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Biomaterial strategies for limiting the impact of secondary events following spinal cord injury

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Abstract

The nature of traumatic spinal cord injury (SCI) often involves limited recovery and long-term quality of life complications. The initial injury sets off a variety of secondary cascades, which result in an expanded lesion area. Ultimately, the native tissue fails to regenerate. As treatments are developed in the laboratory, the management of this secondary cascade is an important first step in achieving recovery of normal function. Current literature identifies four broad targets for intervention: inflammation, oxidative stress, disruption of the blood-spinal cord barrier, and formation of an inhibitory glial scar. Because of the complexity and interconnected nature of these events, strategies that combine multiple therapies together show much promise. Specifically, approaches that rely on biomaterials to perform a variety of functions are generating intense research interest. In this review, we examine each target and discuss how biomaterials are currently used to address them. Overall, we show that there are an impressive amount of biomaterials and combinatorial treatments which show good promise for slowing secondary events and improving outcomes. If more emphasis is placed on growing our understanding of how materials can manage secondary events, treatments for SCI can be designed in an increasingly rational manner, ultimately improving their potential for translation to the clinic.

Keywords

Biomaterials; spinal cord injury; inflammation; oxidative stress; blood-spinal cord barrier; glial scar

1. Introduction

The inability of the central nervous system (CNS) to regenerate following injury is well known. Clinically, there is no evidence to suggest that regeneration of damaged adult human CNS tissue is innately possible [2]. Specifically, traumatic spinal cord injury (SCI) (occurring when the spine experiences damaging mechanical forces) is an area of focused research interest [3, 4, 5, 6, 7]. Due to the complex nature of the pathophysiological events following the primary injury (e.g., secondary events, Figure 1), it is evident that the challenge of CNS regeneration will not be met by a therapy based on a single drug or surgery, but rather by a combinatorial treatment (one which combines multiple treatment

paradigms) that simultaneously addresses the myriad of challenges presented by SCI. As such, engineered biomaterials are poised to play a key role in treating SCI secondary damage, as they are capable of incorporating multiple functionalities. Considerable basic research effort to date focuses on stimulating axonal outgrowth with the goal of demonstrating functional improvement in lower-order animal models (e.g., mice and rats). While this is certainly a critical research need, for any treatment to have a significant chance of clinical translation, it should also halt the progression of secondary events and shift the injury site (and surrounding tissue) towards a pro-regenerative environment rather than a chronic one.

In the initial stages following primary injury (Figure 1), vascular changes allow circulating immune cells (leukocytes and other bone-marrow derived cells) to infiltrate; these immune cells adopt an inflammatory phenotype which may persist indefinitely [8]. Free radical formation (from mitochondrial dysfunction and phagocytes, and the release of cell components due to apoptosis) leads to oxidative stress, which is particularly damaging to neurons and glia [9]. Endogenous cell populations are lost due to glutamate excitotoxicity, apoptosis, and a disruption of the homeostatic ion balance [6]. Crucially, secondary damage progression enters a chronic phase wherein a scar, rich in inhibitory chondroitin sulfate proteoglycans (CSPGs), is deposited by a panoply of cell types, both CNS-intrinsic and extrinsic [10]. This scar provides a physical and chemical barrier to future axon regeneration [11]. In this review, we will divide the progression of secondary events into key treatment targets (Table 1) and identify ways in which these targets can be conquered by various interventions, specifically those that include functionalized materials.

2. Managing inflammation

Following SCI, infiltrating leukocytes and resident microglia initially react to the injury environment and adopt a pro-inflammatory (M1) phenotype [3, 6, 8, 40, 41]. Over time, these cells are capable of adopting a pro-regenerative phenotype (M2), which research suggests is beneficial for the reparation and remodeling of CNS tissue (Figure 2) [40, 41, 42, 43]. The progression of this response can be divided into broad phases akin to wound healing: phase 1 (cell necrosis and inflammation) lasts from 0 to 2 weeks, phase 2 (tissue replacement) lasts from 0.5 to 2 weeks, and phase 3 (tissue remodeling) lasts from 1.5 weeks to 1 year and beyond [10]. The majority of current research regarding inflammation in SCI focuses on evaluating the activation profile (e.g., M1 or M2 phenotype) of immune cells. At the time of this review, there is significant debate regarding the spectrum of M1/M2 activation, what the optimal timeframes are, and even the classification of types/subtypes of immune cells. Rather than enter that arena, this review will give a brief overview regarding the signaling pathways involved in inflammation following SCI and then discuss the ways in which biomaterials have been used to reduce inflammation. A more comprehensive review of inflammation following SCI can be found in [44].

2.1. Signaling pathways involved in inflammation following SCI

There are many pathways implicated in M1 or M2 activation; this review will highlight recent research specifically using SCI animal models. A common strategy for investigating

cell signaling and inflammation within the spinal cord is to alter the expression of specific receptors (knockouts, RNA interference, antibodies, chemical inhibitors, etc.), induce SCI, and then sample the damaged region for changes in activation pathways.

The Notch signaling pathway is known to regulate T and B cell differentiation, activation, and function [45] and neural stem cell (NSC) activation and self-renewal [46]. The effect of Notch signaling on SCI inflammation is not straightforward: globally blocking Notch signaling was found to increase the inflammation cascade, while inhibiting myeloid-specific Notch signaling decreased inflammation, resulting in increased functional outcomes [47]. Extracellular ATP, which is a strong activator of the innate immune system, is increased following SCI and is a major driver of allodynia (experiencing pain from non-painful stimuli) [48]. This mechanism acts through purinergic receptors, namely P2X₄, which is highly expressed within the CNS and is observed to increase following SCI [49]. P2X₄ knockout mice showed significantly less inflammation (including reduced leukocyte infiltration) and improved functional behavior following spinal cord contusion when compared with wild-type controls [50].

Receptor for advanced glycation end products (RAGE) binds a wide variety of ligands (most notably amphoterin, which is upregulated following SCI) and has been implicated in post-SCI inflammation, neurite outgrowth, and Schwann cell growth [51]. RAGE contributes directly to inflammation by upregulating inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) and increasing immune cell migration [51]. Additionally, RAGE deficient mice showed less inflammation and apoptosis through downregulation of the p53/Bax/caspase-3 pathway [52].

Members of the interleukin cytokines have been implicated in regulating inflammation following SCI. IL-6 is a pro-inflammatory cytokine and hallmark of M1 macrophages [53]. Accordingly, if antibodies are used to simply block IL-6 signaling (by inhibiting receptor binding) immediately after SCI, a shift from M1 to M2 is observed alongside increased locomotor scoring [54]. Interleukin-33 (IL-33) is a member of the interleukin-1 (IL-1) family and has been shown to promote M2 macrophage polarization [55]. Administering recombinant IL-33 in a mouse contusion SCI model reduced pro-inflammatory markers and increased the number of M2 (arginase-1+) macrophages while also slowing tissue loss, demyelination, and astrogliosis [56].

The endothelin system is responsible for mediating monocyte migration across the BSCB. Endothelin-1 (ET-1) signals through two receptors, ET_A and ET_B, both of which subsequently play an important role in CNS inflammation [57]. Blocking endothelin receptors A and/or B decreased neutrophil and monocyte infiltration, reducing the associated inflammatory response [58]. However, only when both receptors were simultaneously blocked was locomotor improvement achieved. Overall this research demonstrates that reducing monocyte infiltration engenders an improvement in functional outcomes, and suggests that simply blocking diapedesis (leukocyte infiltration to the spinal cord) is a valid strategy — however, this is not entirely the case. There is a small subpopulation (Ly6C⁺Ly6G⁻) of monocytes that play an important role in moving inflammation beyond the M1

phase and into the M2 phase; depleting this subpopulation resulted in impaired outcomes [59].

2.2. Biomaterial-based approaches to target and reduce inflammation

As our knowledge of the signaling events involved in inflammation increases, the obvious target is to deliver anti-inflammatory treatments. Commonly this consists of loading compounds — typically a pharmaceutical or naturally-derived drug — into a polymeric system and allowing it to be released in a controlled manner into the local environment. For example, minocycline (an antibiotic with anti-inflammatory, anti-apoptotic, and anti-oxidant properties which targets a variety of mechanisms for secondary events in SCI [60]) has shown the ability to modulate inflammation within the CNS and provide neuroprotective effects [12]. When loaded into biodegradable polycaprolactone/polyethylene glycol (PCL/PEG)-based nanoparticles (synthesized via emulsion-free radical polymerization, average diameter ≈ 100 nm, PDI 0.070) and surgically implanted into the spinal cord, minocycline reduced activation/proliferation of microglia and macrophages while reducing IL-6 expression [61]. Minocycline can also be delivered into the intrathecal space using dextran sulfate-minocycline complexes loaded into an agarose gel [13]. The complexes were crosslinked using Mg^{2+} instead of Ca^{2+} , as calcium is neurotoxic. They found that localized delivery reduced ED-1+ cell infiltration, modulated macrophage activation profiles (both M1 and M2, instead of predominantly M1), and improved functional outcomes (forelimb locomotor scale and grid walk) when compared to blank gels and systemic minocycline administration. These studies show the utility of biomaterials for localized, anti-inflammatory drug delivery.

Inducing the expression of different interleukins is also a valid strategy: delivering a lentivirus encoding IL-10 (which is anti-inflammatory and promotes M2 polarization) from heparin-coated poly(lactide-co-glycolide) (PLG) nerve bridges with multiple channels skewed polarization to the M2 phenotype and improved motor function in mice (as measured by the Basso Mouse Scale, BMS) [18]. Interestingly, flavopiridol (a proliferation inhibitor originally developed for the treatment of chronic lymphocytic leukemia [62]), delivered via poly(lactic-co-glycolic acid) (PLGA) nanoparticles, decreased inflammation and glial scarring while improving functional outcomes [14]. PLGA nanoparticles (embedded in an agarose hydrogel) can also be used to deliver methylprednisolone (see section 3) [63]. This approach reduced ED-1+ macrophages, decreased calpain/iNOS expression, and lowered the overall lesion volume. Polymer thin films can be used to deliver anti-inflammatory compounds. p38 and c-Jun N-terminal kinase (JNK) are pro-apoptotic activators of the mitogen-activated protein kinase (MAPK) pathway; delivered via polyvinyl alcohol-polyvinylpyrrolidone (PVA-PVP) thin films, they can reduce inflammatory and apoptotic/necrotic enzymes [15]. In another approach, blocking macrophage migration inhibitory factor (MIF) signaling by delivering liposomes (up to 200 nm in diameter) loaded with Chicago sky blue (CSB, an MIF inhibitor) reduced inflammatory secondary events in rats [16].

Biomaterials themselves can have anti-inflammatory properties. Partially reduced graphene oxide scaffolds were able to reduce M1 (CD80/CD86+) and increase M2 (CD163+ and

Arginase I+) macrophages while supporting angiogenesis [17]. This in turn improved the number of regenerating axons in the scaffolds. Both ferulic acid (FA) and chitosan have innate neuroprotective properties — nanoparticles formed by conjugating FA to glycol chitosan reduced glutamate-induced excitotoxicity of neurons *in vitro* [19]. In a contusion injury model, the FA-glycol chitosan nanoparticles decreased GFAP/ED-1 staining, reduced cavity volume, and improved functional outcomes (BBB score) over 28d. The fact that a non-functionalized material alone can have positive results is also borne out in studies showing the efficacy of PEG to reduce oxidative stress (see section 3), highlighting the value of using biomaterials-based interventions for SCI treatment.

Another area of interest is the use of mesenchymal stem cells (MSCs) to support local cell populations and modulate inflammation. MSCs have been applied to many pathologies and have been shown to alter their secretome (secreted growth factors and cytokines) to modulate their microenvironment; SCI is no exception. Despite being capable of differentiating into various tissues, the secretome of MSCs holds important promise for regenerative therapies [64]. Injecting MSCs into the injured spinal cord strongly influences macrophage polarization (M2 instead of M1), which improved functional outcomes (BBB score), preserved axons, reduced scar tissue, and increased myelin sparing [65]. However, without the use of a carrier device (e.g., hydrogel), the lifespan and localization of MSCs is limited (transfusions of MSCs tend to localize to lung tissue). To protect MSCs from environment stress and improve their survival, they can be encapsulated within a polyurethane-based hydrogel (PEG-poly(serinol hexamethylene urethane)) [20]. This approach increased tissue sparing and improved functional outcomes (BBB score), but interestingly did not affect macrophage polarity. Encapsulating hMSCs within an agarose/carbomer/PEG hydrogel (functionalized with RGD and containing deposited ECM) modulated inflammation and increased M2 macrophages [21]. Importantly, an impermeable cling film was also used to direct the secretome back into the injury site, supporting the notion that secreted factors are the most potent part of MSC-based therapies.

Important lessons regarding the inflammatory or anti-inflammatory properties of biomaterials can be learned from the neural electrode field. Since neural electrodes are intended for long-term implantation, particular attention must be paid to the inflammatory response of the CNS, as inflammation can disrupt the source of the signal or damage the electrode (a review of the field can be found in [66]). For example, electrodes (made from silicon or iridium microwire) can be coated with a pyrrole-hyaluronic acid conjugate (Poly(PyHA)) to reduce reactive gliosis (which is closely related to inflammation; see section 5.1) [67]. However, simply reducing the recruitment of astrocytes is not sufficient to substantially reduce inflammation: coating silicon electrodes with a microgel (consisting of multilayered poly(N-isopropylacrylamide) (pNIPAm) and acrylic acid particles crosslinked with PEG, which under physiologic conditions would decorate the surface) reduced astrocytic recruitment but did not improve the host response [68]. Despite the widespread use of PEG to reducing fouling in non-CNS implants [69], it did not effectively prevent the CNS host response. This shows how difficult it is to design materials for CNS implantation that are truly anti-inflammatory.

When considering how to design treatments to specifically address post-SCI inflammation, the results from a recent Notch signaling study [47] are particularly informative. Their findings are congruent with the results from many different trials using inhibitors — globally blocking a signaling pathway often leads to either reduced efficacy or unwanted side-effects. Thus, improved localized, targeted delivery of inhibitors and anti-inflammatory compounds is needed, and a materials-based approach could potentially offer this. Additionally, delivering bioactive compounds locally reduces overall dosage and can maintain therapeutic levels for longer. The development of polymeric drug delivery systems is currently a very active research area; as new techniques, such as stimuli-responsive systems [70] and affinity-based systems [71] are developed for other areas, the same principles can be applied to the treatment of inflammation within the spinal cord. This is not always a straightforward endeavor, as the CNS presents unique challenges. However, the advantage of using materials-based systems is their modularity and ability to incorporate new discoveries as our understanding of the field alongside therapeutic targets increases.

3. Reducing oxidative stress

Under normal physiological conditions, the production and elimination of reactive oxygen species (ROS) is carefully controlled. Physiologically relevant ROS include hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radicals (OH). The pathological events that follow SCI result in the release of excessive amounts of ROS into the extracellular environment. Cell necrosis releases components that are normally sequestered within the cell into the tissue surrounding the lesion, neutrophils and macrophages generate ROS as part of the inflammatory cascade, and mitochondrial dysfunction are all responsible for the release of ROS [9]. Perhaps because of the similarities to inflammation, research into reducing oxidative stress in SCI by itself is limited. Oftentimes reducing ROS is a byproduct of reducing inflammation. However, since the inflammatory cascade is not the sole source of ROS, efforts to reduce ROS following SCI are critical for clinical success. Indeed, the most commonly prescribed pharmaceutical treatment for SCI (methylprednisolone, MP) works by reducing oxidative stress; however, there is no clear indication that systemic administration of MP contributes to meaningful clinical outcomes [6]. However, MP can be delivered using biomaterials, leading to substantial improvements in efficacy (improved functional outcomes) and potential side-effects (i.e., the total dosage is much lower). The likely reason for this is localization to the injured tissue, where MP can reduce ROS and inflammation rather than acting systemically. MP has been delivered to the lesion site using carboxymethylchitosan/polyamidoamine dendrimers [24], PCL-based nanoparticles [25], or PLGA-based nanoparticles embedded in an agarose gel [26].

Utilizing the inherent properties of materials to reduce ROS is a potent strategy. Interestingly, solely by injecting an aqueous solution of simple polymer (30% PEG, MW 2000, via IV) into injured spinal cord tissue, the oxidative stress burden is reduced enough to slow the progression of secondary damage and gain functional improvements [23, 72]. PEG itself does not have the ability to scavenge free radicals or suppress the production of superoxides; the likely mechanism for ROS reduction is simply by physically reducing cell membrane disruption [22]. In a follow-up study, PEG-decorated silica nanoparticles were investigated [27]. These nanoparticles helped to seal cells (measured by decreased leakage

of lactate dehydrogenase — and also likely accompanied by decreased neurotransmitter release), reduced ROS (lipid hydroperoxide assay), and improved nerve conduction through the lesion. Likewise, PEG-coated Au nanoparticles improve functional (BMS, beam walking, and inclined ladder) and tissue-level outcomes (inflammation, remyelination, and neural outgrowth) [28]. PEG has also been studied in chronic injuries, where it was shown to have better results (axonal outgrowth and behavioral measures) than alginate hydrogels or matrigel [73]. These results (with PEG), and the inherent stabilization provided by many biomaterials, should be considered in any study investigating the effect of a material on SCI progression: appropriate material-only controls should be included, and some measure of ROS reduction should be included.

Superoxide dismutase (SOD) plays an integral role in the non-pathological management of ROS by catalyzing the conversion of O_2^- into O_2 and H_2O_2 , neither of which are as damaging as the former. Mn(III)tetrakis (4-benzoic acid) porphyrin (MnT-BAP, a superoxide dismutase mimetic), when compared with methylprednisolone, improved functional outcomes and scavenged ROS more effectively [74], suggesting that even simply replacing methylprednisolone in the current paradigm of clinical treatment would be an improvement. However, the potential for soluble delivery of SOD and a related enzyme, catalase (which degrades H_2O_2), is limited by their short biological half-life and the fact that they won't cross cell membranes nor be taken up by neurons [75]. Instead, SOD can be lecithinized [76], CAT can be delivered via PLGA nanoparticles [29], or they can be combined together into PLGA nanoparticles [30].

As with inflammation, much can be gained by using materials that inherently reduce ROS, such as by incorporating ROS-scavenging functionalities [77] or delivering small molecules that alleviate the burden of oxidative stress [78]. An oxygen-reactive polymer (containing a thioether group for ROS scavenging) developed for the treatment of traumatic brain injury reduced the degradation of neurons, astrocyte reactivity, microglial activation, and accumulated in the injury site [31]. Similar materials should be investigated for the treatment of SCI. The idea of using a biomaterial to interface with redox biology and treat ROS-induced stress is not new [79], lessons from other areas of literature should be incorporated into the design of new biomaterials for SCI treatment.

4. Restoring the blood-spinal cord barrier

One of the primary drivers of secondary damage after SCI is the disruption of the blood-spinal cord barrier (BSCB) [4, 6, 80]. Normally, tight junctions highly regulate the diffusion of molecules and cells across the BSCB, allowing the CNS to function as an isolated system [81]. However, following injury, diffusion across the BSCB is uncontrolled; the vascular network is also disrupted and fails to repair itself correctly [80]. This contributes to inflammation and ischemic tissue damage — as such, functionally restoring the BSCB to its pre-injury state is an important prerequisite for any type of treatment strategy. BSCB breakdown occurs in a biphasic manner following SCI [82], suggesting that vascular changes/remodeling are dynamic and occur for a long time (days — months) following SCI. Gray matter is more susceptible than white matter [83]. Overall, BSCB breakdown mediates

secondary and long-term damage, and is an important target for biomaterials-based intervention [84].

Since the BBB/BSCB is maintained by tight junctions between endothelial cells, it stands to reason that increasing angiogenesis would have an effect on BSCB permeability. Various angiogenic factors have been investigated as potential mechanisms to repair damaged blood vessels and restore the BSCB, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiopoietin-1 (ang-1), ang-2, and transforming growth factor (TGF)- β 1 and β 2 [85]. The role of these growth factors in SCI treatment is not as straightforward as one might hope. The reality is often nuanced and contradictory, with a variety of uncontrolled factors affecting the ultimate outcome. For example, inhibiting TGF β 1 suppressed glial scar formation and promoted functional recovery in a rat contusion model [86], while administering TGF β 1 reduced lesion volume in effectively the same model of SCI [87]. VEGF seems to be less contradictory and more universally positive [85].

Delivering VEGF from PLGA nanoparticles[32] or a combination of VEGF, Ang-1, and bFGF from PLGA microparticles[33] has been shown to improve histological and functional outcomes. Promoting endogenous VEGF expression via an adenovirus (non-specifically targeting all CNS cell types) was found to reduce cell necrosis and increase the production of blood vessels, leading to the preservation of axons and improved functional outcomes [88]. Polymeric scaffolds can be used to improve angiogenesis and BSCB recovery. Creating vascular networks in a degradable, two-component implant system (outer portion: oriented PLGA, inner portion: macroporous PEG/poly-L-lysine hydrogel) and loading the scaffolds with endothelial cells + neural progenitor cells substantially increased the number of functional vessels[34]. Importantly, half of the vessels in the co-culture implant stained for endothelial barrier antigen, showing that the BSCB was being re-established. Other studies have shown an improvement in angiogenesis (e.g., partially reduced graphene oxide, see section 2.2) without demonstrating whether the BSCB is being restored. It stands to reason that these studies do have some effect (given the strong link between angiogenesis and BSCB recovery), it would be interesting to see whether indications of BSCB permeability (e.g., by measuring luciferase extravasation [89]) improve or not in these types of studies.

Matrix metalloproteinases (MMPs) also play a large role in BSCB breakdown following SCI — there is an imbalance of MMPs and their corresponding tissue inhibitors of metalloproteinases (TIMPs), resulting in tissue destruction. This process can be mediated in a variety of ways. Ghrelin, a hunger hormone released primarily by the stomach, has been shown to have neuroprotective effects, possibly by protecting against glutamate-induced cytotoxicity [90]. The administration of ghrelin (via IP injection) inhibited the expression and activation of MMP9 (in addition to SUR1 and TrpM4), leading to less BSCB breakdown [91]. In MMP9 knockout mice, remote microglial activation was reduced, TNF- α expression was restored to normal, and enabled motor relearning to improve behavioral outcomes (neither MMP-9 knockout nor motor relearning alone was effective) [92]. MMP3 has been shown to have a variety of negative effects following SCI, such as increasing BSCB permeability, degrading tight junction proteins, increasing blood cell infiltration, and increasing MMP2/9 expression — as such, MMP3 knockout mice showed improved functional outcomes after SCI (increased BMS score) due to a reduction in the aforementioned processes [93]. Even preconditioning rats with sevoflurane inhibited

expression of MMP9 and led to improvement following SCI [94]. Toll-like receptor 4 (TLR4, a transmembrane protein involved in proinflammatory signal transduction) signaling is also implicated; inhibiting TLR4 signaling limited BSCB breakdown through various pathways following ischemia/reperfusion injury [95, 96]. Melatonin is capable of BSCB preservation through scavenging ROS, stabilizing tight junction proteins, and reducing apoptosis. Melatonin treatment has been shown to lessen microvessel loss and edema following SCI in mice [97, 98] in addition to decreasing the activity of MMP9 in a rat ischemic SCI model [99]. Finally, curcumin (a phytochemical) also preserved BSCB integrity by reducing proinflammatory protein expression and increasing tight junction protein expression, ultimately reducing the permeability of the BSCB [100]. Curcumin delivery can be improved by incorporation into the backbone of a polymeric carrier (polyacetal)[35]. This improves bioavailability and stability while allowing for localized delivery via osmotic pump. In combination with ependymal progenitor/stem cells (delivered via intrathecal catheter), this material improved functional recovery (BBB score) in chronic SCI. Similar to inflammation, the secretome of transplanted MSCs can have a positive effect on restoration of the BSCB post-injury (see section 2.2). Both intrathecal transplantation of MSCs [101] and intravenous injection [102] have been shown to improve BSCB leakage and associated outcomes, likely through indirect effects (e.g., by reducing local expression of MMP-9). As with earlier examples (section 2.2), providing the MSCs with a carrier material to improve their survival and redirect their secretome would likely enhance this effect.

Restoration of the BSCB is an interesting and compelling case for the use of functional materials as part of a combinatorial treatment. Given the complex nature and purpose of the BSCB, it is impossible that an inert biomaterial could provide the same functions. However, we can use inert materials (such as a chitosan tube [103]) to provide a crude substitute, protecting the damaged tissue while encouraging restoration of the BSCB through other functionalities. It is also becoming more apparent that cell signaling processes are too complex and intricate to be the sole target of a treatment; this is evidenced by the competing findings of researchers studying TGF β 1 (indeed, this type of contradictory evidence is common across many fields of study). Materials-based treatments for BSCB restoration should provide stabilization of the injury site and incorporate BSCB-stabilizing compounds (e.g., by the covalent immobilization of melatonin, which has already been applied to bone tissue engineering [104]).

5. Reducing glial scarring and endogenous inhibition of axon growth

5.1. Astrocyte reactivity and the glial scar

After SCI, astrocytes, which normally function as support cells, become reactive, proliferative, and rapidly deposit chondroitin sulfate proteoglycans (CSPG), forming a potent barrier to the regrowth of axons [6] (other cells types are involved in the inhibition of regrowth; for a complete review see [11]). In 2002, it was demonstrated that chondroitinase ABC (chABC) promotes recovery by reducing glial scarring [105]. Since then, a significant amount of SCI research has focused on manipulating this barrier, either through changing the behavior of astrocytes or affecting CSPGs (Figure 3). One approach is to alter the activation and phenotype of reactive cells following SCI, influencing them to become pro-

regenerative rather than reactive. Zebrafish, which are capable of marked spinal cord regeneration, have been shown to mediate this process partly through FGF-induced alignment of glial cells (a glial bridge), which guide regenerating axons [106]. In mammals, FGF2 has been shown to affect astrocyte reactivity and reduce CSPG formation by reducing inflammation, decreasing astrocyte reactivity, stimulating astrocyte proliferation (helping them regain neurogenic characteristics), and influencing glia to adopt a bipolar, guiding morphology (similar to the zebrafish) [107]. Ultimately, this supported neurite elongation and improved functional recovery (as measured by modified BBB, mBBB, scale), both alone and in combination with chABC (delivered in soluble form either through injection or via catheter) [107, 108]. As heparin is necessary for canonical FGF-FGF receptor binding [109], it stands to reason that delivering FGF to the injury site via heparin-containing materials would improve outcomes. This has been investigated and shown to be plausible [110], but subsequent research is surely needed. A membrane-permeable peptide mimicking protein tyrosine phosphatase- σ (PTP σ) wedge domain (named intracellular sigma peptide, ISP) was administered after SCI and was found to bind PTP σ (a receptor for inhibitory CSPGs), reducing inhibitory CSPG signal transduction [111]. ISP was delivered by repeated subcutaneous injections directly above the lesion site, relieved CSPG-based scarring over 50 days and improved functional outcomes. In other work, the deletion of leukocyte common antigen related phosphatase (LAR) increased the growth of neurites across the scar, showing that LAR plays a role in the ability of CSPGs to block neurite growth [112]. Connexin 43 also is involved, as its depletion reduced the amount of ATP released by astrocytes, resulting in less scarring and reactivity [113]. Through microtubule stabilization, Taxol reduced CSPGs and encouraged axon growth in part via repression of TGF β signaling [114]. Interestingly, it has been observed that growth factors may reduce glial scarring by guiding neural stem cell (NSC) differentiation towards alternative phenotypes and away from reactive astrocytic lineages [115].

There is also considerable possibility for an implanted or injected material to reduce astrocyte reactivity and inhibitory CSPG deposition, either through intrinsic material properties or the release of therapeutic agents. Intrinsic properties of hydro-gel crosslinking can be used: a composite hydrogel formed from alginate (which uses Ca²⁺ ions to crosslink), chitosan, and genipin sequestered extracellular Ca²⁺ (which causes excitotoxicity following SCI) and reduced astrocyte reactivity *in vitro* [116]. A coacervate system consisting of poly(ethylene argininy laspartate diglyceride):heparin (PEAD:heparin) was able to slowly deliver growth factors (such as sonic hedgehog, Shh) and decrease glial fibrillary acidic protein (GFAP) intensity [39]. A system such as this could be used to deliver FGF (see above) to affect astrocyte reactivity or ISP to directly address inhibitory signal transduction. Aligned, electrospun, fibronectin-coated poly-L-lactic acid (PLLA) fibers controlled astrocyte migration and increased their glutamate uptake (extracellular glutamate causes neuronal cell necrosis) [117], again showing that using a material that sequesters certain molecules can inherently reduce cell death. The chemotactic factor SDF-1 α (or CXCL12) has also been investigated as a possible treatment for SCI in conjunction with chABC. Administering SDF-1 α both alone and with chABC to an impact-compression (via aneurysm clip at T1-2) injury in an effort to recruit endogenous neural precursor cells (NPCs) while degrading the glial scar showed that SDF-1 α had no effect—all recovery was

due to the presence of chABC [36]. Such a finding is interesting, as SDF-1 α has been shown to successfully regenerate mesenchymal tissues (e.g., in myocardial repair) [118]. A probable cause is the lack of a sufficient stem cell population within the adult spinal cord from which to recruit cells. It is also likely that the delivery approach plays an important role — many successful studies have transfected local cells to overexpress SDF-1 α , whereas the above study delivered soluble SDF-1 α from PLGA nanoparticles embedded within a crosslinked methylcellulose hydrogel. Ultimately, more research is needed into the usefulness of SDF-1 α for repairing different types of SCI and the efficacy of various delivery methods. Likewise, chABC and NEP1-40 (a peptide which competitively binds Nogo receptors, blocking signal transduction from myelin-associated inhibitors) when delivered together (from fibrin scaffolds loaded with PLGA microspheres (NEP1-40) and lipid microtubes (chABC)) decreased CSPGs/glia scar and increased axon growth in a dorsal hemisection [37]. However, this study did not include a chABC only control group, so the effect of each compound individually in this system cannot be elucidated. Interestingly, in a similar study, combining NEP1-40/chABC with a variety of other treatments: progenitor motor neurons (pMNs) and/or growth factors (GFs: heparin, antithrombin-III peptide, neurotrophin-3, and platelet-derived growth factor), showed that only GFs alone, pMNs + GFs, and NEP1-40/chABC reduced CSPGs in a dorsal hemisection, while pMNs + NEP1-40/chABC and pMNs + GFs + NEP1-40/chABC increased CSPGs [38].

It is important to note, however, that research is currently being done into the potentially pro-regenerative effects of certain components within the glial scar [119], showing that there are supporting CSPGs and that the true role of the scar may be more nuanced than a simple barrier to regeneration. This provides evidence against the efficacy of any treatment that blocks astrocyte CSPG deposition directly, as their role might be critical for proper protection and regeneration. Instead, focusing on blocking inhibitory signal transduction, as done in ref [111] becomes more compelling. An interesting research study would be to release ISP or a similar compound from a biomaterial or nerve conduit and analyze whether there is a synergistic effect. It seems apparent that administering soluble compounds (e.g., inhibitors or growth factors) could, at worst, be eased by the use of a controlled release system, and at best see bioactivity and subsequent effectiveness greatly enhanced. From the above research, a general picture begins to take shape: a three-pronged strategy of apoptotic molecule uptake (e.g., glutamate or Ca²⁺), astrocyte behavior modulation (i.e., by delivering FGF), and blocking inhibitory signaling (ISP/chABC) would be a very compelling line of inquiry. However, when we consider the above results from various combinatorial strategies, we can see that creating combinatorial treatments is significantly more complex than simply combining existing treatments that work. The possibility of negative synergy must be considered, and publishing negative results like the above studies is key for the field to progress.

5.2. Inhibition from myelin debris

In addition to inhibitory CSPGs, myelin debris contains inhibitory components that prevent regeneration following SCI [120]. These inhibitors include Nogo (which exists in three isoforms: A, B, and C), myelin-associated glycoprotein (Mag), and oligodendrocyte myelin glycoprotein (Omgp). These act through a variety of receptors and signal transduction

pathways to inhibit axonal outgrowth, which are reviewed in [120, 121]. In addition to inhibiting outgrowth, it has been shown that myelin debris influences macrophage polarization, encouraging them to adopt an M1 phenotype instead of an M2 phenotype [122]. Different signaling pathways have been shown to differentially activate M1 or M2 phenotypes (NF- κ B/STAT1 activates M1 and STAT3/STAT6 activates M2). After culturing M2 macrophages with myelin debris, they found that myelin debris inhibited STAT3/STAT6 signaling and increased the activation of NF- κ B. They also observed a decrease in M2 cytokines and an increase in M1 cytokines (specifically, IL-6, MIF, IL-1 β , IP-10, IL-12, and TNF- α). This demonstrates the multifactorial nature of SCI — factors that are commonly thought to be inhibitory may also be pro-inflammatory and contribute to multiple pathologies. Again, this provides motivation for using a combinatorial, biomaterial-based strategy. As above, focus should be placed on inhibiting the inhibitors, and using a material to promote a regenerative microenvironment.

6. The importance of locomotor training

When using functional testing to evaluate a combinatorial approach (which is the predominant direction of the biomaterial-based SCI field [123]), it is important to consider current clinical treatment paradigms. Locomotor training, in a variety of forms, has been shown to positively impact ambulatory function [124], which is cited as a primary concern by patients with incomplete injury [125] (although it is important to note that all groups gave a lower priority to walking than they did to restoration of bowel and bladder control, suggesting that regeneration of the associated tracts should be considered alongside restoration of walking in animal models). In humans, clinical approaches which contain a rehabilitation component have the strongest evidence to support their use [126]. In rats, active engagement of the forelimbs improves hindlimb function following T10 hemisection, likely by reorganizing and re-engaging rostrocaudal spinal interneuronal networks [127]. In general, there is strong evidence to support plasticity of intact circuits following SCI, which can be taken advantage of to improve function [128]. Automated devices have been developed to encourage *ad libitum* training in rats [129], providing an important method for standardizing this approach and lowering the barrier to adoption. When testing the efficacy of biomaterial-based interventions, it is crucial to both a.) choose an injury site which engages the proper tracts affecting hindlimb locomotion and b.) consider the role of locomotor training as a treatment level to evaluate what effect a given intervention has on CNS plasticity.

7. Concluding remarks and future directions

Despite an incredible body of knowledge, a treatment that fully addresses the challenges of SCI continues to be elusive. It is becoming obvious as new research is published that these challenges will not be met by a single drug or material but rather by a strategy that combines many different approaches. As the field moves into more complex research and potential treatments, many specific details will need to be unraveled. For example, it is generally accepted that VEGF contributes to functional recovery, but the reported mechanism by which this is accomplished varies from an acute reduction in BSCB permeability [130] to reducing inflammation by activating autophagy [131]. This highlights our incomplete

understanding of CNS physiology following injury, which poses a large barrier to developing therapeutic strategies for complete functional regeneration.

As more sophisticated materials are developed and combined with different treatment strategies, it is important to focus on controlling the progression of secondary events. In addition to examining changes in functional outcomes, researchers should investigate how biomaterial-based treatments alter macrophage activation and inflammation, reduce oxidative stress, restore the BSCB, reduce glial scar inhibition, and guide neurite outgrowth (Figure 1). There are a variety of materials that address multiple areas of secondary damage, such as PPy, which can reduce inflammation in neural electrodes [67] and increase neurite outgrowth when coated onto PCL and PLGA [132, 133], or chitosan, which can reduce ROS and deliver immobilized growth factors [77, 134].

Interestingly, both ROS reduction and BSCB restoration appear to be under-investigated as targets for intervention. Even inert biomaterials can reduce ROS [22], and a simple thin film or a conduit could go yield significant benefit especially towards protecting the damaged spinal cord from the circulating immune system. As more combinatorial treatments move into animal models, analyses of ROS and BSCB integrity should be incorporated into associated outcome measures.

Additionally, researchers should investigate the role of clinically-relevant strategies, such as locomotor training. Doing so will more closely align laboratory research with clinical practice and give treatments more opportunity to succeed. These practices have been adapted to animal models [108][129][135], and their adoption will likely become more widespread.

Lastly, it is important to published so-called negative results, as done in ref [38]. As more strategies are combined, it is important to have some conception of their potential negative synergies. Additionally, what works in one injury model may not work in another. These data are relevant to all researchers and could affect the translation of different interventions.

Overall, the field is moving in a very promising direction; more focus on secondary events will increase the rate of progress towards the development of a truly effective, biomaterial-based strategy for regeneration following SCI.

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Figure 1.

Progression of secondary events. Various pathologies contribute to an environment where regeneration is difficult, each taking place over different periods of time. Acute: seconds to minutes, sub-acute: minutes to weeks, chronic: months to years [1]. Therapeutic interventions should target each of these pathologies simultaneously in order to maximize efficiency.

Figure 2.

M2 macrophages, which are typically downregulated following SCI, improve axon growth across an inhibitory CSPG gradient. Importantly, this effect is increased in combination with chondroitinase ABC (chABC), lending credibility to the idea of a combinatorial treatment (see section 5.1 for a discussion of chABC in conjunction with other therapies). Reproduced with permission from *Journal of Neuroscience* [43].

Figure 3. chABC and ISP ameliorate the effects of the glial scar and encourage axonal regeneration. A,B: Administering chABC alongside SDF-1 α showed that chABC was primarily responsible for the decrease in CSPGs, as can be seen from the staining: (A) = chABC, (B) = vehicle. Reproduced with permission from *Biomaterials* [36]. C,D: Administration of intracellular σ peptide to a contusion injury encouraged the growth of serotonergic neurons across the lesion (d) when compared to a vehicle control (c). Reproduced with permission from *Nature* [111].

Table 1

Summary of results using biomaterials to target key areas of secondary damage

Pathology	Treatment	Paraphrased Results
Inflammation	Minocycline-loaded PCL/PEG nanoparticles [12]	Reduced macrophage activation over 15 days post-injury
	Dextran sulfate-minocycline complex [13]	Reduced ED-1+ infiltration, modulated macrophage activation
	Flavopiridol-loaded PLGA nanoparticles [14]	Reduced astrocyte inflammatory factor synthesis/cavitation volume
	p38/JNK inhibitor-loaded PVA-PVP films [15]	Lowered active caspase-3 levels
	CSB-loaded liposomes [16]	Altered both pro and anti-inflammatory gene expression
	Partially reduced graphene oxide [17]	Reduced M1/increased M2 macrophages, promoted angiogenesis
	IL-10 via lentivirus from heparin-coated PLG nerve bridges [18]	Promoted M2 phenotype; improved functional outcomes
	Ferulic acid-glycol chitosan nanoparticles [19]	Decreased GFAP/ED-1 staining; improved functional outcomes
	MSCs encapsulated in polyurethane-based hydrogel [20]	Improved functional outcomes
	MSCs encapsulated in agarose/carbomer/PEG, RGD-functionalized hydrogel with impermeable film [21]	Increased M2 macrophages
Oxidative Stress	PEG injection into lesion [22, 23]	Inert material reduced cell membrane disruption
	Delivery of MP via carboxymethylchitosan/polyamidoamine dendrimers [24], PCL nanoparticles [25], or PLGA nanoparticles/agarose gel [26]	Improved functional outcomes
	PEG-decorated silica nanoparticles [27]	Reduced ROS and improved nerve conduction
	PEG-coated Au nanoparticles [28]	Improved functional outcomes
	Delivery of CAT [29] or CAT+SOD [30] via PLGA nanoparticles	Reduced ROS/inflammation
BSCB Breakdown	Oxygen-reactive polymer [31]	Reduced neuronal degradation, astrocyte reactivity, and microglial activation
	Delivery of VEGF from PLGA nanoparticles [32]	Improved histological and functional outcomes
	Delivery of VEGF, Ang-1, and bFGF [33]	Improved histological and functional outcomes
	Engineered vascular network in two-component scaffold and loading with endothelial + neural progenitor cells [34]	Increased endothelial barrier antigen staining
	Curcumin delivery via polyacetal carrier [35]	Improved functional outcomes
Glial Scar	chABC + SDF-1 α from PLGA nanoparticles in methylcellulose hydrogel [36]	SDF-1 α had little effect; chABC degraded CSPGs
	NEP1-40 + chABC from fibrin gel and lipid microtubes [37]	Reduced CSPGs/glia scar, increased axonal growth
	NEP1-40/chABC/GFs/pMNs [38]	GFs, pMNs+GFs, and NEP1-40/chABC reduced CSPGs; pMNs+NEP1-40/chABC and pMNs+GFs+NEP1-40/chABC increased CSPGs
	Growth-factor loaded PEAD-heparin coacervate [39]	Delivered Shh and observed a reduction in GFAP intensity at the injury epicenter