Scaffold-mediated Sequential Drug/Gene Delivery to Promote Nerve Regeneration and Remyelination following Traumatic Nerve Injuries.

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Abstract

Neural tissue regeneration following traumatic injuries is often subpar. As a result, the field of neural tissue engineering has evolved to find therapeutic interventions and has seen promising outcomes. However, robust nerve and myelin regeneration remain elusive. One possible reason may be the fact that tissue regeneration often follows a complex sequence of events in a temporally-controlled manner. Although several other fields of tissue engineering have begun to recognise the importance of delivering two or more biomolecules sequentially for more complete tissue regeneration, such serial delivery of biomolecules in neural tissue engineering remains limited. This review aims to highlight the need for sequential delivery to enhance nerve regeneration and remyelination after traumatic injuries in the central nervous system, using spinal cord injuries as an example. In addition, possible methods to attain temporally-controlled drug/gene delivery are also discussed for effective neural tissue regeneration.

Keywords: Neural Tissue Engineering, Sequential Delivery, Spatiotemporal Delivery, Nerve Regeneration, Remyelination

Contents

1. Introduction

Traumatic central nervous system (CNS) injuries induce hefty neural tissue losses. Such injuries often lead to severe and permanent motor, sensory and autonomic dysfunction due to the inadequate intrinsic regenerative capability of the CNS. Thus, neural tissue engineering methods have been developed in attempt to promote tissue regrowth by utilizing nerve grafts, cell transplantation, biochemical factors and/or tissue engineered scaffolds [1-3].

Traditionally, exogenous biomolecules are delivered systemically via direct injection or oral administration. These routes of delivery often demonstrate suboptimal efficacies due to the poor stability of drugs, rapid biodegradation and clearance from the physiological system. Thus, high drug dosages are frequently required for effective therapeutic outcomes, which may in turn trigger undesirable side effects. To circumvent these problems, scaffold-based delivery systems, where drugs are incorporated within carefully designed carriers, have been developed to deliver high integrity and bioactive molecules in a localized and timely manner to target tissues [4]. Moreover, scaffolds with three-dimensional architecture provide structural support and topographical cues to direct cell fate and tissue regrowth [5-7].

Currently, scaffold-mediated drug/gene delivery largely involves the use of a single therapeutic drug [8]. Unfortunately, tissue regrowth is highly complex and often depends on a multitude of signals ranging from extracellular matrix interactions, to growth factors, cytokines and chemokine signaling [9]. Thus, the inability to fully capture many of these essential biochemical signals for tissue regrowth after traumatic nerve injuries is a probable cause for the limited effectiveness of single drug therapies. Hence, efforts to improve therapeutic outcomes have led to attempts to utilize multiple biomolecules, such as delivering pro-regenerative growth factors in tandem with biomolecules that modify the native inhibitory environment after CNS injuries [10, 11]. Unfortunately, functional recovery after such combinatorial treatments remains sub-optimal.

Besides delivering the essential biomolecules, the temporal sequence by which each of these drugs is delivered could be crucial in CNS injuries [12]. For instance, premature exposure of tissues to late-stage regenerative biomolecules may antagonize other molecules that are necessary

for early stages of regeneration [13]. Thus, the appropriate biomolecules may need to be delivered in a temporally defined fashion during distinct phases of tissue regrowth [12]. Such design considerations have not been adopted in neural tissue engineering methods. However, studies in vascular tissue engineering ([14] Box 2) [15-17], bone and cartilage tissue engineering [18-20] have demonstrated that sequential drug/gene delivery have better efficacies than simultaneous delivery of biomolecules in simulating the intrinsic regeneration pathways to further enhance tissue repair. Hence, useful insights may be drawn from these fields and applied in the context of neural tissue repair and regrowth.

In the following sections, we highlight the prospective application of sequential delivery to promote nerve regeneration following traumatic CNS injuries by using spinal cord injury (SCI) as a case in point to emphasize the temporal sequence of biological responses that often occur following trauma. Specifically, we discuss the biomolecules that are often associated with the discrete stages of tissue responses following trauma. Thereafter, we suggest methods to incorporate engineering knowledge with biological scaffold designs to meet the needs of sequential drug/gene delivery in SCI treatment.

2. Biological Responses after SCI

The acute stage of SCI is characterized by early axonal dieback and demyelination due to extensive apoptotic and necrotic cell death. Following which, activated astrocytes proliferate and surround the lesion to form an astrocytic scar. Although scarring limits tissue loss due to injury [21], it acts as a biochemical and physical barrier to regenerating axons as these axons attempt to regrow in the intermediate and chronic phases of injury [22]. In addition, these axons often do not remyelinate due to failure in the generation, recruitment and/or differentiation of oligodendrocyte

precursor cells (OPCs) into oligodendrocytes (OL) [23]. Understanding the above phases of tissue loss, scarring, regrowth and remyelination would enable us to design strategies to limit tissue damage and/or promote recovery. More robust regeneration may be possible by catering to the temporal needs of the tissues. For example, we could prevent the loss of neural tissues during the acute injury stage and administrating treatments that promote nerve regrowth in the intermediate or chronic stages. This could be followed by promoting remyelination in the final stages of regeneration.

2.1 Biochemical Signaling Associated with Nerve Degeneration after Acute Nerve Injury

During the pathogenesis of SCI [24], trauma ("primary injury") creates a region of impact that is filled with disrupted cells and blood ("primary lesion"). Surrounding the primary lesion are tissues that have been spared from the initial insult [25]. Another wave of cell death soon follows ("secondary injury") within hours due to ischemia, excitotoxicity or inflammation, which leads to massive axonal dieback and demyelination that spread centripetally from the primary lesion ("secondary lesion") [26, 27]. Such widespread nerve degeneration starts a few hours after trauma and lasts up to two or three weeks in all rodents, monkey and human [21, 28-30]. During this time, lipid peroxidation (LP) and neuroinflammatory reactions are two key mechanisms that are responsible for massive cell death and degeneration [25, 31].

2.1.1 Lipid Peroxidation (LP)

LP is consistently regarded as the main culprit linked to tissue degeneration during secondary injuries [31]. LP is the oxidative degradation of cellular membrane and phospholipid-dependent enzymes, and induces substantial oxidative damage to proteins and DNA owing to a chain of free-radical reactions involving reactive oxidative species. Their self-sustainable nature allows propagation of LP reaction, which typically persists for 5 days after SCI in rats [32, 33]. LP

causes extensive damage to the surrounding tissues, and is often exacerbated by the high iron contents in the CNS, which catalyzes the LP reaction [34]. Oxidative damage to essential cellular components is a likely contributor to apoptosis and thus, the early acute axonal dieback that was accounted by Kerschensteriner *et al. [35]*. Such axonal damage further results in the loss of trophic support to myelinating oligodendrocytes, thereby initiating demyelination [36].

2.1.2 Neuroinflammation

Neuroinflammation after SCI influences multiple aspects of secondary injuries, including the induction of neuronal tissue apoptosis. For instance, pro-inflammatory cytokines secreted by leukocytes initiate apoptosis of neurons and OL to exacerbate axonal dieback and demyelination (see review [25]). Thus, understanding leukocytes activation is important in our attempts to halt inflammatory tissue loss. Leukocytes activation after SCI is well-characterized in rats [24, 37, 38] and to a lesser extent, in human [39] (Table 1). The infiltration of leukocytes is temporally regulated in a sequential manner in the spinal cord. Thus, targeting the upstream events in neuroinflammation, by blocking the activators or secreted factors, is likely to have huge impact on modulating the inflammatory response after trauma.

Table 1: Leukocytes Activation and Signaling

Leukocytes Involved	Timeline of Activation and Peak (post-SCI)	Biochemical Signals (Based on Rodent Studies)
Microglia	In rat [24, 37, 38] • Activated immediately in response to trauma • Peak between 3-7 days at lesion epicenter • Plateaued after 2-4 weeks In human [39] • Activated at 1-3 days post injury	• Activated by 1. ATP [40] 2. Glutamate [41] 3. Fractalkines [42] All from injured cells • Secrete 1. pro-inflammatory cytokines (IL-1, IL-6, IL-12 and TNFα) [38]
Neutrophil	In rat [24, 37, 38] • Obvious migration after 8-24 hours	• Attracted by 1. Fractalkines (Eg: CXCL2 and CXCL1) from astrocytes [43] and an array of chemokines

	In human [39] • Extravasation observed 3-4 hours post injury • Obvious infiltration at 1-3 days • Presence decline after 5-10 days	2. Increased intercellular adhesion molecule (ICAM-1) expression due to pro-inflammatory cytokines from microglia [44] • Secrete 1. pro-inflammatory cytokines (IL-1, IL-8, TNF-α) [24] 2. Matrix metalloproteinase Via NFκB translocation pathway [24]
Monocyte/ Macrophage	In rat [24, 37, 38] • Monocytes infiltrate after 2-3 days • Peak at 7 days and is morphologically indistinguishable from microglia after differentiation into resident macrophages • Plateaued after 2-4 weeks In human [39] • Monocytes infiltrate and migrate to lesion between 1-10 days • Presence in large quantity after 5-10 days and is morphologically indistinguishable from microglia after differentiation • Continue to be present in lesion after a year	• Attracted by 1. An array of chemokines including CCL-2 and Macrophage Inflammatory Protein [38] 2. Increased intercellular adhesion molecule (ICAM-1) expression [38] • Secrete 1. Variety of pro-inflammatory cytokines (IL-1, IL-6, TNF-α) [38] 2. Anti-inflammatory cytokines at later time point [45]
Lymphocyte	In rat [24, 37, 38] • Invasion after 1 week • Remain in injury sites for 6-10 weeks In human [39] • Little extravascular lymphocyte presence before 10 days post injury	• Activated by 1. Loss of suppressive ability of regulatory T-cells activates antigen-specific T-cells. 2. Antigen-specific T-cells activate B-cells specific for the same antigens (see [46] Fig 1) • Secrete 1. T-cells secrete inflammatory cytokines (TNF-α, IL-12 etc) [38] 2. B-cells proliferate and differentiate into autoantibody-secreting plasma cells [38]

2.1.3 Biomolecules that Modulate Nerve Degeneration

Since secondary injuries are highly destructive to spinal cord tissues, neuroprotection is crucial at this juncture of SCI. Correspondingly, a wide range of low molecular weight drugs, genetic drugs and proteins have been examined for their neuroprotective effects. Many of these drugs target mechanisms that lead to necrosis-triggering LP or interfere with acute inflammatory signaling cascades that induce massive cell death in the secondary lesion [25].

2.1.3.a Targeting Lipid Peroxidation

A hydrophilic, Low molecular weight (Mw) drug, Methylprednisolone (MP), is the standard treatment for SCI. MP is neuroprotective by its virtue of inhibiting post-traumatic LP [31, 47]. Despite the side effects associated with steroids (MP), there has not been an antioxidant neuroprotective drug with well-defined dose response to rival MP [48]. Edward D. Hall has published multiple reviews detailing the neuroprotective pharmacology of MP [31, 48, 49]. In support of the need for controlled delivery, the efficacy of MP has a strong correlation with its temporal drug profile (Table 2). In particular, the biomarker for LP peaked at no later than a day in animal models across different species [48]. Quick and multiple MP doses within 4 hours of trauma significantly reduced tissue loss and axonal dieback after SCI in rats [50, 51]. Moreover, dosage has negligible effect on the time course or the extent of secondary injuries when at least 30 mg/kg MP was employed [52, 53]. Altogether, a rapid but sustained presence of MP for up to a day may be the most effective in limiting post-traumatic secondary injuries.

Table 2: Methylprednisolone Neuroprotection Outcomes

Effectiveness	MP Dosage	Neuroprotection Outcome
Very Effective Tissue Protection [50]	• T8 transection injury in rats • 30 mg/kg • 5 mins, 2 and 4 h after injury • Intravenous	• At rostral end: 2.4 folds, 2.4 folds and 1.46 folds lower tissue loss at 2, 4 and 8 weeks post-injury respectively up to 1 mm from transection site • At caudal end: 2.4 folds, 2.4 folds and 2 folds lower tissue loss at 2, 4 and 8 weeks post-injury respectively up to 1 mm from transection site • 4 folds lower dieback of vestibule spinal fibres at 2, 4 and 8 weeks post-injury
Very Effective Tissue Protection [51]	• T8 contusion injury in rats • 30 mg/kg • 5 minutes, 2 and 4 h after injury • Intravenous	• 1.9 folds reduction in volume of damaged spinal cord at contused site after 12 weeks • 1.7 folds reduction in volume of lost tissue at contused site after 12 weeks • 1.8 folds increase in volume of tissue spared at perilesion after 12 weeks
Moderately Effective Tissue Protection [54]	• Contusion injury between T9 and T11 • 60 mg/kg 5 min post injury • Continuous delivery 5.4 mg/kg/h for 24 h	• Marginal reduction (19.2% and 18.8%) in lesion volumes at 5 and 14 days but not 42 days post injury • Percentage area of tissue sparing at lesion epicenter remained unaltered

	• Intravenous	
Ineffective Tissue Protection [53]	• T9 incomplete transection injury in rats • 30 or 60 mg/kg • Single dose at 20 mins after injury • Intravenous	• Improve neither the time course nor the extent of secondary cell death • 34.6% and 59.6% lower neutrophils invasion with 30 and 60 mg/kg MP respectively • 67.5% and 72.4% lower macrophages invasion with 30 and 60 mg/kg MP respectively
Ineffective Tissue Protection [52]	• L4 Contusion injury in cats • Single dose ranging from 7.5 mg/kg to 60 mg/kg • Intravenous	• Injury increases lactate and reduces pyruvate concentration • 30 mg/kg dose reduced lactate concentration back to uninjured level. • All dosages increased pyruvate concentration back to uninjured level.

2.1.3.b Targeting Neuroinflammation

Among the anti-inflammatory drugs, minocycline is the most extensively examined low Mw, amphiphilic drug for modulating inflammatory response. It operates through different neuroprotective mechanisms as compared to MP [38]. Specifically, minocycline protects tissues from inflammatory response-induced secondary injuries by decreasing the production of inflammatory cytokines, reducing the chemotaxis of leukocytes, modifying lipid mediator of inflammation and inhibiting MMPs and nitric oxide production from monocytes [55]. However, similar to MP, minocycline needs quick but sustained delivery in the acute injury phase to be effective (Table 3). Specifically, animals that received multiple doses of minocycline for at least 24 hours after induction of SCI saw a reduction in tissue loss and an improvement in functional outcome [56-60].

Comparing the two most commonly employed neuroprotectants, MP appears slightly more potent than minocycline. Generally, 30 mg/kg to 60 mg/kg of MP is needed in comparison with 90 mg/kg of minocycline for effective therapeutic outcomes. Furthermore, MP has been clinically proven to be effective in neuroprotection while minocycline has yet to have conclusive evidence in improving clinical outcome [61, 62]. An additional factor to consider in the design of drug delivery systems is the distinct hydrophilicity differences between the two neuroprotectants. Specifically,

hydrophilic minocycline is more readily entrapped with high degree of latency within an aqueous carrier like a hydrogel without hydrophobic domains or a liposomal drug delivery system. This could provide slow drug release in blood-infiltrated lesions that are filled with plasma proteins [63]. On the other hand, hydrophobic MP may be released rapidly from the same carriers in the same environment, which is key for successful neuroprotection. Thus, the scaffold-drug interactions and surrounding environment could be crucial in achieving the desired rapid release of neuroprotectant.

Table 3: Minocyclin Neuroprotection Outcomes

	Minocyclin Dosage	Neuroprotection Outcome
Very Effective Tissue Protection [56]	• 50 mg/kg 1 hour post-injury, 50 mg/kg 24 hours after • 25 mg/kg every 24 hours for subsequent 5 days • Intraperitoneal • Compression injury at T3/4 level	• 2.4 fold higher BBB score by 28 days • 24% improvement in Inclined Plane score by 28 days • 36% lower lesion area
Very Effective Tissue Protection [59]	• 90 mg/kg immediately after injury • 45 mg/kg at 12 and 24 hours • Intraperitoneal • Contusion injury at T9/10	• 16.7% higher BBB score by 38 days • 2 fold lower lesion area by 38 days • 2.3 fold lower number of apoptotic cells 24 hours post-injury
Very Effective Tissue Protection [57]	• 90 mg/kg 1 hour post-injury • 45 mg/kg every 12 hours for 5 days • Intraperitoneal • Contusion injury at T10	• 30.8% higher BBB score by 28 days • 9.8% improvement in Inclined Plane score by 28 days • Significant sparing of white matter, ventral horn motor neuronsand oligodendrocytes at perilesional areas
Very Effective Tissue Protection [64]	• 50 mg/kg 30 mins post-injury, every 12 hours for 2 days • Intraperitoneal • Dorsal column transection injury at C7/8	• 39.5% lower apoptotic oligodendrocytes at proximal end by 14 days • 43% lower lesion area by 14 days • 7 fold improvement in limb coordination index by 14 days • 4.5 fold improvement in angle of rotation index by 14 days
Very Effective Tissue Protection	• 30 mg/kg 30 mins, 1 and 24 hours post-injury • Or, single dose of 90 mg/kg at 30 mins, 1 or	• 2 fold higher BBB score by 28 days with multiple dose, 1.88 fold higher BBB score by 28 days with single large dose with early administration provide slightly better outcome

[60]	24 hours post-injury • Intraperitoneal • Contusion injury at T9	• 2.8 fold lower lesion area after 28 days • Lower expression of apoptosis marker
Very Effective Tissue Protection [58]	• 90 mg/kg immediately/2 hours post-injury • 45 mg/kg every 12 hours for 3 days • Concurrent MP administration • Intraperitoneal • Contusion injury at T9/10	• 50% higher BBB score and 16% better angle of inclined plane score, 43% lower grid error score by 35 days when administered immediately/2 hours post-injury • 2 fold lower TUNEL+ oligodendrocytes in vitro • 27% more axon at perilesional areas after 38 days
Ineffective Tissue Protection [65]	• 90 mg/kg immediately after injury • 45 mg/kg at 12 and 24 hours • Intraperitoneal • Contusion injury at T9/10	• No improvement in BBB score • No increase in tissue sparing • No decrease in lesion area
Ineffective Tissue Protection [66]	• 90 mg/kg 1 hour post-injury and every day for 3 days • Intraperitoneal • Contusion injury at C5	• No improvement in horizontal ladder test, cylinder rearing test, grooming test, staircase test and sensory test • No increase in tissue sparing

An interesting alternative to minocycline or other low Mw drugs for anti-inflammatory response is induced nitric oxide synthase (iNOS) antisense oligodeoxynucleotides (ASOs). iNOS is a key mediator of inflammatory response after SCI. ASOs selectively inhibits iNOS to reduce secondary neuronal cell death by 50% at the rostral end and 44% at the caudal end after T10 spinal cord contusion injury in rats [67]. However, the stability of such nucleotides *in vivo* and how to deliver them into the cells are major questions to be addressed [68]. While modifications of naked nucleic acids help improve nucleotide resistance towards nucleases [69], a better way to deliver these molecules may be to encapsulate them within scaffolds, which protect the nucleic acids from nuclease degradation *in vivo* for sustained and localized delivery [70-72].

Apart from minocycline and iNOS, two types of immunotherapy for anti-inflammatory neuroprotection have also been examined. During the early years, specific antibodies that selectively neutralize pro-inflammatory cytokines were employed to generate anti-inflammatory response and thwart inflammation-triggered secondary injuries [44, 73-77]. More recently, this immunotherapeutic strategy has evolved in the form of using intravenous immunoglobulin (IVIG) to attenuate neuroinflammation after SCI [78-80]. In contrast to the mode of action by specific antibodies, IVIG regulates $F(ab')_2$ and F_c receptors on monocytes, plasma cells and dendritic cells, which are crucial for the activation of immune response [81]. As IVIG targets cells that secrete cytokines instead of the cytokines per se, this method elicits more potent anti-inflammatory reactions than specific antibodies [78]. Similar to other neuroprotective drugs or nucleic acids, IVIG therapy could be enhanced by localized delivery. In particular, the drug candidate could be chemically conjugated to the drug delivery system or confined by specific antigen-antibody interactions for successful drug loading [82-84]. Early challenges in antibody conjugations [85] have largely been overcome in recent years [83, 84]. Thus, their controlled delivery now represents a promising treatment in other diseases and should be further explored in the field of neural tissue engineering.

Although inflammatory response-induced secondary injuries exacerbate tissue loss after initial trauma, this reaction is also associated with at least partial pro-regenerative outcomes [86]. Thus, the sustained presence of anti-inflammatory factors may not be optimal in SCI treatment. An attenuation of the availability of anti-inflammatory drugs, coupled with sequential administration of neuroregenerative agents, may be a better approach for regeneration.

2.2 Biochemical Signaling Associated with the Blockade and Advancement in Nerve Regeneration

In stark contrast to the extensive cell necrosis and apoptosis that occur during the degenerative phase, the subsequent phase of injury is marked by attempts to limit tissue loss and initiate regeneration. Consequently, astroglia scar formation and abortive sprouting by severed axons are seen in these intermediate and chronic stages of injury, which lasts from three weeks up to months in both rodents and human [21, 28, 29].

2.2.1 Blockade and Advancement in Nerve Regeneration

Myelin debris and astrocytic scars are two key inhibitors of nerve regeneration. Myelin-associated inhibitors play prominent roles in instigating growth cone collapse and hindering neurite growth (see review [87]). Although resident and recruited inflammatory cells clean up these myelin debris, these cells also inevitably activate and promote the proliferation of astrocytes [22]. The proliferation of astrocytes then leads to the production of more inhibitory proteoglycans, which bind to neuronal receptors and inhibit axon outgrowth [88]. Moreover, activated astrocytes form a scar that surrounds the lesion, which restrict neurite outgrowth through similar growth inhibitory receptor mechanisms [88]. Altogether, these cells build an inhibitory environment that blocks nerve regeneration.

However, astrocytic scar in the spinal cord is a double-edge sword. Scarring is also found to protect the spinal cord from further secondary injuries, thereby preserving partial function after SCI. Specifically, the loss of reactive astrocytes exacerbated tissue degeneration, impaired limb functions, increased inflammation and prevented blood-brain barrier repair [21]. In addition, astrocytic scar appears to be necessary for stimulated axon regrowth [89]. Thus, a viable alternative is to enhance the intrinsic neuronal regrowth ability. Although young neurons in the developmental CNS possess

remarkable regenerative capacity, this capacity is lost in adult CNS neurons [90]. Biomolecules could assist the adult axons to recapitulate the regenerative capability to grow past the inhibitory environment after injury.

2.2.2 Biomolecules that Modulate Blockade and Advancement Nerve Regeneration

Chemical digestion or neutralization of the axonal growth inhibitory molecules that are present after SCI could aid neural regeneration. Correspondingly, these approaches often target the inhibitory proteoglycans and myelin-associated inhibitors. On the other hand, regenerative neurotrophins could recapitulate and enhance the intrinsic neuronal regrowth ability to grow past the inhibitory SCI environment.

2.2.2.a Targeting Blockade in Nerve Regeneration

Among the four classes of proteoglycans produced by reactive astrocytes, chondroitin sulphate was identified to be extremely inhibitory to axon outgrowth. Thus, chondroitinase ABC (ChABC) is often employed to digest the glycosaminoglycan chains of this proteoglycan [91]. In combination with other biomolecules, cell transplantation and nerve grafting, the treatment could see regeneration and functional recovery after SCI [10, 11, 92-97]. Similarly, antibodies that neutralize Nogo-A, which is a prominent class of myelin-associated inhibitor, were found to promote nerve regrowth after SCI [98-101]. However, the lack of regeneration in Nogo-deficient mice suggests that the removal of Nogo alone could not promote robust regeneration after injury [102]. Furthermore, the neuron-intrinsic mechanisms of axon regeneration could be of paramount importance in regeneration following SCI [103, 104].

2.2.2.b Targeting Nerve Regeneration

Neurotrophins, including neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF) and nerve growth factor (NGF), have been widely engaged to boost the intrinsic regenerative mechanism in adult neurons to grow past the inhibitory extracellular environment after injury (see Table 4).

Among them, NT-3 enables more consistent axon regeneration and function recovery in both acute [105-107] and chronically injured spinal cords [108]. It was recently used in conjunction with neural stem cell (NSC) transplantation for robust neurite outgrowth, synapse formation and functional recovery [103]. Although early studies utilized genetically-modified fibroblasts to deliver NT-3, it can also be incorporated and delivered through hydrogel-based or electrospun fiber/hydrogel composite scaffolds to promote nerve regeneration [103, 104, 107, 109-111]. The efficacy of these strategies have recently been shown in non-human primate [111], providing a viable alternative to the currently popular cell-based therapy.

Another well-characterized neurotrophin, BDNF, is known to direct NSC neuronal differentiation and survival while stimulating axonal regeneration after SCI [112, 113]. Increasing the availability of BDNF after injury enhanced axonal sprouting [114, 115] and functional recovery in contusion or hemisection rodent injury models [116, 117]. However, there are also reports on poor functional outcomes specifically in cervical level injuries [118, 119].

In comparison to NT3 and BDNF, other neurotrophins could be less efficacious in promoting recovery after SCI. Although NGF and GDNF could enhance axonal growth and sprouting in both chronically injured rodents and non-human primates, there is a lack of functional recovery [120-123].

GDNF-producing Schwann cells transplantation however, has recently seen some promising outcome [124], indicating the possible needs of optimizing the delivery methods of neurotrophins. On the other hand, bFGF exhibited neuroprotective rather than neuroregenerative responses in contused spinal cords [125].

Altogether, it has been proposed that overcoming the inhibitory SCI lesion by boosting the regenerative capacity that is intrinsic to CNS neurons with trophic factors is a better neuroregenerative approach [126]. This is because the removal of extracellular inhibitory environment has thus far, failed to achieve expected robust regeneration. However, it could also be possible that some inhibitory molecules have yet to be identified. Thus, identification of more such factors could open new avenue to this strategy [90].

Table 4: Neurotrophin-induced Neuroregenerative Outcomes

	Dosage	Neuroregenerative Effect	Reference
BDNF	• 100 or 150 µg/day Intrathecally • T9 contusion	• More than 3-fold more animal with >15 BBB score in mild contusion injury 15 days post injury • More than 6-fold more animal with >10 BBB score in intermediate contusion injury 8 days post injury • Consistently 3-fold higher BBB score for 28 days in complete transection injury	[116]
BDNF	• Genetically-engineered Fibroblast Grafting • C3/4 Hemisection	• Enhanced axonal sprouting • 10% increase in frequency of ipsilateral forelimb usage by 4-8 weeks • 2-fold lower deficit score in horizontal rope-crossing test • Poor recovery in other functional test	[114, 118]
BDNF +NT-3 or GDNF +NT-3	• Viral vector infusion • CST Lesion	• BDNF and GDNF group showed 46% and 52% greater number of CST axons crossing midline when delivered with NT-3	[115]
BDNF	• Genetically-	• More axon infiltration into graft	[117]

	engineered Fibroblast Grafting • C3/4 Hemisection	• 41% improvement in BBB score by 12 weeks • 3.6-fold improvement in forelimb function by 12 weeks	
BDNF	• Genetically-engineered Fibroblast Grafting • Contusion injury T8	• 1.8-fold more axonal infiltration into lesion after 10 weeks than control graft • 2-fold more area with myelin-ensheath axon after 10 weeks than control graft • 5-fold more proliferating OPC after 10 weeks than control graft	[127]
BDNF/ NGF/ NT3	• Intramedullary Infusion	• Only BDNF showed 25% improvement in inclined plane test • More preserved axons and smaller cavitation in BDNF treatment group	[128]
BDNF/ NT-3	• Genetically-engineered Fibroblast Grafting 6 weeks post-injury • 188 ng/24 hr of BDNF and 95.4 ng/24 hr of NT-3 • C3/4 hemisection	• 23-fold higher axon length infiltrated into BDNF+NT-3 graft • Poor recovery on motor task, limited recover on sensorimotor task	[129] [119]
NT-3	• Genetically-engineered Fibroblast Grafting • Contusion injury T8	• 2-fold more axonal infiltration into lesion after 10 weeks than control graft • 2.5-fold more area with myelin-ensheath axon after 10 weeks than control graft • 5-fold more proliferating OPC after 10 weeks than control graft	[127]
NT-3	• 1 µg/ul intrathecal • Lower T level transection	• Higher number of CST axon sprouting 2-3 weeks after injury	[105]
NT-3	• Genetically-engineered Fibroblast Grafting • T7 hemisection	• 4.3-fold better functional recovery at 1 month, 2.2-fold at 3 month post injury. • 2.2-fold more CST axonal sprouting at lesion, 4 fold at up to 8 mm distal.	[106]
NT-3	• 0.1 µg/µl in collagen • T9 transection	• 3.1-fold higher CST axonal growth into collagen matrix • 2-fold better functional recovery starting from 2 weeks post injury	[107]
NT-3	• Genetically-engineered Fibroblast Grafting 3 months post-injury	• 14% improvement in BBB score 3 months post transplantation and 6 months post injury • Up to 3-fold more axonal crossing close to lesion site	[108]

	• T7 hemisection		
NT-3	• Chitosan matrices • T8 laminectomy	• Robust de novo axonal regeneration • Somatosensory evoked potential, motor evoked potential, magnetic resonance imaging and kinematics-based analyses revealed sensory and motor recovery	[111]
NGF	• Genetically-engineered Fibroblast Grafting • T7 laminectomy	• 9-fold higher sensory neurite and 5.7-fold higher dopaminergic and noradrenergic neurites penetration into NGF-secreting grafts	[120]
NGF	• Genetically-engineered Fibroblast Grafting 1 to 3 months post-injury • 13.3 ng/g tissue • T7 laminectomy	• More robust penetration of chronically injured axons into grafts • Penetrated axons are mostly dopaminergic and noradrenergic in nature • Little serotonergic or CST axons penetrated into grafts	[121]
NGF	• Genetically-engineered Fibroblast Grafting • T8 dorsal laminectomy • Non-human Primate	• 26-fold higher sensory axon penetration into NGF-secreting grafts • 14-fold higher dopaminergic and noradrenergic axon penetration into NGF-secreting grafts	[122]
bFGF/ CNTF/ NGF	• Infusion • Contusion injury T10	• Only bFGF reduced total zone of injury by 33% and zone of partial preservation by 32%	[125]
GDNF	• Genetically-engineered Fibroblast Grafting • 53.9, 46.4, 35.5 ng/g tissue at 2, 4 and 12 weeks respectively • T7 hemisection or transection	• 13-fold higher dorsal column sensory axon growth into GDNF-secreting cell grafts • 16-fold higher brainstem and propriospinal axons regeneration • Up to 3-fold higher local motor and sensory axon penetration into cell grafts • No pro-regenerative effect with corticospinal axons	[123]
GDNF	• Genetically-engineered Schwann Cell Graft • 4.4, 5.8 ng at 24 and 72 h respectively • T9 transection	• 1.6-fold more regeneration axons • 8-fold higher number of neurons with projected axons • 2-fold and 1.6-fold higher BBB score at 3 weeks and 4 weeks respectively	[124]

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2.3 Biochemical Signaling Associated with Remyelination

Axonal sprouting and neurite regrowth after SCI often serve as indicators of regeneration. While equally important, remyelination, however, is less extensively examined and poorly understood in the field of SCI. Remyelination restores multilamellar, lipid-rich myelin to demyelinated axons, which is essential for axonal metabolic support and conduction of action potential [130, 131]. Following mild contusion injuries, spontaneous remyelination ensues with some degree of long term remodeling and plasticity [132, 133]. However, robust remyelination is never attained. Similarly, chronically injured axons were characterized by significantly lower myelin sheath lengths and higher g-ratios [132]. Crucially, this small degree of spontaneous remyelination enables only minimal functional recovery [134]. Although further disablement of spontaneous remyelination did not aggravate the small recovery following trauma, it is currently not known if an enhancement in remyelination to pre-injury levels would permit better functional outcomes [134]. Thus, detailed remyelination mechanisms following spinal cord trauma has to be thoroughly examined [135]. Although this information is currently missing, much remyelination mechanistic insights may still be drawn from studies dealing with other CNS demyelinating diseases. Specifically, toxins-induced [136, 137] and virus-infected demyelination models [138] offered clues on the sequence and mechanism of events following demyelination, which could be extended to other injury models.

2.3.1 OPC Repopulation, Differentiation and Remyelination

Successful remyelination requires the seamless transition from repopulation of demyelinated lesions by the oligodendrocyte precursor cells (OPCs), to the differentiation of these cells into mature oligodendrocytes, and finally the restoration of myelin cytoarchitecture on axons [23] as summarized in Table 5. Successfully remyelinated spinal cords in lipopolysaccharides (LPS) or ethidium bromide (EtBr) models have found OPCs predominantly around the lesions at 2 days post

LPS and EtBr injection and subsequently, within the lesions after 7 days. This is followed by onset of differentiation, maturation and remyelination by 14 days [139, 140]. Unfortunately, remyelination often fails in SCI due to the dysregulation in one or more stages of necessary activities. Thus, regenerative biomolecules could be necessary in SCI to enhance OPC repopulation for remyelination, especially in chronic injuries [141, 142].

Table 5: OPC Remyelination Timeline and Signals

	Timeline	**Biochemical Signals**

OPC Activation	In demyelinating diseases model
	• Activated rapidly [23]
	
	In SCI
	• Activated rapidly in response to trauma [143]
		• Pro-inflammatory cytokines (TNF-α, TGF-β1, IL-1, IFN-γ) [131, 143, 144]
		  ○ Secreted by resident microglia or circulating macrophages
		  ○ OPC exhibit enlarged cell bodies, hypertrophic process and alteration in wide range of genotypes
		• Astrocytes activated by microglia further amplify OPC activation response, but precise mechanism not known [145]

OPC Proliferation	In demyelinating diseases model
	• Increased proliferation following activation
	• Abnormally quiescent $Ki67^-$ OPC in chronic lesions [142]
	
	In SCI
	• Elevated proliferation following activation and remain elevated for at least 4 weeks [146]
	• Attenuated in chronic injury [146, 147], similar to chronic demyelinating disease.
		• PDGFRα [148, 149]
		  1. Knocking out receptor and ligand PDGF expression impede proliferation after demyelination
		• p27Kip-1 [150]
		  1. Fine-tune OPC responsiveness to mitogens, including PDGF signaling
		• Differentiation regulators (Eg: NOTCH, WNT) [131]
		  1. Transition to cell cycle exit promote differentiation and attenuate proliferation
		  2. Precise transition mechanism is not known

OPC Migration	In demyelinating diseases model
	• Peak at 5 days post lesion [151]
	• Extensive in acute active lesions but attenuated in chronic plaques [152]
	
	In SCI
	• Astrocytic scar impede migration into injury sites [153]
		• Sox 2 [151]
		  1. Genetic ablation of Sox2 reduce OPC migration to lesion
		• Nkx2.2 [154]
		  1. Expression coincide with OPC migration from perilesional areas to lesion epicenter
		• Semophorins [155]
		  1. OPC express Sema3A and Sema3F receptors, while microglia and astrocytes upregulate semophorins to guide OPC migration into active lesions
		• Chemokines (CCL2, IL-1β) [152]
		  1. Overexpression of CCL2 promote OPC migration into lesion

OPC Differentiation and Remyelination	In demyelinating diseases model • Onset of differentiation after 7 days and remyelination after 14 days [154] • In virus infection model, active remyelination is delayed to 4 weeks due to delay in OPC repopulation [156, 157] In SCI • Upregulation of inhibitor signals impede OPC differentiation and remyelination [158]	• PDGFRα 1. Inhibitory to OPC differentiation and final stages of myelin sheath formation during remyelination [159] 2. Nkx2.2 binds to PDGFRα promoter to suppress its expression during remyelination [160] • NOTCH 1. Re-establishment of developmental contact-mediated NOTCH inhibits OPC maturation [161] 2. Jagged1 from reactive astrocytes binds to NOTCH1 from immature OL to induce Hes5 expression, which blocks other helix-loop-helix factors [162] • LINGO1 1. Most crucial contributor of inhibitory environment [163-165] 2. Reduces gelsolin expression to reduce the motility of actin microfilaments that is essential for OPC differentiation [165]

2.3.2 Biomolecules that Modulate Remyelination

Rationally, we may promote remyelination after SCI by delivering biomolecules to promote OPC repopulation, and subsequently, OPC differentiation. Two plausible methods to promote OPC repopulation include 1) Increasing the availability of OPCs in chronic injuries by sustaining the proliferation of OPCs that is often seen in acute injuries, while keeping them viable; and 2) Inducing OPC migration across the glia scar. Following OPC migration, biomolecules could be delivered to promote OPC differentiation for remyelination. It is worth noting that OPC proliferation and differentiation are mutually exclusive [131]. Hence, factors that promote proliferation (Eg PDGF) are almost always inhibitory in nature to differentiation. Thus, temporal control of drug release may be crucial in remyelination.

2.3.2.a Targeting OPC Repopulation

The first approach to promote OPC repopulation of injury site is through enhancing OPC proliferation with mitogens. Five OPC mitogens have been identified, namely, PDGF, bFGF, insulin-like growth factor (IGF-1), type II neuregulin-1 (NRG1) and epidermal growth factor (EGF). Among them, PDGF is the most potent OPC mitogen and was investigated for its efficacy in promoting OPC proliferation and survival in demyelinating models. In particular, transgenic mice with higher PDGF expression have significantly enhanced remyelination in chronic demyelinating lesions [166]. In these mice, higher PDGF expression induced 2.7-fold higher number of OPCs and 7.5-fold more proliferating OPCs in acute cuprizone-induced lesions after 6 weeks of toxin ingestion. These were translated to 1.5-fold more matured OL and significantly more remyelinated axons in the chronic lesions after 18 weeks [166]. In addition, PDGF also acts as a survival factor to protect OPCs from apoptosis. Specifically, enhancing PDGF expression in the same transgenic mice resulted in 2-fold lower apoptotic OPCs in comparison with wild type mice. This suggests that upregulating mitogen PDGF expression can increase OPC availability to repopulate chronic lesions for remyelination [166].

Other OPC mitogens have reported drawbacks that could limit their potential in remyelination therapy: For instance, bFGF was reported to have no impact on OPC proliferation following cuprizone-induced lesions. On the contrary, its absence increases the availability of mature OL by promoting OPC differentiation [167]; Another mitogen, IGF-1, appeared less potent than PDGF in preventing OPCs apoptosis in culture and in developing optic nerves [168, 169]; NRG1 acts on only more matured $O4^+$ pre-oligodendrocyte instead of OPCs [170]. Although EGF was recently shown to be as potent as PDGF in enhancing OPC renewal and survival [171], more extensive *in vivo* studies are needed to verify these findings. Thus, to date, PDGF may be the most promising candidate for increasing the availability of OPCs for remyelination and should be experimented in an SCI model.

The second approach to promote OPC repopulation of injury sites is through inducing OPC migration. The most rational way to induce this migration may be to digest or block the synthesis of proteoglycans, CSPGs, as it is a major component of astrocytic scars [153, 172]. Specifically, digesting CSPGs with 20 mU ChABC enzyme led to more migration of OPCs into a contused spinal cord lesion [153]. On the other hand, blocking the synthesis of CSPG with 50 mg/kg fluorosamine significantly enhanced remyelination in LPS-induced lesion [172]. However, ChABC treatment is not shown to have enhanced remyelination after treatment, while fluorosamine-induced remyelination was associated with OPC maturation rather than migration [172].

Apart from being both a mitogenic and a survival factor, PDGF could induce the chemotaxis of OPCs *in vitro* [173]. However, this PDGF-mediated mechanism is highly dependent on the expression of poly sialic acid-neural cell adhesion molecule (PSA-NCAM) [173]. Although PSA-NCAM expression is highly upregulated in OPCs during early demyelination in demyelinating lesions, and ceases only when remyelination is completed in the spinal cord [151, 174, 175], it is not known if the same occurs after traumatic injuries. If so, PDGF may potentially serve as a mitogenic, survival and pro-migratory factor to increase OPC availability and recruitment, to repopulate the injury site for subsequent remyelination.

2.3.2.b Targeting OPC Differentiation and Remyelination

Overwhelming evidences have shown that remyelination failure is largely associated with OPCs failing to differentiate into myelinating OLs [23]. Such could be due to the presence of an inhibitory environment in the injury sites, the lack of neurotrophic support and/or biochemical cues [176]. Hence, delivering exogenous factors to promote OPC differentiation is vital to successful

remyelination, as shown in demyelinating disease models. Many drugs/genes target NOTCH or LINGO1 signaling pathways due to their crucial roles in constructing the inhibitory environment.

NOTCH-Specific Inhibitor

Delivering γ-secretase inhibitor (MW167) to block pathological NOTCH and Jagged1 expressions was attempted in order to remyelinate the CNS [177]. Intraventricular injection of 1 mM MW167 completely abolished the generation of the intracellular domain of Notch in OLs from murine brains and spinal cords. More importantly, suppressing NOTCH expression was instrumental in giving more remyelinated axons in an EAE demyelination model after 11 days [177]. However, this strategy was effective only if NOTCH was knocked out early in the OPC lineage [178, 179]. Specifically, ablating NOTCH from $Olig1^{+}$ OPCs and OL lineage cells in Cre-LoxP mice promoted OPC differentiation during CNS remyelination [179]. However, eliminating NOTCH from PLP^{+} matured OL in Cre-LoxP recombinant mice had no pro-remyelination effect in a toxin-induced demyelination model [178]. Together, these results suggest that NOTCH expression in OL early progenitors has important implications in their remyelination capability during the matured phase.

LINGO1-Specific Inhibitor

LINGO-1 has profound impact on the early differentiation of OPCs [165], at a greater extent than NOTCH signaling. In particular, 10 μg/ml of anti-LINGO-1 antibody, which is a LINGO-1 antagonist, dramatically increased OPC and pre-OL differentiation into OLs by 40-fold and 10-fold respectively in an axonal co-culture [164]. Anti-LINGO-1 antibody also significantly promoted remyelination in MOG-induced autoimmune and toxin-induced demyelinating models [163, 164]. Most excitingly, this treatment has demonstrated moderate success in phase 1 and phase 2 clinical trials [180, 181]. Thus, LINGO is arguably the single most important target in remyelination therapy.

MicroRNAs

MicroRNAs (miR) is a class of small non-coding RNAs with novel remyelination potential due to their ability to regulate multiple mRNA expressions [182]. OPC differentiation is regulated by the inhibitory PDGFRα [159], NOTCH [161] and LINGO [163] signaling pathways independently (Table 5). Correspondingly, antagonizing a specific pathway may have limited efficacy due to the presence of the other remaining inhibitory signals. Thus, new therapeutics, such as miRs, that could interfere with multiple inhibitory signals are highly valuable [183]. In particular, miR-219 and miR-338 are key regulators for OPC differentiation, myelination and remyelination by targeting the inhibitory pathways [184, 185].

For instance, Hes5 is a critical downstream effector of NOTCH signaling that inhibits OPC differentiation. miR-219 and miR-338 suppress Hes5 expression in this NOTCH pathway to promote OPC maturation. In particular, 50 nM of miR-219 and miR-338 mimics downregulated Hes5 gene expression by 10-fold while 25 nM of LNA that inhibits miR-219 and miR-338 expression resulted in 2-fold higher Hes5 expression and 4-fold lower number of MBP^+ OLs *in vitro* [184]. Viral vector-induced miR-219 and miR-338 overexpression accelerated OL maturation in the development of chick neural tube and mouse cortex. Conversely, the inhibition of miR-219 and miR-338 with antisense LNA blocked OL differentiation in zebra fish [184]. Similarly, the combinatory miR-219/miR-338 treatment suppressed PDGFRα expression in rodents OPCs [186]. Furthermore, miR-219 directly inhibited LINGO-1 transcription and the expressions of OPC differentiation transcriptional regulators (*Nfia*, *Nfib*, *Etv5*) to promote OPC differentiation and remyelination in an LPS-induced demyelination model and functional recovery in EAE mice [185].

Notably, being much smaller in size than proteins, miRs offer better biodistribution when released from small drug carriers [63]. Also, miRs are very hydrophilic and are readily entrapped within aqueous carriers for delayed delivery in plasma rich environments. This is crucial as OPC differentiation should occur late in remyelination. Moreover, scaffold-mediated delivery of miR synergized topographical and biochemical cues to promote OL differentiation *in vitro* [186]. This makes miR-mediated therapeutic strategy particularly attractive in SCI treatment as the scaffold can serve multiple purpose of bridging the transected injury, providing localized delivery of therapeutics and promoting OPC differentiation [187] and remyelination [188].

2.3.3 Differences between SCI and Demyelinating Diseases in Remyelination

Therapeutic strategies targeting demyelinating diseases appear promising. However, these strategies could be ineffectual in SCI due to the distinct differences in the pathophysiology of SCI and demyelinating diseases. An example is in promoting inflammatory responses in chronic inactive demyelinating lesions. Chronic inactive plaques from demyelinating CNS is characterized by minimal inflammation and macrophage infiltration, and is correlated with the absence of active remyelination [189, 190]. However, inflammatory response after SCI is fundamentally different from autoimmune inflammatory diseases. Specifically, neutrophils and macrophages continue to persist in SCI sites even in chronic injuries [191]. Thus, further inducing inflammatory response may not help with remyelination of trauma-induced injuries.

Other factors that are regulated differently between demyelinating diseases and SCI include cell fate-determining Wnt signaling. Wnt signaling was identified to regulate many stages of OPC development in recent decade, mainly during developmental myelination and remyelination following demyelinating diseases [192-194]. In SCI however, Wnt signaling pathway is only known to

regulate axonal regeneration [195]. Last but not least, genetic fate mapping revealed that the limited spontaneous remyelination following traumatic injuries are attributed in part by Schwann cells [131, 196], which is not seen in demyelinating diseases. However, it is not known if this contributes to functional differences. Thus, one should be cautious in translating therapeutics from demyelinating diseases to SCI.

3. Sequential Delivery Timeline and Systems for Temporal Drug/Gene Delivery

Considering aforementioned biochemical signaling pathways for nerve regeneration and remyelination, we propose a general timeline of treatment and design of sequential drug delivery system (DDS) for SCI, as illustrated in Figure 2. Specifically, immediate neuroprotection following SCI is imperative as seen by the temporal needs during the early phase of SCI [61, 197]. Similarly, anti-inflammatory treatment should be administered rapidly following injury. However, the pro-regenerative effects associated with inflammatory response means that these neuroprotective treatments should attenuate before the next phase of injury. Moreover, a localized delivery of anti-inflammatory drugs could reduce the dosage level that is required for effective neuroprotection and reduces the associated side effects [48].

After 24 to 48 hours of neuroprotection, the delivery of regenerative biomolecules for nerve regrowth should ensue. A cocktail of neurotrophic growth factors and OPC mitogen could support axonal regeneration and myelination of regenerated axons [103, 104]. Temporal control is particularly important in this next phrase of remyelination. In particular, it is important to ensure that OPC mitogenic effect is waned when OPC differentiation and myelination begins. Future work should ascertain the time point for the transition between promoting OPC repopulation of lesions and OPC maturation and myelination. Moreover, Anderson *et al.* showed that preventing astrocytic

scar formation or ablation of chronic scar did not enhance axon regrowth [89], in stark contrast with earlier works on chABC treatment. Thus, future work should also ascertain the need of chABC treatment.

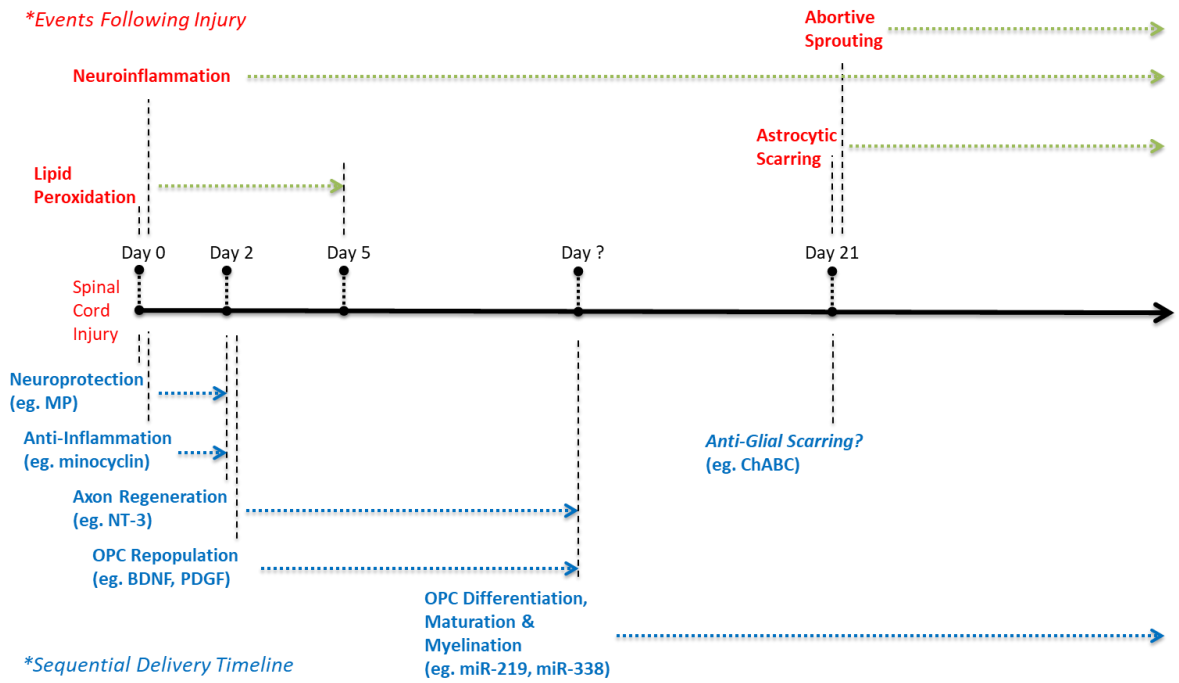

Figure 2: Sequential events and delivery timeline following spinal cord injury

3.1 Sequential Delivery Profiles

To ensure that the effect of aforementioned drugs and genes are acting on the targets in a timely manner, it is important to determine how the molecules are released from their carriers. A typical release profile of a biomolecule that is delivered by a scaffold has a burst release followed by a decay curve. The simplest form of sequential release is to have two such release profiles, with molecule B delivered in a lower quantity than molecule A (Figure 3, Profile 1 [18, 198]). In this profile, the concentration of molecule B is expected to cross the minimal effective drug concentration of the therapeutic window later than the first molecule (molecule A). While it may not exactly be

"sequential" as both biomolecules start their release simultaneously, cellular targets will experience a therapeutically effective dosage of molecule A before B.

A more intuitive sequential delivery release profile is one with immediate release of the first molecule and a delayed release of the second molecule (Figure 3, Profile 2 [199, 200]). This pattern of delivery may be more suitable for applications with antagonistic effects between early and late stage molecules. For instance, to remyelinate the spinal cord, PDGF acts through PDGFRα signaling to promote OPC proliferation. However, miR-219 represses PDGFRα expression in a positive feedback loop [201]. Therefore, the design of the carrier and where the scaffold is going to be implanted are crucial to realize profile 2-like release to avoid the antagonism of factors.

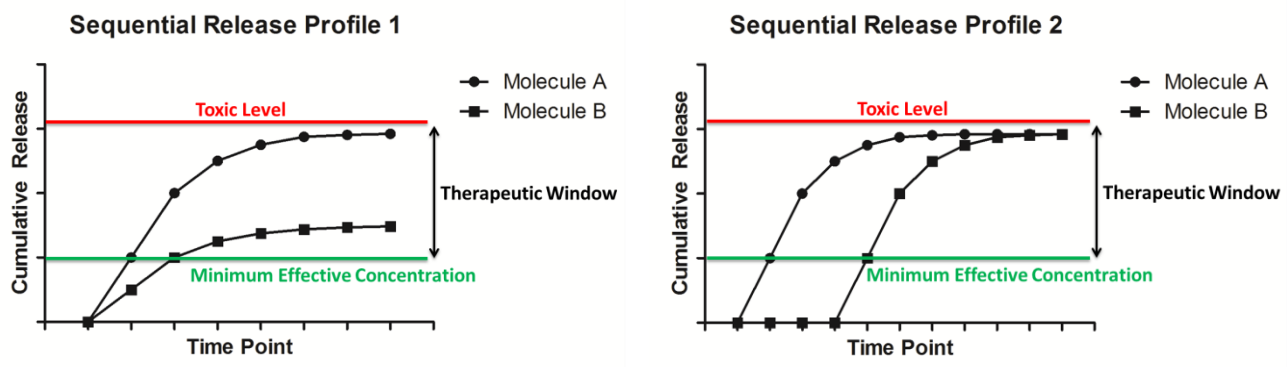

Figure 3: Probable release profiles of sequential delivery systems.

Despite the advancement in sequential delivery in many fields of regenerative medicine and drug/gene delivery, as summarized in Table 6, we have yet to see similar attempts in CNS regeneration therapies. Such attempts are complicated by our incomplete understanding of the highly heterogeneous SCI pathophysiology and crucially, the lack of well-defined dose response relationship of most therapeutics. We shall discuss how carefully designed tools have helped to achieve a variety of sequential release kinetics in other fields, and suggest how these ingenious designs could be applied to SCI therapeutics (Table 7).

1

2

Table 6

Sequentially delivered molecules

1. Low Molecular Weight (Mw) Drugs (< 500 Da), High Molecular Weight (Mw) Drugs (> 500Da)*, Growth Factors, Genetic Material, Cytokine, Ion
2. Hydrophilic vs Hydrophobic

*classified based on rule of 5, a drug with Mw >500 Da may not cross biological barriers in pharmacologically significant amounts and have reduced absorption [202]

Type of Sequential Delivery	Type of Molecule	Drug and Release Sequence	Type of Carrier	Hydrophilicity	Reported Actual (A) Encapsulation (B) Loading and (C) Release Efficiency	In Vitro Outcome of Sequential Release relative to vehicle/plain control	In Vivo Outcome of Sequential Release relative to vehicle/plain control	Reference
Low and High Mw Drug in Cancer Therapy	Low Mw Drug High Mw Drug	CA4 PTX	Coated Micro/nanoparticle	Hydrophobic Hydrophobic	(A) 82% for PTX 52% for CA4 (B) – (C) More than 50% burst release of CA4 and 25% of PTX in first 24 hours. 80% PTX and 60% CA4 accumulated release after 336 hours	1.5 fold increase in endothelial cells elimination after 42 hours 1.5 fold increase in breast tumor cells elimination after 42 hours	-	[203]
	Low Mw Drug High Mw Drug	CA4 PTX	Core-Shell Micro/nanoparticle	Hydrophobic Hydrophobic	(A) – (B) 27.2% for PTX and 20.7% for CA4* (C) 63% CA4 and 40% PTX burst release in the first 24 hours. Around 80% CA4 and 70% PTX accumulated released over 144 hours *Did not mention how the values were obtained	-	3 fold decrease in lung tumor volume after 10 days 2 fold decrease in lung tumor proliferation 1.83 fold increase in lung tumor apoptosis 3 fold increase in animal survival rate after 20 days 10 fold increase in neovasculature inhibition after 7 days No significant tumor metastasis and liver cell death	[204]
	Low Mw Drug Low Mw Drug	CA4 HCPT	Core-Shell Nanofiber	Hydrophobic Hydrophobic	(A) Generally >90% for both drugs (B) 5% for both drugs (C) 90% CA4 release within 3 days and 100% release within 10 days. 40% to 60% HCPT release after 5 days, 50% to 80% accumulated release after 30 days	24 fold decrease in endothelial cells viability after 7 days 12.5 fold decrease in breast tumor cells viability after 7 days	2 fold decrease in breast cancer tumor volume after 10 days and 30 days 3.6 fold decease in breast tumor proliferation after 21 days 7 fold increase in breast tumor apoptosis after 21 days 2.5 fold increase in animal survival rate after 20 days	[205]

							3 fold decrease in vascular density after 7 days, 4 fold after 21 days 6 fold decrease in metastatic colonies after 21 days	
	Low Mw Drug High Mw Drug	CA4 DOX	Coated Micro/nanoparticle	Hydrophobic Hydrophilic	(A) – (B) – (C) Burst release of close to 700 µg CA4 within 30 hours but less than 2 µg Dox release in the first 30 hours	2.5 fold increase in endothelial cell elimination after 12 hours, 5.5 fold increase after 30 hours. 3 fold increase in melanoma cell elimination after 30 hours.	2.86 fold decrease in lung tumor volume after 10 days, 10 fold decrease at 17 days. 16 fold decrease in melanoma tumor volume after 10 days, 100 fold after 17 days 5.75 fold increase in tumor apoptosis. 3 fold decrease in vascular density	[206]
	High Mw Drug Low Mw Drug	DOX CA4	Core-Shell Micro/nanoparticle	Hydrophilic Hydrophobic	(A) More than 90% for both drugs (B) – (C) At pH 7.4, 30% Dox and 15% CA4 release within first two hours, with 70% Dox and 40% CA4 accumulated release. In acidic environment, initial burst of CA4 reduced to 10% and accumulated release reduced to less than 30%	2 fold, 2.5 fold and 4 fold decrease in endothelial cell viability at 24, 48 and 72 hours 2 fold, 3.33 fold and 2.5 fold decrease melanoma cell viability at 24, 48 and 72 hours	-	[207]
	High Mw Drug High Mw Drug	Suramin PTX	Core-Shell Micro/nanoparticle	Hydrophilic Hydrophobic	(A) 80% for Paclitaxel and 50% for Suramin (B) – (C) 70% Suramin burst release within first 3 days and completed release after 30 days. PTX has 20% burst release with only 60% release after 30 days	1.11 fold decrease in glioma cells viability after 1, 2 and 3 days 2 fold, 3.13 fold, 3.53 fold increase in glioma cells apoptosis after 3,6 and 9 days	Significant decrease in BLI signal strength Fewer glioma tumor cells Inhibition of tumor proliferation	[208, 209]
	High Mw Drug Low Mw Drug	DOX CPT	Core-Shell Micro/nanoparticle	Hydrophilic Hydrophobic	(A) 87% for CPT and 45% for DOX (B) – (C) Less than 20% Dox release and almost no CPT release at pH7.4.In acidic environment, more than 80% release of Dox and 50% of CPT within first 4 hours	3.33 and 2.35 fold decrease in breast tumor and drug resistant breast tumor viability after 24 hours 274 and 18 fold increase in breast tumor and drug resistant breast tumor apoptosis after 24 hours	-	[210]
	High Mw Drug Low Mw Drug	DOX Curcumin	Core-Shell Micro/nanoparticle	Hydrophilic Hydrophobic	(A) 90% for curcumin and 20% for Dox (B) – (C) 58% Dox release in 6 hours and more than 75% accumulated release after 2 days. Less than 5% curcumin burst release in 6 hours and more than 70% accumulated release after 1 week	1.5 and 4 fold decrease in concentration of drug needed to achieve 50% inhibition of tumor growth than DOX and curcumin only	-	[211]
	Small Molecule High Mw Drug	LY294002 DOX	Micro/nanoparticle	Hydrophobic Hydrophilic	(A) 53.2% for LY, DOX is chemically conjugated (B) 5.7% for LY (C) 40% LY and 15% Dox burst release in 10 hours. More than 70% DOX and 80% LY accumulated release after 100 hours	1.13, 1.34 and 2.85 fold decrease in oral squamous tumor CAL-27 viability after 12, 24 and 48 hours (relative to DOX only control) 1.13, 1.25 and 2.25 fold decrease in oral squamous tumor HN-6 viability after 12, 24 and 48 hours (relative to DOX only control) 11.9 and 19.3 fold increase in CAL-27 and HN-6	-	[212]

						apoptosis after 48 hours		
	Low Mw Drug High Mw Drug	EGCG PTX	Core-Shell Micro/nanoparticle	Amphiphilic Hydrophobic	(A)96% for Paclitaxel and 77% in the EGCG (B) – (C) Close to 20% EGCG burst release in an hour and 0% Ptx released in the same period. 80% EGCG and less than 40% Ptx accumulated release by 72 hours.	3.33 fold decrease in breast tumor cell viability after 48 hours 12 fold increase in breast tumor cell apoptosis (relative to PTX only control)	-	[213, 214]
	Low Mw Drug Steroid (Low Mw)	Tamoxifen Diosgenin	Multi-layer Micro/nanoparticle	Hydrophobic Hydrophobic	(A) 69% for Tamoxifen and 85% for diosgenin (B) – (C) Close to 30% Tamoxifen and negligible diosgenin release in the first 10 hours. Similar kinetic but delay in diosgenin release, with both have 90% accumulated release	Up to 4 fold decrease in breast tumor growth at optimal combinatory dosage after 48 hours	5 fold decrease in breast tumor volume after 10 days, 17.5 fold decrease after 21 days	[215]
	Low Mw Drug Low Mw Drug	Cisplatin Norcantharidin	Chemical Compound	Amphiphilic Hydrophobic	(A) – (B) – (C) 65% Cis and 15% NCTD burst release within first 5 hours. Less than 60% NCTD and more than 90% Cis release after 96 hours in acidic environment.	1.63 fold decrease in hepatic carcinoma cell viability in dual drug loaded carrier group than cisplatin only control	-	[216]
	Low Mw Drug High Mw Drug	Temozolomide Daunorubicin	Multi-layer Hydrogel	Amphiphilic Amphiphilic	(A) – (B) – (C) Peak elution of TMZ on 20th hour, with peak elution of DNR on 60th hour	-	-	[217]
Low and High Mw Drug in Bone and Cartilage Regeneration	Low Mw Drug High Mw Drug Low Mw Drug	Indomethacin Itraconazole Gentamicin	Multi-layer Array	Hydrophobic Hydrophobic Hydrophilic	(A) (B) 15.8% for Indomethacin and Itraconazole, 12.3% gentamicin (C) About 2 mg of rapid Indomethacin and Itraconazole release for 2-5 days dependent on the composition of carriers. Gentamicin release only after day 5 and complete 7.5 mg release after day 9.	-	-	[218]
Low and High Mw Drug in Periodontal Tissue Engineering	Low Mw Drug Low Mw Drug Low Mw Drug Low Mw Drug	Metronidazole Ketoprofen Doxycycline Simvastatin	Multi-layer Film	Amphiphilic Hydrophobic Hydrophobic Hydrophobic	(A) – (B) – (C) Peak release of Met, Keto, Doxy and Sim at the 10th, 38th, 74th, and 103rd hour respectively	-	-	[219]
	Low Mw Drug	Metronidazole	Multi-layer	Amphiphilic	(A) 96.5% for both (B) –	-	-	[220]

	Low Mw Drug	Ketoprofen	Scaffold	Hydrophobic	(C) Linear release of 95% metronidazole in 9 days compare to just over 60% for ketoprofen. Ketoprofen release more than 80% after 11 days.			
	Low Mw Drug Low Mw Drug	Doxycycline Simvastatin	Multi-layer Scaffold	Hydrophobic Hydrophobic	(A) 96.5% for both (B) – (C) More rapid release of more than 60% of Doxy by day 7 compare to 40% of Simvastatin. Both have almost 100% accumulated release after 13 days.	-	-	[220]
	Low Mw Drug Low Mw Drug	Metronidazole Simvastatin	Core-Shell Scaffold	Amphiphilic Hydrophobic	(A) – (B) 2% for simvastatin (C) 55% to 65% of metronidazole release in first 4 days and slow 4% to 6.7% release per day thereafter. Simvastatin only started to release after 12 days	-	-	[200]
Low and High Mw Drug in Vascular Tissue Engineering	Low Mw Drug High Mw Drug	Acetylsalicylic Acid Paclitaxel	Multi-layer Nanofiber	Hydrophilic Hydrophobic	(A) – (B) – (C) More than 55% ASA burst release in 6 days. 100% accumulated ASA release by 40 days. More sustained and slow release of 10% PTX by 18 days, 30% by 23 days and more than 50% by 60 days.	8.5 fold, 13 fold, and 22 fold decrease in platelet adhesion at day 3, 7 and 14. 1.3 fold increase in HUVEC proliferation after 3 days 1.82 fold, 1.82 fold and 1.54 fold decrease in SMC proliferation at day 14, 28 and 56.	Vasodilation in response to Ach Absence of thrombus and intimal hyperplasia 2 fold increase in number of contractile phenotype SMC	[221]
Low and High Mw Drug in Neuropathic Pain	Low Mw Drug Low Mw Drug	Lidocaine Thalidomide	Graphene	Hydrophilic Hydrophobic	(A) – (B) 55% for LDC and 120% for THD (C) About 90% of THD retained after 72 hours while only about 55% to 70% of LDC retained after the same period.	2.3 fold and 3.5 fold decrease in TNF-α expression after 12 and 24 hours	2.33 fold and 2.67 fold increase in pain threshold through mechanical allodynia at week 2 and 4 2.4 fold increase in pain threshold through hyperalgesic response 2 and 3.5 fold decrease in TNF-α expression after 2 and 4 weeks 1.6 fold decrease in nitrite level after 4 weeks	[222]
Growth Factors in Bone and Cartilage Regeneration	Growth Factor Growth Factor	bFGF BMP-2	Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) Over 70% of bFGF release as opposed to 37% of BMP-2 release after 4 weeks of incubation.	-	1.8 fold lower new bone formation after 4 weeks	[198]
	Growth Factor	bFGF	Micro/ nanofiber in Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) High initial burst release of bFGF at 82.6% and lower initial burst of 42.3% by BMP-2. No	2 fold lower MSC proliferation at day 14 and 21 2.25 fold and 1.75 fold higher MSC osteoblastic differentiation at day 14 and 21	-	[223]

	Growth Factor	BMP-2			significant bFGF detected thereafter. 86.4% of BMP-2 release by D7.	9 fold higher MSC calcium deposition after 21 days		
	Growth Factor Growth Factor	bFGF BMP-2	Core-Shell Micro/nanoparticle	Hydrophilic Hydrophilic	(A) 50% for FGF-2 and 70% for BMP-2 (B) – (C) 95% release of FGF-2 in the first week compare to less than 10% of BMP-2	17 fold higher MSC osteoblastic differentiation after 4 weeks 32 fold higher MSC calcium deposition after 4 weeks	-	[224]
	Growth Factor Growth Factor	bFGF BMP-2	Micro/nanofiber in Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) 50% and 70% bFGF release after 4 and 14 days respectively while about 40% and 50% of BMP-2 release in the same period. Alternatively, it can be tune to release 50% and 65% of BMP-2 after 4 and 14 days respectively with about 20% and 25% of bFGF release in the same period.	-	-	[225]
	Growth Factor Growth Factor	VEGF BMP-2	Core-Shell Micro/nanoparticle	Hydrophilic Hydrophilic	(A) – (B) – (C) Burst release of 35% of VEGF within first day with more than 80% accumulated release. Less than 5% burst release of BMP-2 within first day with about 70% accumulated release	-	1.67 fold, 1.78 fold and 2.33 fold higher bone volume after 2, 4 and 8 weeks. 4 fold or more vascular section after 2, 4 and 8 weeks More advanced formation of new bone, higher activity of blood vessel in-growth, increase bone matrix deposition and maturation of active bone-forming sites	[19]
	Growth Factor Growth Factor	VEGF BMP-2	Micro/nanofiber in Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) Higher initial VEGF release concentration of 750.4 pg/ml compared to BMP-2 136.9 pg/ml. Cumulative VEGF release of 2553 pg/ml compared to BMP-2 5773 pg/ml	1.75 fold higher C2C12 osteoblastic differentiation	3 fold higher bone volume after 4 weeks 2.83 fold higher trabecular number after 4 weeks 3.83 fold lower trabecular spacing after 4 weeks Extensive collagen matrix deposition, increase endochondral ossification process, new osteoid formation	[226]
	Growth Factor Growth Factor	VEGF BMP-2	Micro/nanoparticle in Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) Initial VEGF burst release of 58% in vitro and up to 89.9% in vivo with more sustained release of BMP-2 at 3.3% in vitro and up to 9.5% in vivo	-	2.14 fold higher bone volume after 8 weeks Increase new bone formation and mineralization, new osteoid formation.	[227]
	Growth Factor	PDGF	Micro/nanoparti	Hydrophilic	(A) 55.8% of VEGF (B) 0.1% of VEGF	-	11.7 fold higher bone formation	[228]

		cle in Scaffold	Hydrophilic	(C) 45% and 13% burst release, 75% and 64% accumulated of PDGF and VEGF respectively in vitro. 37% and 13% burst release, 80% and 70% accumulated of PDGF and VEGF respectively in vivo.		2 fold higher mineral apposition rate	
Growth Factor	VEGF						
Growth Factor	FGF-2	Micro/nanoparticle in Core-shell Fiber	Hydrophilic	(A) – (B) Model protein shows up to 13% loading capacity for factor encapsulated in MSN	1.43 fold, 1.18 fold and 1.21 fold higher MSC viability in day 7, 10, 14	1.67 fold higher bone volume after 6 weeks 2.1 fold higher bone surface density after 6 weeks	[229]
Growth Factor	FGF-18		Hydrophilic	(C) 7% FGF-2 model protein release within first 3 days, more than 30% release after 60 days. 5% FGF-18 model protein release within first 3 days with only about 20% accumulated release after 60 days.	1.22 fold, 1.68 fold, 1.43 fold higher MSC osteoblastic differentiation in day 7, 10 and 14 1.8 fold and 1.38 fold higher calcium deposition in day 21 and 28		
Growth Factor	TGF-β1	Micro/nanoparticle in Hydrogel	Hydrophilic	(A) – (B) – (C) Large variation in release kinetics by altering the material phase of growth factors loading.	-	-	[230]
Growth Factor	IGF-1		Hydrophilic				
Growth Factor	TGF-β1	Micro/nanoparticle in Scaffold	Hydrophilic	(A) – (B) 0.025% for IGF-1 and 0.0005% for TGF-β1 (C) 30% burst release of TGF-β1 and no release IGF-1 until after 10 days. Alternatively, release could be configure to be 30% burst release of IGF-1 with no burst release of TGF-β1. TGF-β1 release only after 7 days.	-	-	[199]
Growth Factor	IGF-1		Hydrophilic				
Growth Factor	BMP-2	Micro/nanoparticle in Hydrogel	Hydrophilic	(A) – (B) – (C) More than 15 ng/ml burst release of BMP-2 on day 1 with 35 ng/ml accumulated release by day 7. Less than 10 ng/ml burst release of IGF-1 with about 20 ng/ml accumulated release by day 7.	4.72 fold higher W-20-17 cell osteoblastic differentiation after day 7	-	[18]
Growth Factor	IGF-1		Hydrophilic				
Growth Factor	BMP-2	Multi-layer Coating	Hydrophilic	(A) – (B) – (C) Peak release of 250 ng of BMP-2 and 190 ng of IGF-1 achieve at day 1 and day 6 respectively. Accumulated release of bioactive 50% of BMP-2 and 50% to 60% IGF-1.	1.63 fold and 2.5 fold higher C3H cell osteoblastic differentiation after day 7 and day 10 2.83 fold, 1.67 fold and 1.3 fold higher bone marrow stromal cells osteoblastic differentiation after day 14, 21 and 28 3.5 fold, 1.4 fold and 2.25 fold higher bone marrow stromal cells calcium deposition at D14, D21 and D28	-	[13]
Growth Factor	IGF-1		Hydrophilic				
Growth Factor	BMP-2	Hydrogel	Hydrophilic	(A) – (B) – (C) 62% of BMP-2 release triggered by first wavelength of light, small additional 10%	4.25 fold higher hMSC osteoblastic differentiation	-	[231]
			Hydrophilic				

	Growth Factor	BMP-7			release triggered by second wavelength of light. 40% of BMP-7 release triggered by second wavelength light			
	Growth Factor Growth Factor	BMP-2 BMP-7	Micro/nanoparticle in Scaffold	Hydrophilic Hydrophilic	(A) – (B) – (C) Negligible burst release when PLGA microsphere is encapsulated inside or on fiber with close to 70% and 87% accumulated release respectively. Negligible burst release when PHBV is on the fiber with only 45% accumulated release after 25 days	Up to 2.5 fold, 2.33 fold, 2.25 fold increase in rMSC osteoblastic differentiation at day 7, 14 and 21 compared to BMP-2 only control	-	[232]
	Growth Factor Growth Factor	BMP-2 BMP-7	Micro/nanoparticle in Scaffold	Hydrophilic Hydrophilic	(A) Low entrapment efficiency between 5-10%. (B) – (C) BMP-2 releases all of its content in 10 days, 41% BMP-2 releases in 7 days.	1.32 fold lower rMSC proliferation at day 14 2.5 fold higher rMSC osteoblastic differentiation at day 21	-	[233]
Growth Factors in Angiogenesis	Growth Factor Growth Factor	VEGF PDGF	Multi-layer Micro/nanoparticle in Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) Initial burst release of about 37% VEGF after 3 days follow by accumulated release of about 83% after 40 days. PDGF release was slightly delayed with 23% burst release and accumulated release of about 53% after 40 days	-	1.71 fold higher blood vessel density at 2 weeks 2.67 fold and 1.6 fold higher matured blood vessel at 2 weeks and 6 weeks 3 fold and 2.6 fold higher cross-section area of matured blood vessel at 2 weeks and 6 weeks	[234]
	Growth Factor Growth Factor	VEGF PDGF	Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) Close to 50% release of VEGF and 10% PDGF in the first day. 80% accumulated release of VEGF and 75% accumulated release for PDGF after 30 days.	10 fold higher vascular outgrowth at day 3	1.25 fold higher blood vessel density at 4 weeks 2 fold higher matured blood vessel at 4 weeks 1.75 fold better left ventricular function at 4 weeks	[235]
	Growth Factor Growth Factor	VEGF PDGF	Micro/nanoparticle in Scaffold	Hydrophilic Hydrophilic	(A) – (B) – (C) 1.7 pmol/day of VEGF for first 7 days and close to 25 pmol accumulated release after 30 days.Release rate of PDGF from scaffolds wasvaried from 4.2 pmol/day to 0.10 pmol/day by altering the degradationrate of the polymer	-	4.5 fold and 3.59 fold higher cross-section area of blood vessel at 2 weeks and 4 weeks 2.5 fold higher matured blood vessel at 2 weeks	[236]
	Growth Factor Growth Factor	VEGF PDGF	Micro/nanoparticle in Hydrogel	Hydrophilic Hydrophilic	(A) 87% for VEGF and 97% for PDGF (B) – (C) 44% burst release of VEGF and 14% burst release of PDGF in day 1. 95% VEGF released in a week compare to 40% of PDGF, which has sustained release of up to 75% after three weeks	2.4 fold higher HUVEC proliferation after 48 hours 7 fold higher vessel sprouting area after 6 days	3.7 fold higher matured blood vessel at 4 weeks 1.4 and 1.48 fold better cardiac function recovery at 2 weeks and 4 weeks 2.03 fold thicker left ventricles wall at 4 weeks	[20]

							1.89 fold lower fibrosis at 4 weeks 3.2 fold higher neovessel formation at 4 weeks 1.82 fold higher viability of cardiac muscle at 4 weeks 4.01 fold lower inflammation at 4 weeks	
	Growth Factor Growth Factor	VEGF PDGF	Micro/nanoparticle in Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) 50% of VEGF released within 2 days, 70% by 12 days and 80% by 36 days. PDGF has 10% release within 2 days, 40% by 12 days and 70% by 36 days	-	1.88 fold and 1.67 fold higher blood vessel density at 4 weeks and 6 weeks 2.4 fold, 4 fold and 4.15 fold higher matured blood vessel at 2 weeks, 4 weeks and 6 weeks	[237]
	Growth Factor Growth Factor	VEGF PDGF	Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) Release controlled by the delivery of complementary sequences with up to 10 folds increased in release of growth factor when the right complementary sequence was delivered.	-	-	[238]
	Growth Factor Growth Factor Growth Factor	VEGF PDGF TGF-β1	Hydrogel	Hydrophilic Hydrophilic Hydrophilic	(A) – (B) – (C) Accumulated release of more than 45% of VEGF, 30% of PDGF and slightly lower than 30% of TGF-β1	-	3.86 fold and 4 fold higher blood vessel density at 1 month and 3 months 2.5 fold and 4.5 fold higher matured blood vessel at 1 month and 3 months	[15]
	Growth Factor Growth Factor	bFGF PDGF	Cellulose Fiber	Hydrophilic Hydrophilic	(A) – (B) – (C) Rapid initial bFGF release after injection. bFGF is no longer detectable following saline flushing. Rapid PDGF release after it was subsequently injected	-	2.8 fold higher blood vessel density after 7 days relative to bFGF only 14.5 fold higher matured blood vessel after 7 days relative to bFGF only 6 fold higher red blood cells recruitment after 7 days relative to bFGF only	[17]
	Growth Factor Growth Factor	VEGF HGF	Multi-layer Scaffold	Hydrophilic Hydrophilic	(A) – (B) – (C) Release is dependent on the crosslinking density of polymer and incorporation of high osmotic activity salt.	-	-	[239]
	Protein	TAT-HSP	Micro/n	Hydrophilic	(A) 70% for VEGF in microsphere	-	5.75 fold higher blood vessel density at 4 weeks	[240]

		27	anoparticle in		(B) –			
	Growth Factor	VEGF	Hydrogel	Hydrophilic	(C) Rapid complete release of TAT-HSP27 within 7 days. 55% to 90% accumulated VEGF release after 4 weeks.		11 fold higher matured blood vessels at 4 weeks Higher number of proliferating blood vessels and matured blood vessels at 4 weeks Enhanced rate of limb salvage and reduced rate of limb loss at 4 weeks Muscle protection from ischemia damage by suppressing cell apoptosis at 4 weeks Reduced muscle fibrosis at 4 weeks	
	Growth Factor	IGF-1	Hydrogel	Hydrophilic	(A) – (B) – (C) 50% burst release of IGF-1 during first 6 hours. Almost 100% of IGF-1 released by day 3. No burst release for HGF with slow and sustained release of up to 30% past day 7.	-	1.28 fold and 2 fold higher matured blood vessels at 1 week and 4 weeks 1.71 fold higher scar thickness at 4 weeks 2 fold lower infarct expansion at 4 weeks 2.5 fold and 1.38 fold lower fibrotic area in sequential group in 1 week and 4 weeks 1.67 fold higher cross-section area of matured blood vessel 1.83 fold and 2.25 fold lower apoptotic cardiac cells at 1 week and 4 weeks 2.33 fold higher cardiomyocyte proliferation at 1 week Increase incidence of GATA-4+ cell clusters	[241]
				Hydrophilic				
	Growth Factor	HGF						
Growth Factors for Stroke Treatment	Growth Factor	EGF	Micro/nanoparticle in Hydrogel	Hydrophilic	(A) 54% for EGF and 23% for EPO (B) – (C) Rapid EGF release of >75% and >60% after 4 days in vitro and in vivo respectively without EPO release. EPO release starts after 4 days with about 80% in vitro and almost 100% in vivo release after 21 days		1.25 fold higher proliferating cells in infarct zone after 18 days 1.25 fold higher number of neural precursor in infarct zone after 18 days 2 fold lower apoptotic cells in infarct zone after 18 days 2.5 fold and 7 fold lower apoptotic cells in peri-infarct zone after 18 days and 32 days	[242]
				Hydrophilic				
	Growth Factor	Erythropoietin						

							7.5 fold and 4 fold higher number of neural precursors in peri-infarct zone after 18 days and 32 days. 2.3 fold and 3.33 fold lower cavity volume after 18 days and 32 days	
Growth Factors for Neurite Outgrowth	Growth Factor Growth Factor	CNTF NT-3	Micro/nanoparticle in Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) Complete burst release of CNTF within 10 days with less than 20% NT-3 release during the same period. Sustained complete release of NT-3 after more than 60 days.	-	-	[243]
Protein/Growth Factor and Drugs for Cancer Therapy	Cytokine High Mw Drug	TNF-TRAIL Dox	Graphene	Hydrophilic Hydrophilic	(A) – (B) – (C)Furin cleavable peptide release TRAIL to act extracellularly while Dox act intracellularly as Dox-loaded graphene internalised by cells	3.52 fold higher apoptotic lung cancer cells than Dox only after 48 hours 10 fold lower lung cancer cells viability than Dox only after 48 hours	2.8 fold lower lung tumor volume after 12 days 2.17 fold lower lung tumor weight after 12 days Remission of tumor cells Elevated apoptosis of tumor cells	[244]
	Cytokine High Mw Drug	TNF-TRAIL Dox	Core-Shell Micro/nanoparticle	Hydrophilic Hydrophilic	(A) 99.5% for Dox, 82% for TNF-TRAIL (B) 5% for Dox (C) HAase dependent release of TRAIL. More than 50% TRAIL release within 1 hour in the present of HAase. Dox released after internalization of carrier by cells.	2.09 fold high apoptotic breast cancer cells than Dox only after 48 hours 7 fold lower breast cancer cells viability than Dox only after 48 hours	4.5 fold lower breast tumor volume after 14 days Remission of tumor cells Elevated apoptosis of tumor cells	[245]
Protein/Growth Factor and Drugs for Periodontal Tissue Engineering	Growth Factor Low Mw Drug	PDGF Simvastatin	Core-Shell Micro/nanoparticle	Hydrophobic Hydrophilic	(A) 60% for PDGF and 85% for Simvastatin (B) – (C) Close to 100% PDGF and 40% simvastatin released by 15 days	-	4.6 fold and 2.25 fold higher bone volume at 2 weeks and 4 weeks 1.6 fold and 1.6 fold higher mineral density at 2 weeks and 4 weeks 1.43 fold and 1.4 fold higher trabecular thickness at 2 weeks and 4 weeks 4.25 fold and 1.63 fold higher trabecular number at 2 weeks and 4 weeks 2 fold and 2.38 fold higher defect fill at 2 weeks and 4 weeks 1.33 fold higher osteoblast number at 2 weeks 1.8 fold and 1.5 fold higher TRAP+ cells number at 2 weeks and 4 weeks	[246]

Protein/Growth Factor and Drugs for Vascular Tissue Engineering	Growth Factor High Mw Drug	VEGF Ptx	Micro/nanoparticle in Hydrogel	Hydrophilic Hydrophobic	(A) 50% for PTX and 97% for VEGF (B) 28.6% for PTX and less than 0.1% for VEGF (C) 95% of VEGF released in first 3 days and sustain release of PTX for more than 10 days	Similar level of endothelial cells proliferation in control and VEGF+Ptx from day 1 to 3 1.57 fold, 1.83 fold, 1.71 fold and 4 fold lower SMC viability after 5, 7, 9 and 11 days	-	[247]
Protein/Growth Factor and Drugs for Bone and Cartilage Regeneration	Low Mw Drug Growth Factor Growth Factor	Gentamicin IGF-1 BMP-2	Multi-layer Coating	Hydrophilic Hydrophilic Hydrophilic	(A) – (B) – (C) 38% burst release of gentamicin within first 3 days and plateau at 41%; 26% of IGF-1 released after 3 days followed by sustained accumulated release of 47% at 5th week before plateau; 1% release in 1 day, 3% after 1 week and 6% after 8 weeks for BMP-2.	2.5 fold higher osteogenic differentiation of myoblast cells at 2 weeks 1.3 fold higher osteogenic differentiation of osteoblast-like cells at 2 weeks	-	[248]
Protein/Growth Factor and Drugs for Angiogenesis	Growth Factor Low Mw Drug	VEGF S1P	Cellulose Fiber	Hydrophilic Hydrophobic	(A) – (B) – (C) Rapid initial release of VEGF. VEGF no longer detectable following saline flushing. Rapid S1P release thereafter	-	1.75 fold higher blood vessel density at 1 week than VEGF only 5.42 fold higher matured blood vessel at 1 week than VEGF only	[16]
Protein/Growth Factor and Drugs for Wound Healing	High Mw Drug/Low Mw Drug Growth Factor	PT EGF	Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) Accumulated complete 95% antibiotic released in 12 hours. Sustained EGF release of more than 80% at 250th hour.	-	-	[249]
	Low Mw Drug Growth Factor	Vancomysin VEGF	Micro/nanoparticle in Hydrogel	Hydrophilic Hydrophobic	(A) - (B) 69.8%-73.4% for VEGF dependent on BSA ratio (C) 30%-45% of VEGF and 50%-75% of vancomysin over 7 days dependent on BSA ratio and pH	7.5-fold and 15-fold lower bacteria count than hydrogel and PBS control respectively 1.3-fold higher HUVEC proliferation	6-fold lower wound area after 2 weeks with significantly higher tissue thickness 2.67-fold and 4.5-fold higher Ang-1 and bFGF mRNA expression	[250]
Protein/Growth Factor and Drugs for Biosensor	Low Mw Drug Growth Factor	Dx VEGF	Hydrogel-coated Capillary	Hydrophobic Hydrophilic	(A) – (B) – (C) Both Dex and VEGF follows a first order release kinetics with more rapid Dex release with a release rate constant of 0.5165 then VEGF of 0.3872	-	1.33 fold lower inflammatory cell density after 8 days 1.57 fold higher matured vessel density after 8 days 2 fold higher therapeutic index	[251]
Nucleic Acid/Plasmid and Drugs for Cancer Therapy	Genetic Material High Mw Drug	pCMV-p53 plasmid DNA Dox	Core-Shell Micro/nanoparticle	Hydrophilic Hydrophilic	(A) 32% to 47% Dox encapsulation and 25% to 37% p53 plasmid encapsulation (B) – (C) 2 to 29% burst release of Dox before entering a lag phase of 25 days. Continuous release of Dox of up to 100% after 120 days. Up to 13% quick release of p53 in the first	-	-	[252]

					week and 20% to 35% p53 release after 120 days.			
	Genetic Material High Mw Drug	Bcl-2 siRNA Dox	Graphene	Hydrophilic Hydrophilic	(A) – (B) – (C) –	2.48 fold higher cervival tumor cell cytotoxicity compared to scrambled siRNA in the presence of Dox	-	[253]
	Genetic Material High Mw Drug Low Mw Drug	Bcl-2 siRNA Dox HCPT	Core-Shell Micro/nanoparticle	Hydrophilic Hydrophilic Hydrophobic	(A) – (B) – (C)siRNA was released after endocytosis. In cytoplasm, more than 70% Dox, less than 20% HCPT release in first 10 hours, More than 90% Dox and 60% HCPT accumulated release after 2 days.	-	400 fold lower colon tumor weight at 4 weeks	[254]
	Low Mw Drug Genetic Material	Temozolomide miR-218	Micro/nanoparticle in Hydrogel	Amphiphilic Hydrophilic	(A) – (B) – (C) 60% temozolomide release within first day with close to 100% accumulated release within 2 days. miR-218 mimics was released by place exchange reaction in tumor cells.	1.5 fold higher glioma tumor cell cytotoxicity compared to absence of miR in the present Temozolomide Dosage dependent cytotoxicity of Temozolomide in the presence of miR	40 fold lower glioma tumor weight at 4 weeks	[255]
Cytokines for Bone and Cartilage Regeneration	Cytokine Cytokine	Interferon Gamma Interleukin-4	Decellularised Bone Scaffold	Hydrophilic Hydrophilic	(A) 375 ng IL4 and 325 ng IFNg but only 26.9 ng of IFNg and 153.3 ng of IL4 loaded (B) (C) Less than 1 ng of IFNg release in first 48 hours and less than 8 ng of IL4 release over 6 days	-	4 fold higher blood vessel density at 2 weeks	[256]
Ion and Growth Factor for Bone and Cartilage Regeneration	Ion Growth Factor	Co^{2+} BMP-2	Core-shell Hydrogel	Hydrophilic Hydrophilic	(A) – (B) – (C) Fast complete release of Co2+ within a week while only 21% of BMP-2 was released after 21 days without showing any initial burst effect	2.67 fold, 3.5 fold, 2.78 fold higher CD31, VEGF, HIF 1α gene expression at D6 3.5 fold, 1.92 fold, 1.92 fold, 1.48 fold higher OCN, OPN, BSP, ALP gene expression at D7 2 fold, 3 fold, 1.7 fold, 1.79 fold higher OCN, OPN, BSP, ALP gene expression at D14	2.3 fold higher bone volume 2.08 fold higher bone surface 2.21 fold higher bone surface density Highly calcified neo-formed tissues	[257]
Model Genetic Material	Genetic Material Genetic Material	pEGFP-N1 pDsRed-N1	Multi-layer Film	Hydrophilic Hydrophilic	(A) – (B) – (C)Plot of solution absorbance as a function of time is consistentwith a two-stage, sequential release of two plasmids	-	-	[258]
	Genetic Material Genetic Material	$dsDNA_{TM61}$ $dsDNA_{TM80}$	Micro/nanoparticle	Hydrophilic Hydrophilic	(A) – (B) – (C) More than 30% of ssDNA release from dsDNA with lower melting point after 1 min of exposure to near-IR laser light with no ssDNA release from dsDNA with higher melting	-	-	[259]

					point. 60% of $ssDNA_{TM61}$ and less than 40% of $ssDNA_{TM80}$ release after 5 mins of exposure, 65% of $ssDNA_{TM61}$ and 55% of $ssDNA_{TM80}$ after 10 mins of exposure.			
Model Drugs	Low Mw Drug Low Mw Drug	Benzyl Alcohol 3-phenylpropionoic Acid	Dendrimers	Amphiphilic Hydrophilic	(A) – (B) 5.6% to 11.6% for BA, 8.1% to 15.7% for PPA (C) Consistently faster BA release than PPA. 15% to 70% release of BA by 0.25 hour, 40% to 80% of BA by 1 hour, more than 80% BA after 4 hours. 10% to 40% PPA by 0.25 hour, 25% to 60% of PPA by 1 hour, 55% to 80% PPA after 4 hours, more than 80% PPA by 72nd hour.	-	-	[260]
	Low Mw Drug Low Mw Drug	Gentamicin Naproxen	Micro/nanoparticle-Bioactive Glass Composite	Hydrophilic Hydrophobic	(A) – (B) – (C) At acidic pH, GS completely release in 2 hours while only 17% Nap was released. When pH increased to alkaline condition, 55% Nap release within 2 days and 72% after 10 days.	-	-	[261]
	Low Mw Drug High Mw Drug	Aspirin Doxorubicin	Micro/nanoparticle in Hydrogel	Hydrophilic Hydrophobic	(A) – (B) – (C) 100% burst release of aspirin within 12 hours in all cases. Vary amount of sustain Dox release for 7 days dependent on pH and temperature, ranging from 20% to more than 60% accumulated release after 168 hours.	-	-	[262]
Model Protein	Model Protein Model Protein	Myoglobin Lysozyme	Multi-layer Coating	Hydrophilic Hydrophilic	(A) – (B) – (C) Huge variation in the release of model protein dependent on the PEG MW and PEGT to PBT ratio of the copolymer.	-	-	[263]
	Model Protein Model Protein	TexR-BSA FITC-BSA	Micro/nanoparticle in Hydrogel	Hydrophilic Hydrophilic	(A) Ranging from 22 .0% to 44.6% (B) – (C) 54.2% TexR-BSA and 17.9% FITC-BSA release over first 3 weeks. More than 90% TexR-BSA and 50% FITC-BSA accumulated release after 6 months	-	-	[264]
	Model Protein Model Protein Model	Fluorescein-Ova TexR-Ova Alexa Fluor 555-	Multi-layer Films	Hydrophilic Hydrophilic Hydrophilic	(A) – (B) – (C) Complete accumulated release of ova-FL within hours, complete accumulated release of ova-TR was delayed till the 280th hour while complete accumulated release of ova-AF55 was delayed till 800th hour.	-	-	[265]

	Protein	Ova						
	Dye	Chromazurol B	Multi-layer	Hydrophilic	(A) – (B) –	-	-	[266]
	Dye	TPPS	Micro/nanofiber	Hydrophilic	(C) Sustained release of ChroB lasted for 1.5 hours at the rate of 1.1 µg/cm^2/h without TPPS leakage. 30 mins of release suppression follows due to barrier mesh and followed by release of TPPS for 3 hours at the rate of 0.36 µg/cm^2/h.			

Table 7: SCI Therapeutics

Events	Therapeutics	Chemical Natures	Hydrophilicity
Neuroprotection	Methylprednisolone	Low Mw Drug	Hydrophilic
	Minocycline	Low Mw Drug	Amphiphilic
	iNOS Antisense Oligodeoxynucleotides	Nuclei Acid	Hydrophilic
	Intravenous Immunoglobulin	Protein	Vary
Neuroregeneration	Chondroitinase ABC	Protein	Hydrophilic
	Neurotrophin-3	Protein	Hydrophilic
	Basic Fibroblast Growth Factor	Protein	Hydrophilic
	Nerve Growth Factor	Protein	Hydrophilic
	Brain-derived Neurotrophic Factor	Protein	Hydrophilic
	Glial cells-derived Neurotrophic Factor	Protein	Hydrophilic
	Nerve Growth Factor	Protein	Hydrophilic
	Ciliary Neurotrophic Factor	Protein	Hydrophilic
Remyelination	Platelet-derived Growth Factor	Protein	Hydrophilic
	Insulin-like Growth Factor	Protein	Hydrophilic
	Type II Neuregulin-1	Protein	Hydrophilic
	Epidermal Growth Factor	Protein	Hydrophilic
	Γ-secretase Inhibitor	Low Mw Drug	Hydrophobic
	Anti-Lingo-1 Antibody	Protein	Hydrophilic
	miR-219	Nuclei Acid	Hydrophilic
	miR-338	Nuclei Acid	Hydrophilic

3.2 Sequential Delivery as Controlled by Intrinsic Material Property of Scaffolds

The use of only one material to achieve sequential drug delivery typically involves the passive diffusion of drugs through porous and non-porous materials. Sequential release systems built on the passive diffusion mechanism mainly rely on three modes of action: 1) difference in binding affinity between the drugs and the material; 2) variations in diffusion speed of the drugs from the material; and 3) by controlling the degradation time of the biomaterials.

3.2.1 Differential drug-material binding affinity

Presence of heparin binding domain in growth factors could be cleverly manipulated to allow sequential delivery with simple surface adsorption. Specifically, alginate-sulfate is one of the most commonly described material for controlling growth factors release, since alginate-sulfate could have high specificity binding to heparin binding proteins via the sulfate group [267]. This

specific interaction between the growth factors and materials determines the equilibrium binding constants (K_a), which in turn affects drug release pattern. Together with total molecular concentration in the system, the two factors could be coupled to realize sequential release. For example, vascular endothelial growth factor (VEGF), platelet-derived growth factor-BB (PDGF-BB) and transforming growth factor-β1 (TGF-β1) each possesses a different binding constant to alginate (K_a VEGF: 6.98 x 10^6 M^{-1}, K_a PDGF-BB: 3.53 x 10^7 M^{-1} and K_a TGF- β1: 6.63 x 10^7 M^{-1}). As a result, they have different release profiles from the material, with VEGF being the most rapidly released [15]. In addition, the drug release rate can be enhanced by increasing the quantity of the drug [241]. This was illustrated by using alginate to deliver IGF-1 (K_a = 1.01 x 10^8 M^{-1}) at a molar concentration that was 10-fold higher than hepatocyte growth factor (HGF) (K_a = 5.36 x 10^7 M^{-1}). Despite higher binding efficiency, 95% of IGF-1 was release within 6 hours. Concurrently, no burst release was seen for HGF and a sustained release was achieved for up to 7 days (25 % of release). In addition, the bioconjugation of growth factors with alginate-sulfate protects the growth factors from enzymatic proteolysis [15]. This is crucial after SCI due to the presence of a variety of proteases after trauma [268]. As such, the intrinsic affinity of the growth factors and materials should be examined, and the release of neurotrophins and OPC mitogens could be tuned by varying their bulk concentration in the material.

3.2.2 Differential diffusion speed of drugs

The diffusion coefficient of a drug could modulate its release kinetics. While biomolecule diffusion rates can be controlled by the geometric shape, nature of materials and porosity of the delivery system [269], a huge determining factor is drug hydrophilicity. As compared to hydrophobic drug, hydrophilic drugs entrapped in a material has higher diffusion coefficient as they diffuse much faster from the DDS into the water-based biological environment [221]. Most efficacious

biomolecules in treating SCI are predominantly hydrophilic. Thus, material degradation design becomes more important as discuss in the next section.

3.2.3 Degradation Rate of Material

Controlling the degradation rate of a material is a key factor for adjusting the release of poorly diffusible hydrophobic drugs or higher Mw molecules that are entrapped within porous or non-porous materials. As a large variety of drugs will be needed for the complex SCI treatment, materials with different degradation rates could be utilized to achieve distinct drug release profiles. For instance, neurotrophins, OPC mitogens or other growth factors are significantly larger in size than other drugs such as MP and minocycline. Thus, delaying the release of small, late stage biomolecules through a reduction in material degradation rate could be a useful approach.

One approach to control the degradation rate of DDS with the aim to achieve sequential drug release is through altering the degree of material crosslinking [198, 230, 270]. Increase in crosslinking density increases the resistance of material to hydrolysis and thus, decreases the material degradation time and ultimately the drug release kinetics [271]. This has been shown to be effective in growth factors delivery with gelatin particles [198, 230]. However, the chemical groups that are involved in the crosslinking reactions could be the same as those involved in the binding of the drugs with the DDS. Consequently, drug loading efficiency may be diminished due to reduced drug-material interactions as the crosslinking extent increased [230]. Thus, with the inevitable needs of using drug-laden hydrogels to fill the voids left in the tissue after SCI, the loading efficiency of growth factors should be monitored before implantation or injection into injury site.

Another approach to alter drug release profiles using the degradation mechanism is to graft a drug on a material with hydrolysable bonds. Some studies describe the modification of drugs using carbamate linkers, which have been employed to conjugate growth factors [272]; or ester bonds to control their release kinetics, which are suitable for drugs like MP or minocycline due to the presence of hydroxyl groups [203, 273-275]. While not employed in sequential delivery, grafting is also possible with RNA [276]. In general, the covalent conjugation approach is versatile as most synthetic polymers, like poly-lactic acid, can be chemically modified with the necessary functional groups [203]. Moreover, this approach would significantly delay the release of conjugated molecules. However, chemical grafting may alter the bioactivity of drugs. As such, the bioactivity of the grafted molecules should be examined thoroughly [277].

3.3 Sequential Delivery as Controlled by Physical Entrapment

Besides relying on the intrinsic material properties, having a good control over the physical engineering design of the DDS further allows more precise control over drug release rates. The multifaceted process of nerve regeneration and remyelination would likely benefit from the use of multi-layered scaffolds or micro-/nano- particles-incorporated composite systems for a more carefully orchestrated drug delivery.

3.3.1 Layer-by-Layer (L-b-L)

L-b-L is often the most intuitive and common approach to achieve sequential drug delivery. The first layer of material often acts as a barrier for the diffusion of drugs from the second layer, allowing a sequential delivery profile. A precise sequential release can be obtained by the addition of a blank sacrificial barrier layer or a crosslinked barrier layer with the purpose of limiting the diffusion of drugs and creating a controlled lag time of release. In SCI sequential delivery, it could be the most

straightforward way to delay the release of a couple of therapeutics, especially those that are only required at remyelination late in SCI.

L-b-L architecture has helped to sequentially deliver growth factors [13, 239, 248, 270, 278, 279], low Mw drugs [280-282] and oligonucleotides [283]. Surprisingly, this physical entrapment was shown highly effective in delaying the release of small molecules (low mw drugs and oligonucleotides). Specifically, L-b-L has a 120 times delay in oligonucleotides delivery [283]. Thus, it is not inconceivable that a delayed delivery of miRs for remyelination after SCI could be attained by similar techniques. However, drug loading using the L-b-L approach could possibly reduce loading efficiency [270] and bioactivity [239] due to the crosslinking process. Nonetheless, it could be a highly versatile platform for sequential delivery in SCI, as 1) the distinct layers could be engineered independently to tailor the desired release profile of individual biomolecules; 2) a wide range of synthetic [239, 270, 283] and natural polymers [282] could be engineered into the system.

While layer-by-layer bulk scaffolds are commonly employed in sequential delivery, little has been explored in layer-by-layer nanoparticles. Such system can be obtained by multiple depositions on a solid spherical core or using material with self-assembly properties [284]. This design could be interesting as each nanoparticles could be engineered differently in its payload, dimension, degradation, etc.. Thus, every nanoparticle involved could be a distinct sequential delivery system that is designed to target different cellular populations [285]. Since CNS encompasses close interactions between neurons and the glia, these nanoparticles could be designed to sequentially target cell-specific responses following traumatic injuries.

3.3.2 Core-shell Fibers

DDS with core and shell structure physically entrap biomolecules in the core to achieve sequential drug delivery. This physical entrapment is similar to the layer-by-layer approach, with the exception that the multi-layer is now at the microscale of each individual fiber, which is then assembled to form a macroscale DDS. Co-axial electrospinning and emulsion electrospinning are commonly used processes to produce such core-shell fibers [286] from both synthetic and natural polymers. Interestingly, the method of making these fibers influences the release profile of the drugs [287]. Unfortunately, only few studies have analyzed sequential delivery with core-shell fibers in delivering growth factors [229, 288] and low mw drugs [205].

Although simple compartmentalization of one drug/gene in the shell and the other in the core could achieve sequential delivery [205, 257], different methods were still developed to enhance the differences between the release profiles of the two drugs. These include complexing the late stage drug to an intermediate system, such as particles, to slow the release rate [229]; or complexing the first drug with a hydrophilic carrier, such as cyclodextrin, to accelerate the first burst release [205]. The first strategy could slow the release of neurotrophins while the second strategy could accelerate the release of neuroprotectants to injured tissues. Together, this system could be useful in delaying neurotrophins and regenerative factors delivery following neuroprotection to achieve the desired temporal delivery.

3.3.3 Core-shell Particle

Common synthetic polymers and to a lesser extent, liposomes, have been employed to engineer core-shell particles for sequential delivery purposes [213, 288, 289]. Similar to core-shell fibers, compartmentalization of drugs in the core and in the shell creates a time gap between release of drugs that have been loaded in the core and shell due to physical entrapment. In core-shell

particles, however, the time gap could be enhanced or reduced significantly depending on the hydrophilicity of the drugs and the materials used. For instance, hydrophobic drug, MW167, in a hydrophobic PLGA core is less mobile. Hence, the burst release would be minimal. This would allow late NOTCH inhibition for OPC differentiation. On the contrary, loading hydrophilic drugs, such as MP, in the hydrophobic PLGA shell is ineffective in entrapping the biomolecules [208, 213]. Thus, this drug-material combination could be used to release drugs more rapidly for neuroprotection.

Both particulate carriers and fibrous structure could physically entrap biomolecules to accomplish the desired drug release profiles. In nerve regeneration applications, the two classes of engineering tools have their distinct edge over another. For one, drug-encapsulated nanoparticles could be paired with injectable hydrogels for minimally invasive drug delivery to the delicate CNS [290]. On the other hand, drug-encapsulated nanofibers coupled with hydrogels would require surgical implantation. However, fibers could provide important topographical cues to guide nerve regeneration [109], which may be crucial to re-establish nerve connectivity. Thus, in addition to drug candidates and materials selections, the form which the materials take would require careful consideration for sequential delivery in neural tissues.

3.3.4 Particles-encapsulated Hydrogel

Hydrogels represent a major platform employed in drug delivery due to their biomimicking characteristics. These three dimensional matrices that consist of chemically or physically crosslinked hydrophilic polymer chains are able to hold large quantities of water. Furthermore, hydrogels can be formulated in a variety of physical forms, including slabs, microparticles, nanoparticles, coatings, and films [291]. A variety of polymeric hydrogels have been explored in neural tissue engineering, including collagen, hyaluronic acids, fibrin, etc. [8]. These hydrogels could be tailored to mimic the

extracellular matrixes of the CNS environment, simulate stiffness of the tissues and laden with cells or drugs. The combination of hydrogels with drug-loaded particles is one of the most commonly used systems to achieve sequential drug delivery in many fields of tissue engineering to deliver growth factors [230, 234, 237, 240, 292]. It could be helpful to sequentially deliver neurotrophins and OPC mitogens should that be required.

Hydrophilic molecules, like the neurotrophins, would release rapidly from hydrogels due to the highly porous structure of hydrogels. Similarly, encapsulating low Mw drugs in particles is ineffective in delaying their release from particle-hydrogel system as the high diffusion rate of small drugs through the hydrogel significantly reduced the lag time created by the encapsulation. Thus, encapsulating the second molecule within particles would not guarantee a delayed release. For instance, hydrophilic microparticles in hydrogels would have parallel release for two hydrophilic molecules residing in hydrogels and particles respectively [230]. Only hydrophobic carriers could delay diffusion of the growth factor within the particles to achieve sequential release. In this respect, PLGA microspheres loaded within alginate gel is a well-established system to achieve the desired temporal profile [234, 237, 240]. The release could be further modulated by inducing porous structures in the PLGA carrier, which accelerate drug release from PLGA by 2.4-fold [292]. Alternatively, crosslinking of gelatin particles, as mentioned above, could also be employed to delay the release of the second drug by up to 4.5 times in a particles-in-gel system [230]. Last but not least, if the drugs to be delivered are hydrophobic (Eg. MW167), one could allow hydrophobic macro-domain of an amphiphilic material to interact with hydrophobic drugs and slow drug diffusion through the hydrogel [293].

3.4 Sequential Delivery as Controlled by the Environment

Stimuli-responsive materials release their payloads in response to triggers such as changes in pH [261, 294], temperature [295, 296], mechanical forces, biomolecules (glucose, enzyme), ionic strength [297] and electromagnetic (light, ultra-sound [298] or electric field [299]). Such materials can achieve sequential delivery independently when built into a delivery system like nanoparticles or when built into an intermediate system like particles in hydrogel.

3.4.1 pH-Responsive Delivery

Although not widely documented and well explored, SCI could be accompanied by extracellular acidosis, which could in turn elicit strong GFAP expression in activated astrocytes [300]. pH changes that come along with acidosis could be an environment factor that trigger drug release. Specifically, pH responsive materials elicit changes in configurations, solubility and/or drug binding efficiency in local acidic environments to alter drug release behavior. Sequential delivery can be realized by combining pH sensitive materials such as glutamic propylene oxide-based copolymer, with an inert counterpart, such as poly(vinyl alcohol) [262]. This system accelerates the release of molecules from pH-sensitive materials in low pH environment by 1.5-fold while molecules from the inert material are released through simple diffusion [262]. Other pH-responsive materials that could be built into sequential delivery systems in response to spinal cord acidosis includes chitosan microcapsules [301], poly(propylene oxide)-based micelles [247], graphene oxide nanoparticles [302] and DNA cage [303].

Often, acidic pH responsive materials could be paired with other stimulus as the distinctive triggers can activate the release of one drug independent of another to achieve more precise release. For instance, the combination of acid pH-sensitive and basic pH-sensitive particles (Eg Ca-alginate) that accelerate drug release at a distinct range of pH can help exert further control over the rates of

drug delivery [247]. Alternatively, photothermal sensitive gold nanovesicles could be used to release encapsulated drugs and pH-responsive particles upon irradiation [302], which the free drugs would be release quickly before the degradation of pH-responsive particles.

3.4.2 Redox-Responsive Delivery

Increase in the oxidative stresses in the CNS following traumatic injury due to a surge in concentration of free radicals is long established [304]. This change in environment could be cleverly manipulated to deliver drugs via the cleavage of reducible or electrostatic bonds [305], or by desorption of drugs [255]. For instance, disulfide-poly(amido amine)s are unstable in a highly reductive environment. Thus, sandwiching a non-reducible layer of material between the bioreducible layers allows the sequential release of DNA molecules such as miRs that are encapsulated in two different layers (Zou, 2014 #77}). Otherwise, miRs could be adsorbed onto gold particles, and subsequently desorb in respond to the increase in oxidative stress [255]. This could be paired with non-responsive materials to achieve temporal release profile.

3.4.3 Electromagnetic-triggered Delivery

Electromagnetic triggers could be another powerful tool to externally control the release of therapeutics, not limited to any specific field of tissue engineering. For instance, Hu *et al.* incorporated layer-by-layer design into their photo-dependent smart release system for effective sequential delivery [306]. Silica microspheres coated with poly(ethylenimine) were loaded in layers of a photolabile polycations multilayer system. The system contained different photo-cleavable groups in four layers which were sensitive to specific wavelengths or irradiation doses. The first layer was charged with dialkylaminocoumarin group (sensitive to wavelengths < 450 nm), the second and third layers were loaded with two different nitrobenzyl groups (sensitive to wavelengths < 400 nm at

a dose of 6 or 15 mW/cm2 respectively) and the last layer contained methoxyphenacyl group (sensitive to wavelengths < 320 nm). Consequently, following the irradiation dose and the wavelengths used to irradiate the system, Rhodamine-labeled poly(ethylenimine) was 100% released from each layer within 25 minutes of irradiation.

3.5 Sequential Delivery Method for Neural Tissue Engineering

Current single drug delivery systems for SCI have seen promising but limited success in achieving robust regeneration. Universally, hydrogels were employed to fill the cysts at injury sites and act as a carrier or reservoir for drugs [307]. The incorporation of nanofibers into a drug delivering composite system enabled topographical guidance of regenerated axons, which could be enhanced by miRs [109, 110, 308]. Crucially, multiple drug delivery could significantly enhance axonal regrowth following complete spinal cord lesions [12]. However, desired temporal profile could not be achieved without multiple surgeries or injections [12]. Thus, an amalgamation of knowledge in sequential delivery technique and possible drug targets at sequential stages of nerve regeneration and remyelination could hold promises in achieving robust neural tissue regeneration and functional recovery.

Firstly, MP has to be release quickly and localised delivery could be crucial to reduce the dosage needed for effective neuroprotection. Thus, materials and technique that would allow rapid dissolution in water or with low drug/gene binding affinity are needed. Hydrogels from various polymers that were described above are excellent candidates for such purpose. Subsequent nerve regeneration would likely require slight delay in the release of growth factors. This slight delay could be achieved with simple diffusion kinetics due to the significant differences between the Mw of MP and growth factors. Otherwise, it could be achieved by encapsulation in particles or fibers, and be

loaded into the hydrogel. Last but not least, remyelination drugs would be required to remyelinate the regenerated nerve fibers. A multi-layer particles/fibers with sacrificial barrier layers or smart release systems could be needed to significantly delay their release, especially in the case of low Mw drugs/genes.

4 Pitting Sequential Drugs/Genes Delivery against Cellular Transplantation

Cellular transplantation therapy dominates the current SCI research with the help of scaffolds to provide a hospitable environment for cellular grafts. In particular, Tsuzynski lab has championed the implantation of neural stem cell grafts with fibrin scaffolds and a cocktail of growth factors for SCI treatment [103]. The combinatory approach could tackle diverse inhibitory pathways to promote regeneration [309]. Their lab has recently published promising results in non-human primate [310]. Although how transplantation repairs the spinal cord is largely unknown, present evidences suggest that transplanted cells promote functional recovery through multiple modes of actions (see review [3]). These actions include both neuroprotection and neuroregeneration that are related to the secretion of trophic factors by implanted cells [311]. However, transplanted cells have dynamic responses to cues in the lesion microenvironment [3]. Thus, sequential delivery of trophic factors or drugs could help define the exposure of CNS to a concentration of critical protective and regenerative factors, to have better control over patients' responses to therapy.

5 Summary and Future Outlook

While signaling cascades and sequential DDS application were discussed in the context of SCI, many of these would be applicable to traumatic brain injuries and other nerve injuries. These also highlight the deficiency of our biological understanding of the sequence of events for remyelination following trauma. Demyelinating diseases have contributed immensely to our knowledge in this

complex process. However, recent evidence suggests that Schwann cells of CNS origin, are likely responsible for the limited remyelination following traumatic injury. This supports the notion that remyelination following CNS trauma could differ from their demyelinating disease counterparts.

In the recent decades, sequential DDS has made considerable impact in some fields of tissue engineering. Thus, the current theme issue has summarized important work in this aspect. However, the impact of DDS is far from being felt in neural tissue engineering. Particularly after traumatic injuries, where extensive bleeding and inflammation are likely, fouling or scarring could be a huge problem for sequential DDS to operate in neural tissues. Fouling and scarring alter the microenvironment that surrounds the drug delivery scaffolds, which may in turn reshape the release kinetics of drugs. This is especially important as the current DDS design is highly dependent on *in vitro* release profiles rather than release in the biological environment that follows trauma. Thus, design of a DDS should have anti-fouling or anti-scarring properties. This could be achieved by either loading another drug onto the scaffold or the employment of anti-fouling materials.

Last but not least, the efficacy of DDS in traumatic injuries, particularly at extended time periods, is unclear. Chronic stages of SCI see extensive secondary injury-induced cyst formation at both lesion epicenter and perilesional areas, with cavity that increases in size over the duration of injury. Although many works cited the decrease in cavity sizes as an indication of recovery, only limited studies have look well into chronic phases of SCI (12 weeks post trauma), which by our experience, has significantly more severe cyst formation than early chronic injury (4 weeks) (unpublished observations). Moreover, implanted DDS faces varying degrees of scaffold degradation following extended time points in their host, depending on the materials that are employed in the DDS. Scaffold degradation poses a final risk to host due to the possible toxic by-products. Thus, while

cleverly designed DDS could achieve sequential delivery *in vivo*, extended time point studies are needed to ascertain their effect on cyst formation and scaffold degradation. Currently, end-point studies are commonly used in SCI, which could be costly and inefficient in looking at aforementioned factors. Amalgamating *in vivo* imaging of spinal cord [35], with DDS that allow *in vivo* photonic imaging, such as fluorescence probes, could be useful moving forward.

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References

[1] C.E. Schmidt, J.B. Leach, Neural tissue engineering: Strategies for repair and regeneration, Annual Review of Biomedical Engineering, 5 (2003) 293-347.
[2] S. Thuret, L.D. Moon, F.H. Gage, Therapeutic interventions after spinal cord injury, Nat Rev Neurosci, 7 (2006) 628-643.
[3] P. Assinck, G.J. Duncan, B.J. Hilton, J.R. Plemel, W. Tetzlaff, Cell transplantation therapy for spinal cord injury, Nat Neurosci, 20 (2017) 637-647.
[4] S.D. Putney, P.A. Burke, Improving protein therapeutics with sustained-release formulations, Nature Biotechnology, 16 (1998) 153-157.
[5] D.W. Hutmacher, Scaffolds in tissue engineering bone and cartilage, Biomaterials, 21 (2000) 2529-2543.
[6] J.L. Drury, D.J. Mooney, Hydrogels for tissue engineering: scaffold design variables and applications, Biomaterials, 24 (2003) 4337-4351.
[7] E.S. Place, J.H. George, C.K. Williams, M.M. Stevens, Synthetic polymer scaffolds for tissue engineering, Chemical Society Reviews, 38 (2009) 1139-1151.
[8] S.M. Willerth, S.E. Sakiyama-Elbert, Approaches to neural tissue engineering using scaffolds for drug delivery, Adv Drug Deliv Rev, 59 (2007) 325-338.
[9] P.J. Horner, F.H. Gage, Regenerating the damaged central nervous system, Nature, 407 (2000) 963-970.

[10] W.J. Alilain, K.P. Horn, H. Hu, T.E. Dick, J. Silver, Functional regeneration of respiratory pathways after spinal cord injury, Nature, 475 (2011) 196-200.
[11] G. Garcia-Alias, H.A. Petrosyan, L. Schnell, P.J. Horner, W.J. Bowers, L.M. Mendell, J.W. Fawcett, V.L. Arvanian, Chondroitinase ABC combined with neurotrophin NT-3 secretion and NR2D expression promotes axonal plasticity and functional recovery in rats with lateral hemisection of the spinal cord, J Neurosci, 31 (2011) 17788-17799.
[12] M.A. Anderson, T.M. O'Shea, J.E. Burda, Y. Ao, S.L. Barlatey, A.M. Bernstein, J.H. Kim, N.D. James, A. Rogers, B. Kato, A.L. Wollenberg, R. Kawaguchi, G. Coppola, C. Wang, T.J. Deming, Z. He, G. Courtine, M.V. Sofroniew, Required growth facilitators propel axon regeneration across complete spinal cord injury, Nature, 561 (2018) 396-400.
[13] A.T. Raiche, D.A. Puleo, In vitro effects of combined and sequential delivery of two bone growth factors, Biomaterials, 25 (2004) 677-685.
[14] E.S. Place, N.D. Evans, M.M. Stevens, Complexity in biomaterials for tissue engineering, Nature Materials, 8 (2009) 457-470.
[15] I. Freeman, S. Cohen, The influence of the sequential delivery of angiogenic factors from affinity-binding alginate scaffolds on vascularization, Biomaterials, 30 (2009) 2122-2131.
[16] J.E. Tengood, K.M. Kovach, P.E. Vescovi, A.J. Russell, S.R. Little, Sequential delivery of vascular endothelial growth factor and sphingosine 1-phosphate for angiogenesis, Biomaterials, 31 (2010) 7805-7812.
[17] J.E. Tengood, R. Ridenour, R. Brodsky, A.J. Russell, S.R. Little, Sequential delivery of basic fibroblast growth factor and platelet-derived growth factor for angiogenesis, Tissue Eng Part A, 17 (2011) 1181-1189.
[18] S. Kim, Y. Kang, C.A. Krueger, M. Sen, J.B. Holcomb, D. Chen, J.C. Wenke, Y. Yang, Sequential delivery of BMP-2 and IGF-1 using a chitosan gel with gelatin microspheres enhances early osteoblastic differentiation, Acta Biomater, 8 (2012) 1768-1777.
[19] Y. Wang, Y. Wei, X. Zhang, M. Xu, F. Liu, Q. Ma, Q. Cai, X. Deng, PLGA/PDLLA core–shell submicron spheres sequential release system: Preparation, characterization and promotion of bone regeneration in vitro and in vivo, Chemical Engineering Journal, 273 (2015) 490-501.
[20] H.K. Awada, N.R. Johnson, Y. Wang, Sequential delivery of angiogenic growth factors improves revascularization and heart function after myocardial infarction, J Control Release, 207 (2015) 7-17.
[21] J.R. Faulkner, J.E. Herrmann, M.J. Woo, K.E. Tansey, N.B. Doan, M.V. Sofroniew, Reactive astrocytes protect tissue and preserve function after spinal cord injury, J Neurosci, 24 (2004) 2143-2155.
[22] J. Silver, J.H. Miller, Regeneration beyond the glial scar, Nat Rev Neurosci, 5 (2004) 146-156.
[23] R.J. Franklin, C. Ffrench-Constant, Remyelination in the CNS: from biology to therapy, Nat Rev Neurosci, 9 (2008) 839-855.
[24] C. Profyris, S.S. Cheema, D. Zang, M.F. Azari, K. Boyle, S. Petratos, Degenerative and regenerative mechanisms governing spinal cord injury, Neurobiology of Disease, 15 (2004) 415-436.
[25] M.S. Beattie, Inflammation and apoptosis: linked therapeutic targets in spinal cord injury, Trends Mol Med, 10 (2004) 580-583.
[26] M.S. Beattie, A.A. Farooqui, J.C. Bresnahan, Review of current evidence for apoptosis after spinal cord injury, Journal of Neurotrauma, 17 (2000) 915-925.
[27] T. Hagg, M. Oudega, Degenerative and spontaneous regenerative processes after spinal cord injury, Journal of Neurotrauma, 23 (2006) 264-280.
[28] C.H. Tator, I. Koyanagi, Vascular mechanisms in the pathophysiology of human spinal cord injury, Journal of Neurosurgery, 86 (1997) 483-492.
[29] M.J. Crowe, J.C. Bresnahan, S.L. Shuman, J.N. Masters, M.S. Beattie, Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys, Nature Medicine, 3 (1997) 73-76.
[30] J.E. Herrmann, T. Imura, B. Song, J. Qi, Y. Ao, T.K. Nguyen, R.A. Korsak, K. Takeda, S. Akira, M.V. Sofroniew, STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury, J Neurosci, 28 (2008) 7231-7243.

[31] E.D. Hall, J.E. Springer, Neuroprotection and acute spinal cord injury: A reappraisal, NeuroRX, 1 (2004) 80-100.
[32] L.H.S. Sekhon, M.G. Fehlings, Epidemiology, demographics, and pathophysiology of acute spinal cord injury, Spine, 26 (2001) S2-S12.
[33] S.D. Christie, B. Comeau, T. Myers, D. Sadi, M. Purdy, I. Mendez, Duration of lipid peroxidation after acute spinal cord injury in rats and the effect of methylprednisolone, Neurosurg Focus, 25 (2008) E5.
[34] J.M.C. Gutteridge, B. Halliwell, THE MEASUREMENT AND MECHANISM OF LIPID-PEROXIDATION IN BIOLOGICAL-SYSTEMS, Trends in Biochemical Sciences, 15 (1990) 129-135.
[35] M. Kerschensteiner, M.E. Schwab, J.W. Lichtman, T. Misgeld, In vivo imaging of axonal degeneration and regeneration in the injured spinal cord, Nature Medicine, 11 (2005) 572-577.
[36] S. Casha, W.R. Yu, M.G. Fehlings, Oligodendroglial apoptosis occurs along degenerating axons and is associated with Fas and p75 expression following spinal cord injury in the rat, Neuroscience, 103 (2001) 203-218.
[37] P.G. Popovich, P. Wei, B.T. Stokes, Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats, Journal of Comparative Neurology, 377 (1997) 443-464.
[38] T.B. Jones, E.E. McDaniel, P.G. Popovich, Inflammatory-mediated injury and repair in the traumatically injured spinal cord, Current Pharmaceutical Design, 11 (2005) 1223-1236.
[39] J.C. Fleming, M.D. Norenberg, D.A. Ramsay, G.A. Dekaban, A.E. Marcillo, A.D. Saenz, M. Pasquale-Styles, W.D. Dietrich, L.C. Weaver, The cellular inflammatory response in human spinal cords after injury, Brain, 129 (2006) 3249-3269.
[40] D. Davalos, J. Grutzendler, G. Yang, J.V. Kim, Y. Zuo, S. Jung, D.R. Littman, M.L. Dustin, W.-B. Gan, ATP mediates rapid microglial response to local brain injury in vivo, Nat Neurosci, 8 (2005) 752-758.
[41] G.J. Liu, R. Nagarajah, R.B. Banati, M.R. Bennett, Glutamate induces directed chemotaxis of microglia, Eur J Neurosci, 29 (2009) 1108-1118.
[42] D. Berangere Re, S. Przedborski, Fractalkine: moving from chemotaxis to neuroprotection, Nat Neurosci, 9 (2006) 859-861.
[43] I. Pineau, L. Sun, D. Bastien, S. Lacroix, Astrocytes initiate inflammation in the injured mouse spinal cord by promoting the entry of neutrophils and inflammatory monocytes in an IL-1 receptor/MyD88-dependent fashion, Brain, Behavior, and Immunity, 24 (2010) 540-553.
[44] Y. Hamada, T. Ikata, S. Katoh, K.J. Nakauchi, M. Niwa, Y. Kawai, K.J. Fukuzawa, Involvement of an intercellular adhesion molecule 1-dependent pathway in the pathogenesis of secondary changes after spinal cord injury in rats, Journal of Neurochemistry, 66 (1996) 1525-1531.
[45] K.A. Kigerl, J.C. Gensel, D.P. Ankeny, J.K. Alexander, D.J. Donnelly, P.G. Popovich, Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord, J Neurosci, 29 (2009) 13435-13444.
[46] T.B. Jones, Lymphocytes and autoimmunity after spinal cord injury, Exp Neurol, 258 (2014) 78-90.
[47] M.J. Amador, J.D. Guest, An Appraisal of Ongoing Experimental Procedures in Human Spinal Cord Injury, Journal of Neurologic Physical Therapy, 29 (2005).
[48] E.D. Hall, Antioxidant therapies for acute spinal cord injury, Neurotherapeutics, 8 (2011) 152-167.
[49] E.D. Hall, Lipid antioxidants in acute central nervous system injury, Annals of Emergency Medicine, 22 (1993) 1022-1027.
[50] M. Oudega, C.G. Vargas, A.B. Weber, N. Kleitman, M.B. Bunge, Long-term effects of methylprednisolone following transection of adult rat spinal cord, European Journal of Neuroscience, 11 (1999) 2453-2464.
[51] T. Takami, M. Oudega, J.R. Bethea, P.M. Wood, N. Kleitman, M.B. Bunge, Methylprednisolone and interleukin-10 reduce gray matter damage in the contused Fischer rat thoracic spinal cord but do not improve functional outcome, Journal of Neurotrauma, 19 (2002) 653-666.

[52] J.M. Braughler, E.D. Hall, LACTATE AND PYRUVATE METABOLISM IN INJURED CAT SPINAL-CORD BEFORE AND AFTER A SINGLE LARGE INTRAVENOUS DOSE OF METHYLPREDNISOLONE, Journal of Neurosurgery, 59 (1983) 256-261.
[53] D. Bartholdi, M.E. Schwab, METHYLPREDNISOLONE INHIBITS EARLY INFLAMMATORY PROCESSES BUT NOT ISCHEMIC CELL-DEATH AFTER EXPERIMENTAL SPINAL-CORD LESION IN THE RAT, Brain Research, 672 (1995) 177-186.
[54] A.G. Rabchevsky, I. Fugaccia, P.G. Sullivan, D.A. Blades, S.W. Scheff, Efficacy of methylprednisolone therapy for the injured rat spinal cord, J Neurosci Res, 68 (2002) 7-18.
[55] D.P. Stirling, K.M. Koochesfahani, J.D. Steeves, W. Tetzlaff, Minocycline as a neuroprotective agent, Neuroscientist, 11 (2005) 308-322.
[56] J.E.A. Wells, R.J. Hurlbert, M.G. Fehlings, V.W. Yong, Neuroprotection by minocycline facilitates significant recovery from spinal cord injury in mice, Brain, 126 (2003) 1628-1637.
[57] Y.D. Teng, H. Choi, R.C. Onario, S. Zhu, F.C. Desilets, S. Lan, E.J. Woodard, E.Y. Snyder, M.E. Eichler, R.M. Friedlander, Minocycline inhibits contusion-triggered mitochondrial cytochrome c release and mitigates functional deficits after spinal cord injury, Proceedings of the National Academy of Sciences of the United States of America, 101 (2004) 3071-3076.
[58] T.Y. Yune, J.Y. Lee, G.Y. Jung, S.J. Kim, M.H. Jiang, Y.C. Kim, Y.J. Oh, G.J. Markelonis, T.H. Oh, Minocycline alleviates death of oligodendrocytes by inhibiting pro-nerve growth factor production in microglia after spinal cord injury, J Neurosci, 27 (2007) 7751-7761.
[59] S.M. Lee, T.Y. Yune, S.J. Kim, D.W. Park, Y.K. Lee, Y.C. Kim, Y.J. Oh, G.J. Markelonis, T.H. Oh, Minocycline reduces cell death and improves functional recovery after traumatic spinal cord injury in the rat, J Neurotrauma, 20 (2003) 1017-1027.
[60] B.W. Festoff, S. Ameenuddin, P.M. Arnold, A. Wong, K.S. Santacruz, B.A. Citron, Minocycline neuroprotects, reduces microgliosis, and inhibits caspase protease expression early after spinal cord injury, J Neurochem, 97 (2006) 1314-1326.
[61] M.B. Bracken, M.J. Shepard, T.R. Holford, L. Leo-Summers, E.F. Aldrich, M. Fazl, M.G. Fehlings, D.L. Herr, P.W. Hitchon, L.F. Marshall, R.P. Nockels, V. Pascale, P.L. Perot, J. Piepmeier, V.K.H. Sonntag, F. Wagner, J.E. Wilberger, H.R. Winn, W. Young, Methylprednisolone or tirilazad mesylate administration after acute spinal cord injury: 1-year follow up - Results of the third National Acute Spinal Cord Injury randomized controlled trial, Journal of Neurosurgery, 89 (1998) 699-706.
[62] S. Casha, D. Zygun, M.D. McGowan, I. Bains, V.W. Yong, R.J. Hurlbert, Results of a phase II placebo-controlled randomized trial of minocycline in acute spinal cord injury, Brain, 135 (2012) 1224-1236.
[63] T.M. Allen, P.R. Cullis, Drug Delivery Systems: Entering the Mainstream, American Association for the Advancement of Science, 2004, pp. 1818.
[64] D.P. Stirling, K. Khodarahmi, J. Liu, L.T. McPhail, C.B. McBride, J.D. Steeves, M.S. Ramer, W. Tetzlaff, Minocycline treatment reduces delayed oligodendrocyte death, attenuates axonal dieback, and improves functional outcome after spinal cord injury, J Neurosci, 24 (2004) 2182-2190.
[65] A. Pinzon, A. Marcillo, A. Quintana, S. Stamler, M.B. Bunge, H.M. Bramlett, W.D. Dietrich, A re-assessment of minocycline as a neuroprotective agent in a rat spinal cord contusion model, Brain Res, 1243 (2008) 146-151.
[66] J.H. Lee, S. Tigchelaar, J. Liu, A.M. Stammers, F. Streijger, W. Tetzlaff, B.K. Kwon, Lack of neuroprotective effects of simvastatin and minocycline in a model of cervical spinal cord injury, Exp Neurol, 225 (2010) 219-230.
[67] D.D. Pearse, K. Chatzipanteli, A.E. Marcillo, M.B. Bunge, W.D. Dietrich, Comparison of NOS inhibition by antisense and pharmacological inhibitors after spinal cord injury, Journal of Neuropathology and Experimental Neurology, 62 (2003) 1096-1107.
[68] C.A. Stein, Y.C. Cheng, ANTISENSE OLIGONUCLEOTIDES AS THERAPEUTIC AGENTS - IS THE BULLET REALLY MAGICAL, Science, 261 (1993) 1004-1012.

[69] N. Gupta, N. Fisker, M.-C. Asselin, M. Lindholm, C. Rosenbohm, H. Ørum, J. Elmén, N.G. Seidah, E.M. Straarup, A Locked Nucleic Acid Antisense Oligonucleotide (LNA) Silences PCSK9 and Enhances LDLR Expression In Vitro and In Vivo, PLOS ONE, 5 (2010) e10682.
[70] K. Rahme, J.F. Guo, J.D. Holmes, C.M. O'Driscoll, Evaluation of the physicochemical properties and the biocompatibility of polyethylene glycol-conjugated gold nanoparticles: A formulation strategy for siRNA delivery, Colloids and Surfaces B-Biointerfaces, 135 (2015) 604-612.
[71] Y.T. Sun, Y.R. Zhao, X.T. Zhao, R.J. Lee, L.S. Teng, C.G. Zhou, Enhancing the Therapeutic Delivery of Oligonucleotides by Chemical Modification and Nanoparticle Encapsulation, Molecules, 22 (2017) 26.
[72] S. Antimisiaris, S. Mourtas, K. Papadia, Targeted si-RNA with liposomes and exosomes (extracellular vesicles): How to unlock the potential, International Journal of Pharmaceutics, 525 (2017) 293-312.
[73] Y. Taoka, K. Okajima, M. Uchiba, K. Murakami, S. Kushimoto, M. Johno, M. Naruo, H. Okabe, K. Takatsuki, Role of neutrophils in spinal cord injury in the rat, Neuroscience, 79 (1997) 1177-1182.
[74] Y.B. Lee, T.Y. Yune, S.Y. Baik, Y.H. Shin, S. Du, H. Rhim, E.B. Lee, Y.C. Kim, M.L. Shin, G.J. Markelonis, T.H. Oh, Role of Tumor Necrosis Factor-α in Neuronal and Glial Apoptosis after Spinal Cord Injury, Experimental Neurology, 166 (2000) 190-195.
[75] M. Tuna, S. Polat, T. Erman, F. Ildan, A.I. Gocer, N. Tuna, L. Tamer, M. Kaya, E. Cetinalp, Effect of anti-rat interleukin-6 antibody after spinal cord injury in the rat: inducible nitric oxide synthase expression, sodium- and potassium-activated, magnesium-dependent adenosine-5 '-triphosphatase and superoxide dismutase activation, and ultrastructural changes, Journal of Neurosurgery, 95 (2001) 64-73.
[76] R. Gonzalez, J. Glaser, M.T. Liu, T.E. Lane, H.S. Keirstead, Reducing inflammation decreases secondary degeneration and functional deficit after spinal cord injury, Experimental Neurology, 184 (2003) 456-463.
[77] T.Y. Yune, M.J. Chang, S.J. Kim, Y.B. Lee, S.W. Shin, H.W. Rhim, Y.C. Kim, M.L. Shin, Y.J. Oh, C.T. Han, G.J. Markelonis, T.H. Oh, Increased production of tumor necrosis factor-alpha induces apoptosis after traumatic spinal cord injury in rats, Journal of Neurotrauma, 20 (2003) 207-219.
[78] M.G. Fehlings, D.H. Nguyen, Immunoglobulin G: a potential treatment to attenuate neuroinflammation following spinal cord injury, J Clin Immunol, 30 Suppl 1 (2010) S109-112.
[79] D.H. Nguyen, N. Cho, K. Satkunendrarajah, J.W. Austin, J. Wang, M.G. Fehlings, Immunoglobulin G (IgG) attenuates neuroinflammation and improves neurobehavioral recovery after cervical spinal cord injury, Journal of Neuroinflammation, 9 (2012) 14.
[80] F.H. Brennan, N.D. Kurniawan, J. Vukovic, P.F. Bartlett, F. Kasermann, T.V. Arumugam, M. Basta, M.J. Ruitenberg, IVIg attenuates complement and improves spinal cord injury outcomes in mice, Ann Clin Transl Neurol, 3 (2016) 495-511.
[81] I. Schwab, F. Nimmerjahn, Intravenous immunoglobulin therapy: how does IgG modulate the immune system?, Nat Rev Immunol, 13 (2013) 176-189.
[82] T.G. Park, J.H. Jeong, S.W. Kim, Current status of polymeric gene delivery systems, Adv Drug Deliv Rev, 58 (2006) 467-486.
[83] M. Srinivasarao, C.V. Galliford, P.S. Low, Principles in the design of ligand-targeted cancer therapeutics and imaging agents, Nature Reviews Drug Discovery, 14 (2015) 203-219.
[84] A. Beck, L. Goetsch, C. Dumontet, N. Corvaia, Strategies and challenges for the next generation of antibody drug conjugates, Nature Reviews Drug Discovery, 16 (2017) 315-337.
[85] A.L. Daugherty, R.J. Mrsny, Formulation and delivery issues for monoclonal antibody therapeutics, Adv Drug Deliv Rev, 58 (2006) 686-706.
[86] O. Lazarov-Spiegler, O. Rapalino, G. Agranov, M. Schwartz, Restricted inflammatory reaction in the CNS: a key impediment to axonal regeneration?, Molecular Medicine Today, 4 337-342.
[87] M.T. Filbin, Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS, Nature Reviews Neuroscience, 4 (2003) 703-713.
[88] G. Yiu, Z. He, Glial inhibition of CNS axon regeneration, Nat Rev Neurosci, 7 (2006) 617-627.

[89] M.A. Anderson, J.E. Burda, Y. Ren, Y. Ao, T.M. O'Shea, R. Kawaguchi, G. Coppola, B.S. Khakh, T.J. Deming, M.V. Sofroniew, Astrocyte scar formation aids central nervous system axon regeneration, Nature, 532 (2016) 195-200.
[90] K. Liu, A. Tedeschi, K.K. Park, Z. He, Neuronal intrinsic mechanisms of axon regeneration, Annu Rev Neurosci, 34 (2011) 131-152.
[91] E.J. Bradbury, L.M. Carter, Manipulating the glial scar: chondroitinase ABC as a therapy for spinal cord injury, Brain Res Bull, 84 (2011) 306-316.
[92] K. Fouad, L. Schnell, M.B. Bunge, M.E. Schwab, T. Liebscher, D.D. Pearse, Combining Schwann cell bridges and olfactory-ensheathing glia grafts with chondroitinase promotes locomotor recovery after complete transection of the spinal cord, J Neurosci, 25 (2005) 1169-1178.
[93] M.P. Steinmetz, K.P. Horn, V.J. Tom, J.H. Miller, S.A. Busch, D. Nair, D.J. Silver, J. Silver, Chronic enhancement of the intrinsic growth capacity of sensory neurons combined with the degradation of inhibitory proteoglycans allows functional regeneration of sensory axons through the dorsal root entry zone in the mammalian spinal cord, J Neurosci, 25 (2005) 8066-8076.
[94] A. Mingorance, M. Sole, V. Muneton, A. Martinez, M. Nieto-Sampedro, E. Soriano, J.A. del Rio, Regeneration of lesioned entorhino-hippocampal axons in vitro by combined degradation of inhibitory proteoglycans and blockade of Nogo-66/NgR signaling, FASEB J, 20 (2006) 491-493.
[95] J.D. Houle, V.J. Tom, D. Mayes, G. Wagoner, N. Phillips, J. Silver, Combining an autologous peripheral nervous system "bridge" and matrix modification by chondroitinase allows robust, functional regeneration beyond a hemisection lesion of the adult rat spinal cord, J Neurosci, 26 (2006) 7405-7415.
[96] J.M. Massey, J. Amps, M.S. Viapiano, R.T. Matthews, M.R. Wagoner, C.M. Whitaker, W. Alilain, A.L. Yonkof, A. Khalyfa, N.G. Cooper, J. Silver, S.M. Onifer, Increased chondroitin sulfate proteoglycan expression in denervated brainstem targets following spinal cord injury creates a barrier to axonal regeneration overcome by chondroitinase ABC and neurotrophin-3, Exp Neurol, 209 (2008) 426-445.
[97] S. Karimi-Abdolrezaee, E. Eftekharpour, J. Wang, D. Schut, M.G. Fehlings, Synergistic effects of transplanted adult neural stem/progenitor cells, chondroitinase, and growth factors promote functional repair and plasticity of the chronically injured spinal cord, J Neurosci, 30 (2010) 1657-1676.
[98] E. Hauben, A. Ibarra, T. Mizrahi, R. Barouch, E. Agranov, M. Schwartz, Vaccination with a Nogo-A-derived peptide after incomplete spinal-cord injury promotes recovery via a T-cell-mediated neuroprotective response: Comparison with other myelin antigens, Proceedings of the National Academy of Sciences of the United States of America, 98 (2001) 15173-15178.
[99] D. Merkler, G.A.S. Metz, O. Raineteau, V. Dietz, M.E. Schwab, K. Fouad, Locomotor recovery in spinal cord-injured rats treated with an antibody neutralizing the myelin-associated neurite growth inhibitor Nogo-a, Journal of Neuroscience, 21 (2001) 3665-3673.
[100] S.X. Li, S.M. Strittmatter, Delayed systemic Nogo-66 receptor antagonist promotes recovery from spinal cord injury, Journal of Neuroscience, 23 (2003) 4219-4227.
[101] I.C. Maier, R.M. Ichiyama, G. Courtine, L. Schnell, I. Lavrov, V.R. Edgerton, M.E. Schwab, Differential effects of anti-Nogo-A antibody treatment and treadmill training in rats with incomplete spinal cord injury, Brain, 132 (2009) 1426-1440.
[102] B. Zheng, C. Ho, S. Li, H. Keirstead, O. Steward, M. Tessier-Lavigne, Lack of Enhanced Spinal Regeneration in Nogo-Deficient Mice, Neuron, 38 (2003) 213-224.
[103] P. Lu, Y. Wang, L. Graham, K. McHale, M. Gao, D. Wu, J. Brock, A. Blesch, E.S. Rosenzweig, L.A. Havton, B. Zheng, J.M. Conner, M. Marsala, M.H. Tuszynski, Long-distance growth and connectivity of neural stem cells after severe spinal cord injury, Cell, 150 (2012) 1264-1273.
[104] P. Lu, G. Woodruff, Y. Wang, L. Graham, M. Hunt, D. Wu, E. Boehle, R. Ahmad, G. Poplawski, J. Brock, L.S. Goldstein, M.H. Tuszynski, Long-distance axonal growth from human induced pluripotent stem cells after spinal cord injury, Neuron, 83 (2014) 789-796.
[105] L. Schnell, R. Schneider, R. Kolbeck, Y.A. Barde, M.E. Schwab, NEUROTROPHIN-3 ENHANCES SPROUTING OF CORTICOSPINAL TRACT DURING DEVELOPMENT AND AFTER ADULT SPINAL-CORD LESION, Nature, 367 (1994) 170-173.

[106] R. Grill, K. Murai, A. Blesch, F.H. Gage, M.H. Tuszynski, Cellular delivery of neurotrophin-3 promotes corticospinal axonal growth and partial functional recovery after spinal cord injury, Journal of Neuroscience, 17 (1997) 5560-5572.
[107] D.A. Houweling, A.J. Lankhorst, W.H. Gispen, P.R. Bar, E.A.J. Joosten, Collagen containing neurotrophin-3 (NT-3) attracts regrowing injured corticospinal axons in the adult rat spinal cord and promotes partial functional recovery, Experimental Neurology, 153 (1998) 49-59.
[108] M.H. Tuszynski, R. Grill, L.L. Jones, A. Brant, A. Blesch, K. Löw, S. Lacroix, P. Lu, NT-3 gene delivery elicits growth of chronically injured corticospinal axons and modestly improves functional deficits after chronic scar resection, Experimental Neurology, 181 (2003) 47-56.
[109] U. Milbreta, L.H. Nguyen, H. Diao, J. Lin, W. Wu, C.-Y. Sun, J. Wang, S.Y. Chew, Three-Dimensional Nanofiber Hybrid Scaffold Directs and Enhances Axonal Regeneration after Spinal Cord Injury, ACS Biomaterials Science & Engineering, 2 (2016) 1319-1329.
[110] L.H. Nguyen, M. Gao, J. Lin, W. Wu, J. Wang, S.Y. Chew, Three-dimensional aligned nanofibers-hydrogel scaffold for controlled non-viral drug/gene delivery to direct axon regeneration in spinal cord injury treatment, Sci Rep, 7 (2017) 42212.
[111] J.S. Rao, C. Zhao, A. Zhang, H. Duan, P. Hao, R.H. Wei, J. Shang, W. Zhao, Z. Liu, J. Yu, K.S. Fan, Z. Tian, Q. He, W. Song, Z. Yang, Y.E. Sun, X. Li, NT3-chitosan enables de novo regeneration and functional recovery in monkeys after spinal cord injury, Proc Natl Acad Sci U S A, 115 (2018) E5595-E5604.
[112] S. Ahmed, B.A. Reynolds, S. Weiss, BDNF ENHANCES THE DIFFERENTIATION BUT NOT THE SURVIVAL OF CNS STEM CELL-DERIVED NEURONAL PRECURSORS, Journal of Neuroscience, 15 (1995) 5765-5778.
[113] B. Kirschenbaum, S.A. Goldman, BRAIN-DERIVED NEUROTROPHIC FACTOR PROMOTES THE SURVIVAL OF NEURONS ARISING FROM THE ADULT-RAT FOREBRAIN SUBEPENDYMAL ZONE, Proceedings of the National Academy of Sciences of the United States of America, 92 (1995) 210-214.
[114] Y. Liu, D.H. Kim, B.T. Himes, S.Y. Chow, T. Schallert, M. Murray, A. Tessler, I. Fischer, Transplants of fibroblasts genetically modified to express BDNF promote regeneration of adult rat rubrospinal axons and recovery of forelimb function, Journal of Neuroscience, 19 (1999) 4370-4387.
[115] L.J. Zhou, H.D. Shine, Neurotrophic factors expressed in both cortex and spinal cord induce axonal plasticity after spinal cord injury, Journal of Neuroscience Research, 74 (2003) 221-226.
[116] L.B. Jakeman, P. Wei, Z. Guan, B.T. Stokes, Brain-derived neurotrophic factor stimulates hindlimb stepping and sprouting of cholinergic fibers after spinal cord injury, Experimental Neurology, 154 (1998) 170-184.
[117] E. Schwartz, Ex vivo MR determined apparent diffusion coefficients correlate with motor recovery mediated by intraspinal transplants of fibroblasts genetically modified to express BDNF, Experimental Neurology, 182 (2003) 49-63.
[118] D. Kim, T. Schallert, Y. Liu, T. Browarak, N. Nayeri, A. Tessler, I. Fischer, M. Murray, Transplantation of genetically modified fibroblasts expressing BDNF in adult rats with a subtotal hemisection improves specific motor and sensory functions, Neurorehabilitation and Neural Repair, 15 (2001) 141-150.
[119] J.S. Shumsky, C.A. Tobias, M. Tumolo, W.D. Long, S.F. Giszter, M. Murray, Delayed transplantation of fibroblasts genetically modified to secrete BDNF and NT-3 into a spinal cord injury site is associated with limited recovery of function, Experimental Neurology, 184 (2003) 114-130.
[120] M.H. Tuszynski, K. Gabriel, F.H. Gage, S. Suhr, S. Meyer, A. Rosetti, Nerve growth factor delivery by gene transfer induces differential outgrowth of sensory, motor, and noradrenergic neurites after adult spinal cord injury, Experimental Neurology, 137 (1996) 157-173.
[121] R.J. Grill, A. Blesch, M.H. Tuszynski, Robust growth of chronically injured spinal cord axons induced by grafts of genetically modified NGF-secreting cells, Experimental Neurology, 148 (1997) 444-452.

[122] M.H. Tuszynski, R. Grill, L.L. Jones, H.M. McKay, A. Blesch, Spontaneous and augmented growth of axons in the primate spinal cord: Effects of local injury and nerve growth factor-secreting cell grafts, Journal of Comparative Neurology, 449 (2002) 88-101.
[123] A. Blesch, M.H. Tuszynski, Cellular GDNF delivery promotes growth of motor and dorsal column sensory axons after partial and complete spinal cord transections and induces remyelination, Journal of Comparative Neurology, 467 (2003) 403-417.
[124] B.K. Chen, N.N. Madigan, J.S. Hakim, M. Dadsetan, S.S. McMahon, M.J. Yaszemski, A.J. Windebank, GDNF Schwann cells in hydrogel scaffolds promote regional axon regeneration, remyelination and functional improvement after spinal cord transection in rats, J Tissue Eng Regen Med, 12 (2018) e398-e407.
[125] T.T. Lee, B.A. Green, W.D. Dietrich, R.P. Yezierski, Neuroprotective effects of basic fibroblast growth factor following spinal cord contusion injury in the rat, Journal of Neurotrauma, 16 (1999) 347-356.
[126] S.A. Liddelow, B.A. Barres, REGENERATION Not everything is scary about a glial scar, Nature, 532 (2016) 182-183.
[127] D.M. McTigue, P.J. Horner, B.T. Stokes, F.H. Gage, Neurotrophin-3 and brain-derived neurotrophic factor induce oligodendrocyte proliferation and myelination of regenerating axons in the contused adult rat spinal cord, Journal of Neuroscience, 18 (1998) 5354-5365.
[128] J. Namiki, A. Kojima, C.H. Tator, Effect of brain-derived neurotrophic factor, nerve growth factor, and neurotrophin-3 on functional recovery and regeneration after spinal cord injury in adult rats, Journal of Neurotrauma, 17 (2000) 1219-1230.
[129] C.A. Tobias, J.S. Shumsky, M. Shibata, M.H. Tuszynski, I. Fischer, A. Tessler, M. Murray, Delayed grafting of BDNF and NT-3 producing fibroblasts into the injured spinal cord stimulates sprouting, partially rescues axotomized red nucleus neurons from loss and atrophy, and provides limited regeneration, Experimental Neurology, 184 (2003) 97-113.
[130] R. Nashmi, M.G. Fehlings, Mechanisms of axonal dysfunction after spinal cord injury: with an emphasis on the role of voltage-gated potassium channels, Brain Research Reviews, 38 (2001) 165-191.
[131] R.J.M. Franklin, C. Ffrench-Constant, Regenerating CNS myelin - from mechanisms to experimental medicines, Nat Rev Neurosci, 18 (2017) 753-769.
[132] B.E. Powers, D.L. Sellers, E.A. Lovelett, W. Cheung, S.P. Aalami, N. Zapertov, D.O. Maris, P.J. Horner, Remyelination reporter reveals prolonged refinement of spontaneously regenerated myelin, Proceedings of the National Academy of Sciences, 110 (2013) 4075-4080.
[133] Z.C. Hesp, E.Z. Goldstein, C.J. Miranda, B.K. Kaspar, D.M. McTigue, Chronic oligodendrogenesis and remyelination after spinal cord injury in mice and rats, J Neurosci, 35 (2015) 1274-1290.
[134] G.J. Duncan, S.B. Manesh, B.J. Hilton, P. Assinck, J. Liu, A. Moulson, J.R. Plemel, W. Tetzlaff, Locomotor recovery following contusive spinal cord injury does not require oligodendrocyte remyelination, Nat Commun, 9 (2018) 3066.
[135] P. Assinck, G.J. Duncan, J.R. Plemel, M.J. Lee, J.A. Stratton, S.B. Manesh, J. Liu, L.M. Ramer, S.H. Kang, D.E. Bergles, J. Biernaskie, W. Tetzlaff, Myelinogenic Plasticity of Oligodendrocyte Precursor Cells following Spinal Cord Contusion Injury, J Neurosci, 37 (2017) 8635-8654.
[136] W.F. Blakemore, R.J.M. Franklin, Remyelination in experimental models of toxin-induced demyelination, in: M. Rodriguez (Ed.) Advances in Multiple Sclerosis and Experimental Demyelinating Diseases, Springer-Verlag Berlin, Berlin, 2008, pp. 193-212.
[137] M. Kipp, T. Clarner, J. Dang, S. Copray, C. Beyer, The cuprizone animal model: new insights into an old story, Acta Neuropathologica, 118 (2009) 723-736.
[138] M. Mecha, F.J. Carrillo-Salinas, L. Mestre, A. Feliu, C. Guaza, Viral models of multiple sclerosis: Neurodegeneration and demyelination in mice infected with Theiler's virus, Progress in Neurobiology, 101 (2013) 46-64.
[139] M. Watanabe, Y. Toyama, A. Nishiyama, Differentiation of proliferated NG2-positive glial progenitor cells in a remyelinating lesion, J Neurosci Res, 69 (2002) 826-836.

[140] J.F. Talbott, D.N. Loy, Y. Liu, M.S. Qiu, M.B. Bunge, M.S. Rao, S.R. Whittemore, Endogenous Nkx2.2+/Olig2+ oligodendrocyte precursor cells fail to remyelinate the demyelinated adult rat spinal cord in the absence of astrocytes, Exp Neurol, 192 (2005) 11-24.
[141] G. Wolswijk, Chronic stage multiple sclerosis lesions contain a relatively quiescent population of oligodendrocyte precursor cells, Journal of Neuroscience, 18 (1998) 601-609.
[142] J.L. Mason, A. Toews, J.D. Hostettler, P. Morell, K. Suzuki, J.E. Goldman, G.K. Matsushima, Oligodendrocytes and progenitors become progressively depleted within chronically demyelinated lesions, The American Journal Of Pathology, 164 (2004) 1673-1682.
[143] K.E. Rhodes, G. Raivich, J.W. Fawcett, Cellular neuroscience: The injury response of oligodendrocyte precursor cells is induced by platelets, macrophages and inflammation-associated cytokines, Neuroscience, 140 (2006) 87-100.
[144] J.M. Levine, R. Reynolds, J.W. Fawcett, The oligodendrocyte precursor cell in health and disease, Trends in Neurosciences, 24 (2001) 39-47.
[145] S.A. Liddelow, K.A. Guttenplan, L.E. Clarke, F.C. Bennett, C.J. Bohlen, L. Schirmer, M.L. Bennett, A.E. Munch, W.S. Chung, T.C. Peterson, D.K. Wilton, A. Frouin, B.A. Napier, N. Panicker, M. Kumar, M.S. Buckwalter, D.H. Rowitch, V.L. Dawson, T.M. Dawson, B. Stevens, B.A. Barres, Neurotoxic reactive astrocytes are induced by activated microglia, Nature, 541 (2017) 481-487.
[146] D.M. McTigue, P. Wei, B.T. Stokes, Proliferation of NG2-positive cells and altered oligodendrocyte numbers in the contused rat spinal cord, Journal of Neuroscience, 21 (2001) 3392-3400.
[147] M.O. Totoiu, H.S. Keirstead, Spinal cord injury is accompanied by chronic progressive demyelination, J Comp Neurol, 486 (2005) 373-383.
[148] R.H. Woodruff, M. Fruttiger, W.D. Richardson, R.J.M. Franklin, Platelet-derived growth factor regulates oligodendrocyte progenitor numbers in adult CNS and their response following CNS demyelination, Molecular and Cellular Neuroscience, 25 (2004) 252-262.
[149] J.C. Murtie, Y.X. Zhou, T.Q. Le, A.C. Vana, R.C. Armstrong, PDGF and FGF2 pathways regulate distinct oligodendrocyte lineage responses in experimental demyelination with spontaneous remyelination, Neurobiol Dis, 19 (2005) 171-182.
[150] D.P. Crockett, M. Burshteyn, C. Garcia, M. Muggironi, P. Casaccia-Bonnefil, Number of oligodendrocyte progenitors recruited to the lesioned spinal cord is modulated by the levels of the cell cycle regulatory protein p27Kip-1, Glia, 49 (2005) 301-308.
[151] C. Zhao, D. Ma, M. Zawadzka, S.P. Fancy, L. Elis-Williams, G. Bouvier, J.H. Stockley, G.M. de Castro, B. Wang, S. Jacobs, P. Casaccia, R.J. Franklin, Sox2 Sustains Recruitment of Oligodendrocyte Progenitor Cells following CNS Demyelination and Primes Them for Differentiation during Remyelination, J Neurosci, 35 (2015) 11482-11499.
[152] S. Moyon, A.L. Dubessy, M.S. Aigrot, M. Trotter, J.K. Huang, L. Dauphinot, M.C. Potier, C. Kerninon, S. Melik Parsadaniantz, R.J. Franklin, C. Lubetzki, Demyelination causes adult CNS progenitors to revert to an immature state and express immune cues that support their migration, J Neurosci, 35 (2015) 4-20.
[153] J.R. Siebert, D.J. Stelzner, D.J. Osterhout, Chondroitinase treatment following spinal contusion injury increases migration of oligodendrocyte progenitor cells, Exp Neurol, 231 (2011) 19-29.
[154] M. Watanabe, T. Hadzic, A. Nishiyama, Transient upregulation of Nkx2.2 expression in oligodendrocyte lineage cells during remyelination, Glia, 46 (2004) 311-322.
[155] A. Williams, G. Piaton, M.-S. Aigrot, A. Belhadi, M. Théaudin, F. Petermann, J.-L. Thomas, B. Zalc, C. Lubetzki, Semaphorin 3A and 3F: key players in myelin repair in multiple sclerosis?, Brain: A Journal Of Neurology, 130 (2007) 2554-2565.
[156] J.M. Redwine, R.C. Armstrong, In vivo proliferation of oligodendrocyte progenitors expressing PDGF alpha R during early remyelination, Journal of Neurobiology, 37 (1998) 413-428.
[157] A.C. Vana, C.F. Lucchinetti, T.Q. Le, R.C. Armstrong, Myelin transcription factor 1 (Myt1) expression in demyelinated lesions of rodent and human CNS, Glia, 55 (2007) 687-697.

[158] S. Mi, R.B. Pepinsky, D. Cadavid, Blocking LINGO-1 as a therapy to promote CNS repair: from concept to the clinic, CNS Drugs, 27 (2013) 493-503.
[159] Z. Wang, H. Colognato, C. Ffrench-Constant, Contrasting effects of mitogenic growth factors on myelination in neuron-oligodendrocyte co-cultures, Glia, 55 (2007) 537-545.
[160] Q. Zhu, X. Zhao, K. Zheng, H. Li, H. Huang, Z. Zhang, T. Mastracci, M. Wegner, Y. Chen, L. Sussel, M. Qiu, Genetic evidence that Nkx2.2 and Pdgfra are major determinants of the timing of oligodendrocyte differentiation in the developing CNS, Development, 141 (2014) 548-555.
[161] T.R. Hammond, A. Gadea, J. Dupree, C. Kerninon, B. Nait-Oumesmar, A. Aguirre, V. Gallo, Astrocyte-derived endothelin-1 inhibits remyelination through notch activation, Neuron, 81 (2014) 588-602.
[162] G.R. John, S.L. Shankar, B. Shafit-Zagardo, A. Massimi, S.C. Lee, C.S. Raine, C.F. Brosnan, Multiple sclerosis: re-expression of a developmental pathway that restricts oligodendrocyte maturation, Nat Med, 8 (2002) 1115-1121.
[163] S. Mi, B. Hu, K. Hahm, Y. Luo, E.S. Kam Hui, Q. Yuan, W.M. Wong, L. Wang, H. Su, T.H. Chu, J. Guo, W. Zhang, K.F. So, B. Pepinsky, Z. Shao, C. Graff, E. Garber, V. Jung, E.X. Wu, W. Wu, LINGO-1 antagonist promotes spinal cord remyelination and axonal integrity in MOG-induced experimental autoimmune encephalomyelitis, Nat Med, 13 (2007) 1228-1233.
[164] S. Mi, R.H. Miller, W. Tang, X. Lee, B. Hu, W. Wu, Y. Zhang, C.B. Shields, Y. Zhang, S. Miklasz, D. Shea, J. Mason, R.J. Franklin, B. Ji, Z. Shao, A. Chedotal, F. Bernard, A. Roulois, J. Xu, V. Jung, B. Pepinsky, Promotion of central nervous system remyelination by induced differentiation of oligodendrocyte precursor cells, Ann Neurol, 65 (2009) 304-315.
[165] Z. Shao, X. Lee, G. Huang, G. Sheng, C.E. Henderson, D. Louvard, J. Sohn, B. Pepinsky, S. Mi, LINGO-1 Regulates Oligodendrocyte Differentiation through the Cytoplasmic Gelsolin Signaling Pathway, J Neurosci, 37 (2017) 3127-3137.
[166] A.C. Vana, N.C. Flint, N.E. Harwood, T.Q. Le, M. Fruttiger, R.C. Armstrong, Platelet-derived growth factor promotes repair of chronically demyelinated white matter, Journal Of Neuropathology And Experimental Neurology, 66 (2007) 975-988.
[167] R.C. Armstrong, T.Q. Le, E.E. Frost, R.C. Borke, A.C. Vana, Absence of fibroblast growth factor 2 promotes oligodendroglial repopulation of demyelinated white matter, Journal of Neuroscience, 22 (2002) 8574-8585.
[168] B.A. Barres, I.K. Hart, H.S. Coles, J.F. Burne, J.T. Voyvodic, W.D. Richardson, M.C. Raff, Cell death and control of cell survival in the oligodendrocyte lineage, Cell, 70 (1992) 31-46.
[169] P.A. Fernandez, D.G. Tang, L. Cheng, A. Prochiantz, A.W. Mudge, M.C. Raff, Evidence that axon-derived neuregulin promotes oligodendrocyte survival in the developing rat optic nerve, Neuron, 28 (2000) 81-90.
[170] P.D. Canoll, J.M. Musacchio, R. Hardy, R. Reynolds, M.A. Marchionni, J.L. Salzer, GGF/neuregulin is a neuronal signal that promotes the proliferation and survival and inhibits the differentiation of oligodendrocyte progenitors, Neuron, 17 (1996) 229-243.
[171] J.L. Yang, X.J. Cheng, J.J. Qi, B.H. Xie, X.F. Zhao, K. Zheng, Z.Y. Zhang, M.S. Qiu, EGF Enhances Oligodendrogenesis from Glial Progenitor Cells, Frontiers in Molecular Neuroscience, 10 (2017) 10.
[172] M.B. Keough, J.A. Rogers, P. Zhang, S.K. Jensen, E.L. Stephenson, T. Chen, M.G. Hurlbert, L.W. Lau, K.S. Rawji, J.R. Plemel, M. Koch, C.C. Ling, V.W. Yong, An inhibitor of chondroitin sulfate proteoglycan synthesis promotes central nervous system remyelination, Nat Commun, 7 (2016) 11312.
[173] H. Zhang, L. Vutskits, V. Calaora, P. Durbec, J.Z. Kiss, A role for the polysialic acid - neural cell adhesion molecule in PDGF-induced chemotaxis of oligodendrocyte precursor cells, Journal of Cell Science, 117 (2004) 93-103.
[174] B.N. Oumesmar, L. Vignais, E. Duhamelclerin, V. Avellanaadalid, G. Rougon, A. Baronvanevercooren, EXPRESSION OF THE HIGHLY POLYSIALYLATED NEURAL CELL-ADHESION MOLECULE DURING POSTNATAL MYELINATION AND FOLLOWING CHEMICALLY-INDUCED

DEMYELINATION OF THE ADULT-MOUSE SPINAL-CORD, European Journal of Neuroscience, 7 (1995) 480-491.
[175] B. Nait-Oumesmar, N. Picard-Riera, C. Kerninon, L. Decker, D. Seilhean, G.U. Hoglinger, E.C. Hirsch, R. Reynolds, A. Baron-Van Evercooren, Activation of the subventricular zone in multiple sclerosis: evidence for early glial progenitors, Proc Natl Acad Sci U S A, 104 (2007) 4694-4699.
[176] M. Mekhail, G. Almazan, M. Tabrizian, Oligodendrocyte-protection and remyelination post-spinal cord injuries: a review, Prog Neurobiol, 96 (2012) 322-339.
[177] M. Jurynczyk, A. Jurewicz, B. Bielecki, C.S. Raine, K. Selmaj, Inhibition of Notch signaling enhances tissue repair in an animal model of multiple sclerosis, J Neuroimmunol, 170 (2005) 3-10.
[178] M.F. Stidworthy, S. Genoud, W.W. Li, D.P. Leone, N. Mantei, U. Suter, R.J. Franklin, Notch1 and Jagged1 are expressed after CNS demyelination, but are not a major rate-determining factor during remyelination, Brain, 127 (2004) 1928-1941.
[179] Y. Zhang, A.T. Argaw, B.T. Gurfein, A. Zameer, B.J. Snyder, C. Ge, Q.R. Lu, D.H. Rowitch, C.S. Raine, C.F. Brosnan, G.R. John, Notch1 signaling plays a role in regulating precursor differentiation during CNS remyelination, Proc Natl Acad Sci U S A, 106 (2009) 19162-19167.
[180] M. Mellion, K.R. Edwards, R. Hupperts, J. Drulović, X. Montalban, H.-P. Hartung, B. Brochet, P.A. Calabresi, R. Rudick, A. Ibrahim, Y. Zhang, L. Xu, D. Cadavid, Efficacy Results from the Phase 2b SYNERGY Study: Treatment of Disabling Multiple Sclerosis with the Anti-LINGO-1 Monoclonal Antibody Opicinumab (S33.004), Neurology, 88 (2017) S33.004.
[181] A. Ranger, S. Ray, S. Szak, A. Dearth, N. Allaire, R. Murray, R. Gardner, D. Cadavid, S. Mi, Anti-LINGO-1 has no detectable immunomodulatory effects in preclinical and phase 1 studies, Neurology-Neuroimmunology & Neuroinflammation, 5 (2018) 10.
[182] A.E. Pasquinelli, MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship, Nat Rev Genet, 13 (2012) 271-282.
[183] J.K.W. Lam, M.Y.T. Chow, Y. Zhang, S.W.S. Leung, siRNA Versus miRNA as Therapeutics for Gene Silencing, Molecular Therapy-Nucleic Acids, 4 (2015) 20.
[184] X. Zhao, X. He, X. Han, Y. Yu, F. Ye, Y. Chen, T. Hoang, X. Xu, Q.S. Mi, M. Xin, F. Wang, B. Appel, Q.R. Lu, MicroRNA-mediated control of oligodendrocyte differentiation, Neuron, 65 (2010) 612-626.
[185] H.B. Wang, A.L. Moyano, Z.Y. Ma, Y.Q. Deng, Y.F. Lin, C.T. Zhao, L.G. Zhang, M.Q. Jiang, X.L. He, Z.X. Ma, F.H. Lu, M. Xin, W.H. Zhou, S.O. Yoon, E.R. Bongarzone, Q.R. Lu, miR-219 Cooperates with miR-338 in Myelination and Promotes Myelin Repair in the CNS, Developmental Cell, 40 (2017) 566-+.
[186] H.J. Diao, W.C. Low, Q.R. Lu, S.Y. Chew, Topographical effects on fiber-mediated microRNA delivery to control oligodendroglial precursor cells development, Biomaterials, 70 (2015) 105-114.
[187] U. Milbreta, J.Q. Lin, C. Pinese, W. Ong, J.S. Chin, H. Shirahama, R.F. Mi, A. Williams, M.E. Bechler, J. Wang, C. Ffrench-Constant, A. Hoke, S.Y. Chew, Scaffold-Mediated Sustained, Non-viral Delivery of miR-219/miR-338 Promotes CNS Remyelination, Molecular Therapy, 27 (2019) 411-423.
[188] W. Ong, J. Lin, M.E. Bechler, K. Wang, M. Wang, C. Ffrench-Constant, S.Y. Chew, Microfiber drug/gene delivery platform for study of myelination, Acta Biomater, 75 (2018) 152-160.
[189] D. Katz, J.K. Taubenberger, B. Cannella, D.E. McFarlin, C.S. Raine, H.F. McFarland, CORRELATION BETWEEN MAGNETIC-RESONANCE-IMAGING FINDINGS AND LESION DEVELOPMENT IN CHRONIC, ACTIVE MULTIPLE-SCLEROSIS, Annals of Neurology, 34 (1993) 661-669.
[190] J.W. Peterson, L. Bo, S. Mork, A. Chang, B.D. Trapp, Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions, Annals of Neurology, 50 (2001) 389-400.
[191] K.D. Beck, H.X. Nguyen, M.D. Galvan, D.L. Salazar, T.M. Woodruff, A.J. Anderson, Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment, Brain, 133 (2010) 433-447.
[192] S.P. Fancy, E.P. Harrington, T.J. Yuen, J.C. Silbereis, C. Zhao, S.E. Baranzini, C.C. Bruce, J.J. Otero, E.J. Huang, R. Nusse, R.J. Franklin, D.H. Rowitch, Axin2 as regulatory and therapeutic target in newborn brain injury and remyelination, Nat Neurosci, 14 (2011) 1009-1016.

[193] S.P. Fancy, E.P. Harrington, S.E. Baranzini, J.C. Silbereis, L.R. Shiow, T.J. Yuen, E.J. Huang, S. Lomvardas, D.H. Rowitch, Parallel states of pathological Wnt signaling in neonatal brain injury and colon cancer, Nat Neurosci, 17 (2014) 506-512.
[194] H.K. Lee, L.S. Chaboub, W. Zhu, D. Zollinger, M.N. Rasband, S.P. Fancy, B. Deneen, Daam2-PIP5K is a regulatory pathway for Wnt signaling and therapeutic target for remyelination in the CNS, Neuron, 85 (2015) 1227-1243.
[195] Y. Zou, Wnt Signaling in Spinal Cord Injury, (2015) 237-244.
[196] D. Ma, B. Wang, M. Zawadzka, G. Gonzalez, Z. Wu, B. Yu, E.L. Rawlins, R.J.M. Franklin, C. Zhao, A Subpopulation of Foxj1-Expressing, Nonmyelinating Schwann Cells of the Peripheral Nervous System Contribute to Schwann Cell Remyelination in the Central Nervous System, J Neurosci, 38 (2018) 9228-9239.
[197] M.B. Bracken, M.J. Shepard, T.R. Holford, L. LeoSummers, E.F. Aldrich, M. Fazl, M. Fehlings, D.L. Herr, P.W. Hitchon, L.F. Marshall, R.P. Nockels, V. Pascale, P.L. Perot, J. Piepmeier, V.K.H. Sonntag, F. Wagner, J.E. Wilberger, H.R. Winn, W. Young, Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury - Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial, Jama-Journal of the American Medical Association, 277 (1997) 1597-1604.
[198] H. Wang, Q. Zou, O.C. Boerman, A.W. Nijhuis, J.A. Jansen, Y. Li, S.C. Leeuwenburgh, Combined delivery of BMP-2 and bFGF from nanostructured colloidal gelatin gels and its effect on bone regeneration in vivo, J Control Release, 166 (2013) 172-181.
[199] A. Jaklenec, A. Hinckfuss, B. Bilgen, D.M. Ciombor, R. Aaron, E. Mathiowitz, Sequential release of bioactive IGF-I and TGF-beta 1 from PLGA microsphere-based scaffolds, Biomaterials, 29 (2008) 1518-1525.
[200] B.R. Orellana, D.A. Puleo, Tailored sequential drug release from bilayered calcium sulfate composites, Mater Sci Eng C Mater Biol Appl, 43 (2014) 243-252.
[201] J.C. Dugas, T.L. Cuellar, A. Scholze, B. Ason, A. Ibrahim, B. Emery, J.L. Zamanian, L.C. Foo, M.T. McManus, B.A. Barres, Dicer1 and miR-219 Are required for normal oligodendrocyte differentiation and myelination, Neuron, 65 (2010) 597-611.
[202] W.M. Pardridge, Drug and Gene Delivery to the Brain: The Vascular Route, Neuron, (2002).
[203] Z. Wang, P.C. Ho, A nanocapsular combinatorial sequential drug delivery system for antiangiogenesis and anticancer activities, Biomaterials, 31 (2010) 7115-7123.
[204] Z. Wang, P.C. Ho, Self-assembled core-shell vascular-targeted nanocapsules for temporal antivasculature and anticancer activities, Small, 6 (2010) 2576-2583.
[205] X. Luo, H. Zhang, M. Chen, J. Wei, Y. Zhang, X. Li, Antimetastasis and antitumor efficacy promoted by sequential release of vascular disrupting and chemotherapeutic agents from electrospun fibers, Int J Pharm, 475 (2014) 438-449.
[206] S. Sengupta, D. Eavarone, I. Capila, G. Zhao, N. Watson, T. Kiziltepe, R. Sasisekharan, Temporal targeting of tumour cells and neovasculature with a nanoscale delivery system, Nature, 436 (2005) 568-572.
[207] Y. Cao, B. Wang, Y. Wang, D. Lou, Polymer-controlled core–shell nanoparticles: a novel strategy for sequential drug release, RSC Advances, 4 (2014) 30430.
[208] H. Nie, Z. Dong, D.Y. Arifin, Y. Hu, C.H. Wang, Core/shell microspheres via coaxial electrohydrodynamic atomization for sequential and parallel release of drugs, J Biomed Mater Res A, 95 (2010) 709-716.
[209] H. Nie, Y. Fu, C.H. Wang, Paclitaxel and suramin-loaded core/shell microspheres in the treatment of brain tumors, Biomaterials, 31 (2010) 8732-8740.
[210] W.M. Li, C.W. Su, Y.W. Chen, S.Y. Chen, In situ DOX-calcium phosphate mineralized CPT-amphiphilic gelatin nanoparticle for intracellular controlled sequential release of multiple drugs, Acta Biomater, 15 (2015) 191-199.

[211] V. Sharma, S. Anandhakumar, M. Sasidharan, Self-degrading niosomes for encapsulation of hydrophilic and hydrophobic drugs: An efficient carrier for cancer multi-drug delivery, Mater Sci Eng C Mater Biol Appl, 56 (2015) 393-400.
[212] W. Saiyin, D. Wang, L. Li, L. Zhu, B. Liu, L. Sheng, Y. Li, B. Zhu, L. Mao, G. Li, X. Zhu, Sequential release of autophagy inhibitor and chemotherapeutic drug with polymeric delivery system for oral squamous cell carcinoma therapy, Mol Pharm, 11 (2014) 1662-1675.
[213] S. Narayanan, M. Pavithran, A. Viswanath, D. Narayanan, C.C. Mohan, K. Manzoor, D. Menon, Sequentially releasing dual-drug-loaded PLGA-casein core/shell nanomedicine: design, synthesis, biocompatibility and pharmacokinetics, Acta Biomater, 10 (2014) 2112-2124.
[214] S. Narayanan, U. Mony, D.K. Vijaykumar, M. Koyakutty, B. Paul-Prasanth, D. Menon, Sequential release of epigallocatechin gallate and paclitaxel from PLGA-casein core/shell nanoparticles sensitizes drug-resistant breast cancer cells, Nanomedicine, 11 (2015) 1399-1406.
[215] B.N.P. Kumar, N. Puvvada, S. Rajput, S. Sarkar, S.K. Das, L. Emdad, D. Sarkar, P. Venkatesan, I. Pal, G. Dey, S. Konar, K.R. Brunt, R.R. Rao, A. Mazumdar, S.C. Kundu, A. Pathak, P.B. Fisher, M. Mandal, Sequential release of drugs from hollow manganese ferrite nanocarriers for breast cancer therapy, J. Mater. Chem. B, 3 (2015) 90-101.
[216] M. Chen, X. Zhu, D. Yan, Sequential drug release for synergistic cancer treatment and immunity promotion, RSC Advances, 3 (2013) 13399.
[217] H.B. Man, R. Lam, M. Chen, E. Osawa, D. Ho, Nanodiamond-therapeutic complexes embedded within poly(ethylene glycol) diacrylate hydrogels mediating sequential drug elution, physica status solidi (a), 209 (2012) 1811-1818.
[218] M.S. Aw, J. Addai-Mensah, D. Losic, A multi-drug delivery system with sequential release using titania nanotube arrays, Chem Commun (Camb), 48 (2012) 3348-3350.
[219] S.C. Sundararaj, M.V. Thomas, R. Peyyala, T.D. Dziubla, D.A. Puleo, Design of a multiple drug delivery system directed at periodontitis, Biomaterials, 34 (2013) 8835-8842.
[220] S.C. Sundararaj, M.V. Thomas, T.D. Dziubla, D.A. Puleo, Bioerodible system for sequential release of multiple drugs, Acta Biomater, 10 (2014) 115-125.
[221] C.H. Lee, C.Y. Yu, S.H. Chang, K.C. Hung, S.J. Liu, C.J. Wang, M.Y. Hsu, I.C. Hsieh, W.J. Chen, Y.S. Ko, M.S. Wen, Promoting endothelial recovery and reducing neointimal hyperplasia using sequential-like release of acetylsalicylic acid and paclitaxel-loaded biodegradable stents, Int J Nanomedicine, 9 (2014) 4117-4133.
[222] T. Song, K. Gu, W. Wang, H. Wang, Y. Yang, L. Yang, P. Ma, X. Ma, J. Zhao, R. Yan, J. Guan, C. Wang, Y. Qi, J. Ya, Prolonged Suppression of Neuropathic Pain by Sequential Delivery of Lidocaine and Thalidomide Drugs Using PEGylated Graphene Oxide, J Pharm Sci, 104 (2015) 3851-3860.
[223] H.J. Lee, W.G. Koh, Hydrogel micropattern-incorporated fibrous scaffolds capable of sequential growth factor delivery for enhanced osteogenesis of hMSCs, ACS Appl Mater Interfaces, 6 (2014) 9338-9348.
[224] L. Lei, S. Wang, H. Wu, W. Ju, J. Peng, A.S. Qahtan, C. Chen, Y. Lu, J. Peng, X. Zhang, H. Nie, Optimization of release pattern of FGF-2 and BMP-2 for osteogenic differentiation of low-population density hMSCs, J Biomed Mater Res A, 103 (2015) 252-261.
[225] K. Cho, H.J. Lee, S.W. Han, J.H. Min, H. Park, W.G. Koh, Multi-Compartmental Hydrogel Microparticles Fabricated by Combination of Sequential Electrospinning and Photopatterning, Angew Chem Int Ed Engl, 54 (2015) 11511-11515.
[226] J.M. Kanczler, P.J. Ginty, L. White, N.M. Clarke, S.M. Howdle, K.M. Shakesheff, R.O. Oreffo, The effect of the delivery of vascular endothelial growth factor and bone morphogenic protein-2 to osteoprogenitor cell populations on bone formation, Biomaterials, 31 (2010) 1242-1250.
[227] D.H. Kempen, L. Lu, A. Heijink, T.E. Hefferan, L.B. Creemers, A. Maran, M.J. Yaszemski, W.J. Dhert, Effect of local sequential VEGF and BMP-2 delivery on ectopic and orthotopic bone regeneration, Biomaterials, 30 (2009) 2816-2825.

[228] B. De la Riva, E. Sánchez, A. Hernández, R. Reyes, F. Tamimi, E. López-Cabarcos, A. Delgado, C. Évora, Local controlled release of VEGF and PDGF from a combined brushite–chitosan system enhances bone regeneration, Journal of Controlled Release, 143 (2010) 45-52.
[229] M.S. Kang, J.H. Kim, R.K. Singh, J.H. Jang, H.W. Kim, Therapeutic-designed electrospun bone scaffolds: mesoporous bioactive nanocarriers in hollow fiber composites to sequentially deliver dual growth factors, Acta Biomater, 16 (2015) 103-116.
[230] T.A. Holland, Y. Tabata, A.G. Mikos, Dual growth factor delivery from degradable oligo(poly(ethylene glycol) fumarate) hydrogel scaffolds for cartilage tissue engineering, J Control Release, 101 (2005) 111-125.
[231] M.A. Azagarsamy, K.S. Anseth, Wavelength-Controlled Photocleavage for the Orthogonal and Sequential Release of Multiple Proteins, Angewandte Chemie International Edition, 52 (2013) 13803-13807.
[232] P. Yilgor, K. Tuzlakoglu, R.L. Reis, N. Hasirci, V. Hasirci, Incorporation of a sequential BMP-2/BMP-7 delivery system into chitosan-based scaffolds for bone tissue engineering, Biomaterials, 30 (2009) 3551-3559.
[233] F.B. Basmanav, G.T. Kose, V. Hasirci, Sequential growth factor delivery from complexed microspheres for bone tissue engineering, Biomaterials, 29 (2008) 4195-4204.
[234] R.R. Chen, E.A. Silva, W.W. Yuen, D.J. Mooney, Spatio-temporal VEGF and PDGF delivery patterns blood vessel formation and maturation, Pharm Res, 24 (2007) 258-264.
[235] X. Hao, E.A. Silva, A. Mansson-Broberg, K.H. Grinnemo, A.J. Siddiqui, G. Dellgren, E. Wardell, L.A. Brodin, D.J. Mooney, C. Sylven, Angiogenic effects of sequential release of VEGF-A165 and PDGF-BB with alginate hydrogels after myocardial infarction, Cardiovasc Res, 75 (2007) 178-185.
[236] T.P. Richardson, M.C. Peters, A.B. Ennett, D.J. Mooney, Polymeric system for dual growth factor delivery, Nature Biotechnology, 19 (2001) 1029-1034.
[237] Q. Sun, E.A. Silva, A. Wang, J.C. Fritton, D.J. Mooney, M.B. Schaffler, P.M. Grossman, S. Rajagopalan, Sustained release of multiple growth factors from injectable polymeric system as a novel therapeutic approach towards angiogenesis, Pharm Res, 27 (2010) 264-271.
[238] M.R. Battig, B. Soontornworajit, Y. Wang, Programmable release of multiple protein drugs from aptamer-functionalized hydrogels via nucleic acid hybridization, J Am Chem Soc, 134 (2012) 12410-12413.
[239] R. Chapanian, B.G. Amsden, Combined and sequential delivery of bioactive VEGF165 and HGF from poly(trimethylene carbonate) based photo-cross-linked elastomers, J Control Release, 143 (2010) 53-63.
[240] S.H. Shin, J. Lee, K.S. Lim, T. Rhim, S.K. Lee, Y.H. Kim, K.Y. Lee, Sequential delivery of TAT-HSP27 and VEGF using microsphere/hydrogel hybrid systems for therapeutic angiogenesis, J Control Release, 166 (2013) 38-45.
[241] E. Ruvinov, J. Leor, S. Cohen, The promotion of myocardial repair by the sequential delivery of IGF-1 and HGF from an injectable alginate biomaterial in a model of acute myocardial infarction, Biomaterials, 32 (2011) 565-578.
[242] Y. Wang, M.J. Cooke, N. Sachewsky, C.M. Morshead, M.S. Shoichet, Bioengineered sequential growth factor delivery stimulates brain tissue regeneration after stroke, J Control Release, 172 (2013) 1-11.
[243] J.A. Burdick, M. Ward, E. Liang, M.J. Young, R. Langer, Stimulation of neurite outgrowth by neurotrophins delivered from degradable hydrogels, Biomaterials, 27 (2006) 452-459.
[244] T. Jiang, W. Sun, Q. Zhu, N.A. Burns, S.A. Khan, R. Mo, Z. Gu, Furin-mediated sequential delivery of anticancer cytokine and small-molecule drug shuttled by graphene, Adv Mater, 27 (2015) 1021-1028.
[245] T. Jiang, R. Mo, A. Bellotti, J. Zhou, Z. Gu, Gel-Liposome-Mediated Co-Delivery of Anticancer Membrane-Associated Proteins and Small-Molecule Drugs for Enhanced Therapeutic Efficacy, Advanced Functional Materials, 24 (2014) 2295-2304.

[246] P.C. Chang, L.Y. Chong, A.S. Dovban, L.P. Lim, J.C. Lim, M.Y. Kuo, C.H. Wang, Sequential platelet-derived growth factor-simvastatin release promotes dentoalveolar regeneration, Tissue Eng Part A, 20 (2014) 356-364.
[247] L. Wei, J. Lin, C. Cai, Z. Fang, W. Fu, Drug-carrier/hydrogel scaffold for controlled growth of cells, Eur J Pharm Biopharm, 78 (2011) 346-354.
[248] C. Strobel, N. Bormann, A. Kadow-Romacker, G. Schmidmaier, B. Wildemann, Sequential release kinetics of two (gentamicin and BMP-2) or three (gentamicin, IGF-I and BMP-2) substances from a one-component polymeric coating on implants, J Control Release, 156 (2011) 37-45.
[249] M. Pulat, A.S. Kahraman, N. Tan, M. Gumusderelioglu, Sequential antibiotic and growth factor releasing chitosan-PAAm semi-IPN hydrogel as a novel wound dressing, J Biomater Sci Polym Ed, 24 (2013) 807-819.
[250] J. Huang, J. Ren, G. Chen, Z. Li, Y. Liu, G. Wang, X. Wu, Tunable sequential drug delivery system based on chitosan/hyaluronic acid hydrogels and PLGA microspheres for management of non-healing infected wounds, Mater Sci Eng C Mater Biol Appl, 89 (2018) 213-222.
[251] J. Sung, P.W. Barone, H. Kong, M.S. Strano, Sequential delivery of dexamethasone and VEGF to control local tissue response for carbon nanotube fluorescence based micro-capillary implantable sensors, Biomaterials, 30 (2009) 622-631.
[252] Q. Xu, Y. Xia, C.H. Wang, D.W. Pack, Monodisperse double-walled microspheres loaded with chitosan-p53 nanoparticles and doxorubicin for combined gene therapy and chemotherapy, J Control Release, 163 (2012) 130-135.
[253] L. Zhang, Z. Lu, Q. Zhao, J. Huang, H. Shen, Z. Zhang, Enhanced chemotherapy efficacy by sequential delivery of siRNA and anticancer drugs using PEI-grafted graphene oxide, Small, 7 (2011) 460-464.
[254] L. Fan, Y. Zhang, F. Wang, Q. Yang, J. Tan, G. Renata, H. Wu, C. Song, B. Jin, Multifunctional all-in-one drug delivery systems for tumor targeting and sequential release of three different anti-tumor drugs, Biomaterials, (2015).
[255] L. Fan, Q. Yang, J. Tan, Y. Qiao, Q. Wang, J. He, H. Wu, Y. Zhang, Dual loading miR-218 mimics and Temozolomide using AuCOOH@FA-CS drug delivery system: promising targeted anti-tumor drug delivery system with sequential release functions, J Exp Clin Cancer Res, 34 (2015) 106.
[256] K.L. Spiller, S. Nassiri, C.E. Witherel, R.R. Anfang, J. Ng, K.R. Nakazawa, T. Yu, G. Vunjak-Novakovic, Sequential delivery of immunomodulatory cytokines to facilitate the M1-to-M2 transition of macrophages and enhance vascularization of bone scaffolds, Biomaterials, 37 (2015) 194-207.
[257] R.A. Perez, J.H. Kim, J.O. Buitrago, I.B. Wall, H.W. Kim, Novel therapeutic core-shell hydrogel scaffolds with sequential delivery of cobalt and bone morphogenetic protein-2 for synergistic bone regeneration, Acta Biomater, 23 (2015) 295-308.
[258] B. Sun, D.M. Lynn, Release of DNA from polyelectrolyte multilayers fabricated using 'charge-shifting' cationic polymers: tunable temporal control and sequential, multi-agent release, J Control Release, 148 (2010) 91-100.
[259] H. Fukushima, S. Yamashita, T. Mori, Y. Katayama, T. Niidome, Sequential Release of Single-stranded DNAs from Gold Nanorods Triggered by Near-infrared Light Irradiation, Chemistry Letters, 41 (2012) 711-712.
[260] A.L. Acton, C. Fante, B. Flatley, S. Burattini, I.W. Hamley, Z. Wang, F. Greco, W. Hayes, Janus PEG-based dendrimers for use in combination therapy: controlled multi-drug loading and sequential release, Biomacromolecules, 14 (2013) 564-574.
[261] W. Xia, J. Chang, J. Lin, J. Zhu, The pH-controlled dual-drug release from mesoporous bioactive glass/polypeptide graft copolymer nanomicelle composites, Eur J Pharm Biopharm, 69 (2008) 546-552.
[262] L. Wei, C. Cai, J. Lin, T. Chen, Dual-drug delivery system based on hydrogel/micelle composites, Biomaterials, 30 (2009) 2606-2613.
[263] J. Sohier, T.J. Vlugt, N. Cabrol, C. Van Blitterswijk, K. de Groot, J.M. Bezemer, Dual release of proteins from porous polymeric scaffolds, J Control Release, 111 (2006) 95-106.

[264] D.M. Nelson, Z. Ma, C.E. Leeson, W.R. Wagner, Extended and sequential delivery of protein from injectable thermoresponsive hydrogels, J Biomed Mater Res A, 100 (2012) 776-785.
[265] J. Hong, N.J. Shah, A.C. Drake, P.C. DeMuth, J.B. Lee, J.Z. Chen, P.T. Hammond, Graphene Multilayers as Gates for Multi-Week Sequential Release of Proteins from Surfaces, ACS NANO, 6 (2012) 81-88.
[266] T. Okuda, K. Tominaga, S. Kidoaki, Time-programmed dual release formulation by multilayered drug-loaded nanofiber meshes, J Control Release, 143 (2010) 258-264.
[267] I. Freeman, A. Kedem, S. Cohen, The effect of sulfation of alginate hydrogels on the specific binding and controlled release of heparin-binding proteins, Biomaterials, 29 (2008) 3260-3268.
[268] K.K. Veeravalli, V.R. Dasari, J.S. Rao, Regulation of proteases after spinal cord injury, J Neurotrauma, 29 (2012) 2251-2262.
[269] A.L. Iordanskii, A.A. Ol'khov, S.G. Karpova, E.L. Kucherenko, R.Y. Kosenko, S.Z. Rogovina, A.E. Chalykh, A.A. Berlin, Influence of the Structure and Morphology of Ultrathin Poly(3-hydroxybutyrate) Fibers on the Diffusion Kinetics and Transport of Drugs, Polymer Science Series A, 59 (2017) 352-362.
[270] B.B. Hsu, K.S. Jamieson, S.R. Hagerman, E. Holler, J.Y. Ljubimova, P.T. Hammond, Ordered and Kinetically Discrete Sequential Protein Release from Biodegradable Thin Films, Angewandte Chemie, 126 (2014) 8231-8236.
[271] W.E. Hennink, C.F. van Nostrum, Novel crosslinking methods to design hydrogels, Advanced Drug Delivery Reviews, 64 (2012) 223-236.
[272] N. Hammer, F.P. Brandl, S. Kirchhof, A.M. Goepferich, Cleavable carbamate linkers for controlled protein delivery from hydrogels, Journal of Controlled Release, 183 (2014) 67-76.
[273] M. Biondi, F. Ungaro, F. Quaglia, P.A. Netti, Controlled drug delivery in tissue engineering, Adv Drug Deliv Rev, 60 (2008) 229-242.
[274] V. Luginbuehl, L. Meinel, H.P. Merkle, B. Gander, Localized delivery of growth factors for bone repair, Eur J Pharm Biopharm, 58 (2004) 197-208.
[275] R. Vasita, D.S. Katti, Growth factor-delivery systems for tissue engineering: a materials perspective, Expert review of medical devices, 3 (2006) 29-47.
[276] E.W. Lin, H.D. Maynard, Grafting from Small Interfering Ribonucleic Acid (siRNA) as an Alternative Synthesis Route to siRNA-Polymer Conjugates, Macromolecules, 48 (2015) 5640-5647.
[277] C.D. Spicer, E.T. Pashuck, M.M. Stevens, Achieving Controlled Biomolecule-Biomaterial Conjugation, Chemical Reviews, 118 (2018) 7702-7743.
[278] K.C. Wood, H.F. Chuang, R.D. Batten, D.M. Lynn, P.T. Hammond, Controlling interlayer diffusion to achieve sustained, multiagent delivery from layer-by-layer thin films, Proc Natl Acad Sci U S A, 103 (2006) 10207-10212.
[279] J. Min, R.D. Braatz, P.T. Hammond, Tunable staged release of therapeutics from layer-by-layer coatings with clay interlayer barrier, Biomaterials, 35 (2014) 2507-2517.
[280] B.R. Orellana, D.A. Puleo, Tailored sequential drug release from bilayered calcium sulfate composites, Materials Science and Engineering: C, 43 (2014) 243-252.
[281] P.K. Mandapalli, S. Labala, J. Bojja, V.V.K. Venuganti, Effect of pirfenidone delivered using layer-by-layer thin film on excisional wound healing, European Journal of Pharmaceutical Sciences, 83 (2016) 166-174.
[282] S.C. Sundararaj, M. Al-Sabbagh, C.L. Rabek, T.D. Dziubla, M.V. Thomas, D.A. Puleo, Comparison of sequential drug release in vitro and in vivo, Journal of Biomedical Materials Research Part B: Applied Biomaterials, 104 (2016) 1302-1310.
[283] Y. Zou, L. Xie, S. Carroll, M. Muniz, H. Gibson, W.Z. Wei, H. Liu, G. Mao, Layer-by-layer films with bioreducible and nonbioreducible polycations for sequential DNA release, Biomacromolecules, 15 (2014) 3965-3975.
[284] S. Correa, E.C. Dreaden, L. Gu, P.T. Hammond, Engineering nanolayered particles for modular drug delivery, Journal of Controlled Release, 240 (2016) 364-386.
[285] R. Weissleder, K. Kelly, E.Y. Sun, T. Shtatland, L. Josephson, Cell-specific targeting of nanoparticles by multivalent attachment of small molecules, Nat Biotechnol, 23 (2005) 1418-1423.

[286] L.W. Elahi MF, Guoping G, Khan F, Core-shell Fibers for Biomedical Applications-A Review, J Bioeng Biomed Sci (2013) 121.
[287] X. Luo, C. Xie, H. Wang, C. Liu, S. Yan, X. Li, Antitumor activities of emulsion electrospun fibers with core loading of hydroxycamptothecin via intratumoral implantation, International journal of pharmaceutics, 425 (2012) 19-28.
[288] R.A. Perez, H.W. Kim, Core-shell designed scaffolds for drug delivery and tissue engineering, Acta Biomater, 21 (2015) 2-19.
[289] W. Dai, W. Jin, J. Zhang, X. Wang, J. Wang, X. Zhang, Y. Wan, Q. Zhang, Spatiotemporally controlled co-delivery of anti-vasculature agent and cytotoxic drug by octreotide-modified stealth liposomes, Pharm Res, 29 (2012) 2902-2911.
[290] B. Baumann, R. Wittig, M. Linden, Mesoporous silica nanoparticles in injectable hydrogels: factors influencing cellular uptake and viability, Nanoscale, 9 (2017) 12379-12390.
[291] T.R. Hoare, D.S. Kohane, Hydrogels in drug delivery: Progress and challenges, Polymer, 49 (2008) 1993-2007.
[292] J. Lee, K.Y. Lee, Local and sustained vascular endothelial growth factor delivery for angiogenesis using an injectable system, Pharm Res, 26 (2009) 1739-1744.
[293] X.J. Zhang, L.M. Ke, J. Yang, L.W. Lin, E.S. Xue, Y. Wang, L.Y. Yu, Z.K. Chen, Development, characterization and anti-tumor effect of a sequential sustained-release preparation containing ricin and Cobra venom cytotoxin, Pharmazie, 67 (2012) 618-621.
[294] L.A. Connal, Q. Li, J.F. Quinn, E. Tjipto, F. Caruso, G.G. Qiao, pH-Responsive Poly(acrylic acid) Core Cross-Linked Star Polymers: Morphology Transitions in Solution and Multilayer Thin Films, Macromolecules, 41 (2008) 2620-2626.
[295] M.D. Kurkuri, M.R. Nussio, A. Deslandes, N.H. Voelcker, Thermosensitive copolymer coatings with enhanced wettability switching, Langmuir, 24 (2008) 4238-4244.
[296] S. Chen, Y. Li, C. Guo, J. Wang, J. Ma, X. Liang, L.-R. Yang, H.-Z. Liu, Temperature-Responsive Magnetite/PEO−PPO−PEO Block Copolymer Nanoparticles for Controlled Drug Targeting Delivery, Langmuir, 23 (2007) 12669-12676.
[297] N.M. Shah, M.D. Pool, A.T. Metters, Influence of network structure on the degradation of photo-cross-linked PLA-b-PEG-b-PLA hydrogels, Biomacromolecules, 7 (2006) 3171-3177.
[298] M.H. Lee, H.Y. Lin, H.C. Chen, J.L. Thomas, Ultrasound mediates the release of curcumin from microemulsions, Langmuir, 24 (2008) 1707-1713.
[299] K. Nolkrantz, C. Farre, A. Brederlau, R.I.D. Karlsson, C. Brennan, P.S. Eriksson, S.G. Weber, M. Sandberg, O. Orwar, Electroporation of Single Cells and Tissues with an Electrolyte-filled Capillary, Analytical Chemistry, 73 (2001) 4469-4477.
[300] T.H. Oh, G.J. Markelonis, J.R. Vonvisger, B. Baik, M.T. Shipley, ACIDIC PH RAPIDLY INCREASES IMMUNOREACTIVITY OF GLIAL FIBRILLARY ACIDIC PROTEIN IN CULTURED ASTROCYTES, Glia, 13 (1995) 319-322.
[301] X.-L. Yang, X.-J. Ju, X.-T. Mu, W. Wang, R. Xie, Z. Liu, L.-Y. Chu, Core–Shell Chitosan Microcapsules for Programmed Sequential Drug Release, ACS Applied Materials & Interfaces, 8 (2016) 10524-10534.
[302] J. Song, X. Yang, O. Jacobson, L. Lin, P. Huang, G. Niu, Q. Ma, X. Chen, Sequential Drug Release and Enhanced Photothermal and Photoacoustic Effect of Hybrid Reduced Graphene Oxide-Loaded Ultrasmall Gold Nanorod Vesicles for Cancer Therapy, ACS Nano, 9 (2015) 9199-9209.
[303] W. Sun, T. Jiang, Y. Lu, M. Reiff, R. Mo, Z. Gu, Cocoon-Like Self-Degradable DNA Nanoclew for Anticancer Drug Delivery, Journal of the American Chemical Society, 136 (2014) 14722-14725.
[304] B. Halliwell, REACTIVE OXYGEN SPECIES AND THE CENTRAL-NERVOUS-SYSTEM, Journal of Neurochemistry, 59 (1992) 1609-1623.
[305] H. Deng, Y. Zhang, X. Wang, Jianhuazhang, Y. Cao, J. Liu, J. Liu, L. Deng, A. Dong, Balancing the stability and drug release of polymer micelles by the coordination of dual-sensitive cleavable bonds in cross-linked core, Acta Biomaterialia, 11 (2015) 126-136.

[306] X. Hu, Z. Qureishi, S.W. Thomas, Light-Controlled Selective Disruption, Multilevel Patterning, and Sequential Release with Polyelectrolyte Multilayer Films Incorporating Four Photocleavable Chromophores, Chemistry of Materials, 29 (2017) 2951-2960.
[307] G. Perale, F. Rossi, E. Sundstrom, S. Bacchiega, M. Masi, G. Forloni, P. Veglianese, Hydrogels in Spinal Cord Injury Repair Strategies, Acs Chemical Neuroscience, 2 (2011) 336-345.
[308] Z. Na, M. Ulla, C. Jiah Shin, P. Coline, S. Hitomi, L. Junquan, W. Wutian, J. Wei, L. Hang, C. Sing Yian, Biomimicking fiber scaffold as an effective *in vitro* & *in vivo* microRNA screening platform for directing tissue regeneration, Advanced Science, Accepted (2019).
[309] R. Requejo-Aguilar, A. Alastrue-Agudo, M. Cases-Villar, E. Lopez-Mocholi, R. England, M.J. Vicent, V. Moreno-Manzano, Combined polymer-curcumin conjugate and ependymal progenitor/stem cell treatment enhances spinal cord injury functional recovery, Biomaterials, 113 (2017) 18-30.
[310] E.S. Rosenzweig, J.H. Brock, P. Lu, H. Kumamaru, E.A. Salegio, K. Kadoya, J.L. Weber, J.J. Liang, R. Moseanko, S. Hawbecker, J.R. Huie, L.A. Havton, Y.S. Nout-Lomas, A.R. Ferguson, M.S. Beattie, J.C. Bresnahan, M.H. Tuszynski, Restorative effects of human neural stem cell grafts on the primate spinal cord, Nat Med, 24 (2018) 484-490.
[311] G.W.J. Hawryluk, A. Mothe, J. Wang, S. Wang, C. Tator, M.G. Fehlings, An In Vivo Characterization of Trophic Factor Production Following Neural Precursor Cell or Bone Marrow Stromal Cell Transplantation for Spinal Cord Injury, Stem Cells and Development, 21 (2012) 2222-2238.