Injectable scaffold-systems for the regeneration of spinal cord: advances of the last decade

Sofia Santi[‡,ǂ,§,*], *Ilaria Corridori*[‡,†,*], *Nicola M. Pugno*[†,+], *Antonella Motta*[ǂ,§], *Claudio Migliaresi*[ǂ,§]

[ǂ] BIOTech Research Center and European Institute of Excellence on Tissue Engineering and Regenerative Medicine, University of Trento, Via delle Regole 101, 38123 Trento, Italy

[§] Department of Industrial Engineering, University of Trento, Via Sommarive 9, 38123 Trento, Italy

[†] Laboratory of Bio-inspired, Bionic, Nano, Meta Materials & Mechanics, Department of Civil, Environmental and Mechanical Engineering, University of Trento, Via Mesiano 77, 38123 Trento, Italy

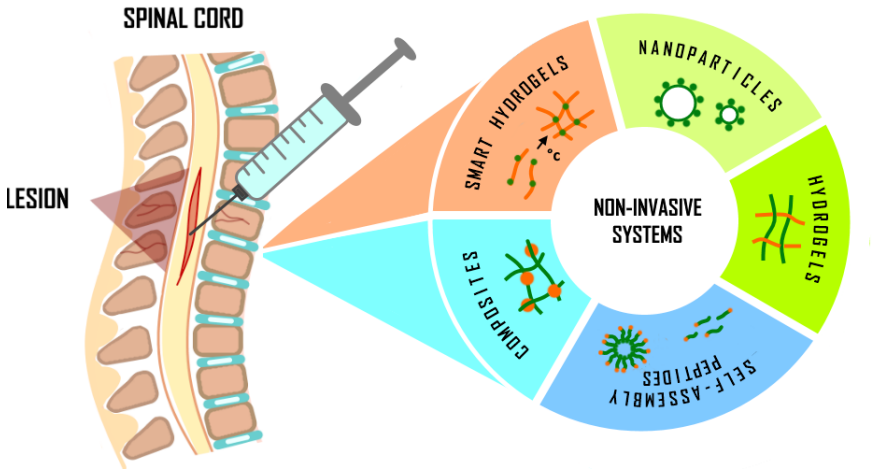
[+] School of Engineering and Material Science, Queen Mary University of London, Mile End Road, E1 4NS London, United Kingdom

[‡] equally contributed

* corresponding authors, e-mail: sofia.santi@unitn.it, ilaria.corridori@unitn.it

Abstract. Nowadays, whenever is possible and as alternative to open spine surgery, minimally invasive procedures are preferred to treat spinal cord injuries (SCI), with percutaneous injections or small incisions, that are faster, less traumatic and require less recovery time. Injectable repair systems are based on materials that can be injected in the lesion site, can eventually be loaded with drugs or even cells, and act as scaffolds for the lesion repair. The review analyzed papers written from 2010 onwards on injectable materials/systems used/proposed for the regenerative and combinatorial therapies of SCI, and discusses the *in vivo* models that have been used to validate them.

Graphical Abstract.



Keywords. Injectable hydrogel, smart hydrogel, injectable composite, injectable nanoparticles, injectable self-assembling peptides. All the listed keywords were linked to the spinal cord injury topic.

1. Introduction

The incidence of spinal cord injury (SCI) is approximately 17,730 new cases each year in the United States [1]. The leading causes of injury are vehicle crashes, followed by falls and the case of violence. Damages of the spinal cord often lead to permanent functional and sensory loss due to the limited regenerative capacity of the central nervous system (CNS).

The clinical therapeutic guidelines of neurorestoration in the case of SCI are focused on alleviating secondary injury. They consist of restricting active and passive movement, early fixation, combined extramedullary and intramedullary decompression, suitable cell therapies, early rehabilitation, or electric stimulation therapy [2]. In particular, repetitive and rhythmical movements during early rehabilitation activate the spinal networks thanks to the sensorimotor information, which allows functional recovery and remodels the function of the cerebral cortex [3]. The neuroprotection aims to minimize secondary

injuries by pharmacological therapy (i.e. erythropoietin, ibuprofen, indomethacin, anti-oxidants) [4] to avoid cellular apoptosis or necrosis and promoting neuronal survival. These clinical guidelines are very important to facilitate treatments by using prostheses or scaffolds to promote the regeneration of neural cells. The neuroregenerative therapies, instead, differ from the neuroprotective ones since they aim to create the best conditions for the neural tissue to maximally express its regenerative potential. The neuroregenerative approach is a younger discipline and thus few clinical trials have been performed. However, neuroregenerative methods seem to have less side effects than neuroprotective techniques. Given the advantages of both methodologies, the research is going toward combinatorial and interdisciplinary approach[5–7].

Currently, most of the clinical trials are based on stem cell therapy [6] or *in situ* pharmacological treatments. The main difficulty of the cell transplantation regards the inhospitality of the environment at and around the damaged tissue: inhibitory molecules and an inflammatory status prevent tissue regeneration, limit the cell survival, and the clinical efficiency of cell therapy. Instead, pharmacological treatments such as i) neuroprotective agents [8] (i.e. sodium salicylate, polyphenols, aspirin), ii) growth factors as well as iii) suppressors of inhibitory molecules of the inflammatory response (i.e. suppressors of NOGO-A, myelin-associated glycoprotein (MAG), oligodendrocyte-myelin glycoprotein (OMgp), and chondroitin sulfate proteoglycans (CSPGs) digestion with the administration of chondroitinase ABC (ChABC) or hyaluronidase) [9–12] are hindered by the blood-brain barrier or blood spinal cord barrier (BSB) that limit their diffusion. Furthermore, high systemic doses to reach a therapeutic concentration at the site of the injury could induce tumor formation, fibrosis or other negative effects caused by the off-target of the molecules injected [13].

Researchers are pushing towards solutions avoiding further damages to the tissue. Non-invasive injectable biomaterials can be precisely positioned in the lesion site, and eventually repetitively injected to obtain the complete regeneration of the tissue. Moreover, the therapeutic advantages of directly

injecting therapies in the parenchyma of the spinal cord were proven superior concerning the systemic delivery of materials. [14].

Some examples of injectable systems are i) self-assembling peptide materials (SAPs), whose gelation process is charge dependent; ii) amphiphilic diblock copolypeptide hydrogels (DCHs), which have a shear-thinning property, allowing the injection; iii) gel containing multiple tryptophans and proline-rich peptide domains, which undergo a sol-gel phase transition upon mixing or iv) injectable thermosensitive hydrogel of PEG–PLGA–PEG triblock copolymers [15,16]. The aim of this review is to provide an overview on the *in situ* injectable scaffold-systems. The possible injectable solutions analysed belong to four categories: hydrogels, nanoparticles, self-assembly peptides, and composites.

2. Pathophysiology

The SCI following trauma is characterized by four subsequent stages: immediate, acute (0-7 days), sub-acute (7-14 days), and chronic (months) (Figure 1).

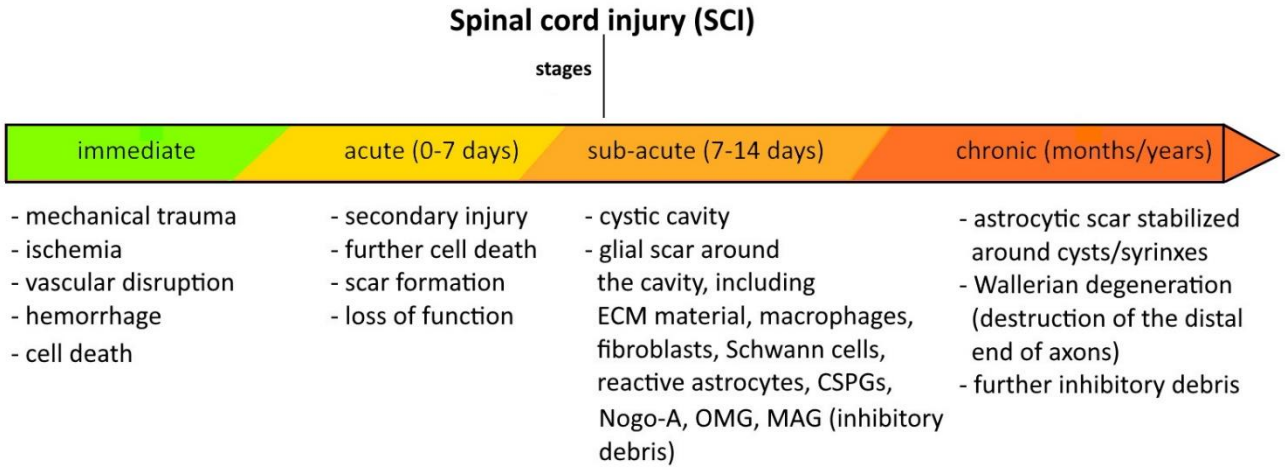


Figure 1 Timeline of the events following a SCI. Four stages characterize the injury progression: immediate, acute (0-7 days), sub-acute (7-14 days), and chronic (months).

In particular, a spinal cord contusion leads to an inflammatory reaction at the lesion site with the infiltration of leukocytes and activation of glial cells which limit the damage by reestablishing blood brain barrier and ionic homeostasis [17] (Figure 2). However, the dense scar and inhibitory molecules such as chondroitin sulphate proteoglycans (CSPGs), Nogo-A, OMgp, MAG, which appear at later stages, are detrimental towards regeneration [18–20] (Figure 2). In particular, CSPGs interact with proteins in the extracellular matrix due to its negative charges and these interactions could inhibit the neurite outgrowth following CNS injury [20]. Thus, the inhibition of CSPGs by using the bacterial enzyme ChABC seems to be very promising for enhancing axonal regeneration [21].

In the CNS, microglial cells are much slower compared to the peripheral nervous system (PNS) in clearing this debris, which may be present as long as three years post-injury [15].

External the CNS, macrophages derived from circulating monocytes reach injured tissues and some of them seem to represent controlled recruitment needed for repair [22] (Figure 2).

M1 monocyte macrophages were found to derive from monocytes that entered the injured spinal cord (SC) via monocyte chemoattractant protein 1 (MCP1) through the adjacent SC leptomeninges [22]. M1s possess proinflammatory, phagocytic, and proteolytic functions, essential for damaged tissue digestion and debris removal. M2 macrophages instead, came from monocytes that transit through the brain-ventricular choroid plexus (CP), via VCAM-1-VLA-4 adhesion molecules and epithelial CD73 enzyme [22,23]. Along with the CP, leukocytes extravasate across the endothelium, interact with the tightly connected epithelial cells, and enter the blood-cerebrospinal-fluid (CSF), facilitating the CNS immunosurveillance [22,24]. M2 possess anti-inflammatory functions and are involved in tissue regeneration, growth, angiogenesis, and matrix deposition, supporting tissue remodeling [22,25].

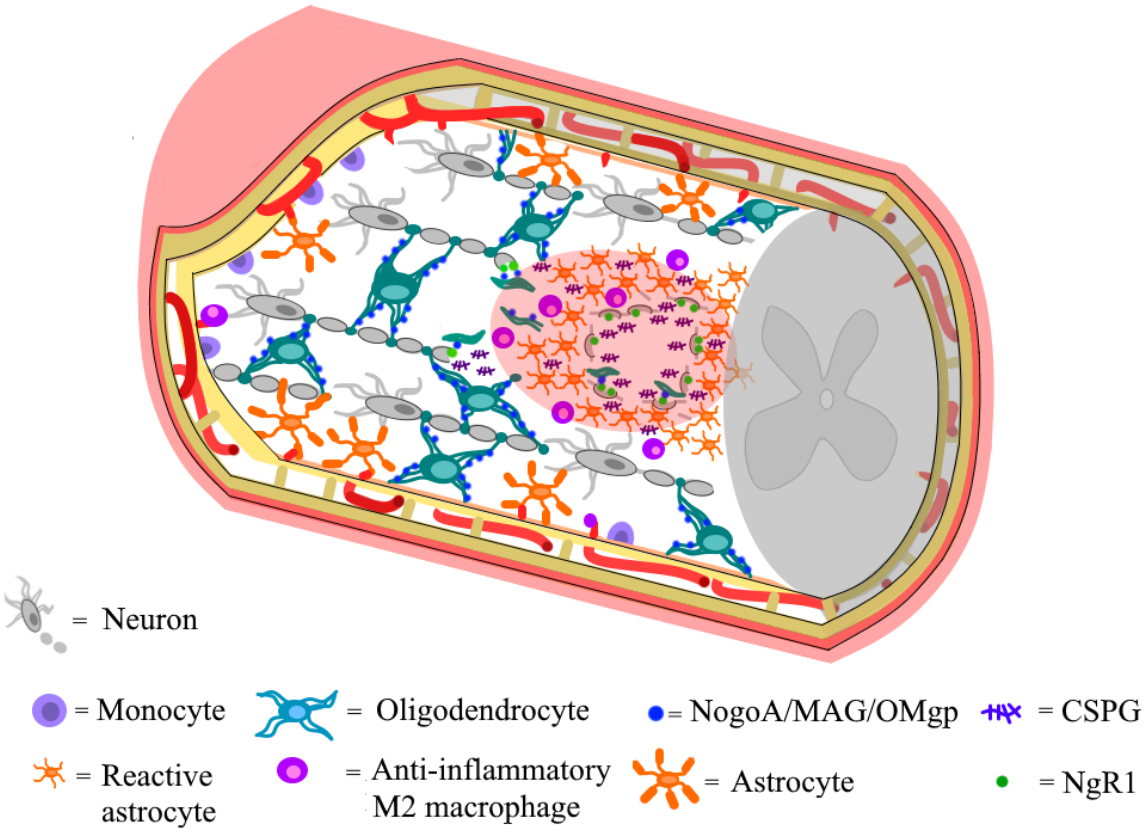


Figure 2. Pathophysiology model during the regenerative phase, involving the polarization of the monocytes in anti-inflammatory M2 macrophages to start the healing process.

During these detrimental phenomena, the neuroplasticity of the SC promoted in some cases spontaneous recovery of locomotor function after SC contusion[3].

The reasons could be the variation of existing neuronal pathways, the formation of new connections, dendritic arborization remodeling, and axonal sprouting, regulating the expression of neurotrophin-3/4 (NT-3, NT-4), brain-derived neurotrophic factor (BDNF), and the glial cell-derived neurotrophic factor (GDNF)[26]. The spontaneous recovery also involves the presence of proliferating ependymal cells at early postinjury times, which later may have contributed to the expansion of the ependymal zone and the formation of cellular trabeculae within the lesion cavity [27]. The cellular trabeculae may serve to guide

fibers from the CNS (like the corticospinal tract) into the center of the lesion. The dorsal roots likely represent the main source for axons and Schwann cells which provide most of the myelin [27].

The presence of several regenerating axons within the lesion matrix after severe contusion injuries strongly suggests that under some conditions, the tissue repair response in the adult provides a substrate for growth.

3. Non-invasive materials for SCI treatment: description and results

A traditional surgery often requires a large incision with intrinsic risks, pain for the patient, perduring functional mobility, long hospital stay, long time of recovery and large costs for the healthcare system.

Minimally invasive surgical procedures have root in the middle of last century, with the experimental use of arthroscopy, but only in the 80's minimally invasive surgery emerged as preferred alternative to open surgery procedures, to reduce trauma, surgery associated risks, pain for the patient and also treatment cost. Nowadays, the use of minimally invasive surgical procedures is considered of paramount importance as also evidenced in international research and innovation roadmaps and programs . For the spinal cord minimally invasive treatments, *in situ* injectable materials such as nanoparticles [21], smart hydrogels [3,28], injectable lipid microtubes [29], self-assembling peptides [30], and self-assembling nanofibers [31–33] have been widely investigated and proposed. Injectable materials allow minimally invasive implantation procedures and present shape versatility; some of them can have stiffness comparable to the human spinal cord and possess water retention [15]. Moreover, they can be injected repeatedly until the complete functional tissue formation. Many of them can be functionalized or combined with adhesion ligands (i.e., IKVAV, RGD, CQAASIKVAV), growth factors (ex. fibroblast growth factor-2, FGF2, Neurotrophin-3, NT3), enzymes (es. chABC), and anti-inflammatory molecules (i.e. minocycline) to allow cell attachment, renewal, sprouting and extra cellular matrix (ECM) regeneration. However several problems have been reported with the use of injectable materials: for instance, the injected materials could form aggregates, creating barriers to the tissue regeneration, as observed for collagen gels stabilized by carbodiimide that causes endogenous collagen deposition; an excessive swelling of the material could increase the local pressure causing secondary damages to the parenchyma [3.34].

The electrical stimulation of neuronal cells [35,36], and the formation of a controlled 3D structure along the longitudinal axis of the spine seem to be important requirements for i) surviving and maintaining the

cells active [37,38], ii) promoting long-distance axonal elongation [39], and iii) achieving oriented axons regeneration [40] for a natural tissue structure. It is still a challenge for injectable materials.

Non-invasive biomaterials-based systems that have been used for SCI therapies can be classified in hydrogels, smart hydrogels, nanoparticles, composites, and self-assembling peptides often combined with cells and specific signals. These systems are discussed below, summarized in Table 1 and the achievements obtained are represented in Figure 3.

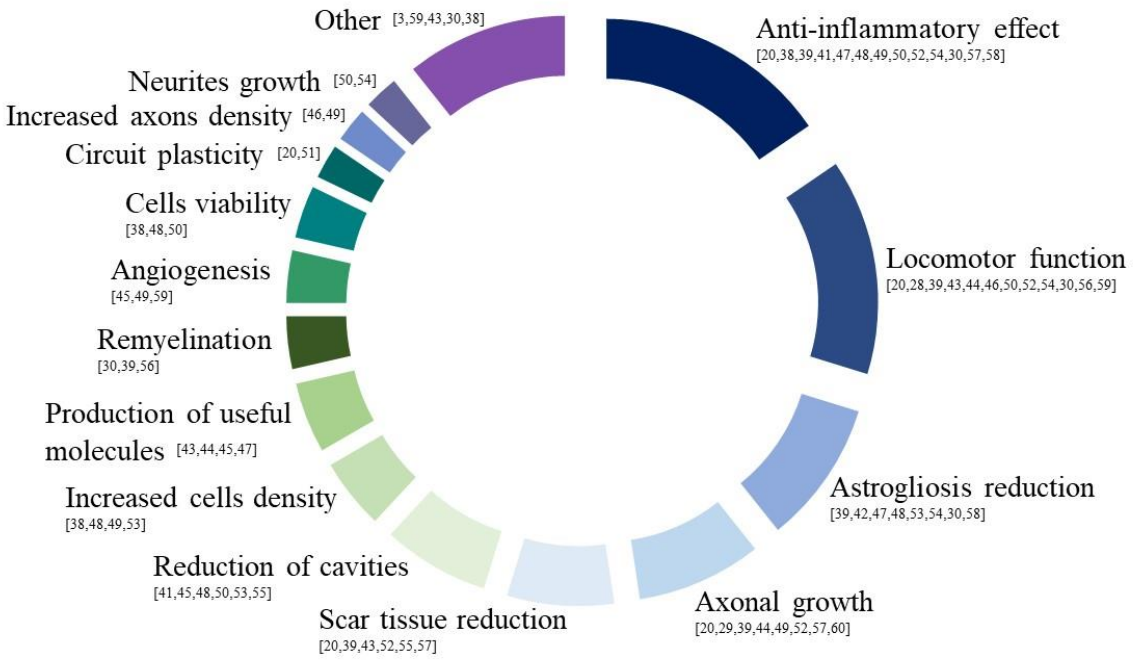


Figure 3. Achievements reported in the cited papers.

Table 1 Non-invasive biomaterials-based scaffold for the treatment of SCI. The table highlights many features of the systems: the animal and related SCI model, the material and the shape of the scaffold, eventual signals and cells used. The obtained achievements and the associated observations are also summarized.

Reference	Animal model	SCI model/location	Material	Shape	Cells	Signals	Achievements	Observations
Azizi, M., et al. (2020) [21]	Rat	Contusion, T10	PLGA	Nano-particles		chABC	↑ Anti-inflammatory M2 macrophages ↑ Axonal growth scar digestion in the injured site ↑ Locomotor function ↑ Circuit plasticity	Failure of extensive axonal regeneration Incomplete cleavage of the CSPG, MAG, OMgp and Nogo
Bonnet, M, et al. (2020) [3]	Rat	Contusion, T10	PNIPAAm-g-PEG	Thermo-responsive hydrogel			Unmodified inflammatory reaction ↓ Spasticity	No sensorimotor benefits after PNIPAAm-g-PEG combined with training Control+training and PNIPAAm-g-PEG+training had similar locomotor and ladder climbing test results.
Führmann, T., et al. (2016) [41]	Rat	Compression, T2	HA + MC	Hydrogel	OPCs	PDGF-A RGD	Cell survival, integration, and differentiation (glial phenotype) ↓ Teratoma	Teratoma formation not avoided Teratoma caused no improvements in motor function
Vismara, I., et al. (2020) [42]	Mice	Compression, T12	PEG + PEI	Nano-structured gel	iPs human-derived	Rolipam	NG internalisation in 24 hours Selective internalisation in pro-inflammatory phenotypes (in vitro) ↓ Pro-inflammatory response NG internalisation in activated astrocytes, few in microglia none in neurons ↓ Production of inflammatory molecules by astrocytes (in vivo) Neuroprotective effect (in vivo)	The motor improvement observed at early stages after injury NG was not internalised in microglia and neurons

Wang, C., et al. (2019) [43]	Rat	Compression, T9	Laponite + heparin hydrogel	Hydrogel	FGF4	↑ Axonal regrowth ↑ Remyelination ↓ Astroglyosis ↓ Glial fibrotic scar ↓ Inflammatory response Motor functional recovery	
Hong, L.T.A., et al. (2017) [44]	Rat	Contusion, T10-11	imidazole-poly (organophosphazenes) (I-5)	Thermo-responsive hydrogel		↓ Cavity spaces Structural stabilisation ↑ Macrophages in the lesion for wound healing processes and ECM remodelling	
Boido, M., et al. (2019) [45]	Mice	transection	Chitosan+ β GP	Thermo-responsive hydrogel	MSCs	Fast gelation ↓ Reactive astrocytes in the injury site ↑ MSCs survival in the injury site	Preliminary in vivo tests Locomotor recovery not evaluated
Li, X., et al. (2019) [46]	Rat	Hemisection, T9	P10R5-LA	Thermo-responsive hydrogel	Cabazi-taxel	↑ Bladder functions ↓ Fibrotic scarring ↓ Axons growth inhibitory molecules ↓ Demyelination adjacent to the injury site Locomotor recovery	
Donaghue, I.E., et al. (2015) [47]	Rat	Impact/ compression, T1-2	HA + MC + PLGA nanoparticoles	Composite	NT-3 Anti-NogoA	Sustained release of NT-3 ↑ Axon density Unmodified inflammatory response ↑ Anti-NogoA with the addition of NT-3 ↑ Locomotor function	Unmodified inflammatory response
Kang, C.E., et al. (2012) [48]	Rat	Compression, T2	HA + MC + PLGA nanoparticles	Composite	FGF2	↑ Angiogenesis, ↓ cavity No proliferative lesion Sustained and long-term release of FGF2	No functional improvements
Ansorena, E.., et al. (2013) [49]	Rat	Hemisection	Alginate + fibrinogen + (PLGA microspheres)	Composite	GDFN	↑ Number of neurofilaments Functional recovery Slow release of GDFN Homogeneous and dense regenerated tissues	Fast release kinetics Growing neurites absent within the lesion Free-GDNF hydrogels induced superior functional recovery compared to GDNF-microspheres hydrogel

Jain, A., et al. (2011) [50]	Rat	Hemisection, T8-10	Agarose + lipid microtubes	Composite		BDNF, CA-Cdc42, or CA-Rac1	↓ Number of astrocytes ↓CSPG deposition presence of neurofilaments across the lesion, ↑ Axons in the CSPG-rich regions	↑ immune/inflammatory response Dosage of the three proteins not optimized
Chen, S., et al. (2020) [51]	Rat	Hemisection, T9-10	Silk fibroin + PDA				↑ Axons length ↑ Cell density ↑ Cell viability ↓ Glial cells High porosity Lesion cavity filled Fibrous distribution of neurons	Further in vivo studies needed Mechanical and morphological properties depend on PDA
Li, X., et al. (2020) [52]	Rat	Contusion, T9	PCL + MAL (fibres) + HA-SH + PEGDA	Composite			Limit spinal cord thinning ↑ Cells infiltration and interaction Suitable porosity Shift of macrophages from M1 to M2 ↑ Neovascularisation ↑ Differentiation of endogenous stem cells in immature neurons ↑ Number of axons	Role of nanofibers not fully known
Wang, C., et al. (2018) [53]	Mouse	Tissue removal, T12	[P(DLLA-co-TMC)) (fibres) + GCP	Composite	MN-ESC		Cell survival and engraftment in vivo NF inducedr ESC differentiation Neurite grew parallel to the axia direction Lesion cavity totally filled ↓ Tissue loss ↓ Inflammatory response Motor function recovery	Slow degrading dynamic
Khaing, Z.Z. et al. (2016) [54]	Rat	Transection, T2	PLGA (nanoparticles)+ HA +MC	Composite		BDNF	Efficient delivery platform of bioactive molecules Material with tunable properties based on the molecular weight of HA Promotion of adaptive plasticity	Inflammatory response not investigated
Nazemi, Z., et al. (2020) [55]	Rat	Hemisection, T8-10	PLGA microspheres + Alginate	Composite		MH PTX	Prolonged release of the drugs (2 months) ↑ Inflammation, ↓ Scar tissue (ECM deposition)	No therapeutic effect on cell death, no alteration of reactive astrocytes

							↑ Axonal regeneration and protection reconnection of neural network Functional improvement	
Marquardt, L.M., (2020) [56]	Rat	Contusion, C5	C7 recombinant engineered peptide + PEG modified with proline-rich peptides + PNIPAM	Thermo-responsive polymer	SC		↓ Secondary injury ↓ Cystic cavitation ↓ Astroglyosis in the peri-lesion space ↑ Cell retention ↑ Neurons and axonal sparing	Unavoidable cell loss Ligands might be necessary Further full dynamic response of immune cells infiltration is necessary
Hu, H.Z., et al. (2018) [29]	Dog	chronic severe SCI, L3-4 L4-5	Lipid microtubes + agarose	Composite		chABC-trehalose	↑ Locomotor function No long-term adverse effects	No improvements in detection of sensory evoked potentials
Tavakol, S., et al. (2015) [57]	Rat	Chronic SCI, T10	(RADA)4-GG-BMHP1	SAP	hEnSCs	BMHP1	↑ Neural differentiation Functional recovery ↓ Reactive astrocytes ↓ Inflammatory response	Axon regeneration and myelination limited to the area around the cavity ↓ cell proliferation in more concentrated gels
Cicognini, D. et al. (2014) [31]	Rat	Contusion, T9-10	B24 and biotin-LDLK12	SAP			Haemostatic effect ↑ Axon sprouting/regeneration	SAP caused compression of the surrounding tissue
Zweckberger, K., et al. (2015) [58]	Rat	Compression/ contusion, C5-6	K2(QL)6K2 (QL6)	SAP	NPC	BDGF EGF FGF	Cavity is bridged ↓ Scar tissue ↑ NPCs survival and migration	Osmotic micro-pump necessary
Tysseling, V.M., et al. (2010) [32]	Mouse/rat	Compression, T10/ Compression, T13	Peptide amphiphile	SAP		IKVAV	Axon elongation Functional recovery Suppressed progression of astrogliosis ↑ Remyelination of axons inside the lesion Regeneration of corticospinal motor fibers and sensory fibers ↑ Number of serotonergic fibers caudal to the lesion significantly ↓ Cells undergoing apoptosis	Behavioural results depending on the compression Small number of regenerating dorsal column fibers
Ye, J.C., et al. (2016) [59]	Rat	Compression, T10	RADA16 (polypeptide)	SAP	NSC from primates		In vitro differentiation in neurons, olygodendrocytes and glial cells ↑ Myelin production ↑ Motor function recovery	Primate NSCs not easily available

Tran, K.A., et al. (2020) [60]	Rat	Contusion, C5	RADA16I	SAP	Human cerebral microvascular endothelial cells		↓ Inflammatory response ↓ Glial scar Axon growth across the lesion	Not oriented structure New axons could origin from sprouting Perfusion in the microvessel not tested
Hassanejad, Z., et al. (2019) [30]	Rat	Compression, T7-8	$CH_3(CH_2)_{14}CO$-AAAAGGGEIKVAV	SAP		IKVAV BDNF	No inflammatory reaction ↓ Astroglyosis ↑ Migration of astrocytes and oligodendrocytes in the injury Sustained release of BDFN for 21 days	Functional recovery was not statistically significant
Cicognini, D., et al. (2011) [61]	Rat	Contusion, T9-10	RADA16-4G	SAP		BMHP1	Remodelling of ECM Basement membrane deposition ↑ Angiogenesis ↑ Migration of neurons, microglia cells and olygodendrocytes precursors ↑ Locomotor functions recovery	Inflammatory response No cyst and cavities ↓ Partial filling of the injury cavity New axons could origin from sprouting
Sun, Y., et al. (2016) [62]	Rat	Transection, T	(Ac-(RADA)4-$CONH_2$)	SAP	NSC/NPC	IKVAV RGD	↑ Cell viability and survval Differentiation of stem cells into neural and glial phenotypes in vitro Mild assembling process that presrvve the environment	Slow in vivo gelation Axons growth along the graft and not across it
Sever-Bahcekapili, M, et al. (2020) [33]	Rat	Hemisection, T9-10	Peptide amphiphile	SAP		IKVAV heparan sulfate-mimetic epitope	↑ Tissue integrity ↓ Cell loss Neurons toward injection site ↑ Locomotor functions recovery	

*3.1 Hydrogel*s. They are materials characterised by a three-dimensional network with a hydrophilic structure that holds large amounts of water. Hydrogels can be injected before crosslinking with it happening *in situ* in some seconds/minutes. Some hydrogels can display the so called "smart" behaviour, with a non-reversible or reversible transition from the state of sol to the state of gel following the application of external stimuli. The main component of the extracellular matrix of the spinal cord is hyaluronic acid (or hyaluronan HA) [63], thus this natural polymer is widely used in SCI regenerative medicine. For example, hyaluronan-methylcellulose (HAMC) hydrogels, first studied by Gupta et al. [64], loaded with oligodendrocyte progenitor cells (OPCs), platelet-derived growth factor receptors (PDGF-R), and RGD promoted the survival, integration, and differentiation of cells [41]. However, teratoma formation was not avoided but attenuated concerning the cell-therapy (Figure 4).

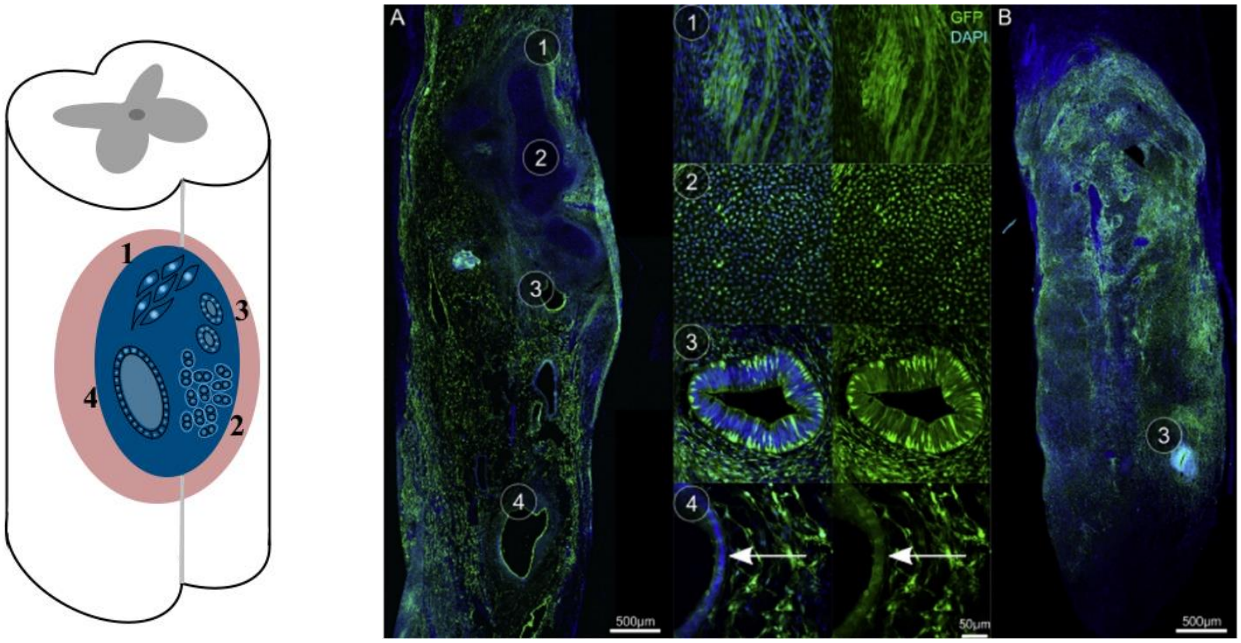


Figure 4. Morphology and immunohistochemistry of spinal cord tissue suggests: (1) muscle, (2) cartilage, (3) intestinal-like, epithelium, and (4) epithelium (arrow) within the teratoma.

Figure 4 (right, A-B) reproduced with permission from ref 41. Copyright 2016 Elsevier.

Other problems could also derive from a "not well cleaned" (full of debris) lesion site, which may impede the gelation of the material [34]. Moreover, considering some injectable hydrogels such as gelling agents (ex. β-glycerophosphate disodium salt hydrate, β-GP) [45] blended with chitosan, imidazole-poly (organophosphazenes) (I-5) [44,65], or poly-N-isopropyl acrylamide-based thermoresponsive hydrogels, the sol-gel transition could be preferentially promoted by physical factors (ex. temperature, physiological pH). to avoid inflammatory response, generally caused by chemical crosslinkers [3].

To avoid the harmful mechanisms activated by reactive astrocytes after SCI, a PEG (polyethylene glycol)-PEI (poly(ethylene imine)) NanoGel (NG) delivering Rolipram (antidepressant drug) was injected in a mice compression model [42]. The results showed a selective internalization of NG in activated astrocytes, few in microglia, and none in neurons. Motor functional improvement was possibly caused by a reduced production of inflammatory molecules by astrocytes and its consequent neuroprotective effect. This result was observed in the early stage after injury [42].

A biopolymer largely proposed in regenerative therapy strategies is silk fibroin. Chen et al. [51] produced a material coupling the silk fibroin with the quinone structure of oxidized dopamine (DA). DA is a mussel adhesion protein recently studied as a cross-linking medium to obtain injectable hydrogels [66]. Indeed, DA goes towards a self-polymerization of free DA generating an injectable silk fibroin/polydopamine (SF/PDA) hydrogel. This material has favoured neurite growth and neuronal differentiation. This scaffold presents tunable properties by varying the concentration of DA. *In vivo* tests showed repair of SC tissue after hemisection in rats but further investigation is needed to evaluate possible clinical studies. Recently, a synthetic smectite clay (Laponite XLG, $Na_{+0.7}[(Si_8Mg_{5.5}Li_{0.3})O_{20}\ (OH)_4]^{-0.7}$) was also used for SCI repair due to its capacity to aggregate in solution. Laponite XLG consists of interlayer cations (Na^+) that balance the net negative charge of a single crystal of Laponite. Its ability in binding heparin is exploited in regenerative medicine applications. Indeed, Wang C., et al. [43] produced an injectable

heparin-Laponite hydrogel loaded with a novel neuroprotective factor, the fibroblast growth factor 4 (FGF4). The results showed a motor functional recovery, reduced fibrotic scar tissue, and the inflammatory response with consequent remyelination.

Smart hydrogel polymers present a sol-gel transition responsive to different external stimuli: temperature, light, pH, ionic concentration, magnetic and electrical fields, and chemicals [67]. For thermo-responsive polymers, the transition occurs at specific threshold points: above the critical solution temperature (ex. LCST polymers) or below (ex. UCST polymers). The most common smart hydrogels used in SCI repair are thermoresponsive, such as poly(N-isopropyl acrylamide) (PNIPAAm) [3] or triblock copolymers based on polyethylene oxide, polyethylene glycol, polypropylene glycol, or polylactic acid (ex. PEO-PEG-PEO or PLA grafted on PPG-PEG-PPG) [68].

PNIPAAm-g-PEG (poly(N-isopropyl acrylamide-g-polyethylene glycol), introduced by Comolli et al. [69], was investigated in contusion SCI cases and results showed an unvaried inflammatory response [3]. The locomotor recovery improved when PNIPAAm-g-PEG was combined with an exercise training program. The electrophysiological recordings indicated reduced spasticity of treated animals, but this benefit was not recorded when the polymer treatment was coupled with exercise.

PPG(polypropylene glycol)-PEG-PPG (P10R5) has been used to deliver a chemotherapic agent (Cabazitaxel) to the injured area [46]. Besides the inhibiting role of Cabazitaxel on prostate cancer, this chemotherapeutic drug was proven to be capable of supporting the neurite extension of cortical neurons *in vitro*. The treatment creates a protective environment leading to an improvement of bladder and locomotor functions.

Different delivery systems based on PNIPAM are produced to allow cells to reach the target site. Marquardt et al. [56] synthesized an engineered protein (C7), composed by repetitive motives $CC_{43}WW$ (Figure 5, left). The motives were separated by a multi-arm of 8-armed PEG tethered with proline-rich peptides, PNIPAMs, and cell-adhesive peptides (IKVAV, RGD, YIGSR). The system, called SHIELD

(Shear-thinning Hydrogel for Injectable Encapsulation and Long-term Delivery) [70], represents an example of injectable hydrogel for autologous human Schwann cells (SCs) transplantation. The cell membrane protection promoted by the SHIELD encouraged a decrease of the cystic cavity and an increase of the functional recovery (Figure 5, right).

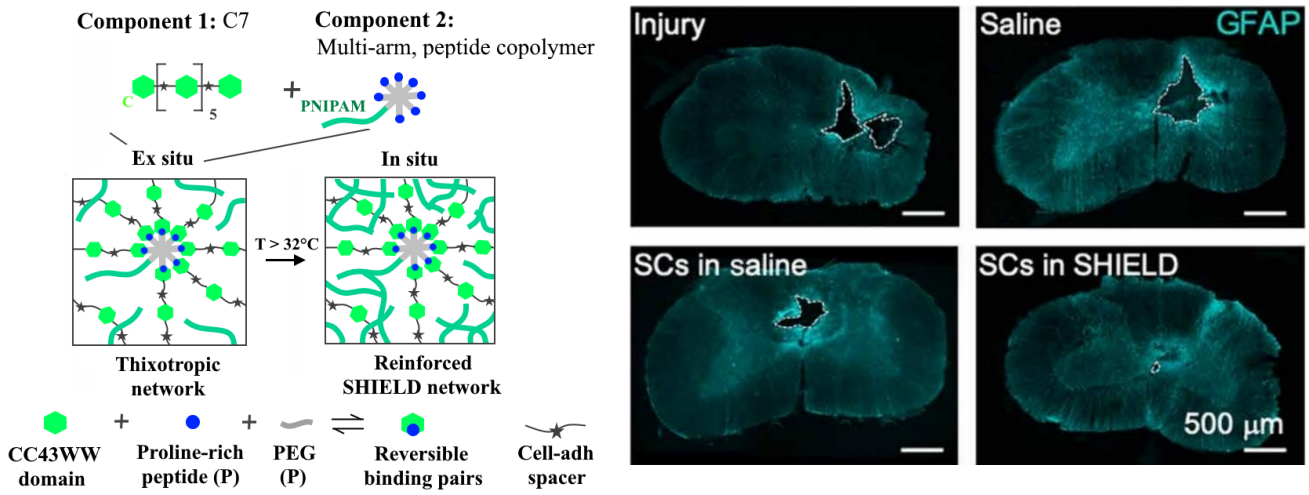


Figure 5. SHIELD design (left) and a scan fluorescent images (right) of spinal cord sections display cavity areas across all groups: untreated lesion (injury), injury treated with saline medium (saline), injury treated with saline medium (saline) and Schwann cells (SC in saline), injury treated with SHIELD and Schwann cells (SC in SHIELD), Cyan, GFAP (right).

Figure 5 reproduced with permission from ref 56. Copyright 2020 American Association for the Advancement of Science.

3.2 Nanoparticles. Given the small dimensions, nanoparticles can be injected in the affected area through the needle of a syringe. The fabrication methods (ex. double emulsion/solvent evaporation technique, thermal decomposition) used to produce these particles give the opportunity of embedding

specific signals or cells. Azizi et al. [21] studied the use of poly(lactic-co-glycolic acid) PLGA nanoparticles (273.5 ± 36.4 nm) embedding chABC in a rat contusion model. The anti-inflammatory response was promoted by the enhancement of regenerative M2 macrophages (in a process described above) and caused axonal regrowth (Figure 6). The improvement of locomotor functions and the enhancement of circuit plasticity were also observed. However, the PLGA nanoparticles failed in obtaining an extensive axonal regeneration and the complete cleavage of the CSPG, MAG, OMgp, and Nogo. These results were obtained through an ”extrinsic” approach, different from the “intrinsic” one [44] described in Figure 7. Additionally, Zhang et al. [71] used nanoparticles to track the position of mesenchymal stem cells (MSCs), investigating their optimal number to transplant in the post-traumatic syrinx caused by SCI. Nanoparticles of 53±9 nm made of ferric oxide (Fe_3O_4) cores were coated with bovine serum albumin (BSA) covalently conjugated with monoclonal antibodies against vascular endothelial growth factor (mAbVEGF). The results showed a precise transplantation strategy of MSCs thanks to the magnetic resonance imaging (MRI) visualization of magnetic nanoparticles.

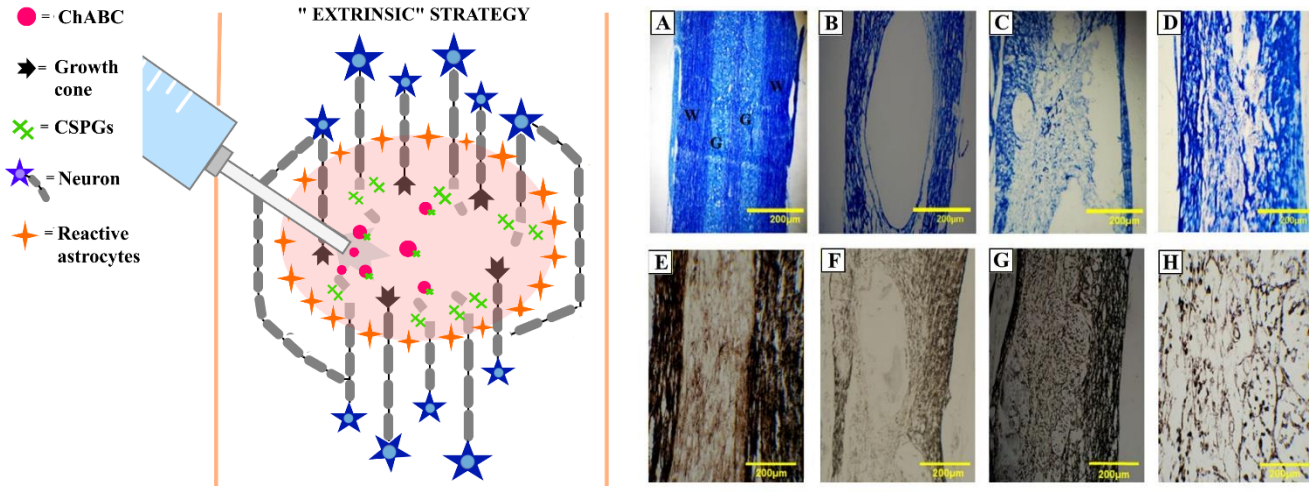


Figure 6. Injected drugs such as ChABC delivered by nanoparticles (NPs) showed a local action/extrinsic strategy of CSPGs removal. The remaining inhibitory molecules are not completely removed, and a pathological status is partially present (left), while a regenerative process (plasticity increasing, axonal growth and elongation) can be observed. Luxol fast blue (LFB) (A–D) and Bielschowsky (E–H) staining of longitudinal sections of the injured spinal cord within 8 weeks after treatment (right). The samples observed are (A, E) the sham group, (B, F) untreated spinal cord after injury, (C, G) PLGA NPs injected without ChABC (D, H) the ChABC particle-treated groups. In the Bielschowsky staining, the axons appear brown to black in color. W and G stand for the white and the gray matter of the spinal cord, respectively.

Figure 6 (right, A-H) reproduced with permission from ref 21. Copyright 2020 Elsevier.

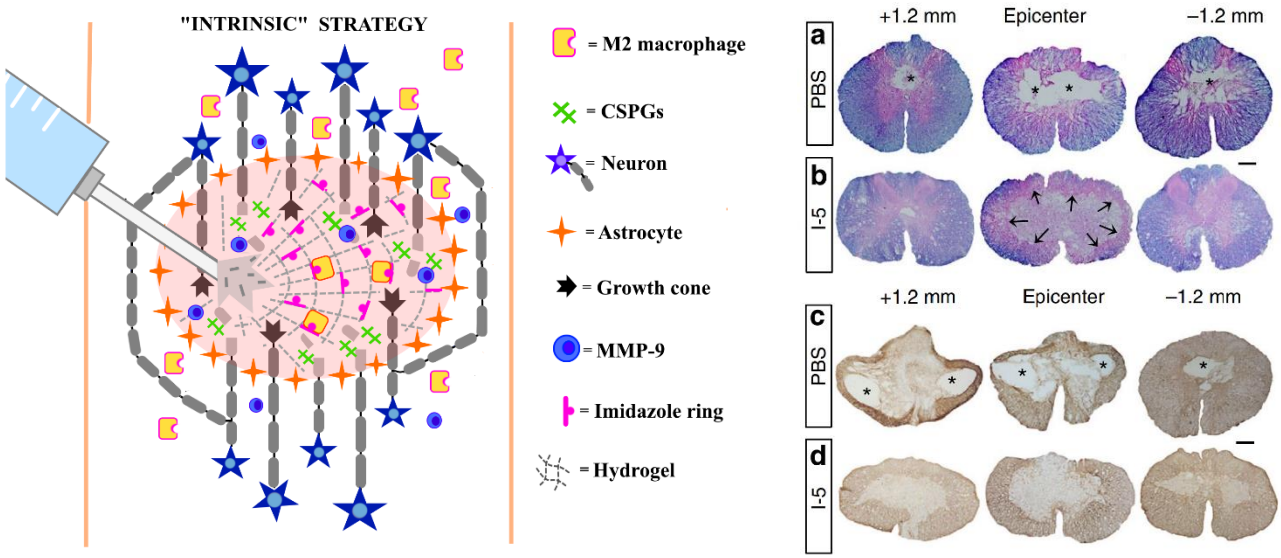


Figure 7. Injection of I-5 hydrogel stimulates an "intrinsic" mechanism of MMP-9 and M2 macrophages recruitment come from the surrounded tissue. The imidazole rings located in the hydrogel matrix interact with the histamine receptors on macrophages that linger for a prolonged time enhancing a wound healing

mechanism (left). On the right (a-d) the effects of I-5 injection can be evinced: a cystic cavity reduction, ECM remodelling and inflammatory response decrease. Representative images of transverse spinal cord sections stained with eriochrome cyanine and eosin (a, b) or GFAP antibodies (c, d). Spinal cord sections were obtained from animals 4 weeks after PBS (a, c) or I-5 injection (b, d). The sections shown are from the epicenter and 1.2mm rostral (+1.2 mm) or caudal (−1.2 mm) to it. Asterisks indicate cystic, cystic boundary are indicated by black arrows (b). Scale bars represent 200 μm.

Figure 7 (right, a-d) reproduced with permission from ref 44. Copyright 2017 Springer Nature.

3.3 Composites. The term refers to the combination of two or more materials with different physical properties resulting in a new material with improved tailored characteristics. Injectable composites for SCI repair are generally made of nanotubes/nanoparticles/nanofibers/microtubes embedded in hydrogels. In particular, microtubes are generated by self-assembly of glycolipids, phospholipids, and other amphiphilic molecules. Variation in concentration, pH, or temperature influences the supramolecular assembly, which is driven by van Der Waals, electrostatic forces, or hydrogen bonding. Microtubes embedded in hydrogels are investigated as drug carriers for SCI repair applications. For example, hemi-sectioned chronic severe SCI in dogs was treated with a hydrogel-lipid microtube based delivery system of chABC combined with trehalose [29], TS-ChABC. Trehalose was found to stabilize chABC activity at 37 °C but the mechanisms are not well known [19]. The thermal stabilization of chABC demonstrated: sustained drug delivery, CSPG inhibition, improvements in locomotor function in dogs with chronic severe SCI, and some sensory recovery, which increased if chABC and NT3 were combined. The chABC long-distance diffusion required into the injured human spinal cord could be a limitation. Generally, chABC is administrated continuously by invasive pumps implanted *in vivo* but TS-chABC via hydrogel-microtube system could be a less invasive option.

Lipid microtubes associated with different axonal growth cones factors (Cdc42, Rac,1, and BDNF) were also coupled with agarose hydrogel by Jain et al. [50]. In a dorsal over-hemisection lesion, the treated rats showed a reduced number of reactive astrocytes in the injured area, a reduced CSPG deposition, the presence of neurofilaments across the lesion, and a higher percentage of axons in the CSPG-rich regions. The dosage of the three growth factors was not optimal for stimulating the axonal growth in the CST.

Specific molecules proven to be useful in healing and regenerating the spinal cord can also be loaded in nanoparticles, which are eventually embedded in hydrogels to allow a sustained release of drugs or factors. A composite of HA and methylcellulose (MC) hydrogel with PLGA nanoparticles was used to localise the nanoparticles in the specific site of injection and for the sustained release of neurotrophic factors and inflammatory molecules suppressors (embedded in the nanoparticles). [47] The BBB score of treated rats with a compression injury increased with respect to controls, but the inflammatory response was notable. The same composite structure was also coupled with FGF2 [48]. Thanks to the degradation kinetics of the materials, a sustained and long-term release of FGF2 was obtained. Results showed improved angiogenesis, a decrease of the cavity volume, and proliferative lesion, but functional improvements were not recorded.

Ansorena et al. [49] demonstrated that a composite scaffold made of alginate, fibrinogen, and PLGA microspheres increased the number of neurofilaments, gained functional recovery, and more homogeneous and dense regenerated tissues through the slow release of GDFN. However, the functional recovery in hemisected rats was higher with free-microsphere hydrogels.

Instead, a nanofibre-hydrogel composite was produced by mixing fragments of polycaprolactone (PCL) fibers with surface grafted maleimide (MAL) groups, and a gel of thiolated hyaluronic acid (HA-SH) and polyethylene glycol diacrylate (PEGDA) [52,72]. The material provided mechanical support for spinal cord regeneration and a suitable porosity for cell infiltration. The spinal cord thinning because of the progressive loss of neural tissue following contusion was controlled by the presence of the composite.

The authors reported a shift of M1 macrophages to M2, limited to the lesion site, possibly linked to the presence of the PCL fibres. The role of fibres needs to be further investigated, but their presence seemed to encourage neo-vascularisation and differentiation of endogenous stem cells in immature neurons.

A minimally invasive method to deliver mouse embryonic stem cell (mESC) in the injury site was studied by Wang et al. [53]. They embedded an aligned electrospun nanomesh of poly (D,L-lactic acid-co-trimethyl carbonate (P(DLLA-co-TMC)) in gelatin-acrylated β-cyclodextrin (β-CD) polyethylene glycol (GCP) hydrogel, which was formed by the photo-crosslinking process. The high stretchability of this material allows it to be injected. Motor neurons derived from embryonic stem cells (MN-ESC) were also embedded in the composite. Results showed an oriented neurite growth, a dendritic development, a decreased loss of tissue and inflammatory response, synapses formation, and motor function recovery. The material filled the injury cavity, but the degradation kinetics was not optimal.

A hydrogel of hyaluronic acid and methylcelluloseenriched with PLGA microparticles and BDNF was tested in a rat transection model [54]. This material had tunable mechanical properties, gelation, and biological activity by changing the molecular weight of HA. The inflammatory response was not investigated but the scaffold improved the adaptive plasticity.

Nazemi et al. [55] used microspheres of PLGA for the delivery of hydrophobic drugs such as the paclitaxel (PTX). PTX is an anti-cancer drug that leads to axonal growth, functional outcomes, and a reduction of the fibrotic scar when injected at the lesion site of rat's spinal cord. The microspheres loaded with PTX were included in an alginate hydrogel that interacted electrostatically and by metal-ion chelation with minocycline hydrochloride (MH). This composite showed a prolonged drug release (2 months), a decrease of the inflammation response and scar tissue, an increase of axonal regeneration, protection, and functional improvement.

3.4 Self-assembling peptides (SAP). Amphiphilic molecules can show self-assembly capacity through noncovalent interactions forming 3D structures. Amphiphile peptides (AP) can be designed alternating

hydrophilic and hydrophobic amino acids or positively and negatively charged amino acids that can undergo to self-complementary assembly. For example, Yuqiao Sun et al. [62] presented a strategy to create nanofiber hydrogels using two oppositely charged SAPs conjugated with bioactive peptides motifs such as IKVAV [73] or RDG. The use of peptide amphiphile coupled with IKVAV in rat spinal cord transection gave many beneficial results: axon elongation, functional recovery, suppressed the progression of astrogliosis, facilitated remyelination of axons inside the lesion, regeneration of corticospinal motor and sensory fibers, increasing the number of serotonergic fibers caudal to the lesion, and decreased cells apoptosis. Unfortunately, there was a small number of regenerating dorsal column fibers. Tysseling et al. [32,74] verified also the effect of IKVAV peptide conjugated with amphiphile peptide (PA) on rat spinal cord compression model with similar results.This SAP-based scaffold was first studied by Silva et al. [73], obtaining an efficient differentiation of stem cells into neurons. PA nanofibers were also studied when displaying the heparan sulfate mimetic and laminin mimetic epitopes [33]. The *in vivo* studies showed this injectable scaffold as a valid ECM substitute after SCI. Indeed, an overall tissue integrity was obtained and the locomotor functions of the treated animals improved.

Cicognini et al. [31] investigated the use of two SAPs B24 and biotin-LDLK12 for the treatment of a contusion injury in rats. The SAP B24 is derived from the functional motif of the bone marrow homing peptide 1 (BMHP1), whereas biotin-LDLK12 is an ionic SAP (motif: biotin-LDLKLDLKLDLK-$CONH_2$). At the same concentration (1.12 mM), B24 resulted in less viscous than the biotin-LDLK12, which was less permissive to water, free radicals, and immune cell infiltration than B24. Thus, Biotin-LDLK12 formed a dense scaffold after injection without diffusing within the injured tissue as opposed to B24 and showed a slower degradation rate. For these reasons, the hematoma reabsorption was faster where biotin-LDLK12 was injected (Figure 8). However, SAPs swelling caused the compression of the surrounding tissue.

Self-assembling peptide (RADA16) nanofiber containing IKVAV motif were also studied for central nervous system applications [75,76]. Cicognini et al. functionalized RADA 16-I to BMHP1 by using a 4-glycine bridge and observed an increase of vascularization and migration of glial precursor cells, a remodelling of the ECM with consequent decrease of cyst area [61].

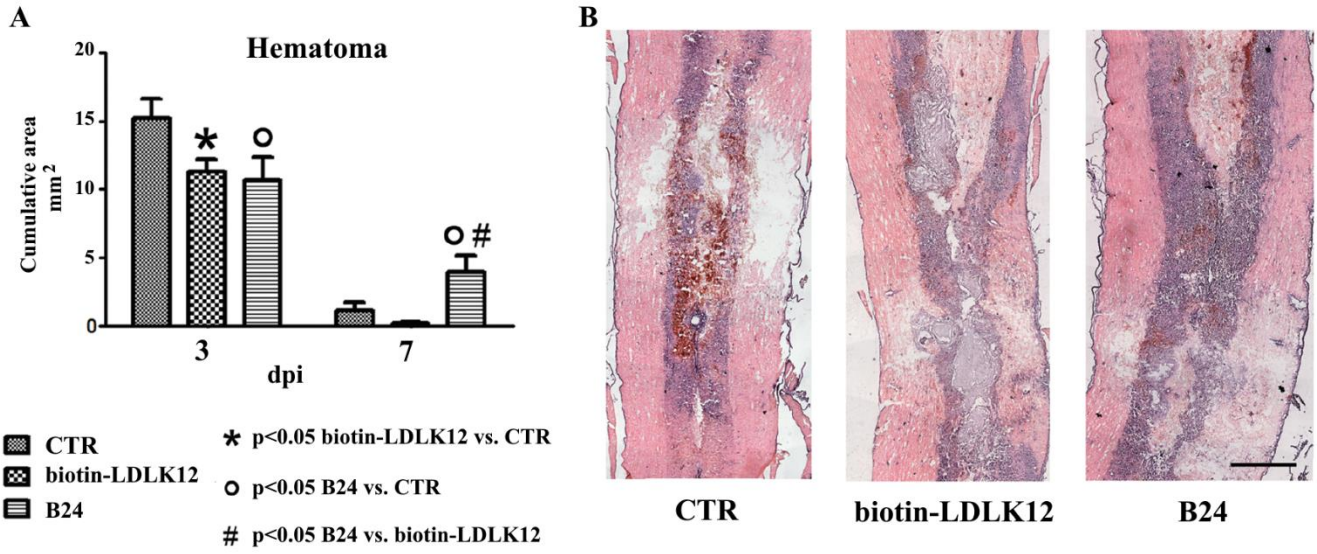


Figure 8 Quantification of hematoma. (A) At 3 day after injury (dpi) both SAP-treated groups had a significant lower leakage of red blood cells in comparison with controls. At 7 dpi biotin-LDLK12-treated animals showed the lowest content of red blood cells, while B24 the highest one. (B) Longitudinal sections stained with hematoxylin/eosin showed the presence of extravasated red blood cells (red-brownish coloured). Scale bar: 700 μm.

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Tran et al. [60], investigated instead the use of pH-responsive self-assembly hydrogel, RADA-16I (Ac-$RADA_4$-$CONH_2$), to primarily provide a favourable environment for capillary formation. Indeed, the damage of the blood spinal barrier (BSCB) causes inflammation and glial scar formation that inhibit tissue regeneration. The results showed the presence of microvessels from 9.0 ± 3.1 μm to 100 ± 46 μm

within the RADA-161 hydrogel, depending on the cell density condition. The formation of the BSCB within the RADA-161 hydrogel reduced inflammatory response and scar formation and increased axon infiltration into the SCI site. An improvement of this system could be the control of microvessels orientation because the axon growth specifically in the rostral-caudal direction could be essential for SCI treatment. Arginine-alanine-acid aspartic-alanine (RADA)4 SAP was also used by Tavakol et al. [57] in combination with IKVAV or with a longer laminin motif (CQAASIKVAV (CQIK)) [57] to form a hydrogel-based material with nanofiber structure. CQIK resulted in improved cellular response compared to IKVAV peptide due to the greater similarity to the laminin active site. In both cases, it was observed neurite outgrowth, myelination, and inhibited astrogliosis. The locomotor recovery was significantly less than (RADA)4 combined with bone marrow homing peptides (BMHP).

Ye et al. [59] cultured isolated primate NSCs in polypeptide RADA16 (AcN-RADARADARADARADA-CNH2) that can be assembled at physiological pH. The *in vivo* tests on the rat compression model showed differentiation of NSCs to neurons, oligodendrocytes and astrocytes, myelin production, and motor function recovery.

A type of SAP used to minimize SCI damages is also represented by K2(QL)6K2 or QL6 [58]. It is characterized by alternating ionic hydrophilic and hydrophobic amino acids that self-assembly into beta-sheet at physiological pH. After the acute stage, QL6 was injected into the center of the lesion, whereas the neural precursor cells (NPC) into adjacent dorsal columns. The cell survival is promoted by continuous subdural administration of growth factors through an osmotic micropump for 7 days. The presence of the scaffold improved the inhibitory environment, reducing the scar tissue and the inflammation with consequent cell survival and differentiation up to functional recovery.

Amphiphilic peptides ($CH_3(CH_2)_{14}$CO-AAAAGGGEIKVAV PA) functionalized with the laminin motif IKVAV were investigated by Hassannejad et al. [30] in order to produce an injectable hydrogel for a sustained release (21 days) of brain-derived neurotrophic factor (BDNF). This latter neuroprotective

protein-enhanced neurite outgrowth from the dorsal root ganglion (DRG) explants and the presence of the hydrogel resulted in considerable axon preservation at 6 weeks post-injury. The functional recovery was not statistically significant between IKVAV-PA hydrogel injected and saline-injected animals.

4. Spinal cord injury models

Different types of trauma can be simulated *in vivo,* generally on the spinal cord of adult rats. These injuries are contusions, compressions, hemisections, or complete sections of the spinal cord and they are performed following standard protocols to obtain reproducible results.

4.1 Severe and moderate contusion model. A reproducible contusion model on rat spinal cord via the weight-dropping method could be performed by using a stereotactic frame and computer-controlled impactor. A severe contusion is, generally, caused by an impactor tip of 3.0 mm at a speed of 4 cm/s with a depth of 2 mm and a dwell time of 0.3 sec towards an exposed and well stabilized spinal cord surface [77–79]. A severe contusion induce the highest gray matter losing, few white matter sparing, and biochemical changes such as free radicals, prostaglandins, calcium-activated proteases, loss of myelin proteins [80], extracellular potassium and calcium concentrations changes. Blight and Decrescito (1986) [81] had observed that large myelinated axons are more damaged at the nodes of Ranvier than the axons closest to the pial surface. Damaged axons caused calcium entry through tetrodotoxin-sensitive channels and consequent secondary reactions due to the calcium entry [82, 83]. A moderate contusion model, instead, preserves most of the ventral and ventral-lateral descending pathways that led to post-injury locomotor recovery in both trained and untrained animals [3].

Mechanical disruption via weight-dropping method was universally accepted to be clinically relevant [77,78], because it simulates human contusion due to their ability to mimic both primary mechanical damage and secondary reactive phase of injury.

4.2 Compression model. The compression is generally induced by an aneurysm clip closed around the exposed spinal cord of rats for 1 min until the generation of an extradural compression of 30 xg pressure. This model also simulates clinical conditions observed in several human cases but causes moderately severe acute compression injury [84].

4.3 Unilateral and hemisection model. The unilateral and hemisection of the spinal cord simulate an injury clinically observed in human cases and allow to compare injured and healthy fibers in the same animal [77] but shows disadvantages concerning model uniformity [85].

4.4 Complete model. The complete spinal cord injury model is generally performed at the T9-T10 segment and a gap of 2 or 4 mm allows the insertion of the scaffold [63]. The complete transection interrupts axon fibers and propriospinal neurons resulting in permanent paralysis. This model is considered by many researchers as the gold standard for validating axonal regeneration but it is not clinically relevant and it shows high variability in the results [85,78].

5. Severe contusion model as the new gold standard: how?

During the past decade, the complete transection SCI model has been used for validating axonal regeneration but this model is far from a real case of human spinal cord injury [86,77,82]. A contusion model, instead, reflects a traumatic human SCI but it is unsuitable for validating axonal regeneration because it is difficult to discriminate the contribution of the scaffold to the axonal regeneration from the spontaneous regeneration of the tissue [77,85]. Thus, matching a reproducible and reliable axonal regeneration with the use of a realistic SCI model is still a challenge. Some researchers proposed studies based on the contusion model to have clinical relevance (as described above) and *in vitro* models to obtain reliable results on the neuronal regeneration.

The commonly used in *vitro* cell culture models of SCI include i) primary isolated neurons, oligodendrocytes, astrocytes, or microglia cells; ii) co-culture of neuronal cells with different cell types,

which are present in the glial scar; iii) co-cultivation with meningeal cells in *in vitro* scar formation model, called scratch model; iv) rat spinal cord cells onto a confluent monolayer of neurosphere derived astrocytes for investigating the CNS axonal myelination; v) neurite outgrowth assays for the phenotypic expression of regeneration progress [87].

However, the *in vitro* evaluation lacks the complexity and physiological relevance of *in vivo* system but the results are reliable and reproducible. Unfortunately, animal studies offer complexity, which is very difficult to model *in vitro,* and high variability, which prevents reproducible studies [88,89]. For this reason, organotypic cultures of spinal cord explants could be an option to obtain reproducible results after a contusion injury [87,90]. The spinal cord explants are cut to obtain slices, called organotypic slices (OTSs), that preserve the basic structural and connective organization of their original tissue (organotypic). OTSs represent an interim system sharing the properties of the cell culture *in vitro* and an animal *in vivo* model. Organotypic spinal cord slice (OTS-SC) are generally cultivated on a semi-porous membrane at the air-medium interface to allow nutrition and gas exchanges, under appropriate conditions the slices can survive for a week to months [87].

The OTS-SC model is suitable for axonal growth evaluation because the typical ventrodorsal polarity of the SC is maintained after a culture period of 2 weeks, and intrinsic spinal cord axons formed a strong fiber tract extending along the longitudinal axis of the slice [87,90] (Figure 9).

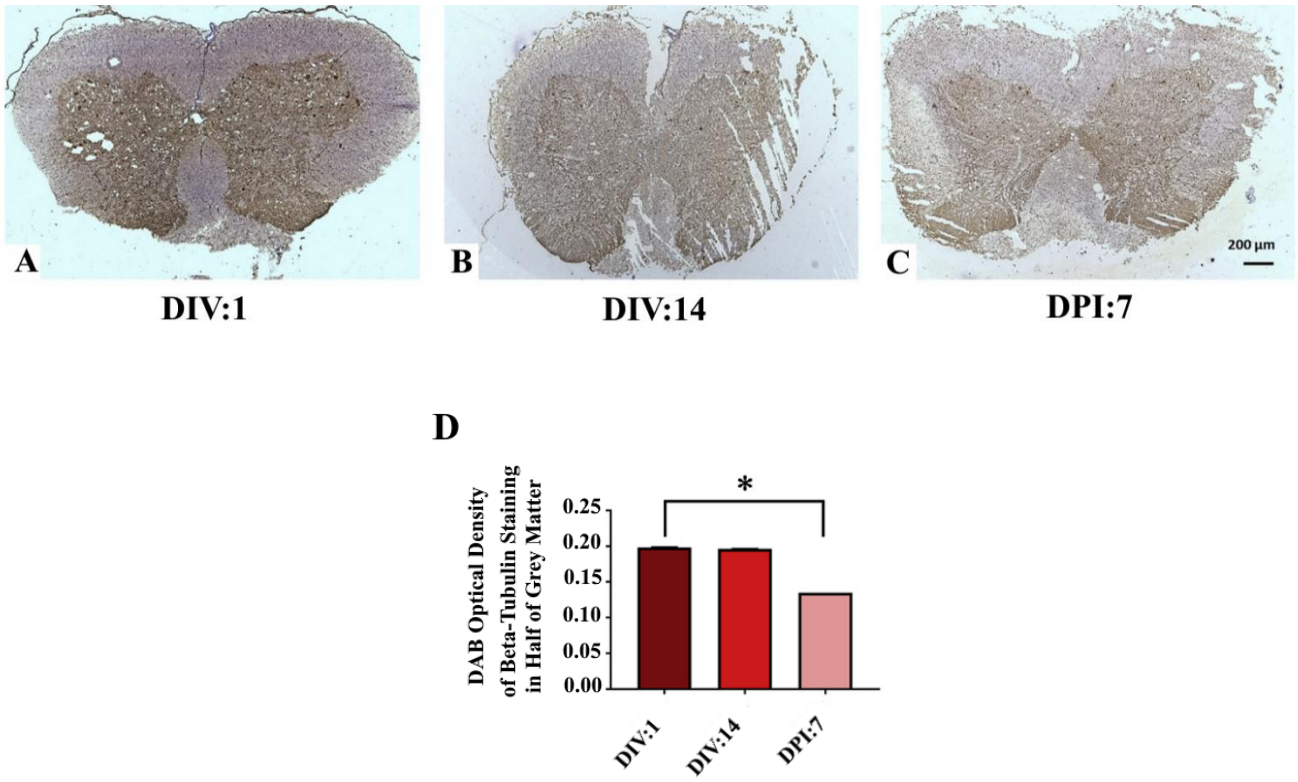

Figure 9. The first day of OTS-SC culture (A) is compared with the slice observed seven days after injury (DPI:7) (B) and its uninjured counterpart (DIV:14) (C). In the diagram (D) is reported the expression of βIII Tubulin in spinal cord slices

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A well defined *in vitro* evaluation could contribute to understanding the results of *in vivo* analysis, for example, Basso, Beattie, and Bresnahan locomotor score (BBB), ladder climbing test, electrophysiological recordings such as the rate-dependent depression (RDD) of the Hoffmann's reflex (H-reflex), defined as "the decrease in reflex magnitude relative to repetition rate" [3], suitable for evaluating sensorimotor improvements and spasticity. Moreover, this model could highlight the real contribution to the neuroregeneration of the material implanted, because the mechanism leading to post-lesional adaptive plasticity might be avoided [3,50,90] . Mechanical disruption via weight-dropping was also

tested on OTSs [90]. The results confirmed the use of this model as a simulation of a human contusion due to their ability to mimic both primary mechanical damage and the pathophysiological mechanisms after SCI. However, OTSs are harvested from animals and their treatment is expensive and time consuming [87,92]. These few limitations allow the use of OTS as a relevant platform before *in-vivo* testing [90,93].

The validation of injectable biomaterials for an eventual advanced therapy medical product (ATMP) or medical device passes through different levels, including a period of nonclinical and pre-clinical research studies, involving parametric data collection and analysis in well-defined systems[94]. In the future, we will need to establish high throughput test platforms for biomaterials, that comprise standardised testing protocols for *ex vivo*, *in vivo*, pre-clinical, and clinical testing. In the case of the SCI contusion model, there is a standardised method to elicit the lesion [77,78,82,95] but the effects of biomaterials are not completely understood once tested *in vivo* due to the high neural tissue complexity. To reach the application of biomaterials on patients, OTC-SC or 3D neural cell culture models could be used to validate the results obtained from BBB locomotor score, ladder climbing test, electrophysiological recordings and acquire reliable data in well-defined system.

6. Conclusions

Non-invasive materials for SCI treatments have gained interest due to their in situ safe procedure of administration, which might be done more than once until the complete ECM formation. Repetitive injections, still now, are expected just for cell therapy, failing the cooperation with supporting materials.

Future studies might be focused on the optimization of injectable material production such as i) swelling control, which may cause secondary damages, ii) use of non-toxic crosslinkers, iii) gradients applied on the deposited scaffold to induce a growth directionality, iv) more precise mimicking of the ECM composition and mechanical properties; v) combination of multiple fabrication methods, vi) control of the 3 D structure, vii) non-invasive electrical stimulation.

These non-invasive systems are generally injected during the sub-acute stage, when the injured site is characterized by a cystic cavity surrounded by a glial scar of ECM material, macrophages, cell, and inhibitory debris and a spontaneous regeneration process begins. *In vivo* models show the variability of outcomes even if the defects are standardized. Moreover, the SCI pathology in this model is sensibly different from humans, and the regeneration shows distinct differences in terms of axon elongation and mechanisms of sprouting [34]. Thus, *in vivo* results could be accompanied by *ex vivo* validation such as OTC-SC after compression model or 3D neural cell culture models that might give more reliable data.

Clinical trials are difficult to reach mainly due to the high cost and variability of *in vivo* testing and the complexity of the biological environment where materials are tested.

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Corresponding Authors

*sofia.santi@unitn.it, ilaria.corridori@unitn.it

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

Orcid

Sofia Santi 0000-0002-5338-2730

Ilaria Corridori 0000-0002-7348-9578

Nicola M. Pugno 0000-0003-2136-2396

Antonella Motta 0000-0003-4893-6863

Claudio Migliaresi 0000-0002-0628-0053

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Sofia Santi, Ilaria Corridori, Nicola M. Pugno, Antonella Motta, Claudio Migliaresi