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Journal
Cells Tissues Organs, 202(1-2)

ISSN
1422-6405

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Publication Date
2016

DOI
10.1159/000446697

Peer reviewed

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Running Title: Spinal Cord Injury, Injectable Biomaterials, and Tissue Swelling

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Abstract

Spinal cord injury (SCI) is a devastating condition that leaves patients with limited motor and sensory function at and below the injury site, with little to no hope of meaningful recovery. Because of their ability to mimic multiple features of central nervous system (CNS) tissues, injectable hydrogels are being developed that can participate as therapeutic agents in reducing secondary injury and in regeneration of spinal cord tissue. Injectable biomaterials can provide a supportive substrate for tissue regeneration, deliver therapeutic factors, and regulate local tissue physiology. Recent reports of increasing intraspinal pressure after SCI suggest that this physiological change can contribute to injury expansion, also known as secondary injury. Hydrogels contain high water content similar to native tissue, and many hydrogels absorb water and swell after formation. In the case of injectable hydrogels for spinal cord, this process often occurs in or around the spinal cord tissue, and thus may affect intraspinal pressure. In the future, predictable swelling properties of hydrogels may be leveraged to control intraspinal pressure after injury. Here, we review the physiology of SCI, with special attention to current clinical and experimental literature, underscoring the importance of controlling intraspinal pressure after SCI. We then discuss how hydrogel fabrication, injection, and swelling can impact intraspinal pressure in the context of developing injectable biomaterials for SCI treatment.

I. Introduction

Spinal cord injury (SCI) currently affects 270,000 Americans with roughly 15,000 new injuries each year (Fehlings, Singh et al. 2014), resulting in partial or complete loss of neurological function at and below the level of injury. In addition to loss of muscle innervation and function, SCI compromises immune and endocrine function, necessitating clinical management as a chronic disease rather than a purely physical disability. Unfortunately, SCI is incurable and current treatments – which include anti-inflammatory steroids, spinal decompression of bony impingements, and physical rehabilitation – only marginally improve neurological recovery.

Biomaterial-based strategies have generated substantial excitement for SCI treatment (427 citations in *Pubmed* using keywords "biomaterials" and "spinal cord injury"), but implantable materials are less attractive for clinical translation than injectable systems because they require the removal of potentially spared tissue. This is in part due to the irregularly sized defect that occurs after injury to the spinal cord. Researchers are therefore developing injectable biomaterials that can be delivered into, and integrate with, the injured tissue to mediate repair. Biomaterials can be custom designed to reduce inflammation, aid in re-sealing of the blood-brain barrier and/or promote regeneration of spinal cord tissue through incorporation of bio-responsive elements such as cell adhesion peptides. In addition, biomaterials can be used as localized delivery vehicles for biological therapeutics, including genes, proteins, drugs and even stem cells. The ability to incorporate multiple factors within similar delivery vehicles provides potential for complex combinatorial therapies to address multiple barriers to repair that exist after SCI. Localized delivery is particularly important for applications in the spinal cord, as systemically delivered therapeutics are typically inefficient at crossing the blood-brain barrier and/or glial scar to reach tissue in the central nervous system (CNS). Readers are referred to the following excellent articles for more general reviews of biomaterial-based strategies for SCI treatment (Potter, Kalil et al. 2008, Cui, Webber et al. 2010, Rubert Perez, Stephanopoulos et al. 2015).

Here, we focus on recent developments in injectable hydrogels and specifically how these materials may affect secondary injury by influencing local tissue swelling. Although initial studies have demonstrated promise, very little is known about how injectable materials contribute to the swelling and pressure at the injection site, and integrate with the native tissue. Despite these unknowns, there are common standards for injection volume and rate that are thought to cause minimal damage to the spinal cord, at least in experimental animal models (Baumann, Kang et al. 2009, Patel, Joseph et al. 2010, Fehlings, Singh et al. 2014). Here, we review recent developments in injectable materials for SCI with a focus on the importance of maintaining physiological tissue pressure. Along these lines, special attention is paid to hydrogel swelling and mesh size as we discuss individual biomaterials. In the following section, we first examine current knowledge of intraspinal pressure build-up after traumatic SCI (*see* **Figure 1**), and its potential contribution to the development of secondary damage after SCI.

II. Pressure-induced damage to spinal cord after the primary injury

The spinal cord is rarely severed by the initial traumatic insult, and experimental studies demonstrate that primary gross tissue damage is remarkably limited in most cases, where

although hemorrhage is seen in the central gray matter, the surrounding white matter is morphologically intact within the first hour following injury (Balentine 1978, Noble and Wrathall 1989). However, the primary insult is followed by a cascade of events known as secondary injury, which include release of free radicals and excitatory neurotransmitters, inflammation, edema, and ischemia that results in widespread death of cells that survived the primary injury. Limiting secondary damage to the spinal cord tissue continues to be an active area of research for improving functional outcomes in patients with SCI. Currently, clinical treatments for acute SCI, including steroids (Kuchner, Hansebout et al. 2000) and hypothermia (Romodanov, Mikhailovskii et al. 1979, Janssen and Hansebout 1989, Alkabie and Boileau 2016), seek to prevent or mitigate secondary injury by reducing inflammation and, indirectly, edema. For example, a recent review study reported evidence that localized and systemic hypothermia reduces many of events associated with the secondary damage after SCI, such as ischemia, oxidative stress, apoptosis, inflammation, and edema (Alkabie and Boileau 2016).

It has long been recognized that immediately after injury to the brain or spinal cord, there is a significant amount of swelling within the local tissue. Indeed, for patients with traumatic brain injury (TBI), there are specific clinical guidelines for intensive monitoring of brain tissue pressure combined with pharmacological and surgical interventions with the goal of maintaining adequate brain tissue perfusion and oxygenation to minimize secondary injury (Brain Trauma, American Association of Neurological et al. 2007). This treatment paradigm has resulted in significant reduction of mortality and morbidity associated with TBI and is attributed to the significant improvements in clinical outcomes for these patients (Marshall, Smith et al. 1979, Miller, Butterworth et al. 1981). A few recent studies have suggested that increases in tissue pressure after SCI are associated with negative functional outcomes (Phang, Werndle et al. 2015, Varsos, Werndle et al. 2015) (**Figure 1A**), and surgical alleviation of this pressure improves functional outcomes in experimental animals (Yang, Li et al. 2013) and in patients (Phang, Werndle et al. 2015). However, currently there are no clear clinical guidelines for monitoring and managing intraspinal pressure after SCI. Experimental data from our lab using a rodent model of SCI in the thoracic cord showed that immediately after a moderate contusive injury, there was a significant increase in intraspinal pressure (up to five times the normal intraspinal pressure; 1.8 ± 0.4 mmHg [~240 Pa] vs. 8.9 ± 1.1 mmHg [~1200 Pa]) (**Figure 1B**) (Khaing, Cates et al. 2016). In this set of experiments, a highly sensitive micropressure probe (300 μm Milar® probe) was inserted directly into the spinal cord to obtain accurate tissue pressure recording at 1kHz. Hence, as we develop new therapies using injectable biomaterials for implantation into the spinal cord, swelling of these materials *in vivo* is an important consideration.

Edema, abnormalities in the location and amount of water, is associated with many pathological conditions of the CNS including traumatic SCI, TBI, and stroke (Simard, Woo et al. 2012, Kurland, Tosun et al. 2013). Edema leads to an increase in local pressure, potentially causing tissue ischemia and eventually cell death (Simard, Woo et al. 2012, Kurland, Tosun et al. 2013). Aquaporins (AQPs), a family of transmembrane proteins that serve as channels for water, are expressed throughout the brain and spinal cord (Manley, Binder et al. 2004, Bradl and Lassmann 2008, Benfenati and Ferroni 2010, Hinson, McKeon et al. 2010, MacAulay and Zeuthen 2010, Saadoun and Papadopoulos 2010, Verkman, Ratelade et al. 2011, Brosnan and Raine 2013, Delgado, Maldonado et al. 2014, Freitas and Guimaraes 2015). Anatomically,

AQP4 – the most abundant AQP in the spinal cord (Shibuya, Hara et al. 2008) – is situated to be a key player in pathological edema. AQP4 is highly expressed in astrocytic end feet in direct contact with blood vessels (Frigeri, Gropper et al. 1995, Nielsen, Nagelhus et al. 1997) and osmoregulatory centers of the brain including the hypothalamic magnocellular nuclei (Jung, Bhat et al. 1994, Nielsen, King et al. 1997). Hence, AQP biology is likely to be important for maintaining fluid homeostasis in the CNS. Interestingly, several recent reports have found that decreased AQP4 function correlates with improved functional recovery after TBI (Fukuda, Adami et al. 2013, Dardiotis, Paterakis et al. 2014), and SCI (Kimura, Hsu et al. 2010, Jing, Wu et al. 2014, Sato, Callegaro et al. 2014, Wu, Zhang et al. 2014, Hu, Li et al. 2015), suggesting that edema associated with injury to the CNS is likely to be mediated in part by AQP receptors. Despite these discoveries, the cellular mechanisms of swelling-induced damage in the CNS remain elusive.

III. Design Considerations for Hydrogels Promoting SCI Repair

Several hydrogel properties are considered relevant to their application to SCI treatment, particularly in injectable systems, including injection method, injection location, mechanical properties of hydrogel materials before and after *in situ*-crosslinking, and hydrogel swelling. One of these is gelation properties which must be compatible with the injection conditions necessary to avoid additional injury to the cord tissue: a syringe capable of delivering small volumes at a controlled rate equipped with a small needle. Specific sizes depend upon the injury model used with as low as 33G needles for rodent studies (Gupta, Tator et al. 2006, Sontag, Uchida et al. 2014, Führmann, Obermeyer et al. 2015), and up to as large as 23G in human clinical trials (Oraee-Yazdani, Hafizi et al. 2015, Shin, Kim et al. 2015) have been utilized. These requirements dictate that the precursor must be liquid or highly deformable.

For biomaterial applications *in vivo*, it will be critical to determine optimal therapeutic volume that may be added into the spinal cord without causing tissue damage. This threshold for injection volume may depend on the location of injection and spinal cord tissue blood perfusion in addition to intraspinal tissue pressure. Limiting the local tissue blood perfusion is associated with negative functional outcomes after traumatic SCI (Hall and Wolf 1987, Alshareef, Krishna et al. 2014). Conventionally, it was thought that the anatomy of the spinal column allows for substantial room for tissue swelling, and therefore standard clinical guidelines for examining and maintaining intraspinal pressure after injury do not yet exist. Despite a consistent lack of consideration for relieving increases in tissue pressure after SCI, recent data suggest that in a large population of SCI patients (84%), there is significant intraspinal swelling and rise in pressure after injury that results in negative clinical outcomes (Werndle, Saadoun et al. 2014). Therefore, swelling of biomaterials exogenously applied to the spinal cord is likely to be key determinant of therapeutic success.

While it is generally assumed that injection of large volumes into the spinal cord will cause damage to the tissue, there is minimal research to quantitatively determine the range of acceptable injection volumes. There are, however, common accepted standards for volumes of cell solutions or hydrogels injected per site of 1-2 μL into the mouse cord (Oraee-Yazdani, Hafizi et al. 2015, Shin, Kim et al. 2015) or 5-10 μL in the rat spinal cord (Yang, Song et al. 2009, Lu, Wang et al. 2012, Song, Song et al. 2012, Führmann, Obermeyer et al. 2015). Complicating the

situation, the acceptable range for injection volumes is likely specific to the unique swelling properties of each hydrogel formulation. The pressure exerted by a highly swelling hydrogel can potentially exacerbate the already increased intraspinal pressure after acute SCI, resulting in the loss of tissue perfusion and can further damage the surrounding soft tissue. However, at this time, we are not aware of any studies in which local tissue pressure is determined after biomaterial application *in vivo*.

Given the various volumes that different injection sites can accommodate, injection location is an important consideration. Normally, the spinal cord is freely floating within the spinal column, and therefore materials injected into the subdural space are likely to exert less physical compression and be better tolerated. However, this decision will depend on the therapeutic goal of the hydrogel. For instance, as a scaffold for axonal growth and/or cell transplantation, hydrogels may be injected directly into the spinal cord parenchyma (or into the cyst after SCI), while hydrogels designed for drug delivery or osmotic transport to relieve swelling may be more effective when injected into the subarachnoid (also known as "intrathecal") space (see **Figure 2B**). In humans, the cross-sectional area of the spinal cord tissue at the cervical level is about 120 mm^2 at its largest region (Sherman, Nassaux et al. 1990). The subarachnoid space, between the dural and the pial membranes (see **Figure 2**), is approximately 5.5 mm in the cervical spine (Ulbrich, Schraner et al. 2014) and about 7.6 mm in the thoracic spine (Gellad, Rao et al. 1983). Therefore, there is some room for swelling within human spinal cord before creating significant increase in tissue pressure. However, in rats, the most commonly used experimental animal model of SCI, the largest area of the spinal cord is only about 28 mm^2 and the subarachnoid space is less than 1 mm. Therefore, there is limited space available for application of therapeutic materials. **Table 1** summarizes recently developed injectable biomaterials that are being tested in pre-clinical models. Note that the list includes materials that swell up to 50% more than its original volume. While, intraspinal pressure measurements have not directly been recorded after *in vivo* application and swelling of hydrogels, we expect that significant swelling within the spinal cord parenchyma will likely be detrimental to the surrounding soft spinal tissue. Although little systematic research has been done regarding this issue, there are instances in the clinic where exogenously applied biomaterials have caused negative neurological outcomes (*see complications associated with DuraSeal® below*). In future studies, it will be important to determine the effects of swelling on local tissue pressure once *in vivo*.

As edema is one of the common features after injury to the CNS (both SCI and TBI), and is thought to be associated with negative functional outcomes. It is intriguing to explore the use of hydrogels that can absorb excess liquid locally and potentially restore fluid homeostasis after CNS injury. A recent report by McBride et al., 2014 utilizes an osmotic transport device (OTD) containing hollow-fibers – filled with an osmotic agent that will create a chemical potential pressure for water – that are embedded within a highly swelling hydrogel material that can locally draw out excess water from the underlying brain tissue (McBride, Szu et al. 2014). The authors showed that application of the OTD after TBI reduced brain edema better than craniotomy alone. A limitation of this study is that the authors did not examine the local intracranial pressure after the excess water was reduced using the OTD and whether this resulted in neurological outcomes. For application in the spinal cord, this type of fluid absorbing

(i.e., swelling) hydrogels should only be applied to injection locations (e.g., intrathecal space instead of directly into the spinal cord) in which the swelling of the material would have limited negative impact on the surrounding tissue.

It is well known that cells sense and respond to their mechanical environment, which regulate a wide range of biological processes. Hydrogels exhibiting elastic moduli within the range of healthy CNS tissues – roughly below 1kPa by rheological measurements or below 10kPa by linear compressive measurements – better support neuronal cell survival and functional phenotype than stiffer substrates (Georges, Miller et al. 2006, Leipzig and Shoichet 2009, Seidlits, Khaing et al. 2010, Man, Davis et al. 2011). Implantation of hydrogels exhibiting compressive moduli similar to native spinal cord (~2-4 kPa) into rodent spinal cords after acute injury has been reported to reduce numbers of inflammatory cells and glial scar deposition (Bakshi, Fisher et al. 2004, Khaing, Milman et al. 2011). *In vitro* studies have shown that hydrogel scaffold mechanics greatly influence neurite outgrowth – a necessary process for spinal cord regeneration – where maximum neurite migration occurs on the softest substrate evaluated (typical compressive moduli of 0.5-5 kPa) (Balgude, Yu et al. 2001, Flanagan, Ju et al. 2002, Willits and Skornia 2004, Gunn, Turner et al. 2005, Georges, Miller et al. 2006, Lampe, Mooney et al. 2010, Man, Davis et al. 2011). It is important to note that in the majority of studies reporting effects of mechanical cues on CNS biology, biomaterial mechanics and swelling were not decoupled. Beyond elastic modulus, the viscous properties of injectable hydrogels should be considered, as they will determine ease of injection through a small-gauge needle and the extent that hydrogel precursors diffuse from the injection site prior to gelation. Shear-thinning materials, like HA, are particularly appealing for their ability to relax in response to increased applied pressures, acting to protect encapsulated therapeutic agents from shear-induced damage (Aguado, Mulyasasmita et al. 2011) and perhaps minimize pressures exerted during swelling after crosslinking *in situ*.

For *in vivo* delivery to the spinal cord, injectable hydrogels must be designed to balance the requirement for soft materials that mimic native tissue with that for controlled swelling. Studies in rodents have suggested that providing physical support via biomaterials in the acute phase after SCI may prevent cyst formation and reduce glial scar deposition (Prang, Müller et al. 2006, Yang, Laporte et al. 2009, Tuinstra, Margul et al. 2013, Pawar, Prang et al. 2015). In the case of hydrogel biomaterials, this support may be provided by the mechanical strength or swelling pressure exerted on the surrounding tissue to hold the implant in position and form a tight interface with the host tissue. Given these requirements, independent control of hydrogel swelling pressure and mechanics will be necessary to provide both good apposition to host tissue and appropriate biological cues for CNS cell function, respectively. Although a few methods have been developed recently that at least partially decouple scaffold mechanics, swelling and mesh size (Jia, Yeo et al. 2006, Lampe, Mooney et al. 2010, Browning, Wilems et al. 2011, Bearat, Lee et al. 2012, Griffin, Weaver et al. 2015, Schweller and West 2015), further advancements to expand the range over which these properties can be independently controlled will likely be required for applications in CNS tissues, which are very soft yet highly sensitive to changes in tissue pressure.

IV. Hydrogel Swelling and Clinical Considerations

Swelling properties of hydrogels have not received sufficient attention within the context of biomaterial-based treatment strategies for SCI. Swelling properties can vary significantly based on hydrogel composition and crosslinking technique. Highly hydrophilic polymers, especially those with charged backbones, can swell more than twice their size after formation (e.g., polyethylene glycol (PEG) and hyaluronic acid (HA) hydrogels), whereas protein-based polymers (e.g., collagen and fibrin hydrogels) tend to swell minimally (**Figure 3A**). Indeed, when we examined the pressure exerted during gel swelling, 2% PEG, 1% HA-PEG and collagen-laminin-HA hydrogels showed physiologically compatible pressures (<1000Pa, **Figure 3B**). As stated above, normal spinal cord tissue pressure is ~240 Pa, but approached ~1200Pa after SCI. Therefore, swelling pressures asserted by both 2% HA-PEG (> 2000Pa) and 4% PEG gels (>10,000Pa) are not likely to be optimal for *in vivo* transplantations and/or injections. While most researchers use volumetric or mass swelling ratios (as in **Figure 3A**) to assess whether swelling of an injectable material is appropriate for use *in vivo*, our data show that this measure is not necessarily quantitatively predictive of pressure exerted during swelling (**Figure 3B**). For example, while 1% HA-PEG and 2% PEG hydrogels have similar equilibrium swelling ratios (~30% increase in mass), the peak pressure exerted by 2% PEG hydrogels is approximately double that for 1% HA-PEG hydrogels. In addition, hydrogels containing less than 1% HA, including collagen-HA-laminin and 1% HA-PEG hydrogels, show an initial increase to peak pressures during the first several minutes of swelling and then rapidly decrease. As HA is a highly shear thinning polymer, we posit that HA-containing hydrogels may be able to relax after initial swelling, thus reducing pressures exerted by the material over time. Therefore, direct measurements of local spinal tissue pressure before and after hydrogel application *in vivo* are needed to confirm these *ex vivo* observations.

Some researchers have examined the swelling properties of hydrogels in experimental settings (in pre-clinical models) for applications within the CNS (Baumann, Kang et al. 2009, Bjugstad, Lampe et al. 2010, Kubinova, Horak et al. 2011, Neuman, Radcliff et al. 2012). Indeed, Bjugstad et al., implanted a strand of photocrosslinked PEG gel that were pre-formed, but not pre-swollen into the ventral midbrain (Bjugstad, Lampe et al. 2010). They found that the application of this hydrogel into the CNS parenchyma resulted in 1) tissue “vacancy” increase (5 -20% likely due to swelling) after hydrogel application, 2) *in vitro* swelling behavior of the gels did not match that of the ones implanted *in vivo*, and finally 3) the defect size continued to get smaller overtime in fast degrading gels, presumably due to sufficient infiltration of host cells as the hydrogel degraded. Interestingly, the authors also found that the magnitude of the acute microglia response and the long-term astrocytic scarring response were attenuated when the PEG-based hydrogels were used compared to control animals (Bjugstad, Lampe et al. 2010). In future studies, it will be important to determine the acceptable swelling range for implanted material within the CNS tissue, and to determine the effects of hydrogel swelling on host tissue response.

DuraSeal®, an FDA-approved injectable hydrogel formulation currently in clinical use to help seal the dura, is an example of a biomaterial where its swelling properties have limited its utility. The contraindications for this product suggest that this hydrogel may swell up to 50% of its original size in any dimension –common for hydrogels in development for clinical use (*see* **Table 1**) – therefore warnings exist not to use this hydrogel system in confined structure where nerves are present. This warning should be heeded since a number of clinical cases have been

reported where application of DuraSeal® has subsequently caused neurological symptoms in patients (Mulder, Crosier et al. 2009, Neuman, Radcliff et al. 2012, Lee, Park et al. 2013). Swelling of the DuraSeal® hydrogels at the site of application had adverse effects in the higher cervical level where there is less space between the bony vertebra and the spinal cord parenchyma (Lee, Park et al. 2013), as well as in the cauda equina where there is a relatively larger space between the vertebra and the spinal cord (Mulder, Crosier et al. 2009, Neuman, Radcliff et al. 2012). It is also important to consider that the application of hydrogel material within the subdural space (as mentioned above the spinal cord is freely floating within the spinal column) can migrate away from the site of application, so that the adverse effects may be detected at distant sites (Mulder, Crosier et al. 2009).

In light of substantial data on the importance of maintaining normal intraspinal pressure – combined with these clinical failures associated with hydrogel swelling – it is imperative that swelling be one of the key features to be examined extensively before testing biomaterial applications *in vivo,* and once applied *in vivo*. In the following sections, we will discuss a number of recently developed hydrogels for applications in the CNS with a focus on swelling properties.

V. Fabrication Strategies for Injectable Hydrogels

Although some “shape-memory” or “self-healing” injectable materials have been developed (Bencherif, Sands et al. 2012, Koshy, Ferrante et al. 2014, Hsieh, Tseng et al. 2015), fabrication strategies are typically chosen so that gelation occurs in the aqueous CNS environment only after injection and penetration throughout the tissue defect, creating an intimate interface between injected materials and the healthy tissue that facilitates cell migration and regeneration. Commonly used conditions to trigger *in situ* crosslinking include 1) exposure to a specific wavelength of light (Piantino, Burdick et al. 2006, Khaing, Milman et al. 2011), temperature (Gupta, Tator et al. 2006, Bearat, Lee et al. 2012), or pH (Prang, Müller et al. 2006, Pawar, Prang et al. 2015) and 2) combining two separate components that spontaneously react upon mixing (Johnson, Parker et al. 2010, Nimmo, Owen et al. 2011, Liang, Walczak et al. 2013, Führmann, Obermeyer et al. 2015, Kim, Kong et al. 2016). *In situ* crosslinking of hydrogels in response to various triggers has been achieved by covalent chemistries (e.g., chain-growth polymerization, step-growth polymerization, Michael addition, Diels-Alder cycloaddition, enzymatic) (Johnson, Parker et al. 2010, Liang, Walczak et al. 2013, Führmann, Obermeyer et al. 2015), physical means (e.g., electrostatic and/or hydrophobic interactions) (Gupta, Tator et al. 2006, Piantino, Burdick et al. 2006) or a combination of both (Bearat, Lee et al. 2012, Pakulska, Vulic et al. 2015). Many of these strategies have been used to form hydrogels *in situ* within the spinal cord with good biocompatibility (**Table 1**).

In all of these strategies, common methods are used to control the maximum degree of hydrogel swelling *in situ* (**Figure 4**). Increasing macromer concentration and/or crosslinking density will decrease swelling, but at the expense of increased mechanical strength and decreased permeability which may have adverse effects. Similarly, addition of hydrophilic and/or electrostatically charged moieties will increase water uptake, at the expense of increased network permeability and possible effects on hydrogel mechanics. Shape-memory (Koshy, Ferrante et al. 2014) and microparticle-based (Jia, Yeo et al. 2006, Griffin, Weaver et al. 2015) hydrogels have the important advantage of being pre-swollen prior to *in vivo* delivery. However,

at least in the case of shape-memory materials, reversion to a larger size after injection may cause tissue damage similar to pressures exerted during *in situ* swelling. Alternatively, maximal swelling may be advantageous for hydrogels applied to the subdural space, where they could act to siphon off the excess fluid in the parenchyma that accumulates after SCI to prevent secondary damage, as recently demonstrated in TBI (McBride, Szu et al. 2014). In either case, better strategies to improve control over hydrogel swelling by decoupling this property from other network properties are needed (Browning, Wilems et al. 2011, Bearat, Lee et al. 2012, Shih and Lin 2012).

Non-covalent gelation strategies include materials designed to undergo a phase transition in physiological conditions (often temperature- or pH-induced) such that network formation occurs through physical association of macromers through hydrophobic/hydrophilic or electrostatic interactions. Such gelation methods based on phase transition are highly biocompatible, as they eliminate the need for non-biological chemical reagents. For example, thixotropic, hyaluronic acid-methyl cellulose (HAMC) composite materials form through phase separation at physiological temperature – where hydrophobic regions associate to yield physical crosslinks – after injection into the spinal cord (**Table 1**) (Gupta, Tator et al. 2006, Tam, Cooke et al. 2012, Caicco, Zahir et al. 2013). These hydrogels have been reported to swell to about 50% of their original mass and *in vivo* (intraspinal and intrathecal) applications of this type of gels appears to be tolerated, although intraspinal pressures were not measured after material injection. It is interesting to note that the resulting networks are fundamentally more inhomogeneous than step-growth polymerized hydrogels, containing hydrophobic “pockets” throughout. Other non-covalent crosslinking include calcium-mediated crosslinking of alginate hydrogels (Prang, Muller et al. 2006, Ansorena, De Berdt et al. 2013, des Rieux, De Berdt et al. 2014, Grulova, Slovinska et al. 2015, Gunther, Weidner et al. 2015, Pawar, Prang et al. 2015) and self-assembly of amphiphilic polymers (Silva, Czeisler et al. 2004, Tysseling-Mattiace, Sahni et al. 2008, Song, Song et al. 2012, Yang, Li et al. 2013), with both types of hydrogels reporting swelling ratios of about 40%. Self-assembled hydrogels are often fabricated from designer polypeptides, which provide a high degree of control over network assembly, as each amino acid is user-specified.

Photocrosslinking techniques, including chain-growth and step-growth polymerization, typically involve addition of an initiator that is activated in response to the crosslinking trigger (e.g., light or heat) and induce covalent bonding between polymer precursors. While chain-growth methods (such as acrylate-mediated crosslinking) have been widely investigated, more recently step-growth polymerization methods have gained popularity based on their ability to create better-defined networks with predictable properties (e.g., swelling, mechanics) (Azagarsamy and Anseth 2013, Tibbitt, Kloxin et al. 2013). Step-growth photo-polymerization typically involves “click” chemistry reagents (such as the norbornene-thiol reaction (Shih and Lin 2012, Gramlich, Kim et al. 2013). As step-growth polymerization is less susceptible to oxygen inhibition, it results in faster forming hydrogels with higher degrees of crosslinking. However, swelling ratios still depend on total macromer content, indicating that substantial network non-idealities still occur (Shih and Lin 2012). Photocrosslinking techniques to develop injectable hydrogels for SCI therapy have been limited due to the difficulty of inducing gelation after injection; exposure of the injured spinal cord to radiation and free radicals post-injury also has the potential to induce further inflammation and damage. However, implantation of pre-formed, pre-swollen,

photocrosslinked HA-based hydrogels after acute spinal cord injury resulted in decreased scar formation (Khaing, Milman et al. 2011). Although preformed gels have limitations in their ability to conform to the irregular defect shape, they can be advantageous in that they can be pre-swollen prior to implantation while incorporating architectures to promote cell infiltration and axon guidance (Prang, Muller et al. 2006, Gros, Sakamoto et al. 2010, Griffin, Weaver et al. 2015, Pawar, Prang et al. 2015).

Non-photocrosslinking covalent strategies avoid the need for potentially cytotoxic initiators – previously used methods to fabricate injectable hydrogels include "click" chemistries (Führmann, Obermeyer et al. 2015) and enzymatic reactions (Johnson, Parker et al. 2010, King, Alovskaya et al. 2010, Teixeira, Feijen et al. 2012). The most widely used enzymatic strategies employ thrombin to crosslink fibrin hydrogels, exploiting the natural blood clotting process (Johnson, Parker et al. 2010, Lu, Wang et al. 2012, Sharp, Dickson et al. 2012, Griffin, Weaver et al. 2015). Fibrin hydrogels can have linear compressive modulus of <1000Pa, similar to native spinal cord tissue, and relatively low swelling ratios (<20%) (**Table 1**). Non-photo-induced "click" reactions (e.g., Diels-Alder type) also proceed by step-growth polymerization, enabling more controlled network formation (Hiemstra, van der Aa et al. 2007, Nimmo, Owen et al. 2011, Shih and Lin 2012, Azagarsamy and Anseth 2013, Tibbitt, Kloxin et al. 2013). A recent paper by Fürhmann et al., used HA-based hydrogels produced using a non-photo-induced click chemistry. They report that these hydrogels swell to 40-60% of their original mass and did not cause any damage to the spinal cord after intrathecal injection and *in situ* formation (Führmann, Obermeyer et al. 2015). Both the location of injection and rate of swelling likely affected this favorable outcome. First, the intrathecal site can accommodate larger injection volumes. Second, the swelling rate after hydrogel formation is relatively slow (~20% within the first 12 hours, an additional 20% over the next 12 hours, and a final 20% by 96 hours after injection), which translates to lower pressures exerted on the surrounding tissue.

Multi-arm polyethylene glycol (PEG) macromers end-capped with click reagents have been widely explored as *in situ*-forming hydrogels. Using this strategy, the network properties (including swelling and mechanics) can be controlled by the molecular weight of the PEG arms and the stoichiometry of click functionalities (Elbert and Hubbell 2001, Lutolf and Hubbell 2003, Shikanov, Smith et al. 2011, Kim, Kong et al. 2016). Recent reports indicate that increasing numbers of PEG arms (e.g., 4-arm vs. 8-arm PEG macromers) increases gelation rate, reduces swelling ratios by ~40%, and improves overall predictability and reproducibility of hydrogel networks (Kim, Kong et al. 2016). This reduction of variability is particularly important for injection into the spinal cord, where a relatively narrow range of mechanical and/or swelling properties may be required to create good apposition to host tissue and prevent swelling-induced damage.

Gelation time is a particularly important consideration for non-photo-induced crosslinking methods, as crosslinking is initiated upon first contact with the spinal cord environment. This time must be slow enough first, not to clog the injection needle, and second, to allow for sufficient contact with spared spinal cord tissue prior to gelation. However, gelation time should be fast enough that the hydrogel and any embedded therapeutic agents (e.g., cells, drugs) remain at the local injection site (Tam, Cooke et al. 2012). Previous investigations have reported good tissue apposition with gelation times within approximately 30 minutes (Liang, Walczak et

al. 2013). However, more work needs to be done to identify optimum gelation times for different *in situ*-forming, injectable hydrogels. Recently, Kim et al. demonstrated that faster network formation correlates to decreased swelling ratios *in vitro*, independently of total macromer content (Kim, Kong et al. 2016). However, it remains unclear how gelation time affects pressure exerted during swelling *in vivo*. In general, photocrosslinking strategies permit better user control as hydrogels form only after exposure to an external light source. Thus, photo-gelation times are often relatively fast (e.g., 30 s to 3 min). In general, faster times are desired in this case to prevent adverse effects from the photo-initiators.

VI. Bioactive Hydrogels

Ultimately, researchers are developing hydrogels that are able to interact with cells of the spinal cord at a biological level to actively promote tissue regeneration. Although synthetic strategies typically provide better control over their physical properties, naturally derived materials (e.g. HA, alginate, agarose, collagen, fibrin) have been widely investigated for their ability to offer a biocompatible, recognizable interface with which host cells can interact. Natural materials derived from the native spinal cord tissue (e.g., HA, laminin and fibrin) can interact directly with cell surface receptors – leveraging biologically encoded signals to induce regeneration. King *et al*., compared viscous fibronectin, and collagen I, fibrin, and fibrin/fibronectin hydrogel implants after SCI in rodents and found that while all gels interfaced well with the spinal cord, fibrin best supported axon extension with fibronectin addition increasing this effect (King, Alovskaya et al. 2010). After injury, a provisional matrix, composed of fibrin and fibronectin, forms as a support scaffold that cells can infiltrate and remodel into repaired tissue. While not abundant in the uninjured spinal cord, a fibrin/fibronectin matrix quickly forms after SCI and may promote wound healing through similar mechanisms as in peripheral tissues. One concern with animal-derived sources of proteins with poorly conserved homology is that the biological response in humans may depend on the source. For example, salmon fibrin has been reported to more effectively support neurite outgrowth and SCI recovery in rodents than bovine or human fibrin (Ju, Janmey et al. 2007, Sharp, Dickson et al. 2012). Hydrogels fabricated from HA, the dominant extracellular matrix (ECM) component in the spinal cord and a polysaccharide with very low inter-species variability, has also shown great promise for SCI treatment. Both covalently (Khaing, Milman et al. 2011, Führmann, Obermeyer et al. 2015) and physically (Gupta, Tator et al. 2006, Austin, Kang et al. 2012, Caicco, Zahir et al. 2013) crosslinked HA hydrogels reduce inflammation and glial scar formation after SCI.

Synthetic hydrogels (e.g., PEG, pHEMA) are highly resistant to protein adsorption and generally do not support cell adhesion, providing a “blank slate” to which adhesive and other bioactive factors can be added with a high degree of user control. Many researchers have conjugated short, cell-adhesive peptides derived from the native ECM to synthetic hydrogels. Although these short peptide derivatives may have less potent bioactivity than their full proteins of origin, they can still support robust, cell receptor-specific adhesion (Silva, Czeisler et al. 2004, Tysseling-Mattiace, Sahni et al. 2008, Tam, Cooke et al. 2012, Lam, Carmichael et al. 2014). Furthermore, the density and presentation (spatial orientation) of peptides within the hydrogel network can be precisely defined. The most commonly used of these adhesive peptides is the integrin-binding RGD sequence – found in many ECM proteins. Such hybrid hydrogels –

combining synthetic polymers with naturally derived biomolecules – can provide biologically recognizable cues with a high degree of user control over biomaterial properties.

Recent developments in designer protein or peptide-based hydrogels have attempted to achieve the consistency of purely synthetic hydrogels while retaining beneficial signaling aspects of natural hydrogels. This includes self-assembling or crosslinked polypeptides developed from di-block copolypeptides or elastin-like polypeptides (Wong Po Foo, Lee et al. 2009, Yang, Song et al. 2009, Cai, Dinh et al. 2014, Wang, Zheng et al. 2015). Such hydrogels can easily incorporate a variety of signaling peptide sequences, generally derived from proteins including laminin, fibronectin, or neural cell adhesion molecule (NCAM). For SCI applications, laminin I-derived peptides, including those containing YIGSR or IKVAV, have shown potential to improve neural cell adhesion and neurite extension. Peptide amphiphiles containing the IKVAV sequence have been synthesized and developed to self-assemble into nanofiber-based hydrogels after injection into the CNS (Silva, Czeisler et al. 2004, Tysseling-Mattiace, Sahni et al. 2008). In a rodent model of SCI, IKVAV-containing nanofibers reduced glial scar formation, increased cell survival, and promoted regeneration of both descending motor and ascending sensory tracts (Tysseling-Mattiace, Sahni et al. 2008). *In vitro*, hydrogels bearing a specific combination of RGD, YIGSR and IKVAV maximized neuronal differentiation of cultured neural stem cells (Lam, Carmichael et al. 2014). However, more research is needed to characterize the relative and combinatorial effects of these peptides on regeneration after SCI.

VII. Delivery of Therapeutic Agents

Hydrogels can also be used as delivery vehicles for other therapeutic agents, including proteins, small molecule drugs, genes and live cell transplants. Swelling or changes in mesh size can have significant effects on 1) the dynamics of release of bioactive factors from hydrogels and 2) cell infiltration into hydrogels, which is a pre-requisite for effective gene delivery. One strategy is to incorporate chemoattractive and/or neurotrophic growth factors (e.g., glial-derived growth factor, sonic hedgehog, brain-derived growth factor, neurotrophin-3) that improve cell survival and actively promote axon migration into the hydrogel implant (Piantino, Burdick et al. 2006, Johnson, Tatara et al. 2010, Lampe, Mooney et al. 2010, Lowry, Goderie et al. 2012, Ansorena, De Berdt et al. 2013, Führmann, Obermeyer et al. 2015). Release and bioactivity of these factors is often prolonged through encapsulation in biodegradable, polymeric microparticles embedded within injectable hydrogels (Lampe, Kern et al. 2011, Lowry, Goderie et al. 2012, Ansorena, De Berdt et al. 2013). A similar microparticle-based approach has been used to deliver chondroitinase ABC, an enzyme that degrades the glial scar (Wilems and Sakiyama-Elbert 2015). Alternatively, conjugation of heparin, which acts to locally tether growth factors and prolong their release, to fibrin hydrogels has been successfully employed in rodent SCI (Johnson, Tatara et al. 2010). This approach is interesting since the release of the payload is not dependent on the degradation of the hydrogel scaffold, although the diffusion rate is still dependent on the mesh size of the hydrogel. Similarly, chondroitinase ABC has been delivered from methyl-cellulose hydrogels through affinity release (Pakulska, Vulic et al. 2013). Small molecule drugs, such as the steroid methylprednisolone, can also be delivered via hydrogel-embedded microparticles (Kim, Caldwell et al. 2009, Browne and Pandit 2015). In both strategies, swelling directly affects release profiles, where increased water uptake and larger mesh size result in faster diffusion out of the hydrogel.

Alternatively, genes encoding bioactive proteins can be delivered to the spinal cord using viral and non-viral carriers tethered to hydrogel scaffolds (Seidlits, Gower et al. 2013, Walthers and Seidlits 2015). Gene delivery allows for continued production of bioactive factors by transduced/transfected cells *in situ* without repeated invasive administration. For example, single-dose delivery of chondroitinase ABC-encoding lentivirus after contusive SCI results in significant functional improvement (James, Shea et al. 2015). Delivery of genes encoding other therapeutic biomolecules, such as growth factors and anti-inflammatory cytokines, has also been reported to enhance spinal cord regeneration (Tuinstra, Aviles et al. 2012, Thomas, Seidlits et al. 2014). Efficient, biomaterial-mediated delivery of genes is less dependent on hydrogel swelling than delivery of proteins or other small molecules. Instead, delivery efficiency is dictated by the number of cells that have physically encountered genetic vectors, which is directly affected by the cells' ability to adhere to and migrate through gene-loaded hydrogel implants (Aviles and Shea 2011, Shepard, Virani et al. 2012). Cell infiltration into hydrogels is directly related to material mesh size and swelling – where larger mesh size and higher degrees of swelling typically result in increased numbers of infiltrating cells.

Finally, hydrogels can be used as injectable carriers for therapeutic cell transplants, including neural and embryonic stem cells (Lu, Wang et al. 2012, Liang, Walczak et al. 2013, Mothe, Tam et al. 2013, Shrestha, Coykendall et al. 2014, Assuncao-Silva, Gomes et al. 2015). Alternatively, engineered cells producing specific regenerative factors can be delivered without the need for repeated administration, as would be required for direct delivery of "naked" protein (Conova, Vernengo et al. 2011, Bearat, Lee et al. 2012). The same requirements that make hydrogels suitable for *in situ* gelation – compatibility with aqueous, non-cytotoxic chemistries – enable hydrogel-mediated delivery of live cells, where cells are embedded in the liquid hydrogel precursor prior to injection and *in situ* crosslinking (Lu, Wang et al. 2012, Liang, Walczak et al. 2013, Mothe, Tam et al. 2013). Although cell transplants have shown exciting potential as a clinical therapy for SCI, low survival rates and inefficient differentiation are major barriers to clinical translation. Evidence of poor survival was recently reported by Sontag, et al., who reported that approximately 90% of transplanted human oligodendrocyte progenitor cells died either during or immediately after transplantation when suspended in a buffered solution and injected directly into the spinal cord (Sontag, Uchida et al. 2014). However, recent reports indicate that delivery within hydrogel carriers with shear thinning properties – meaning that viscosity decreases with application of increasing shear stress – act to protect cells from death during injection (Aguado, Mulyasasmita et al. 2011, Liang, Walczak et al. 2013, Mothe, Tam et al. 2013).

Swelling and mesh size affect diffusion of nutrients, waste and other bioactive factors through *in situ*-formed hydrogels, directly affecting survival, growth and phenotype of encapsulated cells. There is also some evidence that hydrogels with relatively large swelling pressures can physically "crush" encapsulated cells, severely limiting their viability (Shikanov, Xu et al. 2009). Dual-network hydrogels may circumvent this risk. For example, cell-mediated degradation of the fibrin component in interpenetrating networks of fibrin and alginate permitting encapsulated ovarian follicles to expand in size while the remaining alginate structure maintained mechanical integrity (Shikanov, Xu et al. 2009). Additional bioactive factors can be included to further enhance the therapeutic benefits of cell transplants. For example, adult rodent neural progenitor

cells delivered via HAMC hydrogels after SCI improved survival and increased oligodendrocyte-lineage differentiation (Mothe, Tam et al. 2013).

VIII. Host-Biomaterial Interface

Successful induction of spinal cord regeneration by hydrogel implants has been limited, at least in part, due to minimal cell infiltration and axon extension into the hydrogels, resulting in poor integration with host tissues (Khaing, Milman et al. 2011, Pakulska, Ballios et al. 2012). Biomaterials that provide structural support and contain cell-scale macropores can prevent cyst formation and promote ample cell infiltration within the first few days of implantation, allowing a permissive interface between the biomaterial and the host spinal cord tissue (Yang, Laporte et al. 2009, Tuinstra, Aviles et al. 2012, Thomas, Kubilius et al. 2013). This early integration results in reduced glial scar deposition, which can allow more regenerating axons to enter, cross and exit the injury site via polymeric bridges (Tuinstra, Aviles et al. 2012, Tuinstra, Ducommun et al. 2013, Pawar, Cummings et al. 2015). Macroporosity may also provide an avenue for rapid revascularization of lesions to further benefit tissue sparing and functional outcomes after SCI. As macroporosity improves infiltration of host cells through hydrogel implants, the same macroporous architecture can support migration of transplanted cells into the surrounding tissue and, potentially, their functional integration with the patient's spinal cord. In the case of gene therapies, macroporosity-facilitated cell infiltration can enable more cells to contact genetic vectors, thereby increasing the delivery efficiency (Aviles and Shea 2011, Shepard, Virani et al. 2012, Thomas, Seidlits et al. 2014). *In situ*-crosslinking and swelling of hydrogels in irregularly shaped injection sites allows for a more intimate interface with host tissue, where some swelling pressure may actually help keep the implant positioned flush against tissue. As discussed throughout this review, this swelling pressure must be regulated so as not to induce physical injury to the host tissue. Typical injectable scaffolds exhibit microscale pores that are much smaller than a migrating cell. Furthermore, altering this microporosity directly affects hydrogel swelling and other properties, and thus is not an ideal route for independently manipulating cell infiltration (**Figure 4**).

A few methods describing strategies to create injectable scaffolds with macroscale pores have been reported using three basic strategies: *in situ* assembly of pre-formed hydrogel "beads" (Jia, Yeo et al. 2006, Griffin, Weaver et al. 2015), macroporous cryogels that return to their original shape after injection (Koshy, Ferrante et al. 2014), and dual-network hydrogels (Shikanov, Xu et al. 2009, Bearat, Lee et al. 2012, Shih and Lin 2012, Pakulska, Vulic et al. 2015, Schweller and West 2015). Dual-network hydrogels can be designed so that one network component degrades to create a porous structure while the other remains intact to preserve mechanical integrity and control hydrogel swelling (Shikanov, Xu et al. 2009, Schweller and West 2015). Alternatively, each network component may be used to independently control material properties, including swelling, mesh size and mechanics (Shih and Lin 2012). Griffin, et al., recently demonstrated assembly of PEG hydrogel beads into a crosslinked biomaterial scaffold using a method based on the fibrin clotting process to yield injectable scaffolds with independent control of mechanics and macroporosity (Griffin, Weaver et al. 2015). Like other macroporous, biomimetic scaffolds, these scaffolds improved cell infiltration and integration with host tissue. In addition, swelling of pre-formed hydrogel beads to physiological equilibrium prior

to *in vivo* injection may be advantageous over crosslinking hydrogels *in situ*, since any potential issues with swelling pressure increasing intraspinal edema are avoided.

IX. Conclusions

Injectable hydrogels are a highly versatile group of biomaterials and their application as SCI therapeutics has gained popularity among a myriad of materials being considered due to their relatively non-invasive administration, biocompatibility, high water content, tissue-like mechanical properties and ability to deliver regenerative factors including proteins, small molecules and even living cells. Recent findings in the acute physiology of the spinal cord showed that dramatic increases in intraspinal pressure occur after a traumatic injury, and clinical data suggest that this increase in local tissue pressure is a likely contributor to the progression of secondary injury. In light of these findings, future studies will require monitoring changes in intraspinal pressure that may occur during injection (pre-crosslinking) and swelling (post-crosslinking) of hydrogel therapeutics. Swelling characteristics of a material can affect (1) delivery specifications, (2) local tissue pressure, (3) apposition to host tissue, and (4) rate of diffusion of the payload from the material, and therefore have significant bearings on the success of biomaterial-based therapeutics *in vivo*. Although excessive hydrogel swelling may cause increased secondary damage when injected into restricted sites such as the parenchyma, hydrogels placed at subdural sites that can absorb excess fluid from the cord after acute SCI may represent a unique opportunity for injectable biomaterials that positively affect functional recovery. As SCI repair is a highly complex process that will likely require combinatorial approaches to address clinically, it is crucial for researchers to continue developing improved hydrogel fabrication strategies to achieve better control over swelling in a manner that is decoupled from other properties with major effects on the physiological response, including mechanics, macromer content and permeability.

Acknowledgements

The authors would like to thank the Department of Neurological Surgery (CPH, ZZK), and the UCLA Henry Samueli School of Engineering (SKS), Applied Sciences UCLA Faculty Career Development Award (SKS), and Dr. Benjamin Wu at UCLA for providing the use of an Instron.

Disclosure Statement

The authors declare there are no conflicts of interest.

List of abbreviation used in this paper

SCI	spinal cord injury
CNS	central nervous system
TBI	traumatic brain injury
AQPs	aquaporins
PEG	polyethylene glycol
HA	hyaluronic acid
HAMC	hyaluronic acid methylcellulose
ECM	extracellular matrix
pHEMA	Poly(2-hydroxyethyl methacrylate)
OTD	osmotic transport device

X. References

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Figure Legends

Figure 1. Local *intraspinal pressures increase significantly after injury to the spinal cord*. **A**. In humans, injury area within the spinal cord was imaged using a magnetic resonance scan (left image) and a schematic showing different compartments within the spinal cord after injury. Increases in ISP (instraspinal pressure) can be measured using subdural pressure probes to estimate tissue pressure (bottom figure). Con = control; tSCI = traumatic spinal cord injury. *** $p < 0.001$. *Adapted from Werndle et al., 2014*. **B**. An ultrasound image of a rat spinal cord 15 minutes post injury detects hemorrhaging at the lesion epicenter. Dotted circle approximate the injury center (top image). In rodents, significant increases in ISP were directly measured using a pressure probe inserted into the lesion epicenter. ISP was measured utilizing a 1 F Millar Mikro-Tip® pressure probe (tip diameter = 330 μm). For insertion of the probe a small dural defect was produced with a # 11 surgical blade. ISP was recorded in 6 intact animals to establish a pre-injury baseline. In injured animals, the probe was inserted into the spinal cord approximately 4 mm caudal to the injury center. The probe was subsequently advanced rostrally into the injury center. For a total of 7 animals, ISP recordings were performed immediately after injury continuously for 4 hrs. *** $p < 0.001$. Adapted from Khaing et al., 2016.

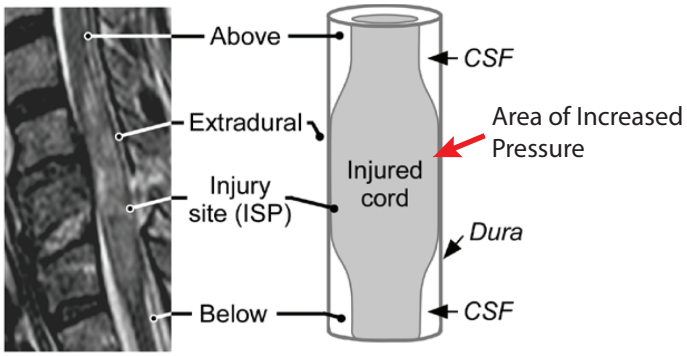
Figure 2. *Schematic illustrations of cross sectional anatomy of the spinal cord, associated membranes, compartments for biomaterial delivery, and their respective dimensions in humans and in rodents.*

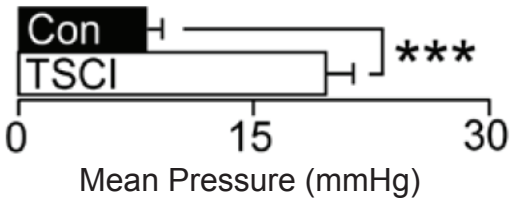
Figure 3. *Hydrogels swelling can produce detectable forces*. **A**. Samples were swelled uniaxially in buffer and resulting swelling forces were measured using a fixed probe on an Instron 5564 linear compressive tester. Formulations were as follows in PBS: 4% PEG (20 mg/mL PEG-vinyl sulfone (VS) and PEG-thiol (SH)), 2% PEG (10 mg/mL PEG-VS and PEG-SH), 2% HA (20 mg/mL HA-SH and PEG-VS), 1% HA (10 mg/mL HA-SH and PEG-VS), HA-Laminin (LN)-Collagen (Col), 3 mg/mL each). **B**. Hydrogels were swelled in buffer until equilibrium was reached. Hydrogel mass was measured at time of gelation and after swelling. Swelling percentage was calculated as the change from initial mass after equilibration in physiological buffer. Formulations were as follows in PBS: 1% HA (10 mg/mL HA-SH and PEG-VS), 2% HA (20 mg/mL HA-SH and PEG-VS), Collagen (3 mg/mL), Fibrin (8 mg/mL), HA-LN-Col (3 mg/mL each), 4% PEG (20 mg/mL PEG-vinyl sulfone (VS) and PEG-thiol (SH)), 2% PEG (10 mg/mL PEG-VS and PEG-SH). All measurements were acquired at room temperature. *Unpublished data from Seidlits and Khaing laboratories*.

Figure 4. *An illustration of theoretical factors that can influence hydrogel swelling*. Changes in macromer concentration, crosslinking density or hydrophilicity can all affect swelling and mesh size.

A

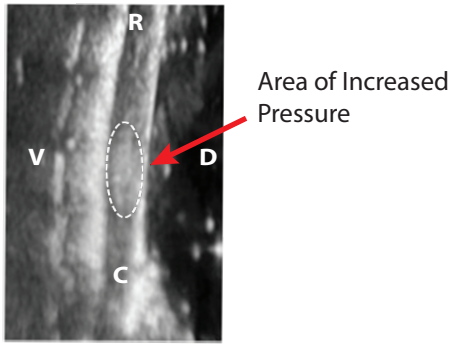
Human traumatic SCI

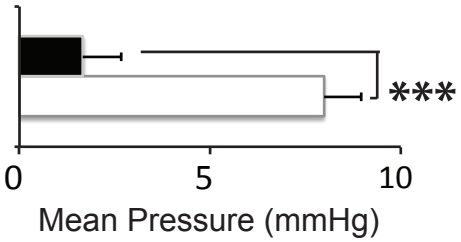


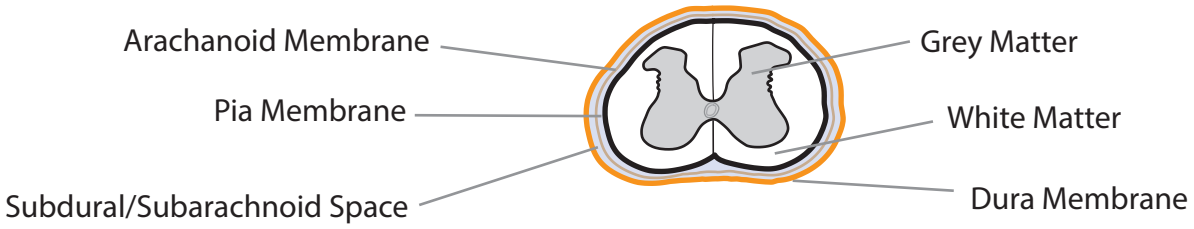


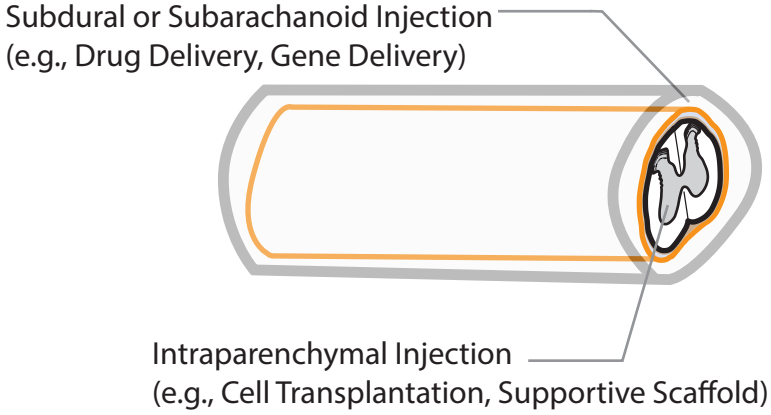
B

Rodent traumatic SCI

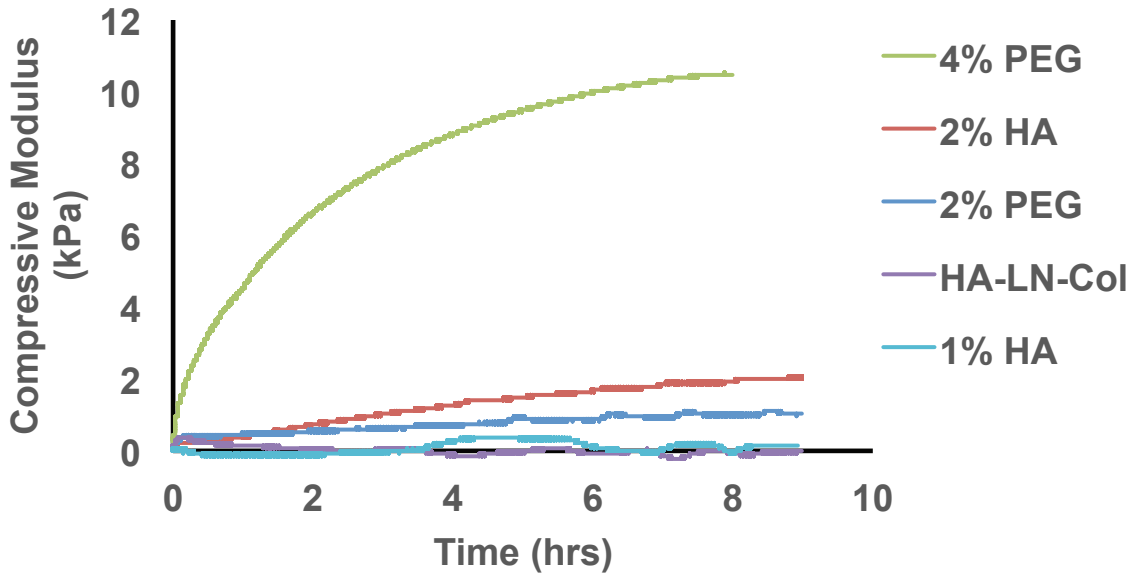


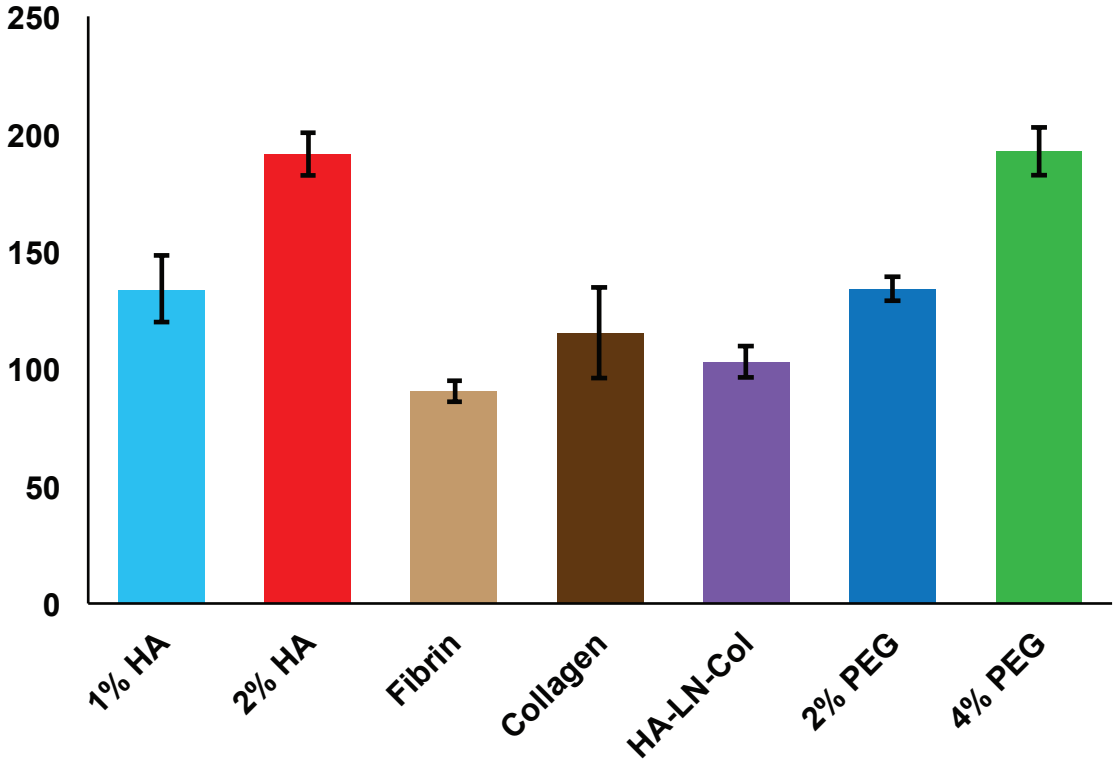






Spinal Cord Dimensions		
	Cross Sectional Area	**Subarachanoid Space**
Human	~ 120 mm^2	~ 5.5 -7.6 mm
Rat	~ 28 mm^2	~ < 1 mm


Compressive Modulus (kPa)
12
10
8
6
4
2
0
0
2
4
6
8
10
Time (hrs)
4% PEG
2% HA
2% PEG
HA-LN-Col
1% HA


250
200
150
100
50
0
1% HA
2% HA
Fibrin
Collagen
HA-LN-Col
2% PEG
4% PEG

Macromer Concentration

Crosslinking Density

Material Hydrophillicity/Ionic Strength

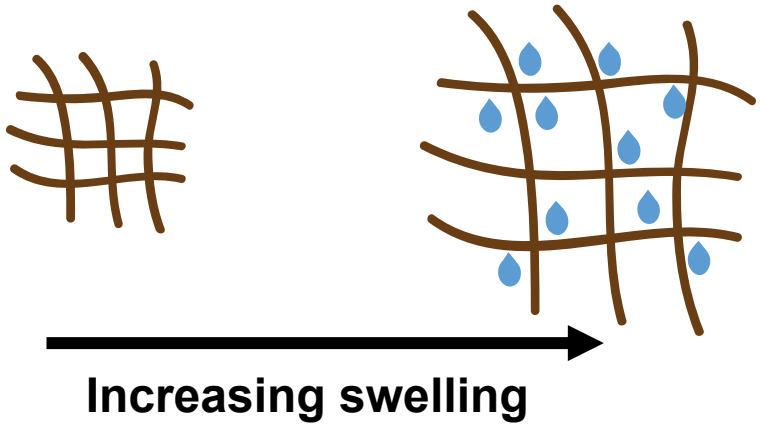


Table 1. *Hydrogels with potential for injection and compatible with SCI therapy.* Reports of stiffness was measured either by rheology (G' – storage modulus, G* - shear modulus) or linear compressive modulus (G). Swelling is reported as an increase in mass or volume between point of gelation and after equilibration in a physiological buffer.

Materials Used	Stiffness & Swelling	Delivery Site	Crosslinking Strategy	Cells and/or Factors Included	References
Hydrogels injected *in vivo:*					
Hyaluronic acid, methyl-cellulose	G' = 1-5 Pa, +50% swelling	T1-2; Intraspinal/ Intrathecal	Phase transition (temperature-responsive)	NPCs, PDGF	Mothe et al., 2013; Gupta et al., 2006; Austin 2012
Hyaluronic acid, PEG	G' = 50 Pa. +40% swelling	T1-2; Intrathecal	Covalent crosslinks (aqueous "click" reaction)	BDNF- PLGA nanoparticles	Führmann et al., 2015; Nimmo et al., 2011
Methyl-cellulose	G' = 400Pa +50% swelling	T1-2; Intrathecal	Phase transition (temperature-responsive), covalent crosslinks	chondroitinase ABC	Pakulska et al., 2013; Pakulska et al., 2015
Self-assembled peptide nanofibers	Not reported	T-10; Intraspinal	Ionic/hydrophobic interactions	IKVAV	Tysseling-Mattiace, et al., 2008
Hydrogels implanted *in vivo* with potential for injection*:*					
Hyaluronic acid	G = 7900 Pa, +40% swelling	T-8; Hemisection	Covalent crosslinking (photo-polymerization)	None	Khaing et al., 2011
Fibrin, fibronectin, collagen	G = <1kPa, +5% swelling	T-8; Transection	Covalent crosslinks (enzymatic), phase transition	None	King et al., 2010; Sargeant et al., 2012; Duong et al., 2009
Fibrin	G = <1kPa, +<20% swelling	T-9; Hemisection	Covalent crosslinks (enzymatic)	NT3, PDGF, NPCs	Johnson et al., 2009; Johnson et al., 2010a; Johnson et al., 2010b; Duong et al., 2009; Sharp et al. 2012
Alginate, fibrin	G' = 130Pa, +0-40% swelling	T-9/10 Hemisection	Ionic (alginate) and covalent (enzymatic) crosslinking	GDNF-PLGA microspheres, VEGF	Ansorena et al., 2013; Kuo, and Ma, 2008; des Rieux et al. 2014
Alginate	Not reported	C-3 Transection	Ionic crosslinking	NPCs	Prang et al., 2006; Pawar et al., 2015; Grulova eta al., 2015; Gunther et al., 2015
Agarose	G* = 130 Pa, +0% swelling	T-10 Hemisection	Phase transition (temperature-responsive)	chondroitinase ABC	Lee et al., 2010; Balgude et al., 2001
PNIPAAm, methyl-cellulose, PEG	G = 1kPa, +40% swelling	C-4/5 Hemisection	Phase transition (temperature-responsive)	BDNF-expressing fibroblasts	Conova et al., 2011; Bearat et al., 2012
PLA, PEG	G = kPa to GPa possible, +0% swelling	T-8 Hemisection	Covalent crosslinking (photo-polymerization)	NT-3	Piantino et al., 2006; Harrane et al., 2011