-b-P/E 1 and lpD-b-P/E 2 and linear pDMAEMA to protect pDNA from degradation by endonucleases (Figure 2.5). For lpD-b-P/E 1 polyplexes, the stability against DNase degradation increased with increasing w/w ratio (Figure 2.5a). This might be because at lower w/w ratios complexes formed are weak and degrade easily under such an environment. However, lpD-b-P/E 2 was able to form tight complexes even at lower weight ratios (Figure 2.5b) showing no degradation under DNase I. In contrast, the naked pDNA which was used as a control was fully degraded by DNase I in 30 minutes. This suggests that both lpD-b-P/E 1 (at high w/w ratios) and lpD-b-P/E 2 (at all ratios) can form stable complexes and can circulate in blood for a longer time without degradation.

The polyplex stability over periods of 2, 7 and 14 days was also determined (Figure 2.6) and both the polymers lpD-b-P/E 1 and lpD-b-P/E 2 were seen to maintain their stability for two weeks. The stability of these polyplexes is critical to ensure safe delivery of the therapeutic plasmid to its target in-vivo. Although linear pDMAEMA formed tight complexes even at lower weight ratios (Figure 2.5c) showing no degradation under DNase I, the polyplexes were not stable in PBS for two weeks (Figure 2.6). This suggests that PEG based hyperbranched unit acts like a protective shield to the polyplexes against salt thereby increasing their stability.

2.3.6 Charge and Size Analysis

Polyplexes were further characterized for size and charge analysis using zetasizer. The surface charge of lpD-b-P/E 1(Figure 2.7) remained constant at different weight ratios (5:1 to 20:1 w/w ratio). High degree of PEGylation of lpD-b-P/E 1 shielded the polyplex surface charge leading to reduced zeta potential.
**Figure 2.6** Polyplex stability of lpD-b-P/E 1, lpD-b-P/E 2 and linear pDMAEMA over time (a) one day (b) seven day and (c) 14 days. Results show that lpD-b-P/E 1 and lpD-b-P/E 2 complexes were stable up to 14 days while linear pDMAEMA complex degraded after seven days of incubation in PBS.
Figure 2.7 Particle size and zeta potential of lPD-b-P/E 1, lPD-b-P/E 2 and linear pDMAEMA at different polymer to pDNA (w/w) ratios. Primary y axis corresponds to the charge and secondary y axis corresponds to the size of the polyplexes. Results indicate that the charge of lPD-b-P/E 1 stayed constant at higher weight ratios (from 5:1 w/w ratio), while the charge of lPD-b-P/E 2 and linear pDMAEMA increased with increasing w/w ratio. Size of the polyplexes decreased with increased polymer to pDNA w/w ratio for lPD-b-P/E 1, lPD-b-P/E 2 and linear pDMAEMA.
However, the surface charge of lpD-b-P/E 2 and linear pDMAEMA increased with increasing weight ratio demonstrating increased electrostatic interaction between the polymer and the plasmid leading to a stronger bond formation.

The size of the polyplexes decreased as the polymer to pDNA weight ratio increased because the higher the w/w ratio the stronger the bond (Figure 2.7). The size of linear pDMAEMA polyplexes was much larger than lpD-b-P/E 1 and lpD-b-P/E 2. In contrast to linear pDMAEMA, hyperbranched polymers (lpD-b-P/E 1 and lpD-b-P/E 2), forms micelle like structure when complexed with a plasmid (Figure 2.1c). This is the reason why hyperbranched polymers showed reduced size when compared to linear pDMAEMA.

2.3.7 Transmission Electron Microscopy (TEM)

Size and shape of the polyplexes were also analysed via Transmission Electron Microscopy (TEM). Figure 2.8 demonstrates the decrease in size of the polyplexes as the ratio increases. At 10:1 w/w ratio, the size of lpD-b-P/E 1 was around 200-250nm, while as the size of lpD-b-P/E 2 was found to be around 50-80nm (Figure 2.8e), which also supports the results from zetasizer.

2.3.8 Cell Metabolism: alamarBlue™ Assay

The effect of polymers on cellular metabolic activity of fibroblasts and ADSCs was analysed via alamarBlue™ reduction method and compared to PEI and dPAMAM (Figure 2.9). It was seen that at all concentrations both the hyperbranched polymers showed a significantly higher cellular metabolic activity when compared to PEI and at higher concentration (100µg/ml), and significantly higher than dPAMAM. However, cellular metabolic activity of lpD-b-P/E 2 was significantly reduced at a higher concentration (100µg/ml) (Figure 2.9) when compared to lpD-b-P/E 1, which indicates that high PEGylation for the polymer can significantly decrease the cytotoxicity of the polymer.

2.3.9 Cell Proliferation: PicoGreen™ Assay

Effect of hyperbranched polymer on cells was further analysed via cell proliferation assay (PicoGreen™) (Figure 2.10).
Figure 2.8 TEM micrograph of polyplexes lpD-b-P/E 1 with different polymer to pDNA weight ratios (magnification 20000x) a) 5:1 b) 8:1 c) 10:1 d) 15:1 and (e) lpD-b-P/E 2 with polymer to pDNA weight ratios 10:1 (magnification 50000x). Results show that the polyplexes are spherical in shape with homogeneous dispersion and also the size of the polyplexes decrease with increasing polymer to pDNA w/w ratios.
Figure 2.9 Effect of lpD-b-P/E 1 and lpD-b-P/E 2 on cellular metabolic activity in (a) fibroblasts, (b) ADSCs after 48 hours of treatment. PEI and dPAMAM were used as controls. Data plotted shows mean ± standard deviation (n=3) * (p<0.05). Results indicate that lpD-b-P/E 1, lpD-b-P/E 2 and dPAMAM does not have an effect on cellular metabolic activity up to 50µg/ml concentration on fibroblasts and ADSCs. In contrast, PEI was toxic from 5µg/ml concentration.
Figure 2.10 Effect of lpD-b-P/E 1 and lpD-b-P/E 2 on cell proliferation in (a) fibroblasts, (b) ADSCs after 48 hours of treatment. Data plotted shows mean ± standard deviation (n=3) *(p<0.05). Results indicate that lpD-b-P/E 1 and lpD-b-P/E 2 did not have an effect on DNA concentration up to 50µg/ml concentration on fibroblasts and 25µg/ml concentration on ADSCs. In contrast, PEI was toxic from 25µg/ml concentration on fibroblast and 5µg/ml concentration on ADSCs and dPAMAM from 25µg/ml concentration on both cell lines.
The results show that both lpD-b-P/E 1 and lpD-b-P/E 2 had no impact on cell proliferation in either of the cell lines. Both PEI and dPAMAM significantly reduced the DNA concentration above 5µg/ml concentration when compared to control cells with no treatment. lpD-b-P/E 1 and lpD-b-P/E 2 by contrast had less impact on DNA concentration levels compared to PEI and dPAMAM suggesting that they had less impact on cell proliferation.

2.3.10 In-Vitro Gene Transfection

Transfection capability of the hyperbranched polymers was assessed using fibroblast cells and ADSCs. To analyse transfection efficiency for the polymer, G-Luc transfection assay kit was used as per protocol. A wide range of weight ratios of polyplexes was analysed for each polymer and the optimum weight ratio for each polymer was determined (Figure 2.11). This step is crucial as the optimum weight ratio of the polymer is different on different cells. PEI showed its optimal transfection at 2:1 w/w ratio for fibroblasts and 1:1 w/w ratio for ADSCs, while dPAMAM showed its optimum transfection at 8:1 (both in fibroblast and ADSCs). lpD-b-P/E 1 showed its optimal transfection at 8:1 w/w ratio in fibroblasts and 10:1 w/w ratio in ADSCs. lpD-b-P/E 2 had its optimal transfection at 10:1 w/w ratio (in both cell lines). Fluorescent microscopic images of ADSCs also confirmed transfection with lpD-b-P/E 1 and lpD-b-P/E 2 (Figure 2.12).

In order to determine the maximum amount of pDNA that the polymer could deliver for a specific number of cells without affecting both transfection and viability, the optimal weight ratio was selected for each polymer, and the transfection capability and cellular metabolic activity was determined as a function of increasing pDNA concentration (Figure 2.13). lpD-b-P/E 1 showed no toxic effects at higher pDNA concentration in either cell lines without compromising transfection efficiency. lpD-b-P/E 1 showed at least a fifteen fold higher cellular metabolic activity when compared to PEI, two fold higher when compared to dPAMAM, and six fold higher than linear pDMAEMA. On the other hand, lpD-b-P/E 2 showed a significantly higher level of toxicity (p<0.05) when compared to vehicle treated cells, but nine fold lower than PEI, 1.5 fold lower than dPAMAM and four to eight fold lower than linear pDMAEMA. PEI is the gold standard among the cationic polymeric systems reported.
Figure 2.11 Transfection capability of lpD-b-P/E 1, lpD-b-P/E 2 compared to PEI, and dPAMAM after 48 hours (polymer/pDNA weight ratio 1:1 to 20:1) (a) fibroblasts cells (b) ADSCs. Data plotted shows mean + standard deviation (n=3). PEI showed its optimal transfection at 2:1 w/w ratio for fibroblasts and 1:1 w/w ratio for ADSCs, while dPAMAM showed its optimum transfection at 8:1 (both in fibroblast and ADSCs). lpD-b-P/E 1 showed its optimal transfection at 8:1 w/w ratio in fibroblasts and 10:1 w/w ratio in ADSCs. lpD-b-P/E 2 had its optimal transfection at 10:1 w/w ratio (in both cell lines).
Figure 2.12 Fluorescent microscopic image of ADSCs after transfection with (a) lpD-b-P/E1 (b) lpD-b-P/E2 (c) PEI and (d) dPAMAM complexed with GFP plasmid. (GFP expression (green) and nucleus staining with DAPI (blue).
Figure 2.13 Transfection ability of lpD-b-P/E 1 and lpD-b-P/E 2 at their optimal polymer to pDNA weight ratios as a function of cellular metabolic activity and pDNA concentration compared to PEI, dPAMAM and linear pDMAEMA (a) fibroblasts cells (b) ADSCs. Data plotted shows mean ± standard deviation (n=3). Results suggest that lpD-b-P/E 1 and lpD-b-P/E 2 maintains its transfection capability even at high doses of pDNA without compromising its cellular metabolic activity in contrast to commercial transfecting agents.
dPAMAM, is widely used as a transfecting agent. Both these polymers showed toxicity issues at high doses. PEI showed less than 20% cellular metabolic activity above 10µg pDNA concentration, which resulted in no transfection at these doses. dPAMAM showed less than 50% cellular metabolic activity at 50µg pDNA concentration. Linear pDMAEMA with no modification showed minimal transfection at all pDNA concentrations. Large polyplex size of linear pDMAEMA (Figure 2.7c) (around 400nm at its optimal transfection w/w ratio ie 20:1), makes it difficult to internalize efficiently leading to low transfection. Also, linear pDMAEMA showed less than 30% cellular metabolic activity above 25µg pDNA concentration, which also resulted in reduced transfection level. Both hyperbranched polymers showed above 70% cellular metabolic activity even at 50µg pDNA concentration. This might be due to this unique structure i.e. with a linear pDMAEMA unit and a PEG based hyperbranched unit. The linear pDMAEMA with the tertiary amine group could easily bind to the phosphate groups of pDNA and form a strong complex which will lead to high transfection efficiency. PEG based hyperbranched unit, by contrast, enhanced the cellular metabolic activity thereby reducing the cytotoxicity while maintaining its transfection efficiency at high doses.

The transfection efficiency of PEI and dPAMAM complexes does not increase proportionally with increasing pDNA dose. These polymers increase toxicity with increasing dose, which indirectly affects their transfection ability. Both lpD-b-P/E 1 and lpD-b-P/E 2 complexes do not affect the toxicity at high doses as compared to PEI and dPAMAM, and therefore lpD-b-P/E 1 and lpD-b-P/E2 maintain their transfection efficiency even at a high dose. This polymeric system can prove favourable for sustained gene therapy applications where long-term and a high DNA dose is required. High doses of polyplex can be loaded into carriers for controlled release.

2.4 Conclusion

A unique structure comprising of a linear pDMAEMA block and hyperbranched PEGMEMA and EGDMA block was synthesized using DE-ATRP approach. This polymer structure is unique as the pDMAEMA unit was able to bind pDNA very efficiently while the hyperbranched unit acted as a shield. This enabled the polymer to maintain high transfection levels without sacrificing cellular viability.
Furthermore, unlike the situation with commercially available transfection agents, the cellular was not affected at high doses. This polymeric system can prove favourable for sustained gene therapy applications, where long-term and a high DNA dose is required.
2.5 References


Chapter 3

Transfection Optimization and Cell Viability *In-Vitro*

The majority of this chapter has been submitted for publication in:

3.1 Introduction

In Chapter two the ability of hyperbranched poly 2-dimethylaminoethyl methacrylate (pDMAEMA) based polymers (lpD-b-P/E 1 and lpD-b-P/E 2) to bind plasmid DNA (pDNA) and transfect fibroblasts and adipose tissue derived stem cells (ADSCs) with minimal toxicity was determined. A more thorough study was performed in this phase. Several studies have shown the effect of molecular weight on transfection efficiency of the polymer [1-8]. Increased molecular weight by adding more PEG unit to the polymer reduces toxicity but adversely affects its transfection. Therefore, it is crucial to determine the optimal PEGylation of the polymer which balances both transfection and toxicity. Previously in Chapter two, hyperbranched pDMAEMA-based polymer with two different molecular weights: lpD-b-P/E 1 (high PEGylation) with 38000Da and lpD-b-P/E 2 (low PEGylation) with 14000Da was examined. In this study another polymer (lpD-b-P/E 3) with a medium PEGylation of molecular weight 24000Da was synthesized. This polymer was compared to previously developed lpD-b-P/E 1 and lpD-b-P/E 2 to determine the optimum molecular weight upon PEGylation for efficient gene delivery with minimal toxicity.

The usefulness of polyamines, particularly for gene delivery, is largely dependent on their ability to possess cationic charges due to the protonation of the amine nitrogen. In comparison to poly ethylenimine (PEI), pDMAEMA has only tertiary amine groups and the charge density is lower than PEI. DMAEMA shows an average pKa of 7.5 [9]. This indicates that at physiological pH, around 50% of side groups of pDMAEMA is positively charged for polyanion complexation [10]. pDNA is condensed and protected by the interaction between positively charged pDMAEMA and negatively charged DNA. pDNA/pDMAEMA complexes (known as polyplexes) are internalized by cell via endocytosis (membrane engulfment mechanism) [11]. It is well accepted that the pH-dependent shift in the protonation state of polyamines plays an important role in enhancing the release of the polyplexes from endosomes into the cell cytosol [12]. “Proton sponge effect” postulates that during the post endocytic trafficking process when the pH of the endosome is lowered from 7.4 to about 5.0, the entry of the H⁺ and Cl⁻ ions into the endosome is amplified by the absorption of protons by the polyamine. This effect increases the osmotic
pressure inside endosomes leading to water entry and ultimately vesicle swelling and bursting [13-16]. Previously Wetering et al proposed that pDMAEMA might have a proton sponge effect similar to PEI and transfects cells by the same mechanism [10]. However, Jones et al later demonstrated that the ability of pDMAEMA to facilitate transfection of cells may not arise from an endosomal disruptive activity and thus that the “‘proton sponge hypothesis’” is probably not applicable [11]. In this study, effect on transfection and cellular metabolic activity of lpD-b-P/E 3 was at various protonation (pH 3.5 (fully protonised), pH 5 (partially protonised) and pH 7 (physiological pH: partially protonised) was studied.

The transfection ability of cationic non-viral gene delivery vectors in-vitro mostly depends on its structure, transfection medium and cell line. High transfection ability of cationic non-viral vectors in-vitro and low transfection in-vivo is due either to serum instability or due to high clearance rate [17]. In-vitro transfection protocols generally require cells that are exposed to polyplexes under serum free conditions [18-23]. Dulbecco’s Modified Eagle Medium (DMEM) contains 6400mg/L NaCl, 3700mg/L NaHCO3 and other positive as well as negative ions, which interacts with peptides or DNA for binding of their counterparts, thereby weakening the electrostatic interaction between peptide and DNA molecules, leading to loosely packed peptide/DNA complexes [24]. Some researchers have modified the structure of the cationic vectors and have performed transfection studies under serum conditions in-vitro to avoid the serum instability factor in-vivo [25-30]. Here, in this phase, the effect of serum and serum free conditions on transfection ability of lpD-b-P/E 3 on various cell types: fibroblasts (3T3 cell line), nucleus pulposus (NP) cells, annulus fibrosus (AF) cells, neu-7 cells and in primary astrocytes was studied. 3T3 cell line is a standard fibroblast cell line and is easy to transfect [31-34]. Transfection of intervertebral disc cells: NP and AF cells are mostly performed either by viral transfection methods or by non-viral transfection such as electroporation, micro bubble enhanced ultrasound gene delivery and by the use gene guns [35-40]. Very few researchers have reported on the cationic polymer based non-viral gene transfection [41]. Neu-7 cell lines are derived from astrocytes and are neuro inhibitory [42]. Astrocytes are star shaped glial cells and are the majority cells in the central nervous system. Astrocytes are involved in a wide range of biological
functions such as physical structuring of the brain, metabolism, and synaptic functioning, as well as in responding to pathological insults [43]. Hence they are attractive targets for gene therapy [44]. In this study the transfection ability of the hyperbranched polymer on NP cells, AF cells, Neu-7 cells and astrocytes was optimized. Results were compared with commercially available transfection vectors such as branched PEI (25000Da) (gold standard for non-viral gene delivery vector) and partially degraded poly (amido amine) dendrimer (dPAMAM; commercial name: SuperFect®).

3.2 Materials and Methods

3.2.1 Chemicals

All reagents including HPLC and analytical solvents, synthesis and cell culture reagents were obtained from Sigma-Aldrich Chemical Co.(Ireland) unless otherwise stated. dPAMAM was purchased from Qiagen, UK. Lipofectin® reagent and Lipofectamine 2000® reagent were purchased from Invitrogen (Ireland). A comprehensive list of materials has been supplied in Appendix A, Table A.1.

3.2.2 Synthesis of Poly (2-dimethyl-aminoethylmethacrylate) (pDMAEMA)-block-Poly Ethylene Glycol Methyl Ether Methacrylate (PEGMEMA)/Ethylene Glycol Dimethacrylate (EGDMA)

lpD-b-P/E 1, lpD-b-P/E 2 and lpD-b-P/E 3 were synthesized as described in section 2.2.3. The reaction of lpD-b-P/E 3 was terminated when a molecular weight of 24000Da was achieved. The polymer then dialyzed against distilled water and was then freeze dried. For detailed description see Appendix (B2).

3.2.3 Protonation

lpD-b-P/E 3 was dissolved in distilled water at a concentration of 1g/ L. Equimolar amounts of HCl according to PDMAEMA units were added to both polymer solutions to obtain a fully protonated polymer. For other pH values, HCl solutions were prepared to adjust the pH value.
3.2.4 Gel Permeation Chromatography (GPC)

Number average molecular weight ($M_n$), weight average molecular weight ($M_w$) and polydispersity ($M_w/M_n$) were obtained by Gel Permeation Chromatography (Varian 920-LC) with a RI detector. The columns (30 cm Polargel M, two in series) were eluted by dimethyl formamide (DMF) and calibrated with poly methyl methacrylate standards. All calibrations and analyses were performed at 40 °C at a flow rate of 1 ml/minute. lpD-b-P/E 3 was dissolved in DMF, and passed through 0.2µm filter before injection to demonstrate the absence of gelation. For detailed description of sample preparation see Appendix (C).

3.2.5 Plasmid Propagation and Isolation

*E. coli* (Stratagene, Agilent, Germany) competent cells were transformed and selected twice in antibiotic containing LB broth and on LB agar plates. Plasmid expansion was performed as recommended in the Giga-Prep (Qiagen, UK) protocol and isolated using that kit. Plasmid purity was confirmed by UV spectroscopy (NanoDrop™ ND1000 Spectrophotometer, Thermo Scientific, UK) and gel electrophoresis. Gaussia Princeps Luciferase (GLuc) plasmid (New England Biolabs USA) and ptdTomato N1 (Clontech USA) were assayed with a kit from the same manufacturer. For detailed description see Appendix (E).

3.2.6 Cell Culture

The transfection ability and effect of polymers on cellular metabolic activity of the synthesised polymer was evaluated on two different cell types. Human fetal foreskin fibroblast cells were purchased from Centre for Applied Microbiology and Research (CAMR, UK). NP cells and AF cells were extracted from five-months old calf bovine fresh tail as described previously [45]. Neu -7 cell line were a kind donation from Prof. J Fawcett, Cambridge UK [42]. Primary astrocytes were extracted from rat brain tissue as described in Appendix (K). Table 3.1 describes the media components for the various cell types.
Table 3.1 Media components for various cell types

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>fibroblasts</td>
<td>DMEM + 2mM Glutamine +10% FBS + 1% Antibiotic</td>
</tr>
<tr>
<td>NP</td>
<td>DMEM + 2mM Glutamine + 10% FBS + 1% Antibiotic</td>
</tr>
<tr>
<td>AF</td>
<td>DMEM + 2mM Glutamine + 10% FBS + 1% Antibiotic</td>
</tr>
<tr>
<td>neu-7</td>
<td>DMEM + 2mM Glutamine + 10% FBS + 1% Antibiotic</td>
</tr>
<tr>
<td>astrocyte</td>
<td>DMEM/F12 suppliment+2mM Glutamine + 10% FBS + 1% Antibiotic</td>
</tr>
</tbody>
</table>

DMEM - Dulbecco's modified eagle medium, FBS – Fetal bovine serum, NP – Nucleus pulposus, AF – Annulus fibrosus
3.2.7 In-Vitro Gene Transfection

The in-vitro gene transfection ability of the polymer/pDNA complexes was assessed via G-luciferase activity. Under usual cell culture sterile conditions, cells were seeded in a 9six well plate with a cell density of fibroblasts (6,500 cells/well), neu-7 (6,500 cells/well), NP (6,500 cells/well), AF cells (6,500 cells/well) and primary astrocytes (10000 cells/well) for 24 hours before the transfection experiment. pDNA (1µg or 50µg per well, 50µl) was mixed with 50µl polymer solutions at their optimal w/w ratios (lpD-b-P/E 1 at 10:1, lpD-b-P/E 2 and lpD-b-P/E 3 at 8:1 w/w ratio). The mixture was then incubated for 30 minutes at room temperature to allow the formation of polymer/pDNA complexes, and 150µl of serum medium or serum free medium was added to the polyplex solution. The cells were washed with Hanks Balanced Salt Solution (HBSS). Complexes in serum medium were added into the wells and incubated at 37°C for 48 hours. For complexes in serum free medium, medium was removed after four hours and replaced by serum medium and incubated for 44 hours at 37°C. After 48 hours at 37°C, the cells were analysed for G luciferase activity. For detailed description of G luciferase assay see Appendix (J). For qualitative analysis astrocytes were transfected with lpD-b-P/E 3 complexed with ptdTomato N1 plasmid at 8:1 w/w ratio under serum condition for 48 hours.

3.2.8 Cell Metabolism: alamarBlue™ Assay

Cellular metabolic activity was assessed by the alamarBlue™ assay, wherein dye reduction was considered proportional to the metabolic activity of the cells. alamarBlue™ assay was also performed on fibroblasts (6,500 cells/well), neu-7 (6,500 cells/well), NP (6,500 cells/well), AF cells (6,500 cells/well) and primary astrocytes (10000 cells/well) after transfection. The assay was performed on cells according to the manufacturer’s instructions. After three-hour incubation, absorbance at 550 and 595nm was determined on a microplate reader (Varioskan™Flash - 4.00.53, Thermo Scientific, Finland) and the percentage reduction of the dye was calculated. For detailed description of alamarBlue™ assay see Appendix (H).

3.2.9 Labelling by Rhodamine B

129.8mg of lpD-b-P/E 3 (Molecular weight 24000, Moles 5.409X10⁻⁶) and 3.86mg of Rhodamine B (Molecular weight 714.77, Moles 5.409X10⁻⁶) was dissolved in
5ml THF in a 25 ml flask equipped with a magnetic stir bar. The flask was then fitted with a rubber cork and degassed by bubbling argon through it for 15 minutes. The reaction was stirred at 50°C for 48 hours. It was then dialysed in distilled water until the water became clear. The labelled polymer was then freeze dried and stored at -20°C until use. For detailed description see Appendix (L).

3.2.10 Uptake Study

GLuc plasmid was labelled with Cy5 (Mirus) as per manufacturer’s instruction. 500µl of media containing 10,000 astrocytes/well were seeded onto the base of chamber slides (Nunc, Thermo Scientific, UK) 24 hours prior to the addition of complexes. lpD-b-P/E 3 was complexed with labelled plasmid and was added on to primary astrocytes as described in section 3.2.11. After four hours of incubation at 37°C, the medium was removed and the cells were washed with HBSS. The cells within the chambers were then washed with PBS and fixed with 4% paraformaldehyde. The chambers were removed from the underlying glass slides and protected with a glass cover slip. Cells were viewed under a fluorescent microscope (Olympus BX51, Mason Technologies, UK). For detailed description of plasmid labelling see Appendix (M).

3.2.11 Statistics

Results are expressed as mean ± standard deviation. Statistical significance was assessed using the analysis of variance (ANOVA). p values of <0.05 were considered statistically significant. In all studies, the minimum sample size was three.

3.3 Results and Discussion

3.3.1 Effect of Molecular Weight upon PEGylation on Transfection and Cellular Metabolic Activity

Hyperbranched polymer with highest degree of PEGylation (lpD-b-P/E 1, 38000Da) showed minimum transfection but at the same time did not affect the cells metabolism. High degree of PEGylation shielded the cationic pDMAEMA unit thereby reducing its toxicity. However, it increased the size of the polyplex (330nm
at 10:1 w/w ratio) which resulted in reduced internalisation and hence reduced transfection. lpD-b-P/E 2 (14000Da) in contrast, had less PEGylation which resulted in higher transfection than highly PEGylated polymer but showed higher toxicity at high concentrations. This suggests the need of optimizing the polymer molecular weight upon PEGylation to obtain maximum transfection with minimal toxicity. Therefore, another hyperbranched polymer (lpD-b-P/E 3) which has similar structure to lpD-b-P/E 1 and lpD-b-P/E 2 was synthesized via deactivation enhanced atom transfer radical polymerisation (DE-ATRP) technique as described in Chapter two. The molecular weight of lpD-b-P/E 3 was restricted to 24,000Da (medium PEGylation) (Table 3.2). As with lpD-b-P/E 1 and lpD-b-P/E 2, lpD-b-P/E 3 showed controlled polymer growth (Figure 3.1). lpD-b-P/E 3 was able to bind pDNA as early as 1:1 w/w ratio (Appendix V.1) showed its optimal transfection at 8:1 w/w ratio (Figure 3.2). The size of lpD-b-P/E 3 complexes at 8:1 w/w ratio was around 80nm (Appendix V.2). The effect of molecular weight or, in other words, the effect of growing hyperbranched PEG chain on pDMAEMA was then studied.

At their optimal weight ratios, lpD-b-P/E 3 showed approximately seven-fold higher transfection than lpD-b-P/E 1 and 2.5 fold higher transfection than lpD-b-P/E 2 when transfected with 1µg of pDNA and eleven-fold higher transfection than lpD-b-P/E 1 and eight-fold higher transfection than lpD-b-P/E 2 when transfected with 50µg of pDNA (Figure 3.3). All three polymers had no effect on cellular metabolic activity at 1µg pDNA dose. However, at high doses, lpD-b-P/E 2 showed significantly higher toxicity. In contrast, both lpD-b-P/E 1 and lpD-b-P/E 3 showed no effect on cellular metabolic activity even at high doses. This indicates that lpD-b-P/E 3 with medium PEGylation was able to bind pDNA effectively and at the same time did not affect the cellular metabolic activity making it the optimal candidate for transfection studies.

3.3.2 Effect of Protonation on Transfection and Cellular Metabolic Activity

Since lpD-b-P/E 3 showed maximum transfection, this polymer was used for further studies. lpD-b-P/E 3 was protonised at various levels by varying the concentration of HCl. At low pH (3.5) the weak basic amine groups of DMAEMA gets protonated leading to a high degree of ionisation. Non-treated lpD-b-P/E 3 (pH 7) was compared to fully protonated (pH 3.5) and partially protonated (pH 5) polymer (Figure 3.4).
**Table 3.2** Weight average molecular weight (Mw), number average molecular weight (Mn) and PDI of lpD-b-P/E 1, lpD-b-P/E 2 and lpD-b-P/E 3 determined by GPC.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Mn(Da)</th>
<th>Mw (Da)</th>
<th>PDI</th>
<th>DMAEMA: PEGMEMA:EGDMA (molar ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lpD-b-P/E 1</td>
<td>37900</td>
<td>44300</td>
<td>1.17</td>
<td>1:12.215:1.104</td>
</tr>
<tr>
<td>lpD-b-P/E 2</td>
<td>13700</td>
<td>16300</td>
<td>1.19</td>
<td>1:0.879:1.12</td>
</tr>
<tr>
<td>lpD-b-P/E 3</td>
<td>24200</td>
<td>26900</td>
<td>1.11</td>
<td>1:7.279:1.19</td>
</tr>
</tbody>
</table>

Mn – Number average molecular weight, Mw – Weight average molecular weight, DMAEMA - 2-dimethyl-aminoethylmethacrylate, pDMAEMA – Poly (2-dimethyl-aminoethylmethacrylate), PEGMEMA - Poly ethylene glycol methyl ether methacrylate, EGDMA - Ethylene dimethacrylate, lpD-b-P/E - Poly (2-dimethyl-aminoethylmethacrylate)-block-poly ethylene glycol methyl ether methacrylate /ethylene dimethacrylate
Figure 3.1 GPC trace showing the controlled growth of lpD-b-P/E 3 over time (1, 2, 4, 6 and 8 hours).
Figure 3.2 Transfection capability of lpD-b-P/E 3 after 48 hours at varying polymer/pDNA weight ratio 1:1 to 20:1. Data plotted shows mean ± standard deviation (n=3) * (p<0.05). lpD-b-P/E 3 showed its optimal transfection at 8:1 w/w ratio in fibroblasts.
Figure 3.3 Effect of molecular weight of the polymer on transfection and cellular metabolic activity on fibroblasts at two different concentrations of pDNA (1µg and 50µg). Data plotted shows mean ± standard deviation (n=3) * (p<0.05). Cellular metabolic activity was normalised to cells alone group. Results indicate that molecular weight has an effect on transfection and cellular metabolic activity. lpD-b-P/E 3 (24000Da) showed significantly higher transfection than lpD-b-P/E 1 (38000Da) and lpD-b-P/E 2 (14000Da). lpD-b-P/E 1 and lpD-b-P/E 3 did not affect cell metabolism even at high dose (50µg). lpD-b-P/E 2 significantly reduced the cellular metabolic activity of fibroblast when transfected with high doses of plasmid.
Figure 3.4 Effect of protonation of lpD-b-P/E 3 on transfection and cellular metabolic activity on fibroblasts when transfected with 1µg of pDNA. Data plotted shows mean ± standard deviation (n=3) * (p<0.05). Cellular metabolic activity was normalised to cells alone group. Results indicate that protonation did not significantly affect transfection. However, fully protonised polymer (pH 3.5) showed significantly lower cellular metabolic activity than partially protonated polymer (physiological pH 7).
It was previously established that at physiological pH, pDMAEMA is partially positively charged [10]. It was seen that when tested on fibroblasts, protonation did not significantly affect transfection (Figure 3.4). However, cellular metabolic activity reduced with decreasing pH. At low pH, weak amine groups of pDMAEMA are protonated resulting in a high degree of ionisation. This high degree of ionisation might have enhanced polyplex uptake but consequently affected the cellular metabolic activity. At physiological pH both cellular metabolic activity and transfection was maintained. These results indicate that pDMAEMA itself (ie additional protonation) has sufficient H⁺ for efficient transfection and PEG based hyperbranched unit shielded the polymer’s toxicity (Chapter two).

3.3.3 Effect of Serum on Transfection and Cellular Metabolic Activity

Serum free transfection conditions did not significantly affect transfection or cellular metabolic activity of fibroblasts (Figure 3.5). However, cellular metabolic activity of the fibroblast was decreased by 1.5 fold when transfected under serum conditions. A similar trend was observed in NP cells (Figure 3.6) and in astrocytes (Figure 3.7).

Under serum free conditions; polyplexes are removed after four hour incubation, thereby reducing the cell’s metabolism. However, AF cells (Figure 3.8) and neu-7 cells (Figure 3.9) did not have any effect on transfection nor cellular metabolic activity. Results indicate that serum or serum free conditions are cell type dependent.

3.3.4 Transfection Ability of Hyperbranched pDMAEMA-based Polymer on Various Cell Types

Previously in Chapter two the transfection ability of the hyperbranched polymer on fibroblasts and ADSCs was discussed. In this phase the transfection ability of hyperbranched polymer on NP cells, AF cells, neu-7 cells, and astrocytes which are generally regarded as “difficult to transfect cells” were investigated. When transfected on NP cells, lpD-b-P/E 3 showed maximum transfection at 2:1 w/w ratio (Figure 3.6). Transfection ability of lpD-b-P/E 3 reduced with increasing w/w ratios.

dPAMAM showed similar transfection to lpD-b-P/E 3, but 2.5 fold lower cellular metabolic activity. PEI showed significantly higher transfection when compared to control groups (cells alone and pDNA alone) but showed a 46 fold lower transfection and a 25 fold reduced cellular metabolic activity than lpD-b-P/E 3.
Figure 3.5 Effect of serum on transfection and cellular metabolic activity on fibroblasts when transfected with 1µg of pDNA. Data plotted shows mean ± standard deviation (n=3) * (p<0.05). Cellular metabolic activity was normalised to cells alone group. Results indicate that when transfection was performed under serum or serum free conditions did not significantly affect transfection ability. However, cellular metabolic activity was significantly reduced when transfected under serum conditions.
Figure 3.6 Transfection capability of lpD-b-P/E 3 on nucleus pulposus (NP) cells compared to PEI and dPAMAM after 48 hours (polymer/pDNA weight/weight (w/w) ratio 2:1 to 10:1). Data plotted shows mean ± standard deviation (n=4) *(p<0.05). Results indicate that lpD-b-P/E 3 showed its maximum transfection with minimal toxicity at 2:1 w/w ratio. Transfection ability of lpD-b-P/E 3 reduced at higher w/w ratio and also significantly reduced cellular metabolic activity. PEI showed significantly higher transfection when compared to cells alone group and pDNA alone group significantly lower than lpD-b-P/E 3 and dPAMAM. PEI was very toxic to NP cells. dPAMAM showed similar transfection as lpD-b-P/E 3 but was toxic to cells.
Transfection Optimization and Cell Viability \textit{In-Vitro}

**Figure 3.7** Transfection capability of lpD-b-P/E 3 on primary astrocytes compared to PEI, and dPAMAM after 48 hours (polymer/pDNA weight/weight (w/w) ratio 2:1 to 10:1). Data plotted shows mean ± standard deviation (n=4) * (p<0.05). Results indicate that lpD-b-P/E 3 showed its maximum transfection with minimal toxicity at 2:1 w/w ratio. lpD-b-P/E 3 transfected under serum condition reduced the transfection ability at higher w/w ratio and also significantly reduced cellular metabolic activity. Polymer transfected under non serum conditions did not affect cells metabolism and maintained its high transfection level. PEI showed significantly higher transfection but was very toxic to NP cells. dPAMAM did not transfect astrocytes and was toxic to cells.
Figure 3.8 Transfection capability of lpD-b-P/E 3 on annulus fibrosus (AF) cells compared to PEI, and dPAMAM after 48 hours (polymer/pDNA weight/weight (w/w) ratio 2:1 to 10:1). Data plotted shows mean ± standard deviation (n=4) * (p<0.05). Results show that lpD-b-P/E 3 and dPAMAM was not able to transfect AF cells. PEI showed significantly higher transfection but inversely affected cellular metabolic activity.
Transfection Optimization and Cell Viability *In-Vitro*

**Figure 3.9** Transfection capability of lpD-b-P/E3 on neu-7 cells compared to PEI and dPAMAM after 48 hours (polymer/pDNA weight/weight (w/w) ratio 2:1 to 10:1). Data plotted shows mean ± standard deviation (n=4) * (p<0.05). Results indicate that lpD-b-P/E3 showed its maximum transfection with minimal toxicity at 8:1 w/w ratio. PEI showed significantly higher transfection than the cells alone group and pDNA alone group was significantly lower than lpD-b-P/E3 and dPAMAM. dPAMAM showed similar transfection to lpD-b-P/E3. Polymers did not affect cellular metabolic activity at all ratios. Serum or serum free conditions did not have a major effect on transfection or in cell metabolism.
These results indicate that lpD-b-P/E 3 is a better transfecting agent for NP cells. However, lpD-b-P/E 3 was not able to transfect AF cells at all ratios, even though it did not affect it cellular metabolic even at higher w/w ratios (Figure 3.8). dPAMAM also did not transfect AF cells. Despite of high toxicity, PEI was able to transfect AF cells. Neu-7, on the other hand, got transfected by lpD-b-P/E 3, dPAMAM and PEI, with dPAMAM showing highest transfection followed by lpD-b-P/E 3 and PEI. Neu-7 cells were viable under all conditions with all polymers (Figure 3.9). This might be because neu-7 cells are dividing cells and are easy to transfect than non dividing cells such as NP, AF and astrocytes. Astrocytes also showed maximum transfection with lpD-b-P/E 3, when transfected at 2:1 w/w ratio (Figure 3.7, 3.10b).

Polyplexes were readily up taken within four hours at this ratio (Figure 3.10a). The cellular metabolic activity of the cells was not affected at this ratio (Figure 3.7). However, the transfection ability and cellular metabolic activity of lpD-b-P/E 3 reduced with increasing weight ratios. dPAMAM was toxic to cells and also did not transfect them. In spite of its high toxicity, PEI transfected astrocytes significantly with values similar to lpD-b-P/E 3.

Lipofectamine 2000® reagent transfected astrocytes 2.5 fold higher than lpD-b-P/E 3 without affecting the cell’s metabolism. Lipofectin® reagent transfected astrocytes six fold lower than lpD-b-P/E 3 with 1.3 fold reduced cellular metabolic activity (Figure 3.11). These results suggest that the transfection activity of cationic polymers is often dependent on their molecular structure, molecular weight, transfection medium and cell line.

3.4 Conclusion

lpD-b-P/E 3 with medium PEGylation (molecular weight 24000Da), showed maximum transfection with minimal toxicity when compared to lpD-b-P/E 1 (high PEGylation; molecular weight 38000Da) and lpD-b-P/E 3 (low PEGylation; molecular weight 14000Da). A high degree of PEGylation reduces cellular toxicity but affects the transfection ability of the polymer. On the other hand, low PEGylation enhances the transfection ability but indirectly reduces cell’s viability.
Figure 3.10 (a) Uptake of polyplex by primary astrocytes after four hour incubation (DAPI: blue, rhodamine labelled polyplex: red) (b) Fluorescent microscopic image primary astrocytes after transfection with lpD-b-P/E 3 complexed with ptdTomato plasmid at 2:1 w/w ratio. (ptd Tomato expression: red).
Figure 3.11 Transfection capability of lpD-b-P/E 3 on primary astrocytes compared to commercial transfecting agents Lipofectin® reagent and Lipofectamine 2000® reagent. Data plotted shows mean ± standard deviation (n=4) * (p<0.05). Results indicate that Lipofectamine 2000® reagent showed maximum transfection followed by lpD-b-P/E 3 and Lipofectin® reagent. Both lpD-b-P/E 3 and Lipofectamine 2000® reagent had no effect on cellular metabolic activity. Lipofectin® reagent showed significantly lower cellular metabolic activity.
Therefore, PEGylation should be balanced to attain maximum transfection with minimal toxicity. pDMAEMA at its physiological pH, has sufficient H\(^+\) ions for efficient transfection. pDMAEMA does not require additional protonation step. Transfection activity of cationic polymers is largely dependent on their molecular structure, molecular weight, transfection medium and cell line. lpD-b-P/E 3 transfected NP cells, neu-7 cells and astrocytes with minimal toxicity when compared to commercial transfection agents such as PEI and dPAMAM. However, lpD-b-P/E was not able to transfect AF cells.
3.5 References


Chapter 4

Ex-Vivo Multiple Sclerosis Model of Inflammatory Demyelination

The majority of this chapter has been previously published in:

4.1 Introduction

Multiple Sclerosis (MS) is the most common cause of neurological disability in young adults and is considered to be a chronic autoimmune, demyelinating disease of the central nervous system (CNS)[1]. MS is characterised, neuropathologically, by multiple foci of inflammation and demyelination in the brain and spinal cord, accompanied by a variable degree of axonal damage in the white matter and neuronal loss in the grey matter [2]. The presence of inflammatory processes in this pathology is well established but determination of the exact pathogenic mechanisms and identification of individual disease mediators continues to be a challenge. Magnetic resonance imaging (MRI) studies and autopsy tissue analysis from patients have made significant contributions towards an understanding of MS pathogenesis, but to fully understand underlying mechanisms of damage, inevitably animal models of MS will be required. The most commonly used animal model of MS is experimental autoimmune encephalomyelitis (EAE) [3] model, which involves the immunisation of genetically susceptible animals with a myelin protein. This results in a progressive clinical course characterised by immune mediated demyelination, primarily in the spinal cord. Although EAE models have been widely used to study disease mechanisms and as a pre-clinical test-bed for new therapeutics, they often fail to predict clinical efficacy in MS patients. This is largely due to their inability to reproduce the location and characteristics of the pathology within the CNS[4-5]. Approaches involving the targeting of EAE lesions to particular anatomical structures and using specific inflammatory mediators are proving helpful in overcoming these limitations [6-7]. However, the complex interactions between the immune and nervous systems in-vivo and the relative expense of EAE models makes using an ex-vivo approach an attractive alternative for studying disease mechanisms and testing drug efficacy.

Organotypic slice culture models have been used previously to investigate demyelination and remyelination with myelin specific antibodies [8-9]. Recent studies have used slices cultured on semi-porous membranes to allow a greater ability to manipulate the molecular environment [10] and for testing remyelination enhancing therapeutic approaches [11]. Recent studies of human post-mortem
MS tissues have suggested an important role for the chronic inflammatory milieu in the CSF of the subarachnoid space that overlies the brain parenchyma [12-13]. In this study we sought to establish a system for testing the role of the chronic presence of pro-inflammatory cytokines in demyelination. To do this, we have used a non-viral gene delivery approach and organotypic slice cultures to transduce cells with genes encoding the pro-inflammatory cytokines TNFα and IFNγ. Both TNFα and IFNγ have been shown to induce demyelination in the rodent CNS [14-15].

Gene transfection of organotypic brain slices has previously been carried out using viral vectors [16-17] or by non-viral methods such as electroporation [18-19], biolistics [20-21], microprojectiles [22] and lipotransfection [18]. Although viral vectors provide good transfection efficiency, their application is limited due to cytotoxicity and immunogenicity [23]. Electroporation can cause severe damage to the tissue and only transfects cells around the edges of a slice [18, 24]. The use of liposomal and polymeric vectors has increased due to their large scale manufacture and lower immunogenicity. However, relatively few studies have used non-viral gene transfection via liposomes in an organotypic brain slice system. In this phase, various parameters for maximum \textit{ex-vivo} transfection have been optimized and evaluated using a hyperbranched PEGmethacrylate linear poly (2-dimethylaminoethylmethacrylate) (lpD-b-P/E 3) (described in Chapter three) [25].

In Chapter three, it was shown that lpD-b-P/E 3 with medium PEGylation (molecular weight 24000Da), showed maximum transfection with minimal toxicity \textit{in-vitro} and demonstrated significantly higher transfection efficiency than commercially available polymeric systems. Hence, lpD-b-P/E 3 polymer was utilized for further optimization studies in an organotypic slice culture system and it was hypothesized that lpD-b-P/E 3 can be used to successfully deliver TNFα and IFNγ genes under optimized conditions for establishing a promising \textit{ex-vivo} model of MS.

\section*{4.2 Materials and Methods}

\subsection*{4.2.1 Chemicals}

All reagents including HPLC and analytical solvents, synthesis and cell culture reagents were obtained from Sigma-Aldrich Chemical Co.(Ireland) unless otherwise stated. A comprehensive list of materials has been supplied in Appendix A, Table A.1.
4.2.2 Synthesis of Poly (2-dimethyl-aminoethylmethacrylate) (pDMAEMA)-block-Poly Ethylene Glycol Methyl Ether Methacrylate (PEGMEMA)/Ethylene Glycol Dimethacrylate (EGDMA)

lpD-b-P/E 3 was synthesized as described in section 2.2.3. The reaction of lpD-b-P/E 3 was terminated when a molecular weight of 24000Da was achieved. The polymer was then dialyzed against distilled water and then freeze dried. For detailed description see Appendix (B2).

4.2.3 Plasmid Preparation and Amplification

Human TNFα and IFNγ cDNAs were cloned from human cDNA clones NM_000594 and NM_000619.2, respectively (Origene, Rockville, USA). The homology of human TNFα and IFNγ was matched with the homology of rat TNFα and IFNγ. Briefly, cDNA clones were digested using Not-I (New England Lab, USA) before amplification of the gene of interest by long proof-read long amplification (Extensor Long Range PCR polymerase Ready Mix kit, Thermo Scientific - Fisher, Ireland) according to the supplier’s instructions (human IFNγ (Forward- TGT CCA ACG CAA AGC AAT AC; Reverse- ATC TGA CTC CTT TTT CGC TTC C), human TNFα (Forward- CCA GGC AGT CAG ATC ATC TTCTC; Reverse- AGC TGG TTA TCT CTC AGC TCC AC) and rat glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (Forward- GTG CCA GCC TCG TCT CAT AGA CAA G; Reverse- GCC GTG GGT AGA GTC ATA CTG GA)). PCR products were then run on a 1% agarose gel, extracted and purified using the MiniElute® kit (Qiagen, Ireland). After purification the PCR product was cloned into a pCR™2.1-TOPO® vector (CMV promoter driven plasmid) following the manufacturer’s instructions. The size of positive clones was checked by electrophoresis on a 1% agarose gel after digestion using Nco I (Promega, Ireland) and by sequencing (MWG-Operon, Germany). After validation, the plasmid was amplified and purified using the Qiagen Plasmid Mega Purification kit (Qiagen, UK). G-Luciferase plasmid (CMV promoter driven plasmid) (New England Lab, USA) was used as an empty vector. In some transfection studies Green Fluorescent protein (GFP) plasmid (CMV promoter driven plasmid) (New England Lab, USA) was also used for transfection analysis. Amplification and purification of the plasmid was performed using the Qiagen® Plasmid Mega Purification kit (Qiagen, UK). For detailed description see Appendix (E).
4.2.4 **Organotypic Brain Slice Culture**

All procedures were performed under a licence issued by the Irish Government Department of Health and were approved by University College Cork Ethics Review board. Embryos (E16) from pregnant Sprague-Dawley rats (Biological Services Unit, University College Cork) were removed and postnatal day 15 (P15) Sprague-Dawley rats were decapitated and the brain was dissected out into ice cold artificial cerebrospinal fluid (ACSF; prepared as described in [26]). The cerebellum and cerebral hemispheres were separated and 250-400µm sections were cut in ice cold ACSF using a vibratome (VT1200, Leica Microsystems). The cerebellum was sectioned in the parasagittal plane to preserve the cerebellar circuitry and the cerebral hemispheres were sectioned in the coronal plane. Slices were positioned on organotypic slice culture inserts (Millipore) in 6-well plates containing 1 ml of culture medium per well (Basal Medium Eagle [Gibco], supplemented with Hanks Balanced Salt Solution [25% v/v], D-glucose [4.5mg/ml], penicillin [100U/ml], streptomycin [100 U/ml], glutamine [0.58mg/ml], and 10% heat inactivated foetal calf serum). Plates containing tissue slices were then placed in an incubator with 5% CO₂ at 37°C. For detailed description of organotypic brain slice culture and ACSF formulation see Appendix (N and O) respectively.

4.2.5 **Gene Transfection**

For optimization experiments cerebellum and cortical slices were cultured for approximately one hour before they were exposed to polymer/plasmid DNA (pDNA) complex (polyplex). Each treatment group contained six wells containing three slices per well. The gene transfection efficiency of the polyplex was assessed via G luciferase activity (New England BioLabs, UK). GFP expression was used for qualitative studies.

4.2.6 **Electroporation**

Embryonic slices were cultured for approximately one hour before electroporation. After one hour of incubation a slice was placed on a glass slide and the residual solution removed. The pulse buffer did not contain any added divalent cations, with the expectation that reduced stability of the membranes would allow easier pore formation. A pair of platinum electrodes (Genetrodes; BTX) was held in place by a frame, which maintained gap size, 2 mm, across the slice. 2µl of GFP (1, 2 or 5µg)
plasmid was added on to the slice. A BTX 830 ECM® Electro Square Porator (BTX) controlled the electroporation parameters. After electroporation, the slice was placed in organotypic cell culture insert supplemented with culture medium and incubated with 5% CO₂ at 37°C for 48 hours.

4.2.7 Application of Polyplex
Polyplexes were either added on top of the slice or into the culture media. Briefly, 15µl of GLuc plasmid (1µg, 2.5 µg or 10µg per slice) was slowly added to 15µl of lpD-b-P/E 3 solution (2:1, 8:1 or 15:1 polymer/pDNA w/w ratio). After brief vortexing, the mixture was then incubated for 30 minutes at room temperature to allow the formation of polyplex. 30µl or 100µl of lpD-b-P/E 3/pDNA complexes were applied on top of each slice above the culture insert membrane and maintained at 37°C in a humidified incubator with 5% CO₂ for 48 hours. The polyplex added into the cell culture media was prepared as follows: 45µl of GLuc plasmid (7.5µg per well) was slowly added to 45µl of lpD-b-P/E 3 solution (8:1 polymer/pDNA w/w ratio). After brief vortexing, the mixture was then incubated for 30 minutes at room temperature to allow the formation of polyplex. 90µl of polyplex was then diluted in 900µl of culture media. Fresh culture medium below the culture insert was replaced with culture media containing polyplex. The culture was maintained at 37°C in a humidified incubator with 5% CO₂ for 48 hours. After 48 hours the tissues were homogenized in the culture media. 50µl medium from all the samples was collected for quantification of luciferase expression. For detailed description of G luciferase assay see Appendix (J).

4.2.8 Commercial Transfecting Agents
All commercial transfection agents (Lipofectin® reagent (Invitrogen, Ireland), Lipofectamine 2000® reagent (Invitrogen, Ireland) and FuGene® reagent (Promega)) /pDNA complexes were prepared as per the manufacturer’s protocol.

4.2.9 Plasmid Uptake
Cy 3 labelled GLuc plasmid was complexed with lpD-b-P/E 3. Polyplexes were applied on top of each slice above the culture insert membrane or into the culture media and maintained at 37°C in a humidified incubator with 5% CO₂ for four hours. Slices were then thoroughly washed and fixed with 4% PFA and later analysed by
immunohistochemistry. For detailed description of plasmid labelling and immunohistochemistry see Appendix (M and P) respectively.

4.2.10 Assessment of Cell Death
Cell death was determined using the fluorescent viability indicator propidium iodide (PI). PI was added on top of the slice at a concentration of 2µg/ml and was incubated at 37°C in a humidified incubator with 5% CO₂ for 15 minutes. Slices were then thoroughly washed with PBS and fixed with 4% PFA. PI fluorescence emission was visualized using an OlympusBX51™ microscope, USA. Image J software was used to analyse the PI density of the whole slice at a set threshold. Cell death was determined by dividing the PI density with the total area of the slice. A minimum of nine slices were used per treatment group.

4.2.11 TNFα and IFNγ Gene Transfection to Primary Astrocytes
Primary astrocytes were extracted from rat brain tissue as described in Appendix (K). Under usual cell culture sterile conditions, cells were seeded in a six well plate with a cell density of 1000000 cells/well for 24 hours before the transfection experiment. TNFα or IFNγ plasmid (1µg per well, 50µl) was mixed with 50µl lpD-b-PE 3 solutions at their optimal w/w ratio. The mixture was then incubated for 30 minutes at room temperature to allow the formation of polymer/pDNA complexes, and 750µl of serum medium was added to the polyplex solution. The culture was maintained at 37°C in a humidified incubator with 5% CO₂ for 48 and 72 hours.

4.2.12 Treatment of Primary Astrocytes with Lipopolysaccharide (LPS) and IFNγ
Primary astrocytes were extracted from rat brain tissue as described in Appendix (K). Under usual cell culture sterile conditions, cells were seeded in a six well plate with a cell density of 1000000 cells/well for 24 hours before the experiment. 0.5µl of LPS (1µg/ml) and 1µl of rIFNγ cytokine (100U/ml) was mixed and 800µl of serum medium was added to the astrocyte culture. The culture was maintained at 37°C in a humidified incubator with 5% CO₂ for 48 and 72 hours.
4.2.13 Demyelination via Gene Transfection and LPC treatment

Cerebellum slices were cultured for approximately 48 hours before they were exposed to polyplexes or lysophosphatidylcholine (lysolecithin, LPC). Each treatment group contained three wells consisting of three slices per well. 15µl of TNFα or IFNγ plasmid (2.5µg per slice) was slowly added to 15µl of lpD-b-P/E 3 solution (8:1 polymer/pDNA w/w ratio). After vortexing, the mixture was then incubated for 30 minutes at room temperature to allow the formation of polymer/DNA complexes. 30µl of lpD-b-P/E 3/pDNA complexes were applied on top of each slice above the culture insert membrane and maintained at 37°C in a humidified incubator with 5% CO₂ for 2,4,7,14 and 21 days. Alternatively, 0.5mg/ml of LPC was added to the medium for 17h, after which slices were returned to normal media. After each time point the slices and media were collected and either fixed with 4% PFA or stored at -80°C until further use. lpD-b-P/E 3 alone, lpD-b-P/E 3 complexed with GLuc plasmid (i.e an empty vector), and Lipofectamine 2000® reagent complexed with TNFα and IFNγ were used as controls. Lipofectamine 2000® complexes were prepared as per manufacturer’s instructions.

4.2.14 Gene Expression

After every time point, total RNA was extracted using a variant of Trizol® isolation. Briefly, TriReagent® (Invitrogen, Ireland) was added to the cerebellum slices or primary astrocytes. Primary astrocytes were directly lysed in the culture dish by scraping the cells using a pipette tip and then by passing the cell lysate several times through the pipette. Slices were mechanically disrupted under an oscillation frequency at 50Hz/s for two minutes using TissueLyser LT (Qiagen, UK). Phase separation was performed using chloroform, and total RNA, from this step, was purified using RNeasy mini kit (Qiagen, UK) according to the supplier’s protocol. Total RNA quantity and purity were determined using an ultraviolet spectrometer (NanoDrop™ 2000 Spectrophotometer, ThermoScientific Inc., USA). Reverse transcription (RT) was performed using the reverse transcription system (Promega, UK) according to the manufacturer’s protocol. Gene transcription was examined using real-time polymerase chain reaction (PCR). Reactions were performed and monitored by StepOnePlus™ detection system (Applied Biosystems, USA) using the QuantiFast SYBR Green gene expression PCR kit (Qiagen, UK) for
the targeted genes (primers described in 4.2.3 above). Gene transcription was
normalised in relation to transcription of the housekeeping rat GAPDH. The $2^{\Delta\Delta Ct}$
method was used to calculate relative gene expression for each target gene according
to Pfaffl method [27] and expressed as fold change relative to the untreated tissue.
For detailed description of RNA extraction, cDNA synthesis and PCR see Appendix
S, T and U respectively.

4.2.15 Immunohistochemistry

Slices were collected at 2, 4, 7, 14 and 21 days in-vitro (DIV) post-treatment, fixed
in 4% paraformaldehyde (PFA; 0.1 M phosphate buffered saline [PBS]) overnight at
4˚C, and washed thoroughly in PBS prior to immunohistochemical staining. Single-
or double-labelling immunofluorescence was performed on floating slices in 24-well
plates. Tissue was blocked in 10% normal horse serum (NHS) and 0.4% Triton™ X
in PBS, for one hour and then incubated in primary antibody (diluted in PBS with
2.5% NHS and 0.4% Triton™ X) for 48 hours at 4˚C. Primary antibodies included:
rabbit anti oligodendrocyte transcription factor (anti-Olig2) (1:500; Millipore, Germany),
rat anti-myelin basic protein (anti-MBP) (1:200; ABD Serotec, UK),
rabbit anti-Iba1 (1:400;Wako Chemicals, USA), mouse anti-βIII-tubulin (1:500;
abcam, UK), mouse anti-NeuN (1:250; Millipore, Germany), goat anti glial fibrillary
acidic protein (anti-GFAP) (1:2000; abcam, UK) and mouse anti neurofilament
heavy chains (anti-NFH) (1:100). After PBS washes, slices were incubated in the
appropriate secondary antibody conjugated to either AlexaFluor® 488, 594 or 633
(1:500; all raised in donkey; Invitrogen, Ireland) in PBS with 2.5% NHS and 0.4%
Triton™ X overnight at 4˚C. Slices were washed in PBS, mounted onto gelatin-
coated slides and cover slipped using PVA-DABCO®.

4.2.16 Cytokine Expression

After induction of demyelination via LPC or by gene transfection, culture media was
collected after 2, 4, 7, 14 and 21 days. TNFα (Thermoscientific, Ireland) and IFNγ
(R&D Systems, Minneapolis, MN, USA) expression was determined by enzyme-
linked immunosorbent assay (ELISA).
4.2.17 Quantification

Confocal microscopy was used to obtain z-stacks of MBP and NFH immunostaining at 1µm thickness intervals (Olympus Fluoview™1000). Co-localization of red (myelin MBP) over green (NFH) was assessed using Image-Pro® Plus (Media Cybernetics, Inc. USA) software via Mander’s coefficient. Mander’s coefficient indicates the overlap of fluorescent channels rather than the intensity distribution of the fluorescent signal (in this case the MBP over NFH immunofluorescence); therefore, representing the true degree of co-localization between myelin protein and axons; giving a measure of axonal myelination [28-29]. An intensity threshold was created for fluorescent images to allow for background correction and was kept constant across all analysed images.

4.2.18 Statistics

Results are expressed as mean ± standard error mean (SEM). Statistical significance was assessed using the analysis of variance (ANOVA) and t-test. \( p \) values of <0.05 were considered significant. In all studies, the minimum sample size was three.

4.3 Results

4.3.1 Polymer Synthesis

Hyperbranched pDMAEMA-based polymer (lpD-b-P/E 3) was synthesized as described previously in Chapter three with a molecular weight of 24,000Da. The transfection capability and cytotoxicity effects of lpD-b-P/E 3 were analysed on primary astrocytes prior to transfecting an organotypic brain slice culture system. lpD-b-P/E 3 transfected astrocytes efficiently with minimal toxicity compared to other commercial transfecting agents (Chapter three, Figure 3.7). Therefore, this polymer was utilized for gene transfer into organotypic brain slice cultures. Since few studies have been performed on non-viral gene delivery in organotypic slice cultures, the optimization of transfection conditions was crucial. The transfection efficiency of cationic gene delivery vectors largely relies on polymer structure [24], but also polymer application, pDNA w/w ratio, concentration and, most importantly, its cytotoxicity [18, 30-31]; therefore these factors required optimization.
4.3.2 Application of Polyplex

The effect of non-viral transfection on embryonic slices at varying pDNA doses was initially investigated. It was seen that the slices were severely damaged at high pDNA doses (5µg), with electroporation causing maximum tissue damage (Figure 4.1 and 4.2). Transfection with lpD-b-P/E 3 caused the least tissue damage when compared to electroporation and lipotransfection. When transfected with 1µg plasmid, lpD-b-P/E3 showed no significant difference when compared to no treatment group. However, with increasing doses significant cell death was observed. GFP positive cells appeared to be more localized near the corpus callosum when transfected with lpD-P/E 3 (Appendix W.1) and was not GFAP positive (Appendix W.2). In contrast, Lipofectin® reagent transfected cells across the brain slice and electroporation transfected cells only around the edges of the slice (Appendix W.1). Further studies were performed on postnatal (P15) brain slices as they were more relevant for this project. At first the effect of slice thickness of P15 brain slices upon transfection was studied. It was seen that 250µm thick slices transfected much efficiently than 400µm thick slices (Figure 4.3). Therefore, for further studies 200µm thick slices were used. Effect of polyplex application on organotypic slices was also studied. Clearly, polyplex when added on top of the slices transfected much more efficiently than in the case when added into media (Figure 4.4). Around 11 fold increase in transfection was observed when the polyplex was applied to the top of the slice. To examine the reason for this effect an uptake study was performed and polyplex uptake was examined for four hours after treatment. Uptake and penetration of the polyplex into the depth of the slice was visualized using confocal microscopy. It was seen that, even in four hours of treatment, the polyplexes penetrated as deep as 15µm. No polyplex was present in the middle depths of the slice. However, the bottom 15µm also showed substantial polyplex uptake (Figure 4.5). When polyplex was added into the media directly, only the bottom surface was exposed, leading to poor transfection. This phenomenon was seen in both cortical (Figure 4.4) and cerebellar slices (Figure 4.6). Commercially available transfecting agent, Lipofectin® reagent, also showed similar results (Figure 4.6). Uptake studies also showed that within four hours the polyplexes were internalized within the cells (Figure 4.7).
Figure 4.1 Representative images of tissue damage in an embryonic organotypic brain slice after non-viral gene delivery with varying plasmid DNA doses. Images indicate that lpD-b-P/E 3 causes least damage to the slices when compared to electroporation and lipotransfection.
**Figure 4.2** Cell death after non-viral gene transfection on embryonic brain slice. Results indicate that cell death was proportional to pDNA dose, with 5µg of plasmid showing the highest cell death. lpD-b-P/E 3 had least effect on cell death compared to lipotransfection and electroporation. Data plotted shows mean ± SEM (n=4) *(p<0.05).*
Figure 4.3 Degree of transfection of lpD-b-P/E 3 and Lipofectin® reagent on 200µm and 400µm thick cerebellar slices. Results indicate that 200µm thick slices transfected much more efficiently than 400µm thick slices. Data plotted shows mean ± SEM (n=3) *(p<0.05).
Figure 4.4 Degree of transfection of cortical slices when polyplex was added either on the top of the slice or directly into the media. A significant increase in the degree of transfection was obtained when polyplexes were added on top of the slice. Data plotted shows mean ± SEM (n=6) *(p<0.05).
Figure 4.5 Uptake of polyplexes at different levels through the slice (top to bottom). Red represents Cy3 labelled pDNA complexed with lpD-b-PE, Blue indicates GFAP positive cells (astrocytes) and green indicates Iba 1 positive cells (macrophages/microglia).
Figure 4.6 Degree of transfection of cerebellar slices when polyplexes were added either on the top of the slice or directly into the media. A significant increase in the degree of transfection was obtained when polyplexes were added on top of the slice. Data plotted shows mean ± SEM (n=6) *(p<0.05).
Figure 4.7 Confocal microscopy images of the internalization of polyplex within the cells after four hours of polyplex treatment. (polyplex (red), GFAP (blue) and macrophages/microglia (Iba 1, green).
4.3.3 Transfection of Cerebral Cortex and Cerebellar Slices

The degree of transfection of both cortical and cerebellar slices at various polymer to pDNA ratios from 2:1 to 15:1 (w/w) was examined. Polymer/plasmid weight ratios were used in this study for increased accuracy over the commonly used nitrogen to phosphate (N/P) quotation because of differences arising in number average molecular weight (Mn) values across varying GPC instruments/calibration.

lpD-b-P/E 3 showed optimal transfection at 8:1 w/w ratio. Cerebellar slices transfected more efficiently than cerebral slices at all ratios (Figure 4.8a). The viability of slices from both brain regions was then examined; propidium iodide (PI), which can only enter cells when they lose their integrity, was used as an indicator of cell viability [32]. Even in control slices that were not transfected, cell death was much higher in cerebral cortex than in cerebellar slices (Figure 4.8b). This suggests that, although cerebral slices take up the polyplexes efficiently (Figure 4.5), the survival of the tissue under ex-vivo culturing conditions is comparatively poor. This may explain why cortical slices show low transfection levels overall.

4.3.4 Concentration and Volume of Polyplex

The effect of alterations of polyplex volume and concentration on degree of transfection and viability of cerebral and cerebellar slices was also examined. Changes in polyplex volume did not have an effect on transfection or viability. However, pDNA concentration did have an effect on transfection in both cortical (Figure 4.9a) and cerebellar slices (Figure 4.10a). Maximum transfection was obtained when slices were transfected with 2.5µg of pDNA per slice and was reduced at higher concentrations of pDNA (10µg). At higher pDNA concentrations, a significant increase in cell death in both cortical (Figure 4.9b) and cerebellar slices (Figure 4.10b) was seen. The higher level of cell death at high pDNA concentrations might have led to the associated low transfection levels.

In summary, the results of optimizations showed that the maximum transfection efficiency of lpD-b-P/E 3 in organotypic slices occurred at 8:1 w/w ratio when transfected with 2.5µg of pDNA on cerebellar slices.
Figure 4.8 (a) Degree of transfection and (b) cell death of lpD-b-P/E 3 on cortical and cerebellar slices at various w/w ratios. Results indicate that lpD-b-P/E 3 has its maximum transfection on cerebellar slices at 8:1 w/w ratio. Data plotted shows mean ± SEM (n=6), * (p<0.05).
Figure 4.9 (a) Degree of transfection and (b) cell death of lpD-b-P/E 3 on cortical slices at various pDNA concentration and doses. Results indicate that lpD-b-P/E 3 has its maximum transfection at 2.5µg pDNA concentration as the cell death significantly increases after 2.5µg pDNA concentration. Data plotted shows mean ± SEM (n=6), * (p<0.05).
Figure 4.10 (a) Degree of transfection and (b) cell death of lpD-b-P/E 3 on cerebellar slices at various pDNA concentration and doses. Results indicate that lpD-b-P/E 3 has its maximum transfection at 2.5µg pDNA concentration as the cell death significantly increases after 2.5µg pDNA concentration. Data plotted shows mean ± SEM (n=6), * (p<0.05).
4.3.5 Comparison with Commercial Transfection Agents

The transfection potential of lpD-b-P/E 3 was compared to liposome mediated gene transfer vectors: Lipofectin® reagent and Lipofectamine 2000® reagent and non liposome mediated gene transfer vector: FuGene® reagent. lpD-b-P/E 3 and Lipofectamine 2000® showed significantly higher transfection levels than Lipofectin® and FuGene® when transfected with 2.5µg of pDNA (Figure 4.11a). Both Lipofectin® and FuGene® showed higher cell death than did lpD-b-P/E 3 and Lipofectamine 2000® reagent (Figure 4.11b).

4.3.6 Transfection of Multiple Cell Types

When transfected with lpD-b-P/E 3, GFP positive cells were found diffusely across all regions of the slices (Figure. 4.12j,k). In the cerebellum, the highest number of transfected cells was seen in the molecular layer and very few cells were found in the Purkinje cell layer (Figure 4.12k,l). In cortical slices, the highest number of transfected cells was found around the dorsal edge of the slice, in the outer cortical layers (Figure 4.12j,m). The transfected, GFP expressing cells displayed a variety of morphologies. In order to determine the potential cell types that were transfected in the slices, immunohistochemistry techniques and established phenotypic markers (GFAP for astrocytes; Olig2 and MBP for oligodendrocytes; Iba1 for microgli/macrophages; and NFH, βIIITub and NeuN for neurons) were used. In the cerebellum, very few cells that displayed Purkinje cell morphology were seen, however there were some NFH (Figure 4.12d-f) or NeuN GFP co-labelled cells in the granular cell layer of the cerebellum, the brainstem and cortical regions, indicating the ability of lpD-b-P/E 3 to transfect neurons. There were substantially more glial cells co-labelled with GFP in all regions of the slices; including GFAP immunopositive astrocytes (often cells displaying Bergman glial-like morphology, see Figure 4.12a-c) and oligodendrocytes (Figure 4.12g-i), but very few transfected Iba-1 immunopositive microglia were found. Unfortunately, some of the GFP positives cells were not co-labelled; however, their morphologies were similar to epithelial cells, granule neurons, fibroblasts etc (Appendix W.3).
Figure 4.11 Degree of transfection (a) and toxicity (b) of lpD-b-P/E 3 on cerebellar slices compared to commercial transfection reagents when transfected with 2.5\( \mu \)g pDNA. Results indicate that lpD-b-P/E 3 showed highest transfection with minimal toxicity. Data plotted shows mean ± SEM (n=6), * (p<0.05).
Figure 4.12 IpD-b-P/E 3 transfects various cell types in the CNS. (a-c) Transfected astrocyte expressing both green fluorescent protein (GFP) shown in green (a) and glial fibrillary acidic protein (GFAP) shown in red (b), merged image shown in (c). (d-f) Transfected neuron expressing both GFP shown in green (d) and NFH shown in red (e), merged image shown in (f). (g-i) Transfected oligodendrocyte expressing both green fluorescent protein (GFP) shown in green (a) and Olig2 transcription factor shown in red (b), merged image shown in (c). GFP positive cells across all
regions of the cortex (j,m) and cerebellum (k, n). (l) Percentage of GFP positive cells across various regions in the cerebellum (n=18 slices). lpD-b-P/E 3 transfects various cell types in all regions of the cerebellum with the highest percentage in the molecular layer. (ml – molecular layer, pcl – purkinje cell layer, gl – granular layer, wm – white matter).
4.3.7 TNFα and IFNγ Expression and Release

Expression and release of the TNFα and IFNγ genes was first analysed in vitro on primary astrocytes (Figure 4.13 and 4.14). It was confirmed that both TNFα and IFNγ genes were expressed when transfected via non-viral gene delivery vectors. Both lpD-b-P/E 3 and Lipofectamine 2000® expressed significantly higher TNFα and IFNγ up to four days after transfection (Figure 4.13a and 4.14a). LPS (1µg/ml) and rIFNγ (100U/ml) was used as the control for TNFα expression and release. ELISA results showed that when transfected with non-viral vectors showed a three fold increase in TNFα release when compared to the TNFα production induced by LPS and rIFNγ (Figure 4.13b). However, IFNγ release was visible only after two day of treatment. Since cerebellum slices showed better survival and good transfection, they were used for further studies. To determine whether the polymer was able to deliver functional TNFα and IFNγ plasmids efficiently to cerebellar slices, real time PCR and ELISA were performed at various time points (2,4,7,14 and 21 days post-transfection). TNFα showed significantly high levels of gene expression up to 14 days post transfection. At day 21, a significantly reduced level of expression was observed (Figure 4.15). Lipofectamine 2000® also exhibited efficient transfection of the TNFα genes and showed similar trends to lpD-b-P/E 3. ELISA results showed that TNFα plasmid when transfected with non-viral vectors showed significant increase in TNFα release up to 14 days (Figure 4.16). TNFα protein levels reached a peak at day four (350pg/ml) and then returned to baseline by day 21 (Figure 4.16). It should be noted that LPC, lpD-b-P/E 3 alone and lpD-b-P/E 3 complexed with empty vector (EV) treated groups showed no TNFα production.

Transfection with non-viral vectors complexed with IFNγ plasmid showed some TNFα release. However, they were significantly lower than the TNFα produced by the transfection with TNFα plasmids. IFNγ genes were also transfected efficiently by non-viral vectors to produce significant IFNγ mRNA expression and release (Figure 4.17). With the exception of day two (75 pg/ml), relatively low levels (approximately 20pg/ml) of IFNγ protein were detected by ELISA (Figure 4.18), indicating that only transient transfection was achieved. Lipofectamine 2000® also promoted transfection of the TNFα and IFNγ genes and showed similar trends to lpD-b-P/E 3.
Figure 4.13 (a) TNFα expression (b) TNFα release on astrocytes after two and four days of transfection with TNFα gene complexed with lpD-b-P/E 3 and Lipofectamine 2000®. Graphs indicate that TNFα gene was successfully transfected via non-viral gene delivery vectors and showed significantly high protein expression and release up to four days after transfection. Data plotted shows mean ± SEM (n=3), * (p<0.05).
**Figure 4.14** (a) IFNγ expression (b) IFNγ release on astrocytes after two and four days of transfection with TNFα gene complexed with IpD-b-P/E 3 and Lipofectamine 2000®. Graphs indicate that TNFα gene was successfully transfected via non-viral gene delivery vectors and showed significantly high protein expression up to four days and release up to two days after transfection. Data plotted shows mean ± SEM (n=3), * (p<0.05).
Figure 4.15 TNFα expression after 2, 4, 7, 14 and 21 days of transfection with the TNFα or IFNγ plasmid complexed with Lipofectamine 2000®, LPC, lpD-b-P/E 3 alone and lpD-b-P/E 3 complexed with empty vector (EV) when compared to TNFα or IFNγ gene complexed with lpD-b-P/E 3. Graphs indicate that TNFα genes were successfully transfected and showed mRNA expression up to 14 days. Data plotted shows mean ± SEM (n=3), * (p<0.05).
Figure 4.16 TNFα release after 2, 4, 7, 14 and 21 days of transfection with the TNFα or IFNγ plasmid complexed with Lipofectamine 2000®, LPC, lpD-b-P/E 3 alone and lpD-b-P/E 3 complexed with empty vector (EV) when compared to TNFα or IFNγ gene complexed with lpD-b-P/E 3. Graphs indicate that TNFα genes were successfully transfected and showed protein expression up to 14 days. Data plotted shows mean ± SEM (n=3), * (p<0.05).
**Figure 4.17** IFNγ expression after 2, 4, 7, 14 and 21 days of transfection with the TNFα or IFNγ plasmid complexed with Lipofectamine 2000®, LPC, lpD-b-P/E 3 alone and lpD-b-P/E 3 complexed with empty vector (EV) when compared to TNFα or IFNγ gene complexed with lpD-b-P/E 3. Graphs indicate that IFN genes were successfully transfected and showed mRNA expression up to seven days. Data plotted shows mean ± SEM (n=3), * (p<0.05).
**Figure 4.18** IFN release after 2, 4, 7, 14 and 21 days of transfection with the TNFα or IFNγ plasmid complexed with Lipofectamine 2000®, LPC, lpD-b-P/E 3 alone and lpD-b-P/E 3 complexed with empty vector (EV) when compared to TNFα or IFNγ gene complexed with lpD-b-P/E 3. Graphs indicate that IFNγ genes were successfully transfected and showed IFN release for two days after transfection. Data plotted shows mean ± SEM (n=3), * (p<0.05).
Although TNFα and IFNγ gene expression was lower compared to lpD-b-P/E 3, protein release was higher. LPC, lpD-b-P/E 3 alone and lpD-b-P/E 3 complexed with empty vector (EV) treated groups showed no IFNγ production.

4.3.8 Demyelination

The effect of TNFα and IFNγ gene expression on myelin in cerebellar slices was examined as both of these cytokines have been implicated in causing demyelination in inflammatory disease in the CNS [14-15]. The slices were cultured for two days, prior to the treatment with the polypeplex carrying the pro-inflammatory genes. This was to recover from the insult caused during the slicing process. The slices maintained their architecture (Figure 4.19). lpD-b-P/E 3 and Lipofectamine 2000® were complexed with TNFα or IFNγ plasmids and added on top of the slices as described in 4.2.13. Optimized transfection conditions were employed in this study. As LPC induced demyelination is a well established in-vivo and ex-vivo model of demyelination [33-34], LPC treated slices were used as the positive control in this study. A no treatment group, lpD-b-P/E 3 alone and lpD-b-P/E 3 complexed with G luciferase plasmid (EV) were used as negative controls. Myelin was identified by the presence of MBP and the co-localization of MBP (myelin sheaths) and NFH (Figure 4.20).

When compared with the group that received no treatment, the slices that were transfected with either the TNFα or IFNγ plasmid showed significant demyelination by two days which continued up to 14 days after treatment. LPC treated slices showed evidence of demyelination by two days, indicated by the lack of MBP expressing myelin-like profiles around NFH⁺ axons and the presence of MBP⁺ debris (Figure 4.21d,e). This was confirmed by the significant reduction in Mander’s coefficient for MBP and NFH immunofluorescence (indicating loss of myelinated axons; Figure 4.22). Treatment with the TNFα-lpD-b-P/E 3 and IFNγ-lpD-b-P/E 3 polypelexes led to a similar degree of myelin loss starting at two days post-treatment and continuing up to seven days (Figure 4.21g-l and 4.22). It should be noted that slices treated with lpD-b-P/E 3 alone showed significant demyelination at day two and recovered within seven days.
Figure 4.19 Confocal microscopic images of cerebellum slice maintaining its architecture
**Figure 4.20** Confocal microscopic images of (a) myelin (MBP staining) (b) oligodendrocyte cell body (Olig 2 staining) (c) axon (NFH staining) and (d) co-localization of MBP (myelin sheaths) and NFH (axons).
Figure 2.21 Effect of TNFα and IFNγ genes on myelin after transfection with lpD-b-P/E 3 compared to no treatment and LPC treatment groups at day two, and seven post-transfection. Immunohistochemistry showing the expression of MBP (myelin) in red, NFH (axons) in green and Olig2 in blue in cerebellar slices. These images indicate that transfection with pro-inflammatory genes; results in demyelination up to seven days when compared to LPC treated groups (Scale bar 50μm).
Figure 2.22 Quantification of the co-localization of MBP and NFH via Mander’s coefficient when transfected with TNFα and IFNγ complexed with Lipofectamine 2000®, lpD-b-P/E 3 alone and lpD-b-P/E 3 complexed with empty vector (EV) when compared to TNFα or IFNγ gene complexed with lpD-b-P/E 3 and LPC treatment. Results indicate that genes transfected with Lipofectamine 2000® showed significant loss of co-localisation up to 14 days of treatment similar to lpD-b-P/E 3. lpD-b-P/E 3 alone showed demyelination within two days. lpD-b-P/E 3 when complexed with empty vector showed no effect on myelin. Data plotted shows mean ± SEM (n=3), * (p<0.05).
This might be because of the initial toxicity of the polymer itself. Complexing polymers with pDNA reduce the toxicity of the polymer; supported by the observations that the lpD-b-P/E 3 complexed with an EV did not affect myelination.

4.4 Discussion

A non-viral gene delivery approach to transfect CNS cells was used. This gene delivery method in a biologically relevant application was used where the transfected cells successfully expressed cytokines in an ex-vivo model of inflammatory demyelination. A hyperbranched poly (2-dimethyl-aminoethylmethacrylate) based polymer(lpD-b-P/E 3), previously in Chapter two and three [25] was used and the transfection protocol for delivering TNFα or IFNγ genes into various cell types in organotypic slice cultures of the cerebellum and cortex was optimised. lpD-b-P/E 3 was able to transfect these genes successfully and with low toxicity to CNS tissue. The resulting expression of pro-inflammatory cytokines and consequent demyelination makes this a promising approach for a gene delivery system for both ex-vivo and potentially in-vivo applications for the further study of disease mechanisms. The lpD-b-P/E 3 polymer (a non-liposomal, non-viral vector) successfully transfected CNS cells with little associated toxicity, are of importance for future gene transfer applications. Previous studies comparing liposomal and non-liposomal transfection of ventral mesencephalic explant cultures reported that liposome mediated gene transfer agents were more toxic due to cell membrane damage, which is a necessity for optimal transfection [31]. lpD-b-P/E 3 showed maximum transfection with minimal toxicity followed by Lipofectamine 2000®, FuGene® and Lipofectin® reagents when transfected with 2.5µg of pDNA. In fact, while FuGene® and Lipofectin® reagents were still able to deliver pDNA, they had a threefold increase in toxicity levels compared with lpD-b-P/E 3 and Lipofectamine 2000® reagent even at low doses.

These findings highlight the effect that the polymeric structure and properties can have on transfection viability. Both Lipofectin® reagent and Lipofectamine 2000® reagent are liposome mediated gene delivery vectors; however, their chemical formulation differs in one important way. Lipofectin® reagent is formed by coupling N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTMA; a cationic lipid) with a neutral helper dioleoylphosphatidylethanolamine (DOPE);
whereas, Lipofectamine 2000® reagent is the product of [1,2-bis(oleoyloxy)-3-(trimethylammonio) propane] (DOTAP) coupled with DOPE. The only difference between these molecules is the presence of ether bonds (DOTMA) rather than ester bonds (DOTAP) linking the chains to the backbone. This ether/ester bond differentiation is important because ester bonds are hydrolysable; potentially rendering the lipid biodegradable and reducing cytotoxicity[35]. The current study supports this assertion as Lipofectamine 2000® reagent showed significantly higher transfection and lower toxicity when compared to Lipofectin® reagent. FuGene® reagent is a lipid-based gene delivery vector, which has successfully been used as a gene delivery vector in-vitro [36]. However, it lacks serum stability and is easily degraded under DNase I[37]. The hyperbranched polymer lpD-b-P/E 3 was designed in such a way that the linear poly(2-dimethyl-aminoethylmethacrylate) unit binds pDNA efficiently while the polyethylene glycol based hyperbranched unit shields the polymer from its toxicity [25]. The current findings convincingly show that lpD-b-P/E 3 was able to transfect cells as effectively as or more effectively than other commercial reagents, while inducing minimal cell death. This polymer has the added advantage of being easy to synthesize and is considerably less expensive than commercial transfecting agents.

The lpD-b-P/E 3 polymer was able to transfect various cell types across all regions of the cerebellum; hence, lpD-b-P/E 3 is a good candidate to transfect genes in an organotypic slice culture disease model. In the current study, this assertion using genes encoding the pro-inflammatory cytokines TNFα and IFNγ was tested. Previous studies have found that TNF-mRNA expression is positively correlated with active demyelinating lesions and oligodendrocyte apoptosis in the MS brain [38] and in EAE [39]. IFNγ also plays an important role in the mechanisms of demyelination MS[40] and has been associated with the up regulation of major histocompatibility complex class II (MHC-II) molecules, gliosis and lymphocytic infiltration in a transgenic mice model. Taken together, this evidence demonstrates IFN’s role in inducing CNS demyelination [41]. Using lpD-b-P/E 3 we were able to transfect both neuroglia (astrocytes and oligodendrocytes) as well as neurons. It should be noted that lpD-b-P/E 3 alone and lpD-b-P/E 3 complexed with an empty vector did not release TNFα or IFNγ, suggesting that polymer itself is not toxic and does not induce
the production of inflammatory cytokines, hence making it a suitable carrier of these genes.

Currently, *ex-vivo* demyelination models are developed either by chemical treatment [33] or by the addition of a demyelinating monoclonal antibody specific for myelin oligodendrocyte glycoprotein (MOG) and complement [42]. Both these methods induce demyelination within one day of treatment followed by complete remyelination within one week. In this study, it was demonstrated that an *ex-vivo* model of pro-inflammatory cytokine induced demyelination resulting in a sustained loss of myelin without remyelination up to seven days after treatment. Using this same model, it was demonstrated that when demyelination was induced using chemical methods (LPC treatment), demyelination was observed within two days of treatment, but there was evidence of remyelination within seven days of treatment (Fig 6 and 7), which is similar to the de/remyelinating time course seen in previous studies [33, 43]. The studies in this phase also show that demyelination induced by LPC was not correlated with TNFα or IFNγ expression. While the LPC model of demyelination may be a useful tool, this method induces demyelination via solubilising the myelin membrane, as opposed to induction by an inflammatory response; hence, the underlying mechanism of demyelination by LPC differs from those in myelin related diseases such as MS. Studies have shown the demyelination induced by the addition of a monoclonal antibody specific for MOG also resulted in complete demyelination in two days and after removal of the antibody, significant remyelination was discernible within two to four days [44]. Therefore, compared to other models, this gene delivery approach resulted in demyelination over a longer period of time, making it a promising *ex-vivo* model for studying the underlying mechanisms of chronic inflammation mediated demyelination. However, more work needs to be done to produce stable transfection over a longer period.

An important characteristic of any *ex-vivo* model of inflammatory demyelination centres on whether the levels of cytokines released are biologically significant. Clinical studies have reported elevated TNFα levels in the CSF of MS patients in the range of 70-247 pg/ml during relapses[45-46], 93-226pg/ml in patients with secondary progressive MS [45] and 4-25pg/ml in remitting patients [45-46], while no TNFα was detected in control groups. The results in this phase of the study show that the transduction of the TNFα gene via hyperbranched polymer produced an
average of 287pg/ml TNFα for up to seven days followed by a decrease to 150pg/ml (day 14) and 50pg/ml (day 21), while the control groups showed an average of 14pg/ml. Thus, the TNFα levels found in the organotypic culture model are within the range of the CSF TNFα concentrations in MS patients during active disease stages; indicating that this model is biologically significant and can be an extremely useful ex-vivo culture system for studying the underlying molecular mechanisms of inflammatory demyelination.

Reported levels of IFNγ in MS patients have shown conflicting results. Some studies reported elevated IFNγ levels (4-256U/ml) in serum of MS patients [47], while other studies have reported no change compared to controls [48]. The studies conducted in this phase showed a transient threefold increase in released IFNγ two days after transfection compared with controls. However, no significant IFNγ release was observed after day two; indicating that IFNγ expression and subsequent levels of IFNγ may not be sufficient for prolonged demyelination when compared with TNFα over expression. The findings that IFNγ induced demyelination after transient expression compared to the prolonged expression of TNFα is in keeping with previous studies in dissociated cultures and in ex-vivo spinal cord slices showing that oligodendrocytes are much more susceptible to the cytotoxic actions of IFNγ than those of TNFα [49-50].

4.5 Conclusion

The hyperbranched polymer lPD-b-P/E 3 transduces various cell types in an organotypic brain slice system with higher efficiency and lower cytotoxicity in cerebellum slices compared with cortical slices. Use of the synthesised non-viral gene delivery system for transfection of TNFα and IFNγ genes resulted in the expression of inflammatory cytokines in cerebellar slices and subsequent demyelination. Hence, using the synthesized non-viral gene delivery system in an ex-vivo model will be a beneficial approach to investigate the underlying mechanisms associated with various changes in inflammatory demyelination without the need to use more complex in-vivo systems. These ex-vivo changes will give an information on the cellular processes taking place during the progression of myelin-related diseases such as MS.
4.6 References

Ex-Vivo Multiple Sclerosis Model


Chapter 5

Summary and Future Directions
5.1 Introduction

Multiple Sclerosis (MS) is one of the leading neurological disabilities that affect young adults. MS is considered to be a chronic autoimmune, inflammatory and demyelinating disease invading the central nervous system (CNS); however, the initial trigger causing this disease is still largely unknown [1]. MS is characterised by the presence of inflammatory demyelinating foci throughout the brain and spinal cord, accompanied by axonal and neuronal damage. Although inflammatory processes are thought to underlie the pathological changes, the individual mediators of this damage are unclear. Magnetic-resonance imaging (MRI) studies and autopsy tissue analysis from patients have made significant contributions towards the understanding of MS [2-7]. However, these studies are limited to post-mortem tissues, rare biopsies or analysis of MRI scans of MS patients who have died with subsequent pathology. These shortcomings have indicated the need to establish animal models that replicate the clinical course and pathology of MS. The most commonly used animal model of MS is Experimental Autoimmune Encephalomyelitis (EAE). At present the most common induction of this model involves the injection of Myelin Oligodendrocyte Glycoprotein (MOG) and proteolipid protein (PLP) followed by intraperitoneal injections of pertussis toxins [8-10].

The complex interactions between the immune and nervous systems in-vivo and the relative expense of EAE models makes the use of an ex-vivo approach an attractive alternative for studying disease mechanisms and testing efficacy. Hence, the use of organotypic brain slices as models for neurodegenerative disease research has gained in importance in recent years. Previous studies have shown that TNFα and IFNγ induce demyelination in the CNS of rodents [11-12]. Current ex-vivo demyelination models induce acute demyelination. The overall aim of this project is to establish an ex-vivo inflammatory-demyelinating model of MS utilizing a novel biomaterial-based non-viral gene transfection agent.

5.2 Summary

5.2.1 Phase One – Non-Viral Gene Delivery Vector Development and Optimization

The objective of phase 1 (Chapter two and three) was to develop and optimize a pDMAEMA-based hyperbranched polymer as a non-viral gene delivery vector for
efficient gene transfection with minimal toxicity. This was achieved by developing a unique hyperbranched polymeric system with a linear poly 2-dimethylaminoethyl methacrylate (pDMAEMA) block and a hyperbranched polyethylene glycol methyl ether methacrylate (PEGMEMA) and ethylene dimethacrylate (EGDMA) block synthesized via deactivation-enhanced atom transfer radical polymerisation (DE-ATRP). The use of the DE-ATRP technique enabled control of the polymer chain growth without early gelation. Two polymers were first synthesized: one with a high PEG content (termed lpD-b-P/E 1: molecular weight 38000Da), and another with a low PEG content (termed as lpD-b-P/E 2: molecular weight 14000Da). Both these polymers were characterized for their structure, molecular weight, size, charge, plasmid DNA (pDNA) binding ability and finally their transfection ability and toxicity in fibroblasts and adipose tissue-derived stem cells (ADSCs) at varying pDNA concentration.

Results indicated that lpD-b-P/E 1 complexed with G-Luciferase pDNA at 10:1 (w/w ratio), whereas lpD-b-P/E 2 complexed at 2:1 (w/w ratio). The size of the polyplex (a complex of polymer and pDNA) was dependent on their polymer to pDNA weight to weight (w/w) ratio as the size of the polyplexes decreased with an increase in polymer to pDNA weight ratio suggesting the higher the w/w ratio, the stronger the bond. On the other hand, the surface charge of lpD-b-P/E 1 remained constant at different weight ratios (5:1 to 20:1 w/w ratio) while the surface charge lpD-b-P/E 2 and linear pDMAEMA increased with increasing weight ratio suggesting that high degree of PEGylation shields the polyplex surface charge leading to reduced zeta potential while low degree of PEGylation leads to an increased electrostatic interaction between the polymer and the plasmid resulting in a stronger bond formation.

Initial studies on in-vitro transfection suggest that optimal weight ratio for each polymer to obtain highest level of transfection is different and depends on the cell line used. It was shown that poly ethyleneimine (PEI: commercial polymer) showed its optimal transfection at 2:1 w/w ratio for fibroblasts and 1:1 w/w ratio for ADSCs, while poly 2-dimethylaminoethyl methacrylate (dPAMAM: commercially available as SuperFect®) showed optimum transfection at 8:1 (both in fibroblast and ADSCs), while, lpD-b-P/E 1 showed optimal transfection at 8:1 w/w ratio in fibroblasts and 10:1 w/w ratio in ADSCs and lpD-b-P/E 2 had its optimal transfection at 10:1 w/w...
ratio (in both cell lines) while linear pDMAEMA showed its optimal transfection at 20:1 w/w ratio (in both cell lines). Having optimized the polymer to pDNA weight ratios on both cell types, the maximum amount of pDNA that the polymer can deliver for a specific number of cells without affecting either transfection or viability was studied. It was demonstrated that the use of this unique structure, ie a linear pDMAEMA block, which efficiently binds to pDNA and hyperbranched poly ethylene glycol (PEG)-based block act as a protective shell allowed maintenance of high transfection levels without sacrificing cellular viability even at high doses while the commercial polymers (PEI and dPAMAM) failed to maintain their viability at high pDNA doses.

To further optimize the hyperbranched polymer, the effect of PEGylation on transfection and viability was then studied. Therefore, a third hyperbranched polymer (lpD-b-P/E 3: molecular weight 24,000Da) was synthesized with the same polymeric structure as the previous ones, but with its PEGylation limited to a medium level. Studies showed that lpD-b-P/E 3 with medium PEGylation (molecular weight 24000Da) showed maximum transfection with minimal toxicity when compared to lpD-b-P/E 1 (high PEGylation; molecular weight 38000Da) and lpD-b-P/E 3 (low PEGylation; molecular weight 14000Da) indicating that high degree of PEGylation reduces cellular toxicity but affects the transfection ability of the polymer. On the other hand, low PEGylation enhances the transfection ability but indirectly reduces cell’s viability. These results suggest that PEGylation should be balanced to attain maximum transfection with minimal toxicity. These studies showed that protonation of lpD-b-P/E 3 did not significantly affect transfection but significantly reduced the cellular metabolic activity at lower pH (pH 3.5; fully protonated state) when compared to the partially protonated polymer (physiological pH 7). These results confirm that pDMAEMA at its physiological pH has sufficient H⁺ ions for efficient transfection and that pDMAEMA does not require an additional protonation step. These studies also confirmed that the transfection activity of cationic polymers is largely dependent on their transfection medium and cell types. In addition, serum-free transfection conditions did not significantly affect transfection or cellular metabolic activity however, the addition of serum significantly reduced cellular metabolic activity by 1.5 fold with no change in transfection in fibroblast, nucleus pulposus (NP) cells and in astrocytes. However,
annulus fibrosus (AF) cells and neu-7 cells did not have any effect on transfection or cellular metabolic activity at both serum and serum free medium. It was also observed that lpD-b-P/E 3 transfected NP cells, neu-7 cells and astrocytes with minimal toxicity when compared to commercial transfection agents such as PEI and dPAMAM. However, neither lpD-b-P/E 3 nor dPAMAM was not able to transfec AF cells, while PEI was able to transfec AF cells. All these studies suggest that transfection activity of cationic polymers such as pDMAEMA is largely dependent on their molecular structure, molecular weight, transfection medium and cell types. However, a more mechanistic study has to be performed to understand this behaviour.

5.2.2 Phase Two – Non-Viral Gene Delivery Optimization Ex-Vivo

The objective of phase two was to optimize the transfection ability of pDMAEMA-based hyperbranched polymer in an organotypic brain slice system. This was achieved by investigating the ability and cytotoxicity of the polyplex in varying organotypic brain slice tissue thickness, tissue section, polyplex application procedure, pDNA concentration, polyplex volume and polyplex ratio. Transfection ability and cytotoxicity of lpD-b-P/E 3 was compared to those of commercial transfecting agents and also to electroporation. The effect of non-viral transfection on embryonic slices at varying pDNA doses was studied. These studies showed that electroporation caused the highest tissue damage followed by lipotransfection and minimal tissue damage with lpD-b-P/E 3. Since localized transfection was observed with embryonic tissue, further studies were performed on postnatal (P15) brain slices. Moreover, postnatal slices were more relevant for the next part of the project viz. to establish an ex-vivo MS model.

The effect of slice thickness of P15 brain slices upon transfection was first studied. These studies showed that 250µm thick slices transfected much more efficiently than 400µm thick slices. Hence, subsequent optimization, studies were performed on 250µm thick slices. These studies also revealed that polypexes, when added on top of the slices, transfected much more efficiently than when added into media, with an overall eleven-fold increase in transfection. It was seen that cerebellar slices transfected more efficiently than did cerebral slices due to their better viability at all polymer to pDNA weight/weight ratios, with maximum transfection being obtained.
at 8:1 weight/weight ratio. Maximum transfection was obtained when slices were transfected with 2.5µg of pDNA per slice and was reduced at higher concentrations of pDNA (10µg) due to increased level of cell death at higher concentrations. However, polyplex volume did not have any effect on transfection or cell death. The comparison study with commercial transfecting agents showed that lpD-b-P/E 3 exhibited the highest transfection with least toxicity.

In summary, the results of the optimization studies showed that the maximum transfection efficiency of lpD-b-P/E 3 in organotypic slices occurred at 8:1 w/w ratio when transfected with polyplex on top of the slice with 2.5µg of pDNA on cerebellar slices. At these optimized conditions, GFP positive cells were found diffusely across all regions of the cerebral slices. The highest numbers of transfected cells were found in the molecular layer of the cerebral slices. Transfected cells were of a variety of morphologies including neurons, astrocytes, oligodendrocytes etc.

5.2.3 Phase Three – Establishment of an Ex-Vivo Model of Multiple Sclerosis via Non-Viral Gene Delivery Approach

The objective of this phase was to establish an ex-vivo model of multiple sclerosis via non-viral gene delivery approach. As mentioned previously, MS is characterised by the presence of inflammatory demyelinating foci throughout the brain and spinal cord accompanied by axonal and neuronal damage. Although inflammatory processes are thought to underlie the pathological changes, the individual mediators of this damage are unclear. In order to study the role of pro-inflammatory cytokines in demyelination in the central nervous system and to thereby establish a biomaterial-based ex-vivo MS model, a non-viral gene transfection agent (lpD-b-P/E 3) developed in phase one and two was utilized.

Organotypic brain slices were successfully transfected with the TNFα or IFNγ genes using lpD-b-P/E 3. Transfection of TNFα and IFNγ plasmid using lpD-b-P/E 3 resulted in significant gene expression, with the highest TNFα and IFNγ expression on day four. By day 21, a reduced level of expression was observed for both cytokines. TNFα protein levels reached a peak at day four and then returned to baseline by day 21. In contrast, IFNγ protein levels reached a peak at day two and then returned to baseline by day four indicating that only transient transfection was
Summary and Future Directions

achieved. It was seen that $\text{lpD-b-P/E } 3$ alone and $\text{lpD-b-P/E } 3$ complexed with an empty vector (EV) treated groups showed no increase in TNF$\alpha$ or IFN$\gamma$ production suggesting that the polymer itself is not toxic and does not induce the production of inflammatory cytokines, thus making it a suitable carrier of these genes. When compared with the group that received no treatment, the slices that were transfected with either the TNF$\alpha$ or IFN$\gamma$ plasmid showed significant demyelination by two days which continued up to fourteen days after treatment while the conventional lysophosphatidylcholine (lysolecithin, LPC) induced model resulted in remyelination within four days. Previously performed clinical studies have reported elevated TNF$\alpha$ levels in the CSF of MS patients in the range of 70-247 pg/ml during relapses[13-14], 93-226 pg/ml in patients with secondary progressive MS [13]and 4-25 pg/ml in remitting patients [13-14], while no TNF$\alpha$ was detected in control groups. These results were within the range of the cerebrospinal fluid (CSF) TNF$\alpha$ concentrations in MS patients during active disease stages. Transduction of the TNF$\alpha$ gene via hyperbranched polymer produced an average of 287 pg/ml TNF$\alpha$ for up to seven days followed by a decrease to 150 pg/ml (day 14) and 50 pg/ml (day 21), while the control groups showed an average of 14 pg/ml. These finding indicate that this model is biologically significant and can be an extremely useful ex-vivo culture system for studying the underlying molecular mechanisms of inflammatory demyelination in myelin-related diseases such as MS.

Figure 5.1 illustrates the three phases of this research project and documents the main outcomes from each phase. The overall aim of this project was to develop an inflammatory demyelinating ex-vivo model of MS via non-viral gene delivery approach. It was hypothesised that the delivery of inflammatory TNF$\alpha$ and IFN$\gamma$ genes into an organotypic brain slice system using a hyperbranched pDMAEMA-based polymer will create inflammation mediated demyelination and these results bear this out.
Figure 5.1 Summary of the main outcomes from each phase of this thesis.
5.3 Limitations

5.3.1 Phase One

In Chapter one and two a hyperbranched pDMAEMA-based polymer with a linear pDMAEMA unit and a hyperbranched PEGmethacrylate unit was synthesized. This polymer was analytically characterized for its structure, molecular weight, size, charge, pDNA binding ability and stability. This polymer was also characterized in-vitro on various cells types under varying conditions. However, this study has two major limitations. One major limitation is the lack of thorough understanding of the molecular mechanism involved in the internalisation process of the hyperbranched pDMAEMA-based polymer, which will also give a deeper understanding of the cell line dependant behaviour of the hyperbranched polymer. Previous studies have shown that pDMAEMA is internalised into cells via fluid-phase endocytosis and does not cause endosomal disruption although it alters the morphology of the late endosomes/lysosomes [15].

This suggests that the cytotoxicity of the polymer is not due to endosomal or lysosomal bursting, which can induce cell death. Hence, a deeper understanding of the molecular mechanism of pDMAEMA and hyperbranched pDMAEMA is necessary to understand the exact process involved in transfection and the process that causes cytotoxicity. The second major drawback of this study is that the transfection efficiency test such as flow cytometric analysis has not been conducted. Flow cytometry allows the measurement of several parameters simultaneously; for example: percentage of transfected cells, changes in cell morphology upon treatment with the polymer and percentage of uptake etc. For example, with fluorescently tagged plasmid DNA, it will be possible to study the percentage of the cells and the type of cells that have uptaken the polyplexes in a co-culture system. For instance, in performing in-vivo transfection it is important to know the type of cell that takes up the polyplex and undergoes transfection. Such knowledge can lead to a greater understanding of where the limitation of transfection lies and can be a useful procedure to use in future studies. Other minor limitation of this phase includes the lack of exploration of other hyperbranched structures, for example a hyperbranched polymer with a linear PEGmethacrylate unit and a hyperbranched pDMAEMA unit. It will be interesting to look at the effect of change in polymeric structure on
transfection and cellular metabolism. In phase one most of the studies performed were compared to commercially available transfecting agents such as PEI and dPAMAM. In the last three years, new transfection reagents have emerged and these have not been compared with the hyperbranched polymer.

5.3.2 Phase Two

Phase two of this project was primarily focussed in optimizing the transfection ability of the pDMAEMA-based hyperbranched polymer in an *ex-vivo* environment. Organotypic slice cultures have been typically prepared from brains of animals at postnatal days 0-15. Brain tissues from these young ages show a high degree of plasticity and resistance to mechanical trauma during the slice preparation, which is important in obtaining viable and healthy cultures routinely. Moreover, previous studies have shown that myelination in rodents starts after postnatal day P10 and is significantly prominent at P15 [16]. Hence, in this study, postnatal (P15) organotypic brain slices were used. However, as mentioned in Chapter one, MS affects young adults with women being affected twice as often as men. Preparation of slice cultures from older animals is technically more challenging as the slices degenerate during long-term culture periods and this degeneration occurs more often in these young adult slices than in neonatal slice cultures [17]. Attempts are made to culture adult rat brain tissues *ex-vivo* for a prolonged culture time via addition of of ascorbic acid, vitamin E or insulin to the culture medium [18]. Such modifications in culture medium have resulted in brain slice culture for at least six days. Therefore, considering the balance of merits and challenges associated with this preparation, mature slice cultures can be more relevant in establishing *ex-vivo* models, following initial outcome in the neonatal culture or evaluation of critical pathways relevant to the aged brain. Hence, an optimization of transfection in adult brain slices could be more beneficial in future studies. Also in this study the transfection conditions on cerebral and cerebellar slices was optimized. It will be interesting to study the transfection on other areas of the CNS such as striatum, hippocampus, spinal cord etc. A further limitation of this study is in determining the transfection efficiency of the transfectant by counting the number of cells that were GFP positive. Cell counting does not provide an accurate representation of the cells that became transfected for the following reasons: unlike mono-layered cell culture, organotypic
slices are 250µm thick and therefore different layers have to be accounted and these numbers are hard to estimate. Also, many of the transfected cells are lost during the slice fixation process. Hence, the transfection ability of the transfecting agents was determined via measuring the G-luciferase expression.

5.3.3 Phase Three

In Phase 3, TNFα and IFNγ genes were delivered to organotypic cerebellar slices via the hyperbranched pDMAEMA-based polymer. The results showed that TNFα and IFNγ genes were expressed up to 14 days after transfection with TNFα showing a significant protein expression up to 14 days and reaching a peak at day four (350pg/ml) and then returning to baseline by day 21. IFNγ protein levels reached a peak at day two (75pg/ml) and then returned to baseline by day four. The relatively low levels (approximately 20pg/ml) of IFNγ protein after day two indicates that only transient transfection was achieved. This might be due to the promoter used in the plasmid. By designing a new promoter for IFNγ plasmid, this issue may be resolved which may lead to a more sustained release of IFNγ. The demyelination effect of TNFα and IFNγ genes were monitored only for seven days after transfection. As MS is a chronic disease, it will be more appropriate to monitor the effect of demyelination and remyelination for a prolonged period. Although organotypic-brain slices have many advantages, they are not without their shortcomings. The first disadvantage of the slice culture is that not all the areas of the CNS are easily amenable to culture [19] and is only ideal for regions with layered structures. Moreover, organotypic cultures do not have a vascular compartment and a CSF flow unlike in-vivo models. Hence, the effect of CSF flow, inflammation and blood brain barrier are neglected when performing transfection studies in an organotypic brain slice cultures, and these are crucial factors determining the transfection efficiency of the vector. Although organotypic brain slices are better models to study the molecular mechanisms involved in inflammatory demyelination, they cannot be used to study disease progression in MS. Hence, in future studies using live rodents will be essential to establish a more accurate model as outlined in section 5.3.2.
5.4 Future Directions

5.4.1 Establishment of Inflammatory Neurodegenerative Ex-Vivo Models via Non-Viral Gene Delivery

Development of neurodegenerative models such as Parkinson’s model, Alzheimer’s model, Huntington’s model and chronic MS models involves many challenges, especially in delivering the test reagents and testing their ability with reasonable throughput and with the access to directly measure the modulation of the molecular targets and signal transduction pathways. Previous studies have shown that these neurodegenerative diseases are associated with chronic inflammation [20-23]. The role of cytokines in inducing inflammation in these diseases is currently under investigation. Identification of these inflammatory inducing cytokines and the sustained delivery of bio molecules that induce pro-inflammatory cytokine expression in organotypic brain slices allow for replication of an inflammatory model and will enable understanding of the molecular mechanism involved during inflammation.

Platform Development

Further modifications of the hyperbranched pDMAEMA-based polymer described in Chapter two and three are needed for the polymer to function as an ideal vector. Polymer degradation is a critical consideration for most in-vivo gene delivery applications where cytotoxic polycations are frequently used. Degradation is typically achieved by the incorporation of one or more cleavable linkers into either the polymer backbone, side-chain, or as a cross-linker or by the addition of a biodegradable monomer. Hyperbranched pDMAEMA has an added advantage of having free vinyl moieties where thiolated RGD sequences can be easily attached via Michael type addition [24]. Change in polymer structure also enhances the transfection efficiency. Similar hyperbranched polymers with a micelle-like structure can also be synthesized for enhanced transfection. For example, micelles with a linear pDMAEMA core and a hyperbranched PEGMEMA and EGDMA shell or micelles with a linear PEGMEMA shell and a hyperbranched PDMAEMA and EGMA core can be synthesized as shown in figure 5.2.
Figure 5.2 (a) Linear pDMAEMA core and hyperbranched PEGMEMA and EGDMA shell (b) Hyperbranched pDMAEMA and EGDMA core and linear PEGMEMA shell.
**Multiple Sclerosis Model – Further Development, Characterisation and Validation**

In Chapter four, the synthesised hyperbranched polymer successfully transfected TNFα and IFNγ genes and showed TNFα and IFNγ gene expression up to 14 days and then returned to the baseline within 21 days. Both the TNFα and IFNγ plasmids used in the study were CMV promoter driven, which is a transient, strong promoter but shows a steady decrease of activity over time [25-26]. This decrease is due to a number of factors: an inhibition of the promoter activity by cytokines or by the binding of a repressor protein, by its methylation, or by the loss of a positive activator [25]. Studies have shown that EF1-α promoter shows 10-fold lower expressions than CMV but its expression will last for more than four weeks [27]. Similarly, CMV-ubiquitin hybrid promoters have shown long-term expression for six months after treatment, with a level of expression equivalent to the CMV promoter [28]. Hence, replacement of the CMV promoters with other promoters (such as β-actin, elongation factor 1-α (EF1-α) or ubiquitin) might confer a more sustained expression which can be utilized in designing a chronic model of MS. This project will be aiming at a chronic response with demyelination for one or two months hence a long term organotypic brain slice culture system will be employed. Hence with subsequent alteration with the polymer structure, promoter and organotypic slice culture system, it can be hypothesized that a chronic *ex-vivo* model can be developed by sustained delivery of TNFα and/or IFNγ genes.

Cortical demyelination in EAE models clearly depends on peripheral immune priming against a myelin protein (MOG or MBP), since injection of pro-inflammatory cytokines in animals immunized with IFA alone was an insufficient trigger for cortical demyelination. However, cytokine injection was necessary in MOG-primed animals, since PBS injection alone caused only minor pathology [29]. Hence, the non-viral *ex-vivo* model of inflammatory demyelination, mentioned in this thesis can further be developed by looking at the effect of TNFα or IFNγ genes alone and/or by looking at their synergic effect on MOG or MBP primed adult rat brains.

Activation of macrophage/microglia during the inflammatory process of MS contributes directly to myelin damage through several mechanisms including production of cytokines, metalloproteinases, production of free radicals, and
phagocytosis. Treatment of TNFα and IFNγ in in-vitro CNS cultures resulted in microglial activation and proliferation [30-31]. The effect of non-viral transfection of TNFα or IFNγ gene on demyelination can further be studied by looking at its effect on microglial activation. Hence, immunostaining for quiescent and activated/phagocytic microglia after non-viral transfection of TNFα and IFNγ genes into brain slices will provide more information on the cellular events taking place during active demyelination. It is also know that treatment of TNFα and IFNγ also induces oligodendrocyte apoptosis [32-33]. The presence of TNFα or IFNγ on oligodendrocytes can be measured by double stained sections for TNFα or IFNγ and CNPase. The co-localization of TNFα or IFNγ and CNPase will indicate the direct cytotoxic effect of TNFα or IFNγ on oligodendrocyte leading to demyelination. Further morphometric analysis by light and electron microscopy will give an insight on the extent of demyelination and remyelination [29].

Therapeutics that impact remyelination in the CNS could be critical determinants of long-term functional outcome in MS. Fingolimod (FTY720), an immunomodulating drug is currently used in clinical trials for treating MS [34]. FTY720 has shown to modulate multiple neuroglial cell responses, resulting in enhanced remyelination in organotypic slice cultures [35]. Certain steroids such as progesterone have shown to stimulate MBP expression and consequently promote remyelination in organotypic slice cultures [36]. Non-viral ex-vivo model of inflammatory demyelination can further be validated using such myelin protectants.

**Inflammatory Ex-Vivo Parkinson’s Model**

Parkinson’s disease (PD) is a progressive-neurodegenerative disease affecting 6.3 million people worldwide [37]. The pathological hallmark of PD is the death of noradrenergic neurons within the locus coeruleus (LC), death of dopamine (DA) neurons within the nigrostriatal system, and the presence of proteinaceous inclusions called Lewy bodies (LB) [38]. Studies show that the pathogenesis for nigral-dopaminergic cell death involves oxidative and nitrative stress, excitotoxicity, inflammation, mitochondrial dysfunction, and altered proteolysis [39]. Previous studies have shown increased levels of pro-inflammatory cytokines such as TNFα [40-41], interleukin (IL)-1beta [40, 42], and IL-6 [40], in the nigrostriatal region of post-mortem brains and/or in the ventricular or lumbar cerebrospinal fluid (CSF) in
patients with sporadic PD, and in animal models of PD. These cytokines have been shown to cause cell death directly by binding death receptors, or indirectly via the production of reactive oxygen/nitrogen (ROS/RNS) species, which converge on mitochondrial dysfunction and activation of intrinsic cell death pathways \[43\]. Current toxin disease Parkinson’s models utilizes 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) \[44\], 6-hydroxydopamine (6-OHDA) \[45\] or lipopolysaccharide (LPS) \[46\]. All these methods initiate inflammation indirectly by the over-expression of inflammatory cytokines. This project will aim at identifying the inflammatory cytokines responsible for nigral-dopaminergic cell death and address their direct role on inflammation which can be achieved by the transfection with genes encoding TNFα, IL-1β or IL-6 or a combination of all via a non-viral gene delivery approach. Nigrostriatal brain slice cultures will be utilized in this study, as described by Kearns et al \[47\]. This culture system has shown an impressive long-term viability with intact cytoarchitecture throughout cortical and sub cortical regions, with intact circuits presents within the slice, interconnecting the striatum, substantia nigra pars compacta (SNc), and cortex and currently utilized for modelling PD in-vitro. This unique method utilizing a non-viral gene delivery approach presents a new strategy for studying the direct effects of inflammatory reaction on dopaminergic system and is also potentially useful for studying PD. Similar strategy could be employed to design other inflammatory models for Alzheimer disease (AD), in which cytokine production is believed to be a key pathologic event in the progression of inflammatory cascades in AD brains with human brains and animal models showing an over expression of TNFα, IL-6 and IL-1β genes \[48-49\]. The hippocampus is known to play a central role in learning and memory and its malfunction has been concerned to be largely responsible for the cognitive deficits manifested in Alzheimer’s patients. Delivery of TNFα, IL-6 and IL-1β genes in to an organotypic hippocampus slice via non viral gene delivery approach will enable a broader understanding to the direct influence to the inflammatory cytokines in AD. Moreover, a comparative study between ex-vivo PD model and AD model can be performed by comparing the effect of TNFα, IL-6 and IL-1β genes on nigrostriatal brain slice cultures and hippocampus brain slice cultures. The major future directions, the current project can take, are summarised in Figure 5.3.
Figure 5.3 Schematic depicting possible future directions of the project.
5.4.2 Development of a Chronic Multiple Sclerosis Rodent Model with the use of a Functional Biomaterial

**Overall Objective**

Development of a slow-release gene delivery method to brain tissue using a collagen/fibrin hydrogels injected into the subarachnoid space in rats. This technology will be used to develop an animal model of chronic CNS inflammation in rats and for development of neuroprotective strategies for treatment of neurodegenerative disease.

**Project Summary**

During the course of Multiple Sclerosis (MS), the body is thought to mount an immune response against the myelin sheath (an insulating, fatty layer surrounding nerves) resulting in myelin loss (demyelination), scarring, inflammation and eventual loss of nerve cells (neurons) in brain and spinal cord. This gives rise to a wide variety of neurological symptoms. An animal model for MS exists, known as Experimental Autoimmune Encephalomyelitis (EAE), which can mimic some of the hallmarks of the disease. This model is induced by immunizing animals with MOG; a protein associated with the myelin sheath, and has proved to be useful in studying inflammatory events and loss of the myelin sheaths during the early stages of MS. However, it is less useful in understanding the events that result from the more chronic effects of the disease, such as progressive loss of neurons in the grey matter. A recently published work has reported the presence of activated immune cells in the membranes surrounding the brain (meninges) in 40% of cases of secondary progressive MS, where loss of neurons is seen [50]. It is thought that the neuronal loss is directly associated with the long-term presence of these immune cells, which can result in the production of neurotoxins and pro-inflammatory cytokines such as TNFα and IFNγ, into the fluid- filled space surrounding the brain. The brain thus becomes bathed in a soup of harmful molecules, leading to slow long-term damage to the underlying cells and the consequent slow loss of brain function that characterises disease progression in MS. This project will exploit this phenomenon to develop an in-vivo model of immune-cell mediated toxicity, whereby the brain is bathed in cytotoxic cytokines. Not only will such a model enable study of the
mechanisms which may lead to neuronal loss in MS, but may also provide information on loss of neurons in other neurodegenerative diseases, such as Alzheimer’s, Parkinson’s and Motor Neuron disease.

A gene delivery approach for bathing the brain in either cytotoxic cytokines or neuroprotective compounds is proposed. By injecting collagen or fibrin hydrogels loaded with polyplex (a non-viral gene delivery vector complexed with the genes of interest) or stably transfected cells, into the cerebrospinal fluid (CSF)-filled area surrounding the brain and spinal cord, known as the subarachnoid space, delivery of molecules to the entire surface of the central nervous system may be achieved. However, this needs to be done in a way that provides a long-term presence of the genes and cells because of the progressive nature of these diseases.

The use of biodegradable collagen or fibrin hydrogels, in which genes can be encapsulated, can further provide a slow-release technology targeted to particular cell types or tissue structures. A schematic representation of the project is shown in Figure 5.4.

**Platform Development**

In the current thesis, it has been demonstrated that the hyperbranched pDMAEMA-based polymer can efficiently transfect TNFα and IFNγ genes *ex-vivo*. However, a major problem with non-viral gene delivery is the lack of efficacy *in-vivo*. While polymeric and liposomal vectors may work well *in-vitro*, performance is often poor *in-vivo*. Hyperbranched pDMAEMA-based polymer with modifications mentioned in section 5.3.1 might be an efficient vector for *in-vivo* applications. Preliminary studies have shown a controlled release of 18% of pDNA from the hydrogel after fourteen days of incubation in artificial CSF (ACSF) (Figure 5.5). The release of the polyplexes can be modified by adjusting the collagen concentration and crosslinker. The size of the microspheres has to be taken into consideration. Microspheres in this scenario acts as a reservoir system and therefore an ideal sphere should not be uptaken by the resident cells. Preliminary studies have shown a size dependent uptake of microspheres by primary astrocytes (Figure 5.6).
**Figure 5.4** Schematic summary of the research steps to be taken to develop a chronic MS rodent model.
Figure 5.5 Percentage of pDNA release from collagen type I hydrogel over time. Results indicate that around 18% pDNA was released from the hydrogel after fourteen days of incubation in ACSF.
Summary and Future Directions

Figure 5.6 Uptake of FITC labelled spheres (a) 100nm (b) 1000nm by primary astrocytes. (FITC labelled spheres (green) and nucleus staining with DAPI (blue).
FTIC labelled collagen type I spheres of 100nm were easily uptaken by astrocytes while spheres of 1000nm were not uptaken (Figure 6.4). Hence, a thorough evaluation of the release of polyplex from microspheres alone, hydrogels alone and a system including both microspheres and hydrogels need to be performed in-vitro on primary astrocytes.

5.4.3 Biomaterials Approach for Biomolecular Therapies for Multiple Sclerosis

Current Therapies and Drawbacks

The exact cause of MS has not yet been identified, hence there is no treatment targeting a causative factor. Current therapies either modulate or suppress the immune system. Interferon Beta (IFN β) (Avonex®, Betaseron® /Betaferon®, and Rebif®) and glatiramer acetate (Copaxone) have been used for a number of years and are known to reduce the frequency of new relapses by 30-40%. IFN β is known to greatly reduce the extent of active MRI lesions and when administered to patients with early course of multiple sclerosis affected the subsequent course of the disease, by decreasing the amount of inflammation [51]. Glatiramer acetate induces the proliferation and activation of a specific population of TH cells, which enter the CNS and express anti-inflammatory and neuroprotective effects [52]. However, their effect on disease progression still remains controversial. Natalizumab (Tysabri®) is an other drug approved for the treatment of MS. It was seen that the treatment with Natalizumab led to fewer inflammatory brain lesions and fewer relapses over a six-month period in patients with relapsing multiple sclerosis [53]. However, none of the available drugs can cure MS. Some studies have shown that substances such as corticosteroids shorten relapse phases in MS. However, they do not influence the long-term disease course [54].

Biomaterials Approach – Controlled Drug/Gene Delivery

A biomaterials-based delivery system can play a crucial role in terms of control delivery of drugs/genes, especially to chronic diseases. It confers the long-term delivery of therapeutic molecules. Hence, a strategy similar to that mentioned in section 5.3.2 can be exploited for the controlled delivery of therapeutic molecules (Figure 5.7).
This approach can also be beneficial for dual drug delivery. With recent studies showing Tysabri® added to IFNβ-1a being significantly more effective than IFNβ-1a alone in patients with relapsing multiple sclerosis [55], controlled release of multiple drugs from a biomaterial based reservoir system will be a promising approach in MS research.
Figure 5.7 Schematic summary of the biomaterials approach for bimolecular therapies for MS.
5.5 References


Summary and Future Directions


Summary and Future Directions


Appendices
## A. List of Agents/Compounds Used

### Table A.1 List of compounds and reagents used in this study

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B. Synthesis of Hyperbranched Polymer

B.1 Synthesis of Poly (2-dimethyl-aminoethylmethacrylate) (pDMAEMA)

Materials

\[
\begin{align*}
\text{DMAEMA} & = 15 \text{g} \ (9.54 \times 10^{-2} \text{ moles}) \\
\text{EBiB} & = 0.146 \text{g} \ (7.51 \times 10^{-4} \text{ moles}) \\
\text{CuCl} & = 0.0371 \text{g} \ (3.75 \times 10^{-4} \text{ moles}) \\
\text{PMDETA} & = 0.0649 \text{g} \ (3.75 \times 10^{-4} \text{ moles}) \\
\text{THF} & = 15 \text{ml}
\end{align*}
\]

Procedure

1. Switch on oil bath to 50°C temperature.
2. Wash equipment to be used with acetone.
3. Weigh out DMAEMA into a 100ml round bottomed flask with two necks.
4. Add EBiB and PMDETA and a stir bar.
5. Bung both necks and bubble argon through for ~20 minutes (gentle flow).
6. Weigh out CuCl and add it quickly into the reaction vessel and keep argon flowing in. (\(O_2\) kills the reaction).
7. Place flask into oil bath at 50°C and stir at medium speed (observe carefully for changes in viscosity).
8. Periodically (half an hour) remove samples for GPC analysis under positive argon pressure.
9. To stop the reaction when required molecular weight is achieved by allowing oxygen into the flask (shake the round bottom flask gently).
10. Take excess of hexane in a beaker (five times THF) equipped with a stirrer. Under stirring condition add drop by drop of the above mentioned reaction mix to hexane.
11. Separate the polymer precipitate from hexane and evaporate any hexane left in a vacuum oven.
12. Store the polymer at 4˚C (for one week) or -20˚C (for > one week).

B.2 Synthesis of Poly (2-dimethyl-aminoethylmethacrylate) (pDMAEMA) block-Poly Ethylene Glycol Methyl Ether Methacrylate (PEGMEMA)/Ethylene Glycol Dimethacrylate (EGDMA)

Materials

\[ p\text{DMAEMA as mentioned in B1 (7.51x10^{-4} moles)} \]

\[ \text{PEGMEMA} = 28.538g (6.008x10^{-2} \text{moles}) \]

\[ \text{EGDMA} = 2.9779g (1.502x10^{-2} \text{ moles}), \]

\[ \text{PMDETA} = 0.0324g (1.87x10^{-4} \text{ moles}) \]

\[ \text{AA} = 0.0066g (3.75x10^{-5} \text{ moles}) \]

\[ \text{THF} = 40 \text{ ml} \]

Procedure

1. Switch on oil bath to 50˚C temperature.
2. Wash equipment to be used with acetone.
3. Dissolve DMAEMA in THF in a 100ml round bottomed flask with two necks.
4. Weigh out PEGMEMA, EGDMA, EBiB and PMDETA and add to the round bottom flask equipped with a stir bar.
5. Bung both necks and bubble argon through for ~20 minutes (gentle flow).
6. Weigh out AA and add it quickly into the reaction vessel and keep argon flowing in. (O\textsubscript{2} kills the reaction).
7. Place flask into oil bath at 50˚C and stir at medium speed (observe carefully for changes in viscosity).
8. Periodically (every hour) remove samples for GPC analysis under positive argon pressure.
9. To stop the reaction when required molecular weight is achieved by allowing oxygen into the flask (shake the round bottom flask gently).
10. Take excess of hexane in a beaker (five times THF) equipped with a stirrer. Under stirring condition add drop by drop of the above mentioned reaction mix to hexane.

11. Separate the polymer precipitate from hexane and evaporate any hexane left in a vacuum oven.

12. Make sure the experiment is done under dark (without lights on).

13. Add the precipitated polymer into dialysis membrane (molecular weight cut-off (MWCO) 8000) and dialysis against highly purified water for three days under dark conditions. Change water every five hours.

14. After three days when the polymer looks transparent, freeze dry the polymer in dark.

15. Store the polymer at 4°C (for one week) or -20°C (for >one week) in dark.
C. Sample Preparation for Gel Permeation Chromatography (GPC)

1. Weigh 10mg of polymer and dilute in 2ml dimethyl formamide (DMF).
2. In a glass pipette put some cotton and add some silica gel until half filled.
3. Add 2ml of sample on the glass pipette (so that it passes through the silica gel and cotton).
4. Collect the filtered polymer solution in a 5ml clean glass beaker.
5. Pass the samples through 0.2µm filter (to demonstrate the absence of gelation) and collect them in GPC vials.
6. Run GPC.
D. Sample Preparation for Proton Nuclear Magnetic Resonance (\(^1\text{HNMR}\))

1. Dissolve 5mg of polymer sample in one ml CDCl\(_3\).
2. In a glass pipette put some cotton.
3. Add 1ml of sample on the glass pipette.
4. Collect the purified polymer solution in \(^1\text{HNMR}\) tube.
5. Run \(^1\text{HNMR}\).
E. **Plasmid Preparation**

E.1 **Transformation**

1. Add 1µg of plasmid to each tube.
2. Prepare an icebox – need to keep this cold.
3. Take XL-1 Blue cells out of -80°C freezer and thaw on ice.
4. Add 50µL of the bacterial suspension to each tube.
5. Mix the tubes well.
6. Incubate two minutes on ice.
8. Incubate another two minutes on ice.
9. Add 1 ml RT LB Broth without any antibiotics (bacteria need to recover a bit).
10. Put this tube in shaker for 30-45 minutes at 37°C.
11. Plate this suspension using a spreader onto LB + antibiotics plate (20 µg/ml concentration).
12. Leave in a humidified 37°C incubator overnight.
13. Pick colonies, do a second selection (ie. Inoculate 1ml of LB + antibiotics, leave eight hours and then streak onto a new plate) (only do if there are very few colonies on the first plate).

E.2 **Propagation (assuming largest scale preparation)**

1. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture.
2. Incubate eight hours at 37°C with vigorous shaking.
3. Transfer 2.5-5ml starter culture into 2.5 L of LB broth.
4. Incubate at 37°C for 12-16 hours with vigorous shaking.

E.3 **Isolation (adapted from Qiagen GIGA-prep protocol, requires components of kit)**

1. Harvest the bacterial cells by centrifuging the broth at >6000g for 15 minutes at 4°C. Remove supernatant.
2. Attach filter to a glass bottle that can be connected to a vacuum.
3. Resuspend pellet in 125 ml buffer P1.
4. Add 125 ml of pre-warmed buffer P2. Invert vigorously four to six times and incubate at room temperature for five minutes.
5. Add 125 ml of cooled buffer P3 and invert four to six times. Mix further if necessary – white, fluffy material should form and lysate should cease to be viscous.
6. Pour lysate into the filter cartridge and incubate at RT for ten minutes.
7. Switch on vacuum until all liquid has passed through filter.
8. Add 50 ml buffer FWB2 to cartridge and gently stir precipitate. Turn on vacuum again.
9. Add 30 ml buffer ER to lysate to remove endotoxins. Invert bottle ten times and put on ice for 30 minutes.
10. Near the end of the 30 minutes, equilibrate Qiagen-tip 10,000 by adding 75 ml buffer QBT and allowing emptying via gravity flow.
11. Add filtered lysate and allow entering resin by gravity flow.
12. Wash the column with a total of 600 ml buffer QC.
13. Elute the DNA from the column with 100 ml buffer QN (can pre- warm to improve efficiency).
14. Precipitate DNA by adding 70 ml of RT isopropanol to eluted solution. Mix and centrifuge immediately at greater than 15 000g for 30 minutes at 4°C.
15. Wash pellet with 10 ml of endotoxin-free RT 70% ethanol and centrifuge again.
16. Air dry pellet and redissolve in suitable volume of buffer TE.

E.4 Characterization

1. UV spectroscopy can be used to estimate the quality of the plasmid by comparing the ratios of the absorbance at 260 and 280 (should be as close as possible).
2. Gel electrophoresis (outlined later) can also be used to determine plasmid quality. The bands observed generally include super-coiled (travels farthest), nicked circles (travels more slowly) and possibly varying forms of cut, linear plasmids.
F. Characterization of Plasmid/Polymer (Polyplexes)

F.1 UV spectroscopy

1. Start-up NanoDrop™.
2. Wipe the pedestal clean with a Kimwipe®.
3. The software will require a distilled water sample to initialize, then will ask for a blank sample – this is a sample with no DNA in it, but any other buffer solutions, etc. Only one-two μl required.
4. Fill in the Sample ID space, then start reading samples.
5. The output of the NanoDrop™ is a spectral representation of the UV absorbance of the sample. Normally, the peak absorbance is at 260nm. If the samples are tightly polyplexed, this peak may be closer to 300nm. Once polyplexed, the concentration measurement is no longer reliable.

F.2 Gel electrophoresis

1. 0.7g agarose in 100ml TAE buffer (for large gel).
2. 0.35g agarose in 50ml TAE buffer (small gel).
3. Add about 2ml extra liquid to account for boiling.
4. Mix in an Erlenmeyer flask, and put into microwave.
5. Leave in microwave until boiling.
6. Remove from microwave with hot mitts or something to protect hands from heat.
7. Allow to cool until can be held comfortably in hand.
8. Add 10μl SYBR® safe dye (do not add until the solution is cool enough or the experiment will be ruined).
9. Pour immediately into plate, add comb and remove any bubbles.
10. Leave to cool and set.

Running Gel

1. Pipette samples into wells.
2. Put on cover, making sure that the orientations are all correct.
3. Attach to power supply.
4. Run gels at 80-100V and leave the current on auto. Check that bubbles are forming along the wires at the bottom.

5. Check after 15 minutes – dye should have moved slightly out of the wells. Check that the direction is right and that all seems to be in order. If you leave it much after this, it’s too late to fix it.

6. Check every half hour/hour. Depending on the gel, dye, etc. it can take three-four hours to run. Make sure to stop it by the time that the dye reaches about ¾ of the way along the gel.

7. Put under the imager and look at it under UV light – save images.

8. Dispose of gel appropriately.
G. Cell Culture

G.1 Aseptic technique

1. Ensure inside of hood is as clean as possible (spray with Virkon® then 70% ethanol).
2. Spray everything entering the hood with 70% ethanol.
3. Assume every exposed surface is contaminated – if anything touches the hood or your hands or lab coat, it most likely will have contamination.
4. The insides of sterile containers are the only clean areas (ie. sterile media, sterile plates, flasks, tubes, etc.).
5. Keep sterile containers sealed.
6. Contamination often starts near the lids of things – never touch lids or necks of flasks or bottles with the tip of your pipette.
7. If something is contaminated, do not open it in the hood.

G.2 Feeding flasks

1. Use aseptic technique (as always).
2. Clean hood, spray all equipment, take flask from incubator, spray with ethanol and wipe clean with tissue.
3. Remove media from flask (Note: when pipetting never let the liquid get up to the cotton at the top of the pipette or the pipette boy will break).
4. Put media in waste container, or use aspirator to remove.
6. Return flask to incubator.

G.3 Cell splitting

1. Remove media from flask.
2. Wash flask with Hanks balanced salt solution or Dulbecco’s PBS by pipetting in five-ten ml, tilting the flask to get the liquid covering the full surface, then removing it.
3. Add enough 0.25% trypsin-EDTA (T/E) to cover the bottom of the flask when it’s lying in the proper configuration (T25: 1 ml, T75: 3ml, T175: seven - ten ml).
4. Put in incubator for five minutes (enzyme is active at 37°C).
5. Take flask out and observe under the microscope. Cells should be rounded and moving around if you tap the flask. If they are still stuck to the bottom, try tapping the side of the flask gently. If they don’t come off, leave it another minute or two.
6. Once all cells are detached from the bottom of the flask, spray the flask and return it to the hood.
7. Add an equal volume of 10% serum media to the flask (ie. if you added five ml T/E, add 5ml media) (deactivates the trypsin).
8. Remove all of the liquid and put in a sterile centrifuge tube (15 or 50ml)
9. Spin for five minutes at 400g.
10. Remove the supernatant and resuspend the pellet (your cells) in new media.
11. Count and seed into new flasks.

G.4 Bringing cells up from liquid nitrogen

1. Take vial from liquid nitrogen.
2. Thaw in the water bath for 30 seconds – one minute but do not submerge the whole vial in the bath (as this can lead to contamination).
3. Spray vial with ethanol before putting into the hood. Must work quickly after the cells have thawed as DMSO is toxic.
4. Transfer cell suspension into a sterile centrifuge tube.
5. Add about five ml 10% serum media drop by drop to the cells in the tube. Add the media slowly to reduce the osmotic shock.
6. Centrifuge at 400-500g for five minutes.
7. Aspirate the media off the cell pellet.
8. Resuspend the cell pellet in two ml 10% serum media.
9. Count the cells.
10. Plate out cells at ~3,000-4,000 cells/cm². So, for a T175 flask plate out 750,000 – 1 x 10⁶ cells per flask. Feed with media (25-30ml for T175 flask) every three - four days.

G.5 Freezing cells

1. When freezing down cells, aspirate the media off cell pellet after it has been centrifuged. Resuspend the pellet in freezing media (freezing media: 45ml FBS, five ml DMSO. Make up freezing media and store at 4°C). Cell number determines amount of freezing media. Generally ~ one ml per vial, 500,000 cells to five million cells per vial.
2. Put cells in Mr. Frosty® container & put into -80°C freezer immediately.
3. Cells can be then put into liquid nitrogen after 24 hours.

G.6 Cell counting

1. Take 50µl of the cell suspension & put into 50µl trypan blue.
2. Add 10µl of this cell/trypsin blue suspension to each side of a haemocytometer (trypan blue is excluded by live cells – blue cells are dead, clear cells are alive).
3. Count cells on both sides and get the average (Figure G1).
4. To calculate the total cell number: average cell no./box x 10⁴ x dilution factor (in this case two) x original volume cells were suspended in (in this case two ml).
**Figure G1.** Grid lines of haemocytometer.
H. alamarBlue™ Assay

1. Wash the cells to be assessed three times with 100μl of Hank’s balanced salt solution (HBSS).
2. Make up the solution of alamarBlue™ in HBSS (ratio 1:9 respectively).
3. Add 100μl per well and add HBSS into four empty wells and the alamarBlue™ solution into four wells for blank subtraction.
4. Incubate for two hours at 37°C.
5. Transfer 100μl of the dye into a clear 9 six well plate.
6. Measure the absorbance at 550nm and 595nm (0.5 seconds per well).
7. Calculate a viability value according to ‘simplified method of calculating percent reduction’ available in the alamarBlue™ handbook.
8. Subtract the absorbance values of HBSS only from the absorbance values of the alamarBlue™ in HBSS (ratio 1:9). AOLW = absorbance of oxidized form at lower wavelength, and AOHW = absorbance of oxidized form at higher wavelength.
   1. Calculate correlation factor: RO.
   \[ \text{RO} = \frac{\text{AOLW}}{\text{AOHW}} \]
   2. To calculate the percent of reduced alamarBlue™:
   \[ \text{ARLW} = \text{ALW} - (\text{AHW} \times \text{RO}) \times 100 \]
I. **Picogreen™ Assay**

**Materials**

1. Clean opaque 9six well plate (black is better, white is ok).
2. Samples.
3. Kit components: 20x TE buffer, 200x Picogreen™ dye, DNA standard (can use for standard curve).
4. (DNA or complexes for standard curve).

**Procedure**

1. Pipette 25-50µl of sample into wells.
2. Pipette standard curves into wells (values depend on DNA concentration) (generally can estimate 5-10pg DNA/cell for eukaryotic organisms).
3. Prepare 1x buffer TE (dilute 20x) (for a full 9six well plate, prepare 15-20 ml).
4. Add 100µl DNAse-free 1x buffer TE/well.
5. (only if breaking polyplexes) Add one µl 1 M NaOH.
7. Add 25 µl 1x Picogreen™ dye to each well (if you expect very low values, can add more dye, but rarely a problem as Picogreen™ can readily detect less than one ng of DNA).
8. Incubate five minutes in dark at room temperature.

(Note that the value for .05ug is still extremely high)

(Note that it’s total mass DNA/well that matters, not the concentrations of the DNA – you must then back-calculate to find your total mass/well)
Example of a standard curve for Picogreen™ assay

<table>
<thead>
<tr>
<th>Mass DNA [ug]</th>
<th>Fluorescence intensity</th>
</tr>
</thead>
<tbody>
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<td>50.00</td>
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</tr>
<tr>
<td>25.00</td>
<td>374884</td>
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<tr>
<td>12.50</td>
<td>370016</td>
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<td>10794</td>
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<td>457</td>
</tr>
</tbody>
</table>
J. Protocol for Transfection *In-Vitro*

1. 24 hours before transfection, seed cells in 96/48/24 flat bottomed, cell+ well plate. Density depends, (approximately 10 000/well for 96 w.p., 15 000/well for 48 w.p., and 20 000/well for 24 w.p.) (note that cell density varies with cell type).
2. Prepare polyplexes in PBS. Generally use between 0.5 and 2ug DNA per well for a 9six well plate, and 1-5ug for a 24 well plate.
3. When polyplexes are ready, remove media from wells and wash with PBS/HBSS.
4. Add polyplex solutions to wells (under serum or serum free condition).
5. Incubate for four hours (only for serum free condition).
6. Remove solution, wash cells with HBSS/PBS and replace full media.
7. Remove media every 24 hours, and assay with GLuc assay system.

J.1 G-Luciferase Transfection Analysis

(As according to New England Biolabs recommended protocol)

1. Prepare 1x GLuc assay solution by dilution buffer 1 to 100.
2. Add 100µl dH2O to black opaque 9six well-plate.
3. Add 50µl of sample into each well.
4. Add 50µl of the GLuc solution to each well.
5. If possible, read within 5-10 seconds.
K. Protocol for Primary Cultures of Astrocytes, and Microglia

The glial cells, also known as neuroglia, consist of: 1- Macroglia, consisting of both astrocytes and oligodendrocytes, and 2- Microglia (resident macrophages of the central nervous system). The functions of these cells are distinct. Oligodendrocytes are the myelin-producing cells in the central nervous system (CNS), analogous to Schwann cells in the peripheral nervous system (PNS). Microglia are similar to macrophages; they remain silent until activated, when they remove cellular debris. Astrocytes serve multiple functions within the CNS including production of growth factors, maintenance of the extracellular environment, and modulation of synaptic transmission.

Note: All steps including cell isolation and, when possible, preparation of solutions should be performed in a laminar flow hood. All solutions and equipment coming into contact with living cells must be sterile, and aseptic technique should be used accordingly. All nondisposable materials should be reserved for cell culture use only and cleaned thoroughly immediately after use. Particular attention need to be given for the proper use of dissection hood in tissue culture room, as this is potentially exposed to pathogens when the sachet is removed, and there is no laminar flow (turned off). Proper cleaning before and after dissection need to be carried by using Virkon®, and 70% ethanol or IMS.

Materials

Dissection solution- HEPES buffered HBSS

1. HBSS (Sigma H 6648 [1X]), with NaHCO₃, D-Glucose, without Ca²⁺, Mg²⁺
2. HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ), 10mM final concentration
3. Penicillin/Streptomycin, 1X
4. with/without L-Glutamine, 1X

Note: Filter sterilize, store @ 4°C, always use cold. Depending on the protocol used, check step 21, warm just before use.
Complete culture medium

1. DMEM (high glucose)/F12 supplement
2. L-Glutamine, 1X
3. 10% FBS
4. 1% Pen/Strep

Note: Filter sterilize, store @ 4°C, always use warm.

Instruments

1. 70% alcohol
2. Microdissection scissors
3. Microdissection forceps
4. Disposable scalpels
5. 60mm petri dishes
6. 35mm petri dishes
7. 50ml tubes
8. 70um and 40um mesh filter
9. T-75 flasks

K.1 Euthanasia and decapitation

1. Anaesthetize rat pups (one to three days old; Sprague-Dawley) by placing them on ice. (Alternatively, it is possible to use dry ice covered with a kitchen towel. This ensures better anaesthesia during the time the animals are transferred to the lab).
2. In the biological prep room, remove one pup at a time from the Styrofoam box. Gently hold pup with thumb and forefinger around thorax and rinse head and neck of pup with 70% ethanol.
3. Using 7-in. curved Mayo scissors, decapitate a pup and place the head on a sterile gauze pad in a new Styrofoam box filled with ice. Place body in a plastic bag for disposal or keep for other uses.

Note: Scissors and forceps should be placed either on a sterile gauze pad or in a beaker of 70% ethanol between decapitations.
K.2 Brain dissection

1. Before beginning dissection, place three to five 60-mm culture dishes containing cold dissection medium on ice.
2. Cover the bottom of a 100-ml beaker with paper towels. Fill the beaker with 70% ethanol. Place the dissection instruments in the beaker.

*Note: Lining the beaker with paper towels protects the tips of the fine forceps.*

3. Secure the head by holding down the snout. With fine-angled microdissecting scissors, cut the skin along the midline from base of the skull to the eyes. Use 0.8-mm forceps to separate the skin and expose the skull as necessary.
4. Use a similar technique to expose the brain by cutting along the midline of the skull with the microdissecting scissors. If desired, cut away the skull flaps to ease removal of the brain.
5. Placing the scissors at a slight upward angle helps prevent inadvertent damage to the underlying brain tissue.
6. With 0.8-mm-tip curved forceps, sever the olfactory bulbs at the anterior end of the brain and the spinal cord at the posterior end. Sever the cerebellum.
7. Gently slip the two sides of 0.4-mm-tip curved forceps under the cortices on either side of the brain so that the forceps are straddling the brain.
8. Gently move forceps from side to side and, with a slight back angle, pull up the cortices.
9. Gently transfer the brain to a 50ml tube filled with cold dissection solution. Leave this tube on ice.
10. Transfer the brains with the hanks solution to a small petri dish.

K.3 Cortical dissociation

11. Place the cortices, one by one petri dish filled with cold dissection solution under the dissecting microscope with the dorsal side up and the anterior (rostral) side facing away.
12. Unfold the cortices and separate the two hemispheres with scissors.
13. Scoop the inside of the hemispheres with the spatula, leaving only the neocortical halves.
14. Only the convexity of the hemispheres should remain.
15. With Dumont forceps, gently tease away the meningeal coverings on the cortical surface.

For meningeal dissociation, jump to section IV

16. Transfer the 2 hemi-cortices to a 50ml falcon filled with dissection solution.

Note: At this stage, either discard the meninges or reserve in dissection solution for meningeal cultures.

17. Working in batches, after dissecting approx. 6 cortices, proceed.
18. Carefully pour tissue into a 10cm dish and gently mince tissue with sterile scissors or razor blade.
19. Transfer tissue to back to 50ml tube and add 5 ml of (1-1.5ml per 2 brains):
   a. 1X trypsin and 50µL DNase for 25 minutes at 37°C.
   b. 0.1% trypsin and 0.02% DNase for 20 minutes at 37°C.
   c. 0.125% trypsin, 0.5 mM EDTA for 30 minutes at 37°C.
20. Swirl tube every five minutes.
21. Wash the cortices with complete medium twice.
22. Dissociate the tissue by gently triturating the cortices through a five ml or two ml pipette, followed by a fire-polished Pasteur pipette, or 1000µl pipette tip.
23. Dilute cell suspension to 10ml of complete medium, and pass through a 40µM cell strainer.
24. Spin down the cells at 1700rpm for five minute.
25. Re-suspend the cells with 10ml of complete medium, and count.
26. Seed 2 x 106 cells/flask in 15ml complete medium.
K.4 Meningeal dissociation

1. After removal of the meninges, pool and transfer to 0.125% trypsin, 0.5 mM EDTA, and 0.25% collagenase.
2. Incubate for 30 minutes at 37°C.
3. Discard dissociation solution.
4. Gently triturate in the presence of 80 μg/ml DNase and 10% FBS/DMEM/1% Pen/Strep (complete meningeal medium).
5. Centrifuge at 1700 rpm for five minutes, and resuspended in complete meningeal medium.
6. Triturate again several times using either 19- and 25-gauge needles, or 1000μl pipette tip.
7. Seed at a density of two brain meninges per 25-cm² flask.
8. Refresh medium the next day.
9. Carry out the first cell passage after seven to ten days.
10. Cells should be used for experiments between passages two and five.
L. Polymer Labelling

Materials

Hyperbranched polymer = 129.8mg (Mw 24000, moles 5.409X10^{-6})

Rhodamine B = 3.86 mg (Mw 714.77, moles 5.409X10^{-6})

THF = 5ml

Procedure

1. Switch on oil bath to 50°C temperature.
2. Wash equipment to be used with acetone.
3. Dissolve hyperbranched polymer and Rhodamine B in THF in a 25 ml round bottom flask equipped with a stir bar.
4. Seal the flask with a rubber cork and purge nitrogen for 20 minutes.
5. Place flask into oil bath at 50°C and stir at medium speed.
6. Stop the reaction after 48 hours.
7. Add the labelled polymer into dialysis membrane (MWCO 8000) and dialysis against highly purified water until water becomes clear. Change water every five hours.
8. Freeze dry the labelled polymer.
9. Store the polymer at 4°C (for one week) or -20°C (for > one week) in dark.

*Note: Polymer labelling should be performed under dark conditions.*
M. Plasmid Labelling

1. Warm vial containing reagent to room temperature. (If new kit is used, add 100µl reconstitution buffer to the vial. Mix, then spin gently).

2. Prepare labelling reaction.

<table>
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<tr>
<th>Component</th>
<th>Max. DNA content</th>
<th>Lower DNA content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, ddH₂O</td>
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<td>35µl</td>
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<tr>
<td>10x labelling buffer A</td>
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<tr>
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<tr>
<td>Label IT® reagent</td>
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</tr>
<tr>
<td>Total volume</td>
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<td>50µl</td>
</tr>
</tbody>
</table>

(Can do multiple at once in multiples of 50µl)

3. Incubate at 37°C for one hour. Spin briefly midway to minimize effects of evaporation.

4. Purify by spinning through micro spin column.
   a. Exactly 50µl per column.
   b. 735 RCF (3000 rpm for 7.3 cm rotor like in small eppendorf centrifuges).
   c. Vortex to suspend resin.
   d. Loosen cap ¼ turn and pull out bottom plug.
   e. Put column into a 1.5 ml centrifuge tube.
   f. Spin for one minute at 735g.
   g. Discard buffer, put column in a new tube.
   h. Apply sample to top of resin.
   i. Spin at 735g for two minutes.

5. Cap the support tube. Sample concentration is approximately 0.2µg/ml.

N. Organotypic Brain Slice Culture

Materials and Tools Required

1. Dissecting microscope
2. Leica vibratome™ VT1200
3. Glue (Loctite® 404™, instant adhesive)
4. Millicell®-CM low height culture plate inserts six well-30 mm & 24 well-12 mm.
5. Six well plate
6. Artificial cerebrospinal fluid (ACSF) (see Appendix O)
7. 4% low melt agarose in distilled water at 50°C until you embed the brain
8. Cell culture medium ((Basal Medium Eagle, supplemented with Hanks Balanced Salt Solution [25% v/v], D-glucose [4.5mg/ml], penicillin [100 U/ml], streptomycin [100 U/ml], glutamine [0.58mg/ml], and 10% heat inactivated foetal calf serum)

N.1 Embryos (E16)

1. Asphyxiate the mother (optimum is E16 timed pregnant) and then sacrifice by cervical dislocation.
2. Spray lower abdomen with 70% EtOH and cut through skin and muscle with a pair of scissors exposing the uterus, intestines and embryos.
3. Immediately put embryos in cold ACSF.
5. Remove brain from skull.
6. Separate cortex and cerebellum.
7. Heat 4% low melt agarose (liquid state).
8. Put some agarose in tissue culture plate.
9. Transfer the brain using a spatula into agarose (make sure that the brain is fully surrounded by agarose).
10. Cover the tissue culture dish and place on ice.
11. Wait until agarose hardens (five to ten minutes).
12. Cut out the brain from the agarose (make sure that brain is not cut through).
13. Put glue on the piece of plastic and put the brain over it and wait until its fixed.
14. Transfer it to the vibratome chamber.
15. Add cold ASCF on the vibratome chamber so that it covers the brain all the time.
16. Adjust parameters in the vibratome (speed, thickness).
17. Discard the first few slices until the desired section is reached.
18. Once the slice is cut transfer it to ACSF in a petri dish.
19. Place Millicell®-CM inserts in a six well plate.
20. Pipette 1ml cell culture media below the Millicell®-CM inserts.
21. Transfer the slice to Millicell®-CM insert.
22. Place the plates containing tissue slices in an incubator with 5% CO₂ at 37°C.
23. Change media every alternate day.

N.2 Pups (Postnatal day P15)

Procedure

1. Asphyxiate rat pups (P15) and then sacrifice by cervical dislocation.
2. Using 7-in. curved Mayo scissors decapitate a pup and place the head on a sterile gauze pad in a new Styrofoam™ box filled with ice. Place body in a plastic bag for disposal or keep for other uses. (Note: Scissors and forceps should be placed either on a sterile gauze pad or in a beaker of 70% ethanol between decapitations).
3. Before beginning dissection, place three to five 60-mm culture dishes containing cold ACSF on ice.
4. Cover the bottom of a 100-ml beaker with paper towels. Fill the beaker with 70% ethanol. Place the dissection instruments in the beaker (Note: Lining the beaker with paper towels protects the tips of the fine forceps).
5. Secure the head by holding down the snout. With fine-angled micro dissecting scissors, cut the skin along the midline from base of the skull to the eyes. Use 0.8-mm forceps to separate the skin and expose the skull as necessary.
6. Use a similar technique to expose the brain by cutting along the midline of the skull with the micro dissecting scissors. If desired, cut away the skull flaps to ease removal of the brain.
7. Placing the scissors at a slight upward angle helps prevent inadvertent damage to the underlying brain tissue.
8. With 0.8-mm-tip curved forceps, sever the olfactory bulbs at the anterior end of the brain and the spinal cord at the posterior end. Sever the cerebellum.
9. Gently slip the two sides of 0.4mm tip curved forceps under the cortices on either side of the brain so that the forceps are straddling the brain.
10. Gently move forceps from side to side and, with a slight back angle, pull up the cortices.
11. Gently transfer the brain to a 50ml tube filled with cold dissection solution. Leave this tube on ice.
12. Transfer the brains with the ACSF to a small petri dish.
13. Separate cortex and cerebellum.
14. Heat 4% low melt agarose (liquid state).
15. Put some agarose in tissue culture plate.
16. Transfer the brain using a spatula into agarose (make sure that the brain is fully surrounded by agarose).
17. Cover the tissue culture dish and place on ice.
18. Wait until agarose hardens (five to ten minutes).
19. Cut out the brain from the agarose (make sure that brain is not cut through).
20. Put glue on the piece of plastic and put the brain over it and wait until it is fixed.
21. Transfer it to the vibratome chamber.
22. Add cold ASCF on the vibratome chamber so that it covers the brain all the time.
23. Adjust parameters in the vibratome (speed, thickness).
24. Discard the first few slices until the desired section is reached.
25. Once the slice is cut transfer it to ACSF in a petri dish.
26. Place Millicell®-CM inserts in a six well plate.
27. Pipette 1ml cell culture media below the Millicell®-CM inserts.
28. Transfer the slice to Millicell®-CM insert.

29. Place the plates containing tissue slices in an incubator with 5% CO₂ at 37°C.

30. Change media every alternate day.

(Note: The cerebellum and cerebral hemispheres were separated and 250-400µm sections were cut in ice cold. The cerebellum was sectioned in the parasagittal plane to preserve the cerebellar circuitry and the cerebral hemispheres were sectioned in the coronal plane).
O. ACSF Formulation (One litre)

Materials

1. 127mM NaCl
2. 2M KCl
3. 10nM Glucose
4. 1.2mM KH2PO4
5. 26mM NaHCO3
6. 2mM MgSO4
7. 2mM CaCl2

Procedure

1. Add all the above reagents into a one litre conical flask.
2. Stir to ensure dissolving.
3. Filter the above solution (do not autoclave).
4. Bubble with 95% O2 and 5% CO2.
P. Staining

P.1 Rhodamine Phalloidin Staining

1. At the appropriate time point remove media from cells.
2. Gently wash the glass cover slips with Hanks balanced salt solution and add 500µl of 4% paraformaldehyde. Paraformaldehyde is prepared in PBS. (Note: heating is required to dissolve paraformaldehyde in PBS).
3. Allow to fix at room temperature for 15 minutes.
4. Remove paraformaldehyde and gently wash sample with PBS.
5. Add 200µl of 0.2% Triton™ X.
6. Allow sit in solution for five minutes then remove Triton™ X.
7. Gently wash sample with PBS.
8. Add rhodamine phalloidin diluted 1:100 in PBS. Alternatively FITC phalloidin can be used.
9. Allow it to sit in solution for one hour then remove rhodamine phalloidin. Note: Protect from light during this procedure and subsequent manipulations.
10. Gently wash sample with PBS.
11. If no further staining is required mount samples onto a histology glass slide.
12. Place glass cover slip in centre of glass slide.
13. Add 1 drop of glycerol to the centre of the samples and place a glass cover slide over the sample. Alternatively, a protective hard set media may be used e.g. VECTASHIELD® (Vector Laboratories, Peterborough, United Kingdom).
14. Seal the edges of the glass cover slide with clear nail polish. This will prevent samples from drying out.

P.2 Organotypic Brain Slice Immunohistochemistry

1. Gently transfer the tissues into a 24 well plate in one ml PBS (transfer gently using a paint brush).
2. Block the tissue for one hour at room temperature under slow agitation with 10% Normal Horse Serum (NHS) and 0.4% Triton™ X (in PBS). Use freshly prepared PBS.

3. After blocking transfer the slices to the next well containing primary antibody (0.5ml) and incubate for two days at 4°C under slow agitation. (primaries are made in 2.5% NHS +0.1% Triton™ X). The concentrations used are (Ms anti NFH 1:100, Rab anti olig 2 1:500, Rb anti GFAP 1:500, Gt anti GFAP 1:2000, Rb anti Iba1 1:400, Ms anti NeuN 1.250 and Ms anti B111 tubulin 1:100).

4. After primary antibody incubation, transfer the tissue to the next well. Wash 3 times in PBS (15 minute each). After each wash gently transfer the tissue to the next well.

5. Incubate overnight at 4°C (dark) with secondary antibody. (Secondaries are made in 2.5% NHS and 0.4 % Triton™ X at a dilution of 1:500).

6. Wash three times again in PBS (15 minutes each) and mount them on a cover slip with PVA as mounting medium.

**Notes:**

- All done in the dark (at least the incubations).
- Negative control – no PRIMARY antibody.
- Mounted and sealed slides stored at 4°C in the dark.
Q. **ELISA for TNFα**

1. Add 50µl of sample diluents to wells.
2. Add 50µl of standards or samples to the well.
3. Cover the plate and incubate at room temperature for one hour.
4. Wash plate three times using the wash buffer.
5. Add 100µl of biotinylated antibody reagent to well.
6. Cover and incubate at room temperature for one hour.
7. Wash plate three times using the wash buffer.
8. Add 100µl of strepavidin HRP reagent to each well.
9. Cover the plate and incubate at room temperature for one hour.
10. Wash plate three times using the wash buffer.
11. Add 100µl of TMB substrate to each well.
12. Develop plate at room temperature in dark for 30 minutes.
13. Add 100µl of STOP solution to each well.
14. Measure absorbance on ELISA plate reader set at 450nm and 550nm.
   Subtract 550nm values from 450nm. This subtraction will correct for optical imperfections in the plate.
15. Calculate concentrations based on standard curve.

(Note: Six well plate, sample diluents, standards, biotinylated antibody reagent, strepavidin HRP reagent, TMB substrate and STOP solution is provided in the ELISA kit purchased. (Note: Homogenise organotypic brain slices in culture supernatant with tissue rupture before the test).
R. ELISA for IFNγ

Reagent Preparation

Bring all reagents to room temperature before use

- **Wash buffer**: If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20ml of wash buffer concentrate into deionised or distilled water to prepare 500ml of wash buffer.

- **Substrate solution**: Colour reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200μl of the resultant mixture is required per well.

- **IFN-γ standard**: Refer to the vial label for reconstitution volume. Reconstitute the IFNγ Standard with calibrator diluent (RD6-21). This reconstitution produces a stock solution of 1000 pg/ml. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

- **Use polypropylene tubes**: Pipette 500μl of calibrator diluent (RD6-21) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted standard serves as the high standard (1000pg/ml). Calibrator diluent (RD6-21) serves as the zero standards (0pg/ml).

Procedure

1. Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

2. Prepare all reagents and working standards as directed in the previous sections. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 100μl of assay diluent RD1-51 to each well.
4. Add 100μl of standard, sample, or control per well. Ensure reagent addition is uninterrupted and completed within 15 minutes. Cover with the adhesive strip provided. Incubate for two hours at room temperature. A plate layout is provided to record standards and samples assayed.

5. Aspirate each well and wash, repeating the process three times for a total of four. Wash by filling each well with wash buffer (400μl) using a squirt bottle, manifold dispenser, or auto washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200μl of IFNγ conjugate to each well. Cover with a new adhesive strip. Incubate for two hours at room temperature.

7. Repeat the aspiration/wash as in step five.

8. Add 200μl of substrate solution to each well. Incubate for 30 minutes at room temperature. Protect from light.

9. Add 50μl of stop solution to each well. The colour in the well should change from blue to yellow. If the colour in the well is green or if the colour change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450nm. If wavelength correction is available, set to 540nm or 570nm. If wavelength correction is not available, subtract readings at 540nm or 570nm from the readings at 450nm. This subtraction will correct for optical imperfections in the plate.
S. Isolation of RNA

1. Homogenize cells with one ml Trizol® by incubating at room temperature for 1 minute and then pipetting.
2. For organotypic brain slices add one ml Trizol® and then homogenize using Tissue Ruptor™ probes (Qiagen, UK).
3. Use fresh probe for each sample. The probes can be sterilized by autoclaving and can be reused later.
4. Incubate the homogenate at room temperature for five minutes to complete dissociation.
5. At this stage, samples can be stored at -80°C and the protocol can be preceded at a later stage.
6. Thaw the frozen samples.
7. Add 200μl of chloroform to one ml of Trizol®.
8. Shake vigorously by inversion several times.
9. Incubate at room temperature for 15 minutes.
10. Centrifuge at 12 000g for 15 minutes at 40°C.
11. Following centrifugation, phase separation takes place creating three distinct layers: Lower phenol chloroform layer, middle inter-phase, upper translucent aqueous phase.
12. Remove translucent aqueous phase (~650μl) and add in a fresh tube.
13. Add one volume (~700μl) of 70% ethanol slowly and carefully mix by inversion.
14. Apply the sample (700μl at a time) to RNeasy® mini spin column (Qiagen), centrifuge for 15 seconds at 8000g and discard flow through.
15. Add 350μl of RW1 buffer to centre of column, centrifuge for 15s at 8000g, discard flow through.
16. Add 10μl DNase stock solution to 70μl buffer RDD and add the DNase incubation mix directly onto the RNeasy column. Incubate at RT for 15 minutes.
17. Add 350μl of RW1 buffer to centre of column, centrifuge for 15 seconds at 8000g, discard the flow through.
18. Transfer column to new two collection tubes.
19. Add 500µl RPE to the centre of column, centrifuge for 15 seconds at 8000g, and discard flow through.

20. Add 500µl of RPE buffer to the centre of column, centrifuge for 15s at 8000g; discard flow through, centrifuge for a further two minutes at 8000g.

21. Transfer column to new 1.5ml tube.

22. Add 10µl RNAse free water onto the column and incubate at room temperature for one minute, centrifuge for one minute at 8000g.

23. Add further 10µl RNAse-free water onto the column, incubate at room temperature for one minute, centrifuge for one minute at 8000 g.

24. Take back the 10µl of eluate and add again onto the column, incubate at room temperature for one minute, centrifuge for one minute at 8000 g.

25. Determine the concentration at the NanoDrop™ and freeze at -80°C.
T. Synthesis of cDNA

This is reverse transcription of RNA to cDNA. This cDNA is then used for real time PCR.

Preparation before reverse transcription

1. Clean the work surface and spray RNAse away.
2. Wipe dry all the pipettes and gloves with RNAse away.
3. Use sterile nuclease free tubes which are pre-chilled on ice.
4. Use 1μg of RNA template and 0.5μg of oligo dT primers and random primers.
5. Denature the target RNA and primers by incubation at 70°C for five minutes.
6. Quick-chill on ice for five minutes.

Recipe for Reverse Transcription Reaction Mix

1. Begin with highest volume.
2. Add the reverse transcriptase enzyme at the end.
3. Prepare the reaction mix on ice and keep on ice until incubation.
4. Follow Table T.1 for volumes.
5. After combining all components, vortex gently to mix.
6. After mixing, place the tube with reaction mix into the reverse transcription machine and run the program as detailed in Table T.2.
7. After reaction is complete proceed with polymerase chain reaction (PCR) or store cDNA at -20°C for future use.
Table T.1 Reaction components and quantities required for a single reverse transcriptase reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>nuclease free water</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>ImProm-II&lt;sup&gt;TM&lt;/sup&gt; 5X reaction buffer</td>
<td>4</td>
<td>1X</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;, 25mM</td>
<td>2.4</td>
<td>3mM</td>
</tr>
<tr>
<td>dNTP mix(10mM each dNTP)</td>
<td>1</td>
<td>0.5mM</td>
</tr>
<tr>
<td>recombinant RNAsin ribonuclease inhibitor</td>
<td>1</td>
<td>1U/µl</td>
</tr>
<tr>
<td>ImProm-II&lt;sup&gt;TM&lt;/sup&gt; reverse transcriptase</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>final volume reaction mix</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

Table T.2 Typical reverse transcription program

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>annealing</td>
<td>25</td>
<td>5 minutes</td>
</tr>
<tr>
<td>extension</td>
<td>42</td>
<td>60 minutes</td>
</tr>
<tr>
<td>heat inactivation reverse transcriptase</td>
<td>70</td>
<td>15 minutes</td>
</tr>
<tr>
<td>end</td>
<td>4</td>
<td>forever</td>
</tr>
</tbody>
</table>
Appendices

U. PCR

1. Dilute cDNA template so as to obtain a final concentration of 20ng per well.
2. Make sure that the cDNA concentration must not exceed 100ng/reaction and 10% of the final volume.
3. Add the components listed in Table U.1 to prepare master mix.
4. For a triplicate reaction, add cDNA template in triplicate and also add a negative control by replacing cDNA with nuclease free water.
5. Mix well the master mix by pipetting and add to each well to obtain final volume of 25µl.
6. Add the plastic cover provided by supplier on the PCR plate.
7. Centrifuge one minute at 1400 rpm.
8. Place the plate in the machine.
9. Open step one software and enter the details to map the plate on the software.
10. Choose SBBR® Green filters for each well.
11. Choose the endogenous control from the plate and enter in the software.
12. Set up the steps in accordance to the gene and melting temperature of the primers. A general program is detailed in Table U.2.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume per reaction (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Quantifast SYBR® Green PCR master mix</td>
<td>12.5</td>
<td>1.5mM</td>
</tr>
<tr>
<td>forward, pmol</td>
<td>0.25</td>
<td>1µM</td>
</tr>
<tr>
<td>reverse, pmol</td>
<td>0.25</td>
<td>1µM</td>
</tr>
<tr>
<td>template DNA</td>
<td>1.49</td>
<td>≤100ng/reaction</td>
</tr>
<tr>
<td>nuclease water (to bring volume to 25µl)</td>
<td>10.51</td>
<td>1U/µl</td>
</tr>
<tr>
<td>final volume</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>
### Table U.2 Typical real time PCR program

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Ramp rate</th>
<th>No of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial step</td>
<td>50</td>
<td>2 minutes</td>
<td>maximal/fast mode</td>
<td>1</td>
</tr>
<tr>
<td>PCR initial activation step</td>
<td>95</td>
<td>5 minutes</td>
<td>maximal/fast mode</td>
<td>1</td>
</tr>
<tr>
<td>two step cycling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>denaturation</td>
<td>95</td>
<td>10 seconds</td>
<td>maximal/fast mode</td>
<td>35</td>
</tr>
<tr>
<td>annealing/extension</td>
<td>60</td>
<td>30 seconds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>final step</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>denaturation</td>
<td>95</td>
<td>15 seconds</td>
<td>maximal/fast mode</td>
<td>1</td>
</tr>
<tr>
<td>annealing/extension</td>
<td>60</td>
<td>20 seconds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>final denaturation</td>
<td>95</td>
<td>15 seconds</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
V. Supplementary Figures for Chapter Three

Figure V.1 pDNA binding ability of the lpD-b-P/E 3 with w/w ratios (1:1 to 15:1) compared to PEI (2:1) and dPAMAM (8:1) at their optimum weight ratios.
Figure V.2 Particle size and zeta potential of IpD-b-P/E 3 at different polymer to pDNA (w/w) ratios.
Figure W.1 Fluorescent microscopic image of embryonic brain slice after transfection with (a) lpD-b-P/E3 (b) Lipofectin® reagent and (c) electroporation. with GFP plasmid. (GFP expression (green)).
**Figure W.2** Fluorescent microscopic image of embryonic brain slice after transfection with lP-D-b-P/E3. GFP plasmid (GFP expression (green) and GFAP red).
Figure W.3 Fluorescent microscopic image of postnatal brain slice after transfection with lpD-b-P/E 3 with GFP plasmid. (a) epithelial cells of the pial membrane (b) cluster of granule neurons (c) fibroblast (d) oligodendrocyte (e) Bergmann glia.
X. List of Publications


Y. List of Conference Proceeding – Podium/Poster Presentation


Appendices

