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INJECTABLE THERMORESPONSIVE HYDROGELS AS DRUG DELIVERY SYSTEM FOR THE TREATMENT OF CENTRAL NERVOUS SYSTEM DISORDERS: A REVIEW

Elena Bellotti[1,*], Andrea L. Schilling[2], Steven R. Little[2,3,4,5,6,7,♣], Paolo Decuzzi[1,♣]

[1]Laboratory of Nanotechnology for Precision Medicine, Istituto Italiano di Tecnologia, Via Morego 30, 16163 Genova, Italy

[2]Department of Chemical Engineering, University of Pittsburgh, 427 Benedum Hall, 3700 O'Hara Street, Pittsburgh, PA, 15261, USA

[3]Department of Bioengineering, University of Pittsburgh, 302 Benedum Hall, 3700 O'Hara Street, Pittsburgh, PA, 15216, USA

[4]Department of Clinical and Translational Science, University of Pittsburgh, Forbes tower, Suite 7057, Pittsburgh, PA, 15213, USA

[5]McGowan Institute of Regenerative Medicine, University of Pittsburgh, 450 Technology Drive, Suite 300, Pittsburgh, PA, 15219, USA

[6]Department of Immunology, University of Pittsburgh, 200 Lothrop Street, Pittsburgh, PA, 15213, USA

[7]Department of Pharmaceutical Science, University of Pittsburgh, 3501 Terrace Street, Pittsburgh, PA, 15213, USA

*corresponding author: elena.bellotti@iit.it

♣ These authors share the senior authorship.

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
aFGF	acidic fibroblast growth factor
AIBA	2,2'-azobis [2-methylpropionamide] dihydrochloride
BBB	blood brain barrier
BBTB	blood brain tumor barrier
BCSFB	blood cerebrospinal fluid barrier
BDNF	brain-derived neurotrophilc factor
bFGF	basic fibroblast growth factor
BMA	butylmethacrylate
BSA	bovine serum albumin
CED	convention enhanced delivery
CMC	carboxymethylcellulose
CNS	central nervous system
CSF	cerebrospinal fluid
DFO	deferoxamine mesylate
DOX	doxorubicin
DPSC	dental pulp stem cells
DSC	differential scanning calorimetry
dscECM	descellularized spinal cord extracellular matrix
ECM	extracellular matrix
EGF	epidermal growth factor
EMA	European Medicine Agency
EPO	erythropoietin
FGF-2	fibroblast growth factor-2
FUS	focused ultrasound
G'	storage modulus
G''	loss modulus
GBM	glioblastoma
GDNF	glial-derived neurotrophic factor
GF	growth factor
HA	hyaluronan
HP	heparin-poloxamer
ICV	intracerebroventricular
IP	intraperitoneal
IT-L	intrathecal-lumbar
LSCT	lower critical solution temperature
MC	methylcellulose
MFG-E8	milk fat globule-epidermal growth factor 8
MSC	mesenchymal stem cells
MSC	microsphere
NGF	nerve growth factor
NIPAAm	N-isopropylacrylamide
NP	nanoparticle
NSC	neural stem cells
NT-3	neurotrophin-3
PAA	poly(amidoamine)
Pcgel	PEG-g-chitosan
PCL	polycaprolactone

PD	Parkinson's disease
PEG	poly(ethylene glycol)
PEGMA	poly(ethylene glycol) methacrylate
PGRN	progranulin
PLA	poly(lactic acid)
PLGA	poly(lactic-co-glycolic acid)
pNIPAAm	poly(N-isopropylacrylamide)
PTX	paclitaxel
RNS	reactive nitrogen species
ROS	reactive oxygen species
SCI	spinal cord injury
TBI	traumatic brain injury
TGP	thermoreversible gelation polymer
TMZ	temozolomide
VEGF	vascular endothelial growth factor
ν	viscosity

ABSTRACT

The central nervous system (CNS), consisting of the brain, spinal cord, and retina, superintends to the acquisition, integration and processing of peripheral information to properly coordinate the activities of the whole body. Neurodegenerative and neurodevelopmental disorders, trauma, stroke, and brain tumors can dramatically affect CNS functions resulting in serious and life-long disabilities. Globally, the societal and economic burden associated with CNS disorders continues to grow with the ageing of the population thus demanding for more effective and definitive treatments.

Despite the variety of clinically available therapeutic molecules, medical interventions on CNS disorders are mostly limited to treat symptoms rather than halting or reversing disease progression. This is attributed to the complexity of the underlying disease mechanisms as well as to the unique biological microenvironment. Given its central importance, multiple barriers, including the blood brain barrier and the blood cerebrospinal fluid barrier, protect the CNS from external agents. This limits the access of drug molecules to the CNS thus contributing to the modest therapeutic successes.

Loco-regional therapies based on the deposition of thermoresponsive hydrogels loaded with therapeutic agents and cells are receiving much attention as an alternative and potentially more effective approach to manage CNS disorders. In this work, the current understanding and challenges in the design of thermoresponsive hydrogels for CNS therapy are reviewed. First, the biological barriers that hinder mass and drug transport to the CNS are described, highlighting the distinct features of each barrier. Then, the realization, characterization and biomedical application of natural and synthetic thermoresponsive hydrogels are critically presented. Advantages and limitations of each design and application are discussed with the objective of identifying general rules that could enhance the effective translation of thermoresponsive hydrogel-based therapies for the treatment of CNS disorders.

Keywords: Injectable gels; drug delivery system; neurological disorders; brain cancer; polymer design.

1. INTRODUCTION

The central nervous system (CNS), consisting of the brain, spinal cord, and retina, is the control panel of the human body and plays a key role in health and well-being. Specifically, the CNS has the unique ability of processing and integrating information to coordinate activities such as movement, cognition, sensing, and emotions [1]. Moreover, it controls vital functions such as heart and respiration rates as well as the body temperature [2,3]. The basic building blocks in the CNS are the billions of neurons representing its functional unit. Neurons store, process, and transfer information across synapses via chemical signals and along axons via electrical impulses [4]. Mature neurons possess a limited regenerative capacity so that any damage would lead to a permanent functional loss, severely impacting the patients' quality of life [5]. Particularly, the CNS can be affected by several conditions such as neurodevelopmental disorders, neurodegeneration, tumors, stroke, as well as traumatic injuries. Millions of people suffer from these conditions, with no age distinction [4], and experience devastating consequences due to the progressive cell degeneration, cell death and ultimately, permanent disabilities and death. In addition to the limited regenerative capacity, the CNS has unique anatomical and physiological features that diminish the therapeutic efficacy of current therapies.

Despite many technological developments, the delivery of drugs to the CNS remains challenging due to the presence of the blood brain barrier and the blood cerebrospinal fluid barrier [6,7], which limit the access of therapeutic molecules to the CNS. Generally, the current clinically relevant approaches for treating CNS disorders are based on the systemic administration of therapeutic agents [8]. However, the effective concentrations reached at the biological targets are modest thus requiring the frequent administration of high drug doses at the risk of severe, systemic toxicity [8]. A commonly used strategy to overcome the drawbacks associated with systemic administration is to bypass the natural biological barriers using surgical methods or injecting the therapeutic molecules directly into the CNS [9,10]. However, these approaches are invasive and increase the risk of complications including neuronal damage and local inflammatory reactions [11]. There is a paramount need to develop minimally invasive and precisely targeted drug delivery methods for efficiently and effectively treating CNS disorders while avoiding off-target effects. To this end, the use of injectable thermoresponsive hydrogels has received much attention as a possible approach to overcome the CNS barriers. Thermoresponsive hydrogels have the unique property of being a free-flowing liquid at room temperature and turning into a viscous gel at body temperature [12–14]. This property is amenable to non-invasive administration, as hydrogels can be deployed in their liquid state, virtually anywhere within the CNS, using a small needle without

requiring any invasive surgery. Then, at body temperature, the viscous hydrogel forms a depot for the sustained release of therapeutic agents.

In addition, they are capable of loading a wide variety of therapeutics including neuroprotective agents, neurotrophic factors, chemo-therapeutics as well as cells [6,15,16]. Moreover, the hydrogel can be designed to be biodegradable or bioresorbable, thus resulting in full elimination without the need for secondary intervention.

The objective of this work is to highlight the advantages introduced by thermoresponsive hydrogels to directly deliver therapeutic molecules to the CNS and overcome the limitations of the methods currently used in the clinic. In this review, we discuss both natural and synthetic thermoresponsive hydrogels that have been proposed for the treatment of CNS disorders. Specifically, the review is structured with an introductory section describing the CNS architecture, the traditional routes of drug administration to the CNS and their limitations. This poses the basis to understand the importance of thermoresponsive hydrogels to target molecules directly to the CNS. In particular, the review highlights the most recent advances in the design and testing of thermoresponsive hydrogels with properties relevant to CNS delivery, providing a specific emphasis not only on their physico-chemical features but also on their applications for the treatment and management of brain tumors, spinal cord and brain injuries, as well as neurodegenerative disorders.

2. BARRIERS TO DELIVERY OF DRUGS TO THE CNS

2.1 The blood brain barrier

The blood brain barrier (BBB) is a natural barrier protecting the brain and controlling the homeostatic, nutritive, and immune environment of the CNS as well as regulating the exchange of molecules between the CNS and blood (**Figure 1**) [17]. The BBB prevents the entrance of potentially toxic substances [9,18,19], however, the same biological mechanism that protects the brain also limits the passage of traditional therapeutic molecules [17,20].

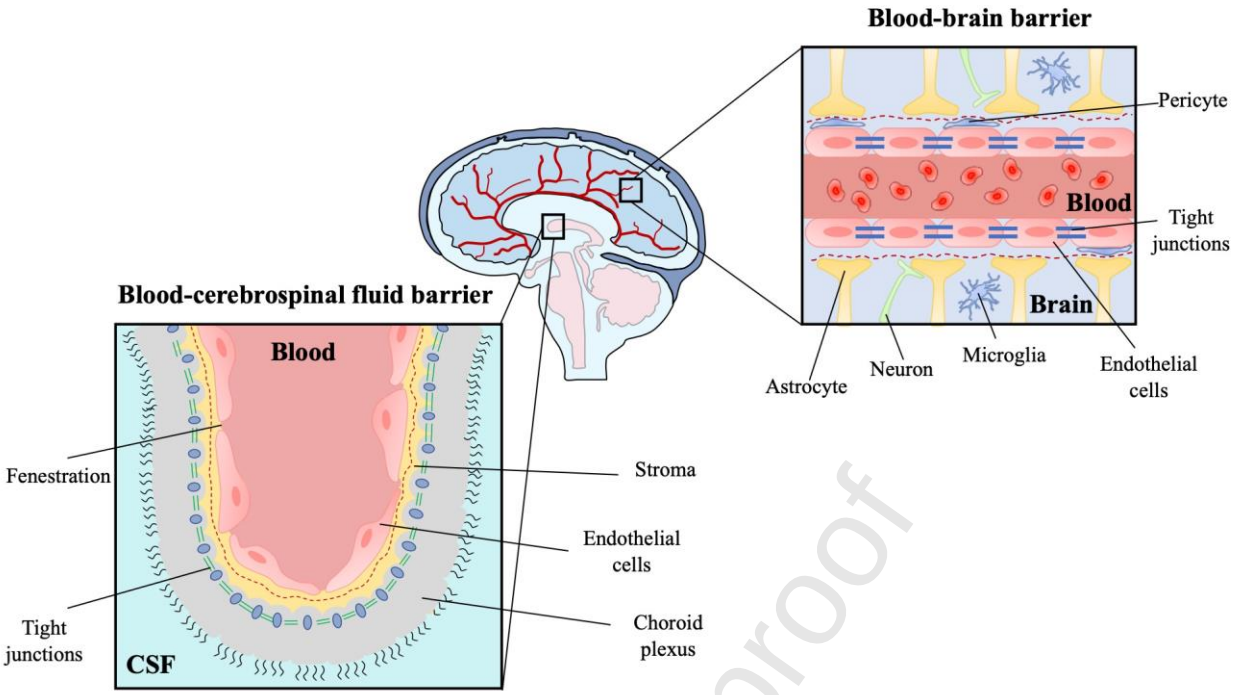


Figure 1: Schematic representations of the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB).

In general, large therapeutics (i.e. monoclonal antibodies, peptides, recombinant proteins, and gene-based therapeutics) and 98% of small molecules cannot cross the BBB [21]. In fact, only small lipophilic molecules (< 500 Da) can cross the BBB and reach the brain at effective concentrations, thus substantially limiting the options for therapeutic candidates that can be used to target CNS cells [22]. The limited access to the brain is mainly regulated by a specialized monolayer of polarized endothelial cells that are connected together to form tight junctions and control the paracellular pathway. The same tight junctions also limit pinocytosis, thus modulating the non-specific transcellular pathway too [23]. Additional obstacles impeding effective therapeutic delivery across the BBB include ecto-enzymes that can inactive many drugs and selective molecular efflux transporters. Ecto-enzymes are expressed at the level of the brain capillary endothelial cells, the capillary pericytes, and the astrocyte end-feet [23], while active efflux transporters (i.e. P-glycoprotein) are expressed at the level of the brain microvasculature for active efflux of molecules from the brain back to the blood [23,24].

2.2 The blood cerebrospinal fluid barrier

The blood cerebrospinal fluid barrier (BCSFB) is the barrier between the ventricular cerebrospinal fluid (CSF) and blood (**Figure 1**) [20]. It is located at the choroid plexuses, where most CSF is secreted [20,25]. The choroid plexuses are highly vascularized structures with a veil-like morphology that are formed by epithelial cells and tight junctions, which creates a physical

barrier [25,26]. Beneath the epithelial cells, there is a stroma containing fenestrated blood vessels (that lack tight junctions), such that large molecules can diffuse from the capillaries through the fenestrae, but are then blocked by the tight junctions of the epithelial cells [7,20]. Moreover, the BCSFB represents a functional interface between the blood and CSF due to the specific acid transporters within the choroid plexus that are capable of eliminating CSF-borne organic acids into the blood [24,27,28]. Consequently, a large number of organic acid-based therapeutics (i.e. anti-neoplastic drugs, antiviral agents) are actively eliminated from the CSF before being able to diffuse into the brain parenchyma [24].

2.3 The blood brain tumor barrier

In cancer, the blood brain tumor barrier (BBTB) originates at the interface between the malignant mass and the blood microvessels and is characterized by highly specialized endothelial cells. Specifically, tumor cells proliferate into the healthy brain tissues during tumor growth. When the tumor reaches a certain size (~ 1 mm) the BBB is damaged both structurally and functionally, and the new BBTB starts to form [29,30]. The high metabolic demand of the tumor leads to an increased expression of vascular endothelial growth factor (VEGF) causing angiogenesis and the formation of abnormal and disorganized blood vessels, and a leaky BBTB [31]. Leakiness and irregular organization are the main features that distinguish the BBTB from a regular BBB (**Figure 2**).

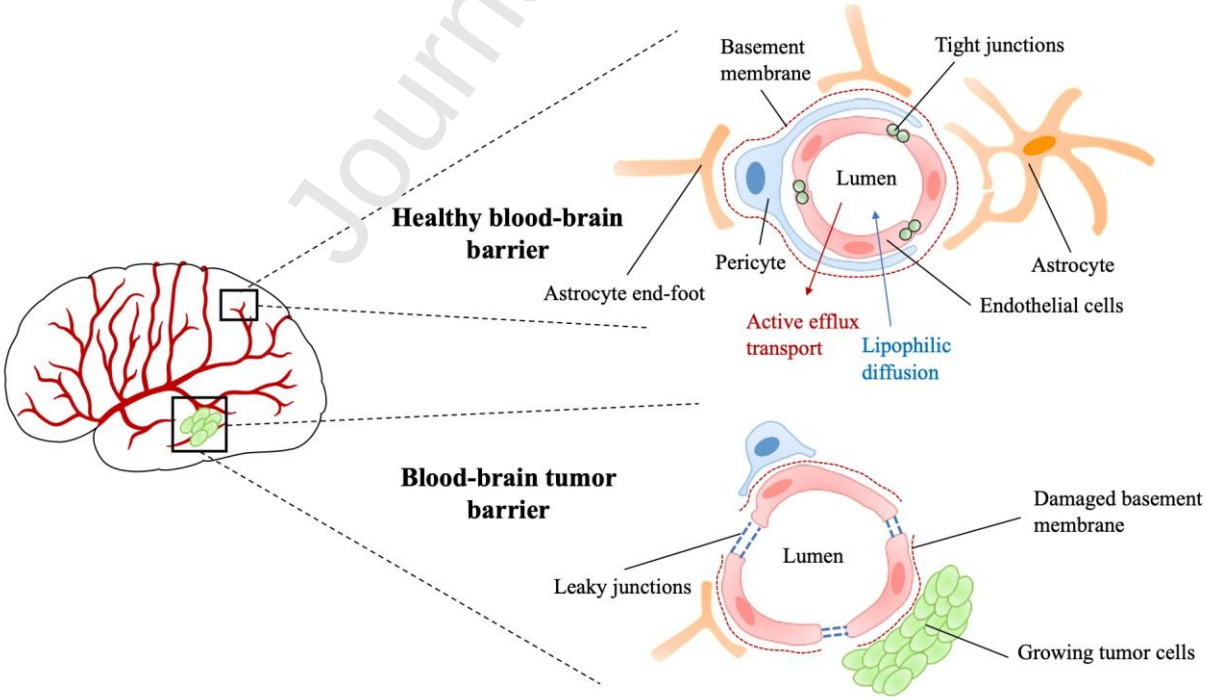


Figure 2: Schematic representations for the healthy blood-brain barrier (BBB) and blood-brain tumor barrier (BBTB).

Despite the presence of a dysfunctional BBTB, its local disruption is not sufficient to guarantee the penetration of drugs at therapeutically relevant concentrations, thus still presenting an obstacle for the treatment of the malignant tissue [29,31].

3. TRADITIONAL STRATEGIES FOR DIRECT DRUG DELIVERY TO THE CNS

Traditional approaches to physically enter the CNS and deliver the therapeutic agent directly at the target site are based on surgical methods. Although direct delivery is an invasive approach, the amount of drug reaching the diseased area is maximized, with minimal involvement of the surrounding healthy tissues. In addition, with these methods, drugs do not enter the systemic circulation, thus reducing peripheral side effects [9]. Examples of direct drug delivery, including intrathecal delivery, convection enhanced delivery, and transient BBB opening, that are utilized in the clinic to date are described in the sequel.

3.1 Intrathecal delivery

Intrathecal delivery consists in the administration of soluble therapeutic agents directly into the CNS by either intracerebroventricular administration (ICV) or intrathecal-lumbar injections (IT-L) [32]. The ICV route delivers the drug directly into the cerebral ventricles via manual compression of a port (Ommaya reservoir) surgically implanted under the scalp (**Figure 3**) [32,33]. On the other hand, single or repeated IT-L injections allow the drug to be directly delivered into the CSF by puncturing the surrounding membrane of the spinal cord (**Figure 3**) [32].

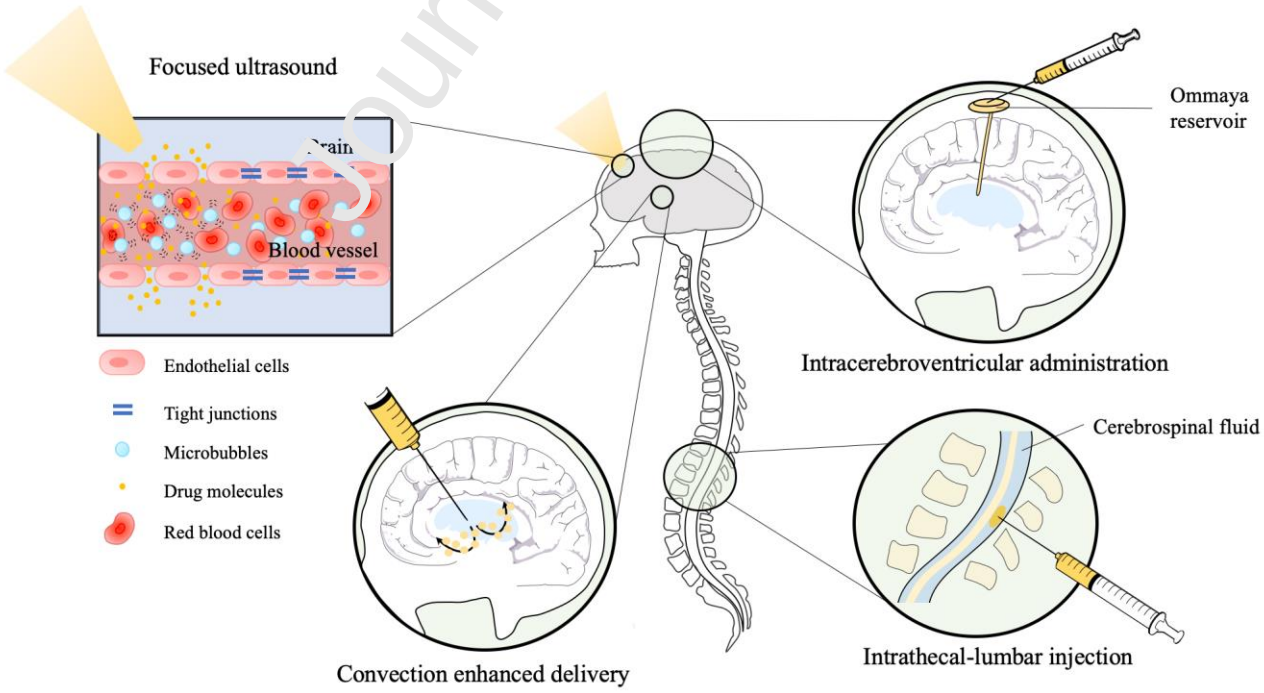


Figure 3: Schematic representation of conventional routes of drug administration for the treatment of CNS disorders: intracerebroventricular administration (ICV), intrathecal-lumbar injection (IT-L), convection enhanced delivery (CED), and focused ultrasound (FUS) combined with the infusion of microbubbles.

Even though intrathecal routes directly bypass the BBB, there are factors limiting its efficacy. Specifically, ICV administration is limited by the slow diffusion of the drug from the ventricles to the brain parenchyma. In fact, the diffusion decreases with the square of the distance, so that a small molecule with a diffusion coefficient of 5×10^{-6} cm^2s^{-1} diffuses only 1 mm in 8 h [6,34]. Since the CSF volume is replaced every 4-5 h, its turnover is much faster than the diffusion rate of a solute into the brain parenchyma. Additionally, CSF flows at approximately 20 ml h^{-1}, leading to a dispersion of the drug throughout the entire CNS or its clearance before even entering the target tissue [6,10]. Consequently, high therapeutic doses and repeated injections are needed [6]. Another limitation of intrathecal delivery is the use of catheters that are surgically implanted and can induce local cell death and infections [35,36].

3.1.1 Direct delivery through the BBTB

In the case of tumors, a convenient approach to overcome the BBTB is to directly deliver chemotherapeutics by injecting them into the resection cavity, the surrounding brain parenchyma, or the ventricles [31,37]. Like ICV and IT-L, this method consists of repeated injections or catheter-based implants connected to a reservoir to allow the continuous delivery of chemotherapeutics. The advantage of this approach is that a large amount of therapeutic agents can be delivered with reduced systemic toxicity [38]. However, drug distribution depends on the concentration gradient and tissue permeability and is generally limited to 3 – 5 mm from the injection site with an exponential decaying concentration. Therefore, this results in a high drug concentration next to the administration site and limited drug density at the target site [37]. Moreover, direct delivery through the BBTB requires invasive surgical procedures that may lead to adverse events including infection, neurotoxicity, and hemorrhage, among others, often resulting from mispositioned or obstructed catheters [31,37].

3.2 Convection enhanced delivery

Convection enhanced delivery (CED) is another invasive technique that bypasses the BBB/BBTB and allows direct and continuous injection of therapeutics using intraparenchymal catheters (**Figure 3**) [9,31]. In CED, a drug is administered via a microcatheter at fixed infusion rates, typically ranging from 0.1 to 10 μl min^{-1}, resulting in an elliptical or spherical drug distribution around the injection site [21,39]. A positive hydrostatic pressure is applied to drive the

therapeutic solution deep into the target tissue, resulting in a more uniformly distributed drug within the target region [9] [40]. Moreover, CED uses microcatheters with minimal tissue damage at the insertion site. CED has been proposed for the treatment of many CNS disorders, including neurodegenerative disorders [41,42] and brain tumors [43–45]. For brain tumors, CED can overcome the poor distribution of chemotherapeutics that characterizes direct injections and can be used after the resection of the primary mass or to treat inoperable malignancies [46]. Despite these advantages, many practical issues, such as optimization of the infusion volume and rate, anatomical features of the infusion site, and backflow are yet to be fully addressed [31].

3.3 Transient opening of the BBB

Another method for enhanced drug delivery to the CNS is by the transient, osmotic opening of the BBB. This can be accomplished through the infusion of hyperosmotic solutions, such as 20% w/v mannitol, through the carotid artery, which causes water to be exchanged between endothelial cells, brain tissue and blood inducing a transient shrinkage of the cells and opening of the BBB [7,21,47]. Consequently, the paracellular transport of molecules from blood into the brain parenchyma is temporarily enhanced. However, this process is not selective and can result in the transport of plasma proteins and other potentially toxic substances from the blood into the CNS. Also, importantly, this procedure is not restricted to a targeted BBB area and it has been associated with cerebral pain and mortality [7].

A less invasive approach relies on the use of focused ultrasound (FUS) combined with the infusion of microbubbles. FUS consists of the application of acoustic energy over a small region of a few millimeters with the systemic injection of microbubbles. When FUS is transcranially applied, the blood-borne microbubbles oscillate only in the target area leading to a highly localized disassembling of the tight junctions and a transient increase in the BBB permeability (**Figure 3**) [48,49]. With this approach, the opening is localized, reversible, and does not cause damages to neuronal cells, so that it can be repeated several times [18,49,50].

This temporary opening of the BBB, or BBTB more specifically, could be employed for the delivery of chemotherapeutics to treat gliomas [30]. However, there are challenges associated with the opening of the BBTB that limit the safe implementation of this technique. In particular, this method is not selective and can affect the BBB as well, leading to uncontrolled passage of molecules to the healthy portion of the brain or an increase of brain fluids that may cause toxic effects [31].

All the aforementioned approaches have limitations associated with patient safety and

therapeutic efficacy. As such, there is still a need for developing alternative and more effective drug delivery strategies to the CNS. One such alternative approach is based on the use of injectable thermoresponsive hydrogels that can be easily administered, in a minimally invasive fashion, and realize localized depot for the long-term, sustained deployment of different therapeutic molecules. Different classes of injectable thermoresponsive hydrogels and their application to the treatment of CNS disorders are described in the following sections.

4. NOTES ON THE MOST COMMON CENTRAL NERVOUS SYSTEM DISORDERS.

4.1. Brain tumors

Glioblastoma (GBM) is the most aggressive brain tumor in adults and is characterized by a rapid growth leading to a poor prognosis with a median survivals of ~15 months [29,51,52]. Current therapies for GBM consist in surgical interventions followed by radio- and chemotherapy, which still do not prevent tumor recurrence in the surrounding brain tissue [52,53]. Moreover, the commonly used therapeutics lack specificity, causing systemic toxicity and off-target side effects [53]. Thus, in the last decades alternative therapeutic strategies have been investigated to improve clinical outcomes. Particularly, thermoresponsive hydrogels are appealing since they can be easily administered in the resection cavity right after surgery and provide a sustained release of the therapeutic molecules in the surrounding tissues.

4.2 Spinal cord and brain injuries

Traumatic spinal cord injury (SCI) is a severe disabling condition resulting in neural tissue damages and consequent loss of locomotor and sensory functions [54,55]. The current treatments for SCI include decompression surgery, injury stabilization, and rehabilitation. However, the recovery of the neurological functions is often limited, leading to permanent disabilities [55]. Cell replacement at the injured site as well as delivery of growth factors might represent a valuable approach to guide neurological recovery and functional repair [54,56]. To this end, thermoresponsive hydrogels represent a valuable approach given their possibility of encapsulating and delivering both cells and growth factors directly at the injured site.

The same approach may be beneficial for treating damages derived from traumatic brain injuries (TBI). Specifically, injuries to the brain often result from external forces damaging neurons, glia cells, and blood vessels. This initiates acute neurodegeneration, followed by a secondary injury, which includes ischemic damages, neuroinflammation, neurotoxicity, oxidative stress, apoptosis, as well as traumatic axonal injuries [57,58]. For all these reasons, TBI is one of the main causes of permanent disabilities with very limited treatment options, and the use of thermoresponsive

hydrogels may be a valid approach to confine therapeutics, cells, and growth factors at the injured site.

4.3 Neurodegenerative diseases

Neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) are chronic and progressive disorders affecting millions of people worldwide [59–61]. These diseases are heterogeneous in their pathophysiology, with symptoms ranging from memory and cognitive impairments to inability to move and speak [62], with severe consequences on the patients' quality of life. Current treatments consist in the administration of therapeutics to alleviate the symptoms. However, anti-AD and anti-PD therapeutics reach ineffective concentrations in the brain and follow difficult administration regimens, which lead to poor patient adherence [63]. *In situ* thermoresponsive hydrogels may offer the opportunity to enhance drug bioavailability at the target site as well as reduce the frequency of administrations, thus helping overcome the drawbacks of traditional therapeutic agents.

5. THERMORESPONSIVE HYDROGELS

Thermoresponsive hydrogels are a specialized class of three-dimensional polymeric networks that undergo a phase change in response to temperature. Due to their dynamic interaction with water molecules, the hydrogel conformation can reversibly change between a liquid and a gel state. Thermoresponsive hydrogels that are in a liquid state at low temperatures and transition to a gel state with a temperature increase exhibit a characteristic lower critical solution temperature (LCST) [64]. These hydrogels are particularly appealing for biomedical applications in that their critical temperatures can be tuned to fall within a physiologically relevant range to enable *in situ* gelation upon *in vivo* application [64,65]. Below the LCST, polymer chains are soluble in physiological solutions, resulting in a free-flowing liquid. Above the LCST, polymer chains collapse as the result of various mechanisms, including hydrophobic interactions, coil-to-helix transitions, or micelle packing [64,66]. The resulting physical connection among the polymer chains creates an insoluble gel [64]. Furthermore, this phase transition is reversible, whereby decreasing the temperature below the LCST will make the polymer chains return to their water soluble configuration [64].

The polymer network constituting thermoresponsive hydrogels, and more in general any hydrogels, is realized via polymerization reactions with crosslinking agents that can be either non-biodegradable or biodegradable. Crosslinking can be achieved by chemical or physical interactions where the type of crosslinking impacts the final hydrogel properties and stability [67], which are

important factors for the target application. Chemical crosslinkers can be added to form covalent bonds, which often result in stiffer hydrogels, but could induce some level of toxicity [16,67]. Conversely, physical crosslinking via chain entanglement or secondary forces can result in hydrogels with reduced stiffness. The selection of the material, polymerization reaction, and gelation conditions must be carefully considered to ensure biocompatibility, especially for injectable systems [65]. Additionally, thermoresponsive hydrogels can be designed to be biodegradable by incorporating weak bonds that are susceptible to hydrolysis or other forms of lysis. Consequently, the cell metabolism and excretion of byproducts resulting from hydrogel dissolution affects the overall safety of the system [66]. In addition to the fabrication method, the type of polymer or polymer mixture can be used to further customize the thermoresponsive hydrogel.

This class of hydrogels offers the unique ability to be injected into a target region as a freely flowing solution which then undergoes in situ gelation conforming to the surrounding environment as temperature increases above the ambient value [65]. As such, the hydrogel LCST, gelation rate, viscosity, and mechanical strength are important parameters to be characterized and tuned for biological applications. For example, the polymer concentration as well as the copolymerization ratio of hydrophobic and hydrophilic monomers can alter the LCST and viscosity of the resulting hydrogel [64,66]. These critical attributes can be finely characterized by in vitro methods, such as spectrometry, differential scanning calorimetry (DSC), rheology, among others [66], and further assessed in preclinical in vivo models. Importantly, thermoresponsive hydrogels for the CNS should also possess a mechanical stiffness matching that of the native neuronal tissues (0.4 – 1.4 kPa for the brain and 5 – 42 kPa for the spinal cord), in order to modulate neural adhesion, proliferation, differentiation, and proper functioning [67,68]. Moreover, an adequate mechanical stiffness is fundamental to withstand in vivo forces, guarantee structural integrity and transmit physiological forces until the hydrogel is fully replaced by the native tissues, thus minimizing the response of the immune system. Generally, the mechanical stiffness of hydrogels can be easily modulated by tuning the polymer concentration and crosslinking density to obtain the optimal mechanical properties for CNS applications [69].

Thermoresponsive hydrogels have been the topic of several reviews, where the main features of a large variety of natural and synthetic hydrogels are presented for a broad range of applications [64–66]. Differently, the present work uniquely focuses on the most recent developments in the design, characterization and preclinical testing of thermoresponsive hydrogels for the treatment and management of CNS disorders. The chemical structures of the main polymers discussed in this review are highlighted in **Table 1**.

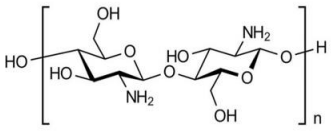
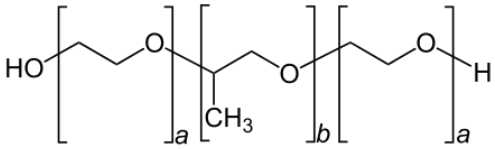
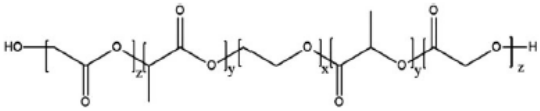
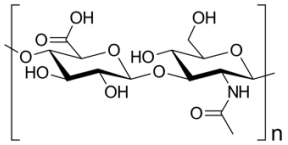
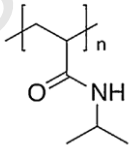
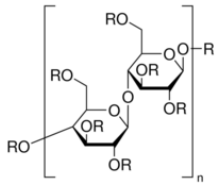
Natural Polymers	Synthetic Polymers
Chitosan 	**Poloxamer**  a: length of PEO b: length of PPO
Collagen 	**PLGA-PEG-PLGA**  x: repeating units of PEG y: lactide z: glycolide
Hyaluronan 	**pNIPAAm** 
Methylcellulose  R: H or CH_3	

Table 1: Chemical structures of natural and synthetic polymers used for the preparation of thermoresponsive hydrogels.

5.1 Natural thermoresponsive hydrogels.

Natural polymers are a class of materials originated by largely available natural products and possessing high biocompatibility and biodegradability [70]. Natural polymers include proteins (i.e. collagen, gelatin), and polysaccharides (i.e. chitosan, hyaluronic acid, methylcellulose) [70], many of which are already used in clinical practice as surgical sponges, lubricant, and dermal fillers [6]. Natural polymers have been demonstrated to be good candidates for developing hydrogels similar to the extracellular matrix (ECM), enabling a significant reduction in the stimulation of chronic inflammation, immunological reactions, and toxicity upon implantation [70,71]. The gelation of natural polymers such as methyl cellulose, collagen, and agarose is temperature driven, making them attractive for *in situ* forming gels for drug delivery and tissue engineering applications [6]. Furthermore, natural polymer-based hydrogels are very attractive for CNS applications because they exhibit mechanical properties that are comparable to CNS tissues and can be processed to have suitable porosity for cell infiltration, transplantation, and axon outgrowth. Moreover, their chemical

properties are suitable for the incorporation of growth factors to enhance cell attachment as well as the ability to encapsulate and deliver different drug molecules [71].

The following sections describe the main natural polymer-based thermoresponsive hydrogels and their application as novel treatments of brain tumors, spinal cord and brain injuries, as well as neurodegenerative diseases. Physico-chemical features and applications of the main natural thermoresponsive hydrogels are summarized in **Table 2**.

Type of polymer	CNS Disorder	Sol-gel transition	Mechanical properties	Degradable	Delivery agent	Status of development	Ref.
Chitosan-based	Brain tumors	$\geq$ 32 °C		Biodegradable	T-cells	*In vitro*	[54]
		34-37 °C		Biodegradable	Ellagic acid	*In vitro*	[75]
	Spinal cord or brain injuries	37 °C			MSC	Mouse	[73]
		37 °C	G' = 200 Pa	Biodegradable	BMSC	Mouse	[78]
		32.6 °C		Biodegradable	Ferulic acid	*In vitro*	[79]
	Neurodegenerative disorders	30-35 °C		Biodegradable	Ibuprofen	*In vitro*	[80]
		37 °C	σ = 140-350 Pa		Ropinirole	Rat	[81]
Collagen-based	Spinal cord or brain injuries	37 °C	G' = 20-45 Pa	Biodegradable	MSC, NSC FGF-2	*In vitro*	[93,94]
		Body temperature		Biodegradable	NT-3	Rat	[95]
	Neurodegenerative disorders	37 °C		Biodegradable	GDNF-MSCs Neurons	Rat	[96,97]
Hyaluronan-Methylcellulose-based	Spinal cord or brain injuries	18-32 °C	$\nu = 10^0$-10^6 Pa•s Shear thinning	Biodegradable		Rat	[13]
		37 °C		Biodegradable	EPO	Rat	[100]
				Biodegradable	EPO	Mouse	[14]
		37 °C		Biodegradable	EPO NPs EGF-PEG NPs	Mouse	[105]
				Biodegradable	cortically specified neuroepithelial stem cells	Rat	[101,104]
				Biodegradable	Peptide (KAFAK) and BDNF	Rat	[102]
		Physiological temperature		Biodegradable	CSa-PLGA MPs	Rat	[103]
			G' = 30-1000 Pa	Biodegradable	PLGA blank-MPs	Rat	[106]

Table 2: Biophysical features and biomedical applications of natural polymer-based thermoresponsive hydrogels

G' = storage modulus, ν = viscosity, σ = mucoadhesive force

5.1.1 Chitosan-based hydrogels

Chitosan is a natural cationic polysaccharide derived by the deacetylation of chitin N-acetyl-glucosamine residues from shellfish [72] (see structure in **Table 1**). Chitosan is an attractive biomaterial due to its well-established biocompatibility, low immunogenicity, easy processability as well as anti-oxidant and anti-inflammatory properties [73]. Typically, chitosan-based hydrogel gelation is pH-dependent. However a temperature-dependent gelation can be achieved by adding glycerophosphate salt or poly(ethylene glycol) (PEG) [53,74,75]. Thanks to its active functional groups, chitosan is suitable for protein binding as well as for encapsulation and delivery of several therapeutics. Furthermore, its inherent positive charge favors the stimulation of cell interaction and differentiation [76]. Given these favorable biomaterial properties, the use of chitosan-based thermoresponsive hydrogels to target the CNS has become of interest over the years.

Drug delivery to brain tumors

Chitosan-based hydrogels have been rationally designed for drug and cell delivery to brain tumors [53,75]. For instance, a biodegradable, thermoresponsive hydrogel based on PEG-g-chitosan (PCgel) has been proposed for the localized delivery of T lymphocytes to glioblastoma cells [53]. PEG/chitosan ratio and gel concentration were adjusted to tailor gelation time and temperature, viscosity and pore size. The resulting hydrogel had a sol-to-gel transition around 32 °C, making it suitable for an *in situ* forming material. PCgel showed good cellular compatibility with T lymphocytes, which retained their anti-tumor activity after being embedded within the gel matrix [53]. Moreover, PCgel had a suitable pore size in the range of 0.1 - 0.5 μm to favor T lymphocyte infiltration into the gel in a higher number compared to the Matrigel control, as well as allowing a steady release of viable cells. Furthermore, T lymphocytes released from PCgel were able to kill glioblastoma cells more effectively than cells released from Matrigel. The study demonstrated that the performances of PCgel are preferable over Matrigel and any other gel of animal origin, given its exceptional biocompatibility, low immunogenicity and pathogen transfer, in addition to low costs and consistent properties from batch to batch. Even though more extended studies on PCgel are recommended, especially to assess its *in vivo* efficacy, the excellent properties highlighted in this study suggest that PCgel depot could offer a valid approach for glioblastoma immunotherapy [53].

Others have proposed a chitosan/β-glycerophosphate thermosensitive gel (Ch/β-GP gel) for the release of ellagic acid, a natural phenol anti-oxidant that has anti-carcinogenic properties [75].

The hydrogel was optimized in terms of composition to ensure gelation around 37 °C, as well as biocompatibility. The hydrogel was enzymatically degradable over time, and the presence of the enzyme significantly increased the ellagic acid release. Furthermore, studies of anti-tumor effects of the ellagic acid-releasing hydrogel on human U87 glioblastoma cells and rat C6 glioma cells, demonstrated the inhibition of cancer cell growth in an agent dependent manner [75]. Although chitosan films with anti-tumor efficacy were already developed by the same group [77], the thermoresponsive Ch/β-GP gel represents an improvement in terms of ease of administration. In fact, while the film needed to be surgically implanted, the Ch/β-GP gel can be administered through a simple injection of a liquid formulation, minimizing the risks associated with the surgery. The *in vitro* characterization showed that the anti-cancer activity was maintained, and even if more *in vivo* studies are needed, the Ch/β-GP gel can be considered a promising delivery method for local cancer treatment.

Delivery to the injured spinal cord and brain

Chitosan-based thermoresponsive hydrogels have been explored to enhance the therapeutic effects of mesenchymal stem cells (MSCs) on spinal cord regeneration by providing 3D support, improving their survival, as well as increasing their retention rate [73,78]. The hydrogels were fabricated in the presence of β-glycerophosphate to ensure the gelation of chitosan around body temperature. Through *in vitro* cytocompatibility assays, MSC viability was reported to be unaffected by the hydrogel. Moreover, the paracrine activity of MSCs, as well as their anti-oxidant properties were maintained after being encapsulated within the gel matrix [73]. Additional *in vivo* studies highlighted that the chitosan hydrogel encapsulating MSCs was easy to inject immediately after murine SCI transection. Moreover, 7 days after the injection, a high number of viable cells was visible both inside and surrounding the lesion [73]. Others have modified the chitosan/β-glycerophosphate hydrogel by adding hydroxyethyl cellulose to accelerate the gelation of the hydrogel and reduce the amount of glycerophosphate. They also included collagen in the hydrogel formulation, given its low immunogenicity [78]. MSCs encapsulated within the hydrogel were viable after 5 days, as showed by *in vitro* cytocompatibility assays. *In vivo* testing showed *in situ* formation of the gel in 5 min, 60% degradation in 21 days, and good histocompatibility as demonstrated by the absence of inflammatory cells at the site of injection, suggesting a minimization of the immune response [78]. Moreover, behavioral studies on SCI mice revealed that hindlimb motor function was gradually recovered over time likely due to inhibition of cell apoptosis and neurotrophic effects [78]. Overall, the positive effects of MSCs in repairing tissues and modulating inflammation is well documented. However, the main drawback is the general low cell

survival after implantation, mainly due to the spinal cord environment. The findings of these studies represent an advancement in the field of spinal cord treatment, showing chitosan-based thermoresponsive hydrogels as a possible potent tool for increasing stem cell survivor and ameliorating cell-based therapies for SCI treatment leading to more positive outcomes.

Similar chitosan-based hydrogels have been proposed as a possible injectable matrix for treating TBI [79]. A chitosan/β-glycerophosphate hydrogel was modified with gelatin and loaded with ferulic acid to investigate its antioxidant and neuroprotective effects to treat H_2O_2 oxidative stress in Neuro-2a cells [79]. Indeed, it has been demonstrated that in TBI a large amount of reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced, which contribute to the progression of the injury. The characterization of the hydrogel revealed a sol-to-gel transition at 32.6 °C, and a gelation time of 75 s. The *in vitro* release assay showed that 85% of ferulic acid was released in the first 24 h, which is compatible with the objective of inhibiting oxidative stress in TBI [79]. In fact, clinical reports have shown a significant increase in the oxidative stress in the first 60 min of TBI, and it is essential to immediately control it to protect neural cells. Cytotoxicity assays of ferulic acid-loaded hydrogel highlighted a high biocompatibility with Neuro-2a cells. Additional analysis revealed a control on H_2O_2 DNA fragmentation, as well as down-regulation of ROS, inflammatory and apoptosis-related markers after ferulic acid release, suggesting that the proposed hydrogel could effectively protect against TBI associated impairments [79].

Drug delivery for neurodegenerative diseases

Given its mucoadhesive properties, chitosan-based thermoresponsive hydrogels have been also proposed for the nasal delivery of molecules involved in the treatment of neurodegenerative disorders such as Alzheimer's and Parkinson's disease [80,81]. Specifically, a chitosan-based thermoresponsive hydrogel has been optimized for the release of ibuprofen through the nasal route [80]. Different molecular weights of chitosan were tested in order to find the optimal formulation for intranasal delivery. Chitosan with a molecular weight of 110-150 kDa met the needs for the development of an optimum nasal spray, with a wide spray area and high surface area coverage [80]. The optimized gel possessed a sol-to-gel transition 2.9 times faster than mucociliary clearance rate and at a temperature in the range of the human nasal cavity, making it suitable for nasal administration [80]. Furthermore, *in vitro* studies revealed no cytotoxic effects on nasal epithelial cells, and a reversible modulation of the tight junctions with an accelerated transport of drug across the epithelium [80].

Others have demonstrated that the use of a nasal formulation based on chitosan hydrogels enhanced the brain delivery of ropinorole, a dopamine D2 agonist used in the management of PD [81]. The optimized formulation transitioned to gel at a temperature suitable for the nasal cavity and had a sufficient mucoadhesive force to aid nasal administration [81]. Intranasal administration of ropinorole hydrogel in albino rats revealed an absolute brain bioavailability of 82%, and the $AUC_{0\text{-}480min}$ in the brain was considerably higher than that obtained after intravenous or intranasal administration of soluble ropinorole [81]. The nose to brain transport was further confirmed by the high direct drug transport percentage (90.36%) and drug targeting index (>1) [81], confirming the chitosan-based thermoresponsive hydrogels to be a valuable approach to improve CNS targeting.

Overall, the chitosan-based thermoresponsive gels have been demonstrated suitable candidates for the encapsulation of different therapeutic molecules, and possess the characteristics to be non-invasively administered through the nasal cavity. The rapid gelation of the formulations, which is faster than the mucociliary clearance rate, along with the mucoadhesive properties offers a great advantage to increase the resident time and drug availability. Thus, chitosan-based gels for nasal delivery could become a potential alternative to other routs of administration to treat CNS pathologies.

5.1.2 Collagen-based hydrogels

Collagen makes up 20-30% of the protein content of the human body and is the principal component of connective tissue [6,82] (see structure in **Table 1**). As a biomaterial, collagen has numerous advantages including bioactivity, biocompatibility, low immunogenicity, and biodegradability [83,84]. These properties make collagen an attractive material for biomedical applications, and its use in drug delivery and tissue engineering is well documented [85–88]. Moreover, collagen is a major candidate for an injectable thermoresponsive hydrogel due to its thermal gelling properties that allow collagen to be injected as an acidic solution that turns into gel at body temperature [6].

Collagen-based hydrogels have shown many advantages as biomaterials for CNS applications. For example, they can be used in the form of injectable scaffolds for the release of therapeutic molecules to protect neurons in neurodegenerative disorders, for cell delivery, as well as for filling the void at the injured site, thus supporting and promoting axonal growth [82].

Drug delivery to the injured spinal cord

Collagen-based hydrogels have shown great potential as materials for CNS injuries both in *in vitro* studies and in *in vivo* models. In fact, collagen-based hydrogels are capable of supporting

growth and differentiation of cortical neural progenitor cells, astrocytes, and cortical neurons [89]. Moreover, the mechanical stiffness of collagen hydrogels can be modulated by changing its concentration to guide cell behavior, including neurite growth and inhibition of glial cell proliferation, thus reducing the formation of glial scars after spinal cord injury [90–92]. However, collagen itself is quite weak, and often cross-linkers, such as genipin, are used to improve its stability and mechanical strength [84]. For example, genipin was added at different concentrations to covalently cross-link a collagen thermoresponsive hydrogel for tuning its degradation profile and mechanical properties to meet the needs of an injectable material for SCI [93]. Particularly, the collagen-based materials gelled in approximately 50 s, and no significant decrease in the gelation point as a function of the increased genipin concentration was detected. On the other hand, the increase of genipin concentration had a significant effect on the storage modulus (G') of the hydrogels (moving from G' = 20.8 ± 0.9 Pa when no genipin was added to G' = 45.1 ± 6.0 Pa, with 1.0 mM genipin) [93], as well as on the degradation resistance. Notably, a 3-fold increase in the remaining weight % was observed for 0.25 mM genipin cross-linked gel (allowed to gel for 1 h prior to exposure to collagenase) as compared to the non-cross-linked one. Furthermore, the degradation resistance was almost 4-fold higher for gels with the lowest amount of genipin and allowed to gel for 27 h prior to exposure to collagenase [93]. In addition, the study demonstrated that the genipin diffusion out of the gel was slow enough to ensure that a significant amount of gel remained within the material. This is relevant for the *in vivo* application of the gel and that substantial cross-linking upon injection is feasible [93]. Furthermore, MSCs and neural stem cells (NSCs) were encapsulated within the gel, since they have been demonstrated to provide therapeutic benefits after SCI in terms of replacement of lost cells, modulation of the wound environment, and reduction of inflammation. Cell viability and proliferation assays showed that MSCs survived the gelation process and remained viable for up to 10 days in gels containing 0.5 mM genipin. On the other hand, NSCs could not tolerate high genipin concentrations, and remained viable only in gels containing up to 0.25 mM genipin [93]. These results demonstrated that, although genipin is necessary for obtaining optimal gel stability and mechanical strength, its concentration must be finely tuned based on the kind of cells to guarantee a prolonged viability and therapeutic efficacy upon administration.

The same group studied the infiltration of astrocyte into the collagen-based hydrogel cross-linked with 0.25 mM genipin and containing fibroblast growth factor-2 (FGF-2) to treat SCI [94]. FGF-2 was loaded into the gel either freely or encapsulated in lipid microtubules. Subsequently, the number of primary rat astrocytes infiltrating into the collagen gel and the infiltrating distance were evaluated *in vitro*. The study showed a significant increase in the number of infiltrating astrocytes,

and a higher penetration distance in the presence of FGF-2 compared to controls without FGF-2. While crosslinking with genipin was shown to decrease the number of infiltrating astrocytes, FGF-2 encapsulated lipid microtubules restored the number of infiltrating cells to levels similar to the non-cross-linked gels with FGF-2 (**Figure 4**) [94].

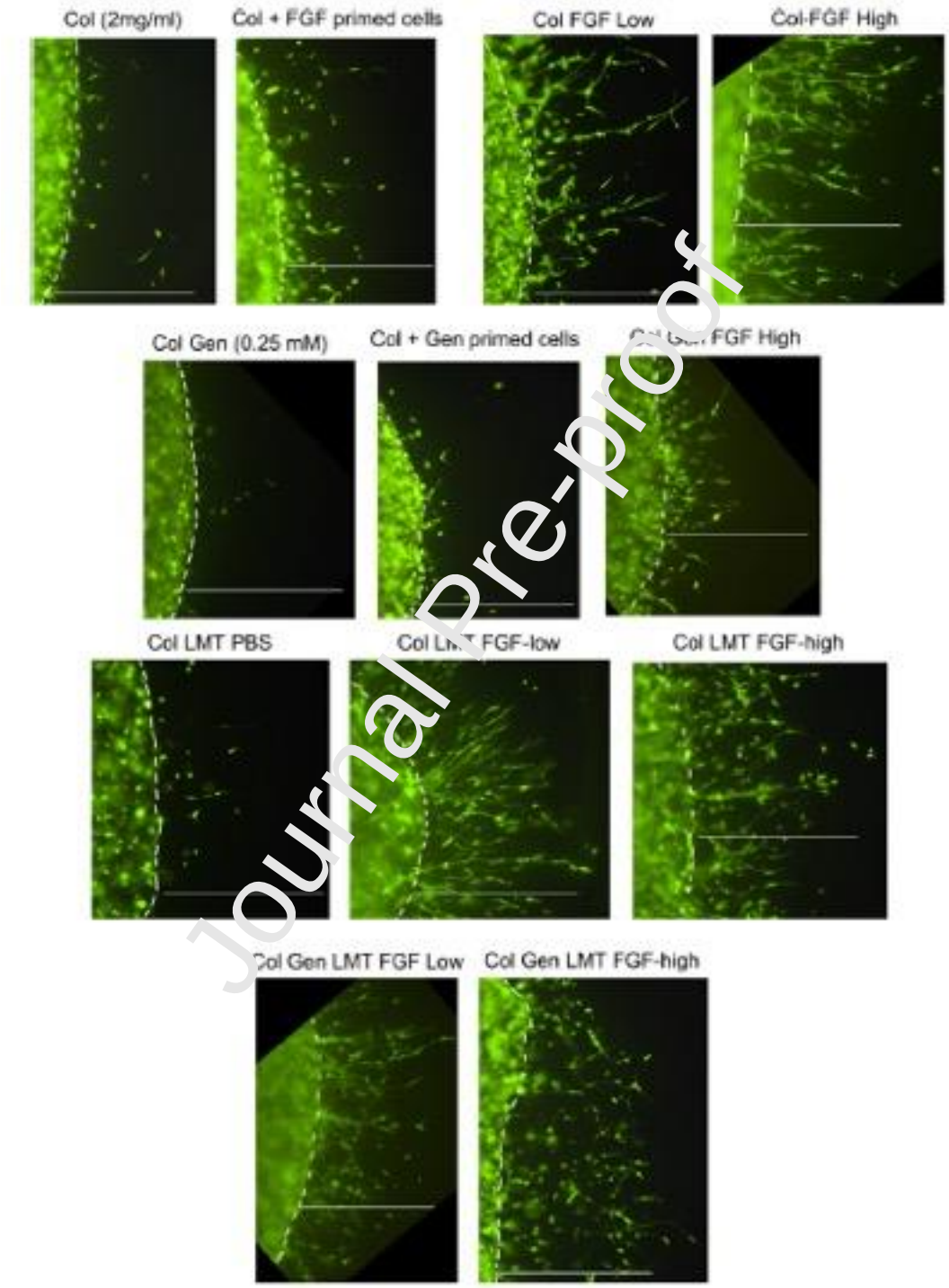


Figure 4: Representative images showing infiltration of astrocytes into the collagen-based hydrogels 10 days post seeding. The images were acquired under a 4x objective lens using a calcein AM viable cell stain and epifluorescence microscopy. Scale bar = 1000 μm. Dotted lines represent the interface. The images refer to the following groups: *Col (2 mg/ml)*: collagen at a concentration of 2 mg/ml; *Col + FGF primed cells*: collagen gel + astrocytes exposed to 150 ng/ml FGF-2 before the assay setup; *Col FGF Low* and *Col FGF High*: collagen gel loaded with 15 ng/ml and 150

ng/ml free FGF-2, respectively; *Col Gen (0.25 mM)*: collagen gel cross-linked with genipin 0.25 mM; *Col + Gen primed cells*: astrocytes exposed to 0.25 mM genipin for 24 h before the assay setup; *Col Gen FGF High*: collagen gel cross-linked with 0.25 mM genipin loaded with 150 ng/ml free FGF-2; *Col LMT PBS*: collagen gel + 0.1 mg/ml lipid microtubules loaded with PBS; *Col LMT FGF-low* and *Col LMT FGF-high*: collagen gel + 0.1 mg/ml lipid microtubules loaded with 0.11 mg/ml and 1 mg/ml FGF 2, respectively; *Col Gen LMT FGF Low* and *Col Gen LMT FGF-high*: collagen gel cross-linked with 0.25 mM genipin + 0.1 mg/ml lipid microtubules loaded with 0.11 mg/ml and 1 mg/ml FGF-2, respectively.
Reprinted with permission from ref. [94].

Others have evaluated the effects of a thermoresponsive collagen-based hydrogel containing neurotrophin 3 (NT-3) loaded microspheres on the recovery after SCI [95]. The hydrogel was cross-linked with pentaerythritol poly(ethylene glycol) ether octasuccinimidyl glutarate, and it was designed to match the compressive modulus of the spinal cord tissues (3-5 kPa) in order to provide structural support and tolerate the external pressure from the vertebral column and surrounding muscles. The administration of the hydrogel in the hemisected spinal cord of rats favored the localized delivery of NT-3. In addition, the treatment promoted axonal growth, and reduced both glial scarring and inflammation [95]. Significant increase in functional recovery was observed in the control group treated with only hydrogel and blank microspheres and was maintained for up to 6 weeks. However, no significant differences were observed in the group treated with the hydrogel containing NT-3 loaded microspheres [95], suggesting that further investigations on the beneficial effects of embedding microsphere within the hydrogel matrix should be carried out.

Drug delivery for neurodegenerative diseases

Collagen-based injectable hydrogels have been proposed for the release of neurotrophic factors to offer neuroprotection in neurodegenerative diseases as well [96,97]. The delivery of neurotrophic factors to the brain via MSCs is a promising neuroprotective strategy, however its use is limited due to the poor cell survival after implantation. Despite the use of collagen-based hydrogels for an intra-cerebral use has not been well established, studies suggest that such hydrogels hold potential to provide a supportive matrix for transplanted cells, thus improving cell survival after implantation. Specifically, the opportunity of using a collagen hydrogel (cross-linked with 4 arm Star-poly(ethylene glycol)) as an intracerebral transplantation matrix for the delivery of glial-derived neurotrophic factor (GDNF) overexpressing MSCs (GDNF-MSCs) was evaluated in rats [96]. The study highlighted that the collagen hydrogel did not affect the viability of the cells and allowed the secretion of the GDNF into the striatum parenchyma. Moreover, transplanting GDNF-MSCs within a collagen gel significantly reduced host brain response to cells, revealed by a reduced recruitment of both microglia and astrocytes at the delivery site (**Figure 5**) [96].

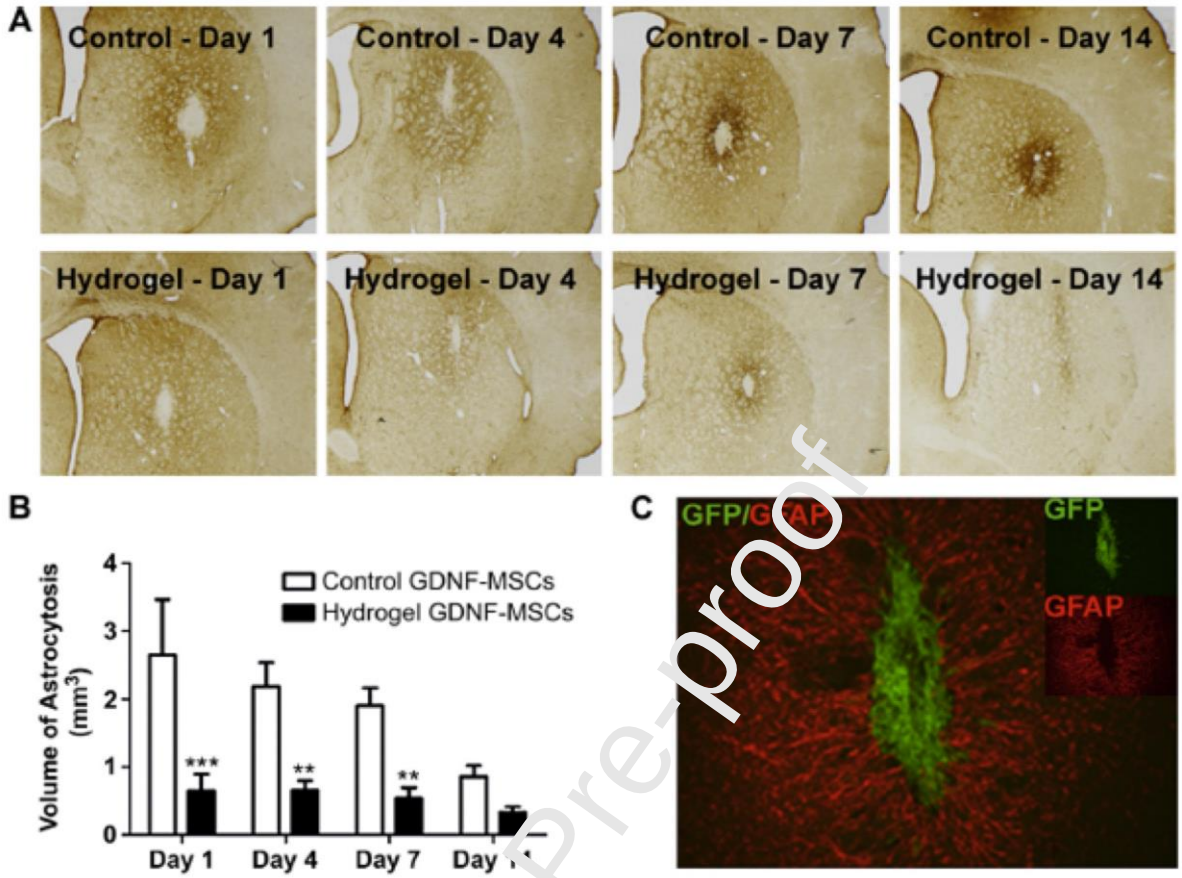


Figure 5: Effect of the collagen hydrogel on the host astrocytic response to GDNF-MSCs in vivo. 30,000 GDNF-MSCs were injected into each striatum either in control medium or in the collagen hydrogel, and the host astrocytic response to the cells was analyzed at Days 1, 4, 7 and 14 after transplantation. (A) A striatal astrocytic reaction to the implanted cells could be visualized using GFAP immunohistochemistry in the host striatum at each time-point examined. A reduction in the host astrocyte response to GDNF-MSCs is clearly visible in the hydrogel group compared to the control group (B) Post-hoc comparison of the group differences revealed that the collagen hydrogel significantly reduced the host astrocytic reaction to the GDNF-MSCs at the Day 1, Day 4 and Day 7 time-points. (C) Qualitative fluorescent staining for OX-42 and GFAP revealed that the astrocytes surrounded but did not infiltrate the GFP-MSC graft site.
Data are presented as mean ± S.E.M and were analyzed by 2-way ANOVA. **P < 0.01, ***P < 0.001 vs. Control GDNF-MSCs.
Reprinted with permission from ref. [96].

A similar GDNF-loaded collagen hydrogel was used to encapsulate primary dopaminergic neurons. The hydrogel was injected in the lesioned striatum of a rat model of PD to assess its efficacy in terms of survival of the primary neurons, re-innervation, and functional recovery [97]. After administration, the hydrogel was well tolerated by the brain, it promoted GDNF retention at the injection site, and protected the transplanted cells from the host immune response. Additionally, the study revealed that the GDNF-collagen hydrogel significantly improved the survival of

dopaminergic neurons and striatal re-innervation, leading to a significant enhancement of the functional recovery [97].

5.1.3 Hyaluronan-Methylcellulose-based hydrogels

Hyaluronan (HA) is a biocompatible, non-immunogenic anionic glycosaminoglycan widely distributed in the connective, epithelial, and neural tissue [98] (see structure in **Table 1**). HA itself does not promote cell adhesion, but it can be combined with other natural polymers to create an ECM-like scaffold for supporting neuronal cells. HA is water soluble and tends to disperse when injected in an aqueous environment, therefore other polymers, such as methylcellulose (MC) must be added to form a physical gel [98].

Methylcellulose is a natural non-immunogenic polysaccharide, which has low viscosity at room temperature and exhibits a sol-to-gel transition at physiological temperatures (see **Table 1**) [98,99]. MC itself forms a weak gel at 37 °C, but the gelling time is not quick enough for drug delivery applications. The combination of MC with other polymers, such as HA reduces the sol-to-gel transition temperature, leading to a more gel-like material than MC alone [98]. Unlike other thermoresponsive hydrogels, those that are HAMC-based are already in a gel-like state at room temperature, but they can be easily injected due to their shear-thinning properties [13]. After the injection, the gel strength is increased by the increased temperature.

Drug delivery to the injured Spinal cord and brain

The use of HAMC-based hydrogels to treat SCI and stroke-related brain injuries is well documented [13,14,100,101]. There are many advantages in using these hydrogels, including the fast gelation to ensure localized administration at the site of action, low cell adhesion to minimize scar formation, biodegradability, and biocompatibility [13]. Additionally, HAMC hydrogels are suitable for the encapsulation of cells and different molecules, such as peptides, proteins, and neurotrophic factors [101–104].

HAMC hydrogels have been proposed for the delivery of neuroprotective molecules, such as erythropoietin (EPO), at the injured site [14,100]. The localized administration from EPO-loaded hydrogels led to an improved residence time and bioavailability of the molecule when compared to intraperitoneal (IP) or direct bolus injection, as well as a significant behavioral recovery in a rat SCI model [100]. Moreover, it was revealed that EPO penetrated deep into the brain, leading to neuroprotective and neuro-regenerative effects in the stroke-injured brain of mice [14]. However, drug delivery from hydrogels is rapid and a sustained release over a long period of time, which is required for tissue repair, is not easy to achieve [105]. To overcome this limitation, the

incorporation of polymeric particles within the hydrogel matrix has been investigated [105,106]. Poly(lactic-co-glycolic) (PLGA)-blank (no drug) nanoparticles designed for achieving a 28-day sustained release were embedded into the HAMC hydrogel, and their effects on the hydrogel stabilization *in vitro*, as well as on the biocompatibility and safety *in vivo* were assessed [106]. The presence of the nanoparticles within the hydrogel caused a significant change to the rheological properties of the gel, leading to an increased stability of the matrix [106]. The combined system was well tolerated in the intrathecal space of SCI rats, with negligible microglia activation and astrocyte response. The safety of the material was further confirmed by similar motor function in rats treated with HAMC gels containing PLGA nanoparticles and controls [106]. Additionally, the same combined system was used for the sequential delivery of epidermal growth factor (EGF)-PEG and EPO in a murine model of stroke-injured brain [105]. The epicortical delivery system provided a prolonged controlled release of both agents and stimulated endogenous neural stem/progenitor cells in the brain of adult mice, achieving neural tissue repair without the typical tissue damages of catheter/minipump systems [105].

The same HAMC hydrogel was used to transplant neural precursor cells in a rat model of stroke-injured brain [101,104]. Even though cell maturity played a key role on transplantation success [101,104], the cell delivery vehicle helped reduce the host response and induced functional recovery of the animals [104], also when injected alone. This suggest that HAMC is a good candidate to promote host tissue repair as well as to function as a cell delivery vehicle.

5.2 Synthetic thermoresponsive hydrogels.

Synthetic polymer-based hydrogels may be more attractive than the natural ones because they can be easily tuned in terms of molar mass, composition, rate of degradation, and mechanical properties [6,71]. Furthermore, the use of synthetic polymers reduces the risk of disease transmission and immunogenic/allergenic reactions [71]. Another advantage in the use of synthetic polymers is the ability to modify or charge the material to favor cell attachment and differentiation [71]. However, not all synthetic polymers offer a suitable biological environment for cell differentiation, proliferation, and tissue regeneration [65].

In order to take advantages of the properties of both natural and synthetic polymer-based hydrogels, a valuable strategy is to create a combined hydrogel. These can be obtained by copolymerization of synthetic and natural macromers, or by forming an interpolymer complex bonded via physical interactions [71]. The advantage of this approach is the combination of the biocompatibility of natural gels with the possibility to tune mechanical properties and degradation of the synthetic ones, leading to a material with customized properties for a variety of applications

[71]. The following sections will describe applications of synthetic polymer-based thermoresponsive hydrogels and their combination with natural polymers for novel treatments of CNS disorders. The characteristics and applications of synthetic thermoresponsive hydrogels are summarized in **Table 3**.

Type of polymer	CNS Disorder	Sol-gel transition	Mechanical properties	Degradable	Delivery agent	Status of development	Ref.
PLGA-PEG	Brain tumors	Body temperature		Biodegradable	OncoGel™ (paclitaxel)	Clinical trials	[119,123-125]
	Spinal cord or brain injuries	30 °C			Atsttrin	Mouse	[126]
		Physiological temperature		Biodegradable	MG-E8	Rat	[127]
pNIPAAm-based	Brain tumors	20 °C			Doxorubicin soluble liposomes or MPs	Mouse	[135,136]
					Camptothecin soluble and MP	Rat	[137-139]
					Vincristine	Rat	[138]
		28 °C		Non-biodegradable	Epirubicin, paclitaxel	Mouse	[140]
		32 °C		Biodegradable	Rhodamine, IgG	*In vitro*	[141]
	Spinal cord or brain injuries	30 °C	E = 1-4 kPa	Non-biodegradable	BDNF, NT-3	*In vitro*	[142]
		33 °C	G' = 25-50 kPa G" = 17-20 kPa	Non-biodegradable	BDNF, NT-3	Rat	[143]
				Non-biodegradable	BDNF	Rat	[144]
		31-32 °C	E = 50-250 kPa	Biodegradable	BDNF, NT-3	Rat	[145-147]
		37 °C	G' = 400 Pa	Biodegradable	DFO	Rat	[148]
	Neurodegenerative disorders	32 °C	$\nu = 10^0$-10^2 Pa•s	Biodegradable	Activin B	Mouse	[60]
Poloxamer-based	Spinal cord or brain injuries	37 °C		Biodegradable	GDNF	Rat	[55]
		37 °C	Strain amplitude = 6.5%		aFGF	Rat	[56]

		35-37 °C	ν = 7-16 Pa•s		FGF-2	Rat	[109-111]
		37 °C			NGF	Rat	[108]
	Neurodegenerative disorders	32-34 °C	0.1 Pa•s		Rivastigmine hydrogen tartrate NPs	*In vitro*	[61]
					Rivastigmine tartrate		[112]
		18-51 °C	σ = 380-1100 Pa	Biodegradable ("corrosion")	Geniposide	*In vitro*	[113]
		22-52 °C	σ = 148-540 Pa		Ropinirole	Mouse	[114]
		34 °C	ν = 1-1000 Pa•s		Amantadine hydrochloride	*In vitro*	[115]
		30-32 °C	σ = 100 Pa		Levodopa NP	Rat	[116]
		28-33 °C			Rasagiline mesylate	Rabbit, Rat	[117]

Table 3: Biophysical features and biomedical applications of synthetic polymer-based thermoresponsive hydrogels.

G' = storage modulus, G" = loss modulus, ν = viscosity, σ = mucoadhesive strength or force, E = compressive modulus

5.2.1 Poloxamer-based hydrogels

Poloxamers or Pluronics® are nonionic triblock copolymers consisting of a central hydrophobic chain of poly(propylene oxide) and two external hydrophilic chains of poly(ethylene oxide) that make the polymer amphiphilic and possess surface active properties (see structure in **Table 1**) [107]. Poloxamer and water solutions undergo sol-to-gel transition at physiological temperatures. In addition, they are non-toxic and non-irritant making them attractive materials for biomedical applications [107]. Many poloxamers are commercially available, with different molecular weights and hydrophobic-hydrophilic ratios, thus allowing the fabrication of thermoresponsive hydrogels with customable properties in terms of gelation time and critical gelation concentration at physiological conditions. Poloxamer water solutions can undergo a sol-to-gel transition around 37 °C and a gel-to-sol transition at 50 °C in a concentration-dependent manner [107]. Furthermore, poloxamers have a proven record of FDA approval and are listed in the US and European Pharmacopeia for uses as stabilizers, emulsifiers, solubilizers, and for topical, parenteral, and oral administration [107].

Drug delivery to the injured spinal cord and brain

Poloxamer-based thermoresponsive hydrogels have been proposed for the encapsulation and release of growth factors (GFs) to the injured spinal cord, in order to increase their physicochemical stability and overcome their intrinsic inability to cross the BCSFB [55,108,109]. Poloxamers are often combined with heparin to increase the affinity for GFs, improve their controlled-release capabilities, maximize the effectiveness of the molecule, as well as minimize the GF biochemical modifications that happen when administered *in vivo* [56]. Several GFs have been loaded into heparin-poloxamer (HP) hydrogels, including nerve growth factor (NGF), GDNF, and acidic fibroblast growth factor (aFGF) [55,56,108]. In general, the HP hydrogels protected the bioactivity of the GFs and enhanced cell uptake [56,108]. Furthermore, the administration of GF-loaded HP hydrogel into the injured spinal cord of rats revealed neuroprotection against cellular apoptosis, reduced reactive astrogliosis, as well as increased neuronal and axonal rehabilitation [55,56,108]. Another positive outcome of the administration of HP hydrogels containing GFs compared to the free administration of the molecules was the gradual restoration of the locomotor function following SCI in rats [55,56,108]. Another study showed the beneficial effect of combining dental pulp stem cells (DPSCs) and basic fibroblast growth factor (bFGF) within an HP hydrogel to provide neuroprotective action as well as neural regeneration and functional recovery after SCI [110]. Specifically, it was demonstrated that SCI animals treated with DPSC-bFGF-HP hydrogels had the highest motor and sensory functional recovery with a significant difference in comparison with SCI animals treated with DPSC-HP hydrogel, bFGF-HP hydrogel, or HP hydrogel [110]. Others have investigated the possibility of increasing GF stability and affinity within the HP hydrogel matrix by adding decellularized spinal cord ECM (dscECM) [109,111]. GFs were first mixed with the dscECM and subsequently added to the HP gel. The addition of dscECM to the hydrogel did not significantly alter the gelation temperature, which was still suitable for administration. Furthermore, *in vitro* release studies highlighted that the initial combination of GFs with the dscECM helped obtain a slower release (lasting up to 7 days) in comparison to that from HP hydrogel alone [109,111]. *In vivo* studies revealed an improvement in neuronal function and tissue morphology with enhanced inhibition of glial scarring. Moreover, the functional recovery of SCI rats was improved over time due to the regeneration of nerve axons and differentiation of neural stem cells [109,111]. Given the promising results of combining heparin with poloxamer to stabilize GFs and increase their cell uptake thus minimizing any possible systemic adverse reaction, further investigation of the long-term effects of GF-loaded HP hydrogels is worthwhile to advance an effective SCI therapy.

Drug delivery for neurodegenerative diseases

The use of poloxamers-based thermoresponsive hydrogels have also been investigated for the encapsulation and release of different therapeutic agents involved in the treatment of AD and PD via nasal administration [61,112–117]. However, poloxamers alone possess low residence time in the nasal cavity due to the rapid mucociliary clearance, which leads to a limited drug absorption [112]. To increase the nasal residence time, poloxamers are often combined with mucoadhesive polymers that ultimately can improve drug absorption. Many mucoadhesive polymers have been utilized to this end, including methylcellulose, chitosan, and Carbopol [112,114,115,117], and the hydrogels have been optimized in terms of poloxamer/mucoadhesive polymer ratio, sol-to-gel transition temperature and time, as well as mucoadhesive strength [112,113,115,117]. It is extremely important to finely balance the ratio between the mucoadhesive component and poloxamer, in order to lead to the optimal characteristics for nasal administration. Overall, the optimized hydrogels possessed a sol-to-gel transition temperature around that of the nasal cavity (32-35 °C) and they transitioned to the gel state faster than the rate of mucociliary clearance, making them suitable for nasal application [112,114,117]. Furthermore, poloxamer-based hydrogels have an adequate mucoadhesive strength to enable nasal residence time suitable for drug release on the order of hours [112–115]. After *in vivo* administration, histological analysis of nasal mucosa revealed that the hydrogels are non-irritant and non-toxic, showing a protective effect on cells with respect to free drug, which led to a moderate cell damage [114,117]. Moreover, the brain bioavailability was significantly enhanced (4- to 6-fold increase) when the drug was administered through the hydrogel compared to oral solution, intranasal solution or IV route [112,114,117].

5.2.2 PLGA-PEG-based hydrogels

PLGA is an hydrophobic copolymer obtained by ring opening polymerization of D,L-lactide and glycolide [118]. PLGA is one of the most studied polymers for drug delivery and biomedical applications because of its biocompatibility and biodegradability. Specifically, PLGA degradation via hydrolysis forms lactic and glycolic acids, which are two endogenous monomers easily metabolized by the body [119]. Consequently, minimal toxicity is associated with the use of PLGA. Furthermore, PLGA-based drug delivery systems have been approved by FDA and European Medicine Agency (EMA) for parenteral administration [119].

PLGA allows the formation of thermoresponsive hydrogels when monomers are polymerized in the presence of PEG. Specifically, the polymerization leads to the formation of either ABA or BAB triblock copolymers with hydrophobic PLGA blocks and hydrophilic PEG blocks coupled via ester links [120]. The thermoresponsive behavior is due to the balance between hydrophobic and hydrophilic interactions. At low temperature, hydrogen bonds between PEG and water molecules

prevail, resulting in polymer dissolution in water [120]. When the temperature increases, the hydrogen bonds become weaker, and the hydrophobic interactions among PLGA segments dominate, leading to sol-to-gel transition [120]. PLGA-PEG-based hydrogels are attractive materials for injectable drug delivery systems due to their biocompatibility, biodegradability, as well as tunable sol-to-gel transition temperature [12,121,122]. Moreover, they are suitable for the encapsulation and release of both hydrophobic and hydrophilic molecules including genes, proteins, and anti-cancer drugs [12,121,122]. The application of PLGA-PEG-based thermoresponsive hydrogels to the CNS will be described in the following sections.

Drug delivery to brain tumors

PLGA-PEG-based hydrogels loaded with paclitaxel (PTX) have been proposed under the name of OncoGel™ to treat solid tumors, including GBM. Specifically, OncoGel™ is a formulation of PTX in ReGel™, which is a solution of PLGA-PEG copolymer with low viscosity at low temperature that becomes a gel-like material for controlled release at body temperature [119,123]. The safety of OncoGel™ has been extensively demonstrated in different tissues (i.e. skin, CNS, and pancreas) and via different administration pathways using rats, dogs, and pigs [123]. To treat glioma, OncoGel™ can be administered either in the proximity of the tumor or injected into the tumor cavity and provides a depot for 6-weeks of controlled release of PTX with high local concentrations [119].

The efficacy of OncoGel™ was investigated in a rat model of GBM [124]. OncoGel™ containing 6.3 mg/ml PTX was intracranially administered along with the implantation of the tumor (Day 0) or after 5 days (Day 5). The median survival significantly increased in the OncoGel™ Day 0 treated group compared to controls (31 days for the Day 0 group, while those in the control group died after 17 days) [124]. Furthermore, OncoGel™ Day 0 group showed a long-term survival at 120 days of 37.5%. OncoGel™ was also administered to GBM rats in combination with radiotherapy, leading to a median survival of 83 days for Day 0 group and 32 days for Day 5 group [124].

Another group investigated the efficacy of the combination of OncoGel™ with temozolomide (TMZ) and radiotherapy in a rodent model of GBM [125]. Specifically, the study revealed that the combination of OncoGel™ with either oral or local TMZ was effective and synergistic in the treatment of the gliosarcoma model. Either alone, or in combination with other therapeutics, OncoGel™ holds great potential as a minimally invasive vehicle for the localized treatment of GBM, thus opening up the possibility of change the way this tumor is treated.

Drug delivery to the injured spinal cord

PLGA-PEG-PLGA thermoresponsive hydrogels have been proposed as a vehicle for the release of molecules for SCI anti-inflammation therapy (see **Table 1**). For instance, it was demonstrated that the release of Atsttrin from a PLGA-PEG-PLGA hydrogel can alleviate neuroinflammation and cellular apoptosis induced by progranulin (PGRN) deficiency in SCI [126]. Specifically, Atsttrin was conjugated to the hydrogel and administered in the intrathecal space of PGRN-deficient mice prior to SCI. The study revealed that the controlled release of Atsttrin reduced the pro-inflammatory and pro-apoptotic effect of PGRN deficiency, thus improving neurological recovery [126]. Similarly, the administration of PLGA-PEG-PLGA containing milk fat globule-epidermal growth factor 8 (MFG-E8) played an anti-inflammatory role in a rat model of SCI [127]. Indeed, the hydrogel provided a controlled release of MFG-E8 over time, which contributed to promoting the anti-inflammatory effect. In addition, the therapeutic efficacy of the MFG-E8 loaded hydrogel was characterized by a reduction of fibrotic scar formation and reduced neural death, which led to the promotion of myelin regeneration and axonal extension with a consequent functional recovery and an overall reduction of SCI severity [127].

5.2.3 pNIPAAm-based hydrogels

Thermoresponsive hydrogels comprised of synthetic N-isopropylacrylamide (NIPAAm) monomers are widely used in biomedical applications due to their LCST around 32 °C [64,128]. In fact, the properties, fabrication, and biomedical applications of poly(N-isopropylacrylamide) (pNIPAAm) (**Table 1**) hydrogels have been the topic of multiple reviews [128–130]. The utility of pNIPAAm-based hydrogels is enhanced by the ability to co-polymerize NIPAAm with other natural and synthetic polymers to tune the LCST, mechanical, and biochemical properties. For example, pNIPAAm-based hydrogel properties can be tuned by altering the concentration and molecular weight of the PEG within the polymer mixture [131,132]. pNIPAAm hydrogels are created by aqueous free radical polymerization, atom transfer radical polymerization, and graft copolymerization using combinations of initiators, accelerators, and chemical or physical crosslinking [128,129]. Below the LCST, the pNIPAAm chains are soluble in water in a random coil conformation. Once the temperature is increased above the LCST, the polymer chains collapse to a globular structure, forming an insoluble gel [64]. These hydrogels have been utilized for long-acting, drug-releasing depots in a range of anatomical regions, including the eye [133] and paranasal sinuses [134], as well as for injectable applications such as the CNS. In the following sections, the design and application of various pNIPAAm-based hydrogels for treating CNS diseases will be reviewed.

Drug delivery to brain tumors

pNIPAAm-based hydrogels for direct injection into brain tumors have been sourced from commercial vendors or fabricated in-house. Several groups have used a hydrogel comprised poly(NIPAAm-co-*n*-butylmethacrylate (BMA)) with hydrophilic PEG purchased from Ikeda Rika Inc. (Toyko, Japan) [135] or Melbio Inc. (Kanagawa, Japan) [136–139]. Another commercially available pNIPAAm (Sigma-Aldrich, St. Louis, MO) was further modified by grafting a bioerodible polymer, carboxymethylcellulose (CMC), to enable the *in vivo* degradation of the hydrogel [140]. Another method of fabricating a biodegradable hydrogel was demonstrated by using atom transfer radical polymerization with a polycaprolactone (PCL)-based macroinitiator to create linear pNIPAAm chains with degradable backbones [141]. For delivering treatments to tumor resection sites in the brain, it is important that a pNIPAAm-based gel is modified to enable its safe degradation so that follow-up procedures are not necessary to remove the material, as this would pose unnecessary risks to patients.

These pNIPAAm-based hydrogels were explored for their utility to localize chemotherapeutic delivery directly to brain tumors over extended periods of time. This strategy of direct injection into the tumor or resected tumor site can achieve a local high concentration of chemotherapeutic, particularly for drugs that do not cross the BBB or have associated toxicity with systemic administration [136,138]. Many brain tumor applications for thermoresponsive hydrogels also combine a controlled release vehicle, such as liposomes [136], bovine serum albumin (BSA)-nanoparticles (NPs) [140] , PLGA microspheres (MSs) [136–139,141] or poly(lactic acid) (PLA) MSs [141]. While direct injection of PLGA MSs alone would result in the diffuse spread of the drug carriers throughout the brain, the addition of a thermoresponsive hydrogel matrix can help localize treatment to the tumor site [138]. Additionally, the controlled release mechanism can extend the therapeutic effect. For example, Arai *et al.* initially conjugated doxorubicin (DOX) to a pNIPAAm-based thermoreversible gelation polymer (TGP) and observed short term anti-tumor efficacy, likely due to the rapid release of the hydrophilic drug [135]. To extend the therapeutic window, PLGA MSs or liposomal DOX were combined with the TGP, which extended drug release for over 30 days and improved anti-tumor efficacy (**Figure 6**) [136].

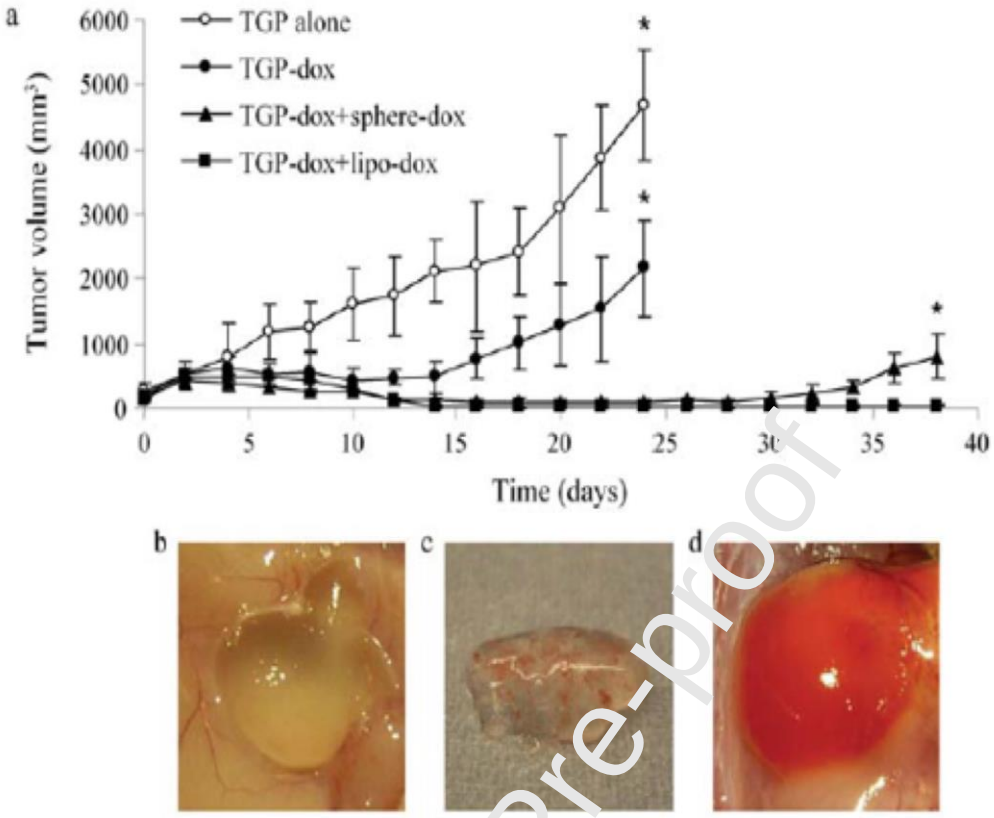


Figure 6: In vivo inhibition of subcutaneous glioma xenograft growth by TGP alone, TGP loaded with DOX (TGP-dox), TGP-dox combined with DOX-loaded microspheres (TGP-dox+sphere-dox), and TGP-dox combined with DOX-loaded liposomes (TGP-dox+lipo-dox).
a) TGP-dox inhibited tumor growth for 14 days, followed by aggressive tumor growth until day 24. TGP-dox+sphere-dox inhibited the tumor growth until day 32, when the tumors started to increase. TGP-dox+lipo-dox inhibited tumor growth up to day 38. Values are presented as the mean (n=5) ± standard deviation. Significant differences between tumor volumes were determined by analysis of variance (ANOVA), * $p<0.01$). Representative pictures of b) TGP-dox, c) TGP-dox+sphere-dox, and (d) TGP-dox+lipo-dox removed from mice at the end of the experiment. TGP-dox was colorless, suggesting that the DOX was completely released from the TGP; TGP-dox+sphere-dox presented light orange spots, suggesting the presence of DOX; TGP-dox+lipo-dox was bright orange in color, suggesting the retention of a large amount of DOX.
Reprinted with permission from ref. [136].

Others have proposed the timed release of multiple chemotherapeutics using different controlled release mechanisms, such as loading a hydrophobic drug in the pNIPAAm gel matrix for faster release and a hydrophilic drug in BSA-NPs for slower release [140]. Another method explored was employing polymeric microspheres with different degradation profiles in combination with pNIPAAm-based gels, specifically PLGA MSs for release over a few weeks and PLA MSs for release over 9 months [141]. While several of these studies have demonstrated effective tumor suppression and enhanced survival [136,138–140], additional characterization of the penetration

depth of the therapeutic into the surrounding tumor and brain tissue is a critical quality of the potential enhanced capabilities of pNIPAAm-based delivery depots in comparison to conventional delivery methods.

Drug delivery to the injured spinal cord and brain

The combination of hydrophilic polymers with pNIPAAm has been explored to produce hydrogels with favorable mechanical properties for injection in the injured spinal cord. However, there are conflicting views on the best ultimate fate of these hydrogels. For spinal cord applications, hydrophilic PEG of various molecular weights has been copolymerized with pNIPAAm using free radical polymerization [142–147]. The addition of PEG helps to reduce the syneresis of the pNIPAAm chains, resulted in improved elasticity and porosity [143]. Alternatively, methylcellulose was also tested as a copolymer, but its addition resulted in a gel with higher viscosity that may pose challenge for uniformly distributing cells for injection [144]. Through mechanical testing, the compressive modulus of a pNIPAAm and PEG (8000 g/mol, at a 700:1 monomer ratio) hydrogel was reported to be in the range of 1-4 kPa, which is similar to that of spinal cord white matter (3-5 kPa) [142]. Others have reported similar storage modulus (G') and loss modulus (G") of a pNIPAAm-co-PEG methacrylate (PEGMA) to the spinal cord [143]. In all cases of these pNIPAAm-PEG hydrogels, the polymer backbone is not highly susceptible to hydrolytic cleavage, thus making it non-biodegradable [142]. Some have proposed that this is preferable as a degradable scaffold could create compressive stress on axons during regeneration leading to cell death. Additionally, the presence of a moving boundary layer during tissue repair and axonal regeneration could increase the inflammatory response or cause glial scar formation [142]. However, others have advised that a degradable hydrogel is preferable because hydrogel calcification and a prolonged inflammatory response could result following injection of a non-degradable polymer [143].

Whether the hydrogels would degrade or not, the properties of pNIPAAm-based hydrogels can be engineered to match the native tissue, mimic the extracellular environment, enable cell migration, nutrient diffusion, and neovascularization, and provide soluble cues to promote axonal regeneration [142,143]. As a demonstration of biocompatibility, human bone marrow stromal cells were shown to attach and proliferate on pNIPAAm-PEG scaffolds [142]. Furthermore, these scaffolds could be loaded with brain-derived neurotrophic factor (BDNF) and NT-3 and the gradual release of these neurotrophic factors over 4 weeks was demonstrated *in vitro* [142]. In addition to showing *in vitro* bioactivity [142], BDNF- and NT-3-loaded scaffolds were tested in rat SCI models [144–146]. Both a synthetic pNIPAAm-g-PEG hydrogel and semi-synthetic pNIPAAm-g-MC hydrogel were shown to gel *in situ* without causing an additional inflammatory response beyond

that caused by the disease model [144]. In a follow-up study, functional recovery was demonstrated in groups treated with and without BDNF in the pNIPAAm-g-PEG scaffold, with the presence of BDNF increasing the recovery rate of fine motor skills [146]. Another study combined treadmill training with the neurotrophin-loaded hydrogel and investigated the treatment efficacy for reducing spasticity that results after SCI [145]. Still others have suggested that a pNIPAAm-g-PEG hydrogel without neurotrophic factors is effective for recovery following SCI, likely by creating a custom-forming, permissive environment for axonal recovery [143]. Overall, these injectable pNIPAAm-PEG hydrogels have shown promise for SCI treatment in rodent models, but longer duration and larger animal studies will be necessary to determine if and when the material should degrade.

An injectable pNIPAAm-based hydrogel has also been explored as a therapy to mitigate brain injury associated with stroke by reducing excess iron that accumulates following intracerebral hemorrhage. The gel was fabricated by grafting keratin to NIPAAm by a 'click' reaction and then using 2,2'-azobis [2-methylpropionamide] dihydrochloride (AIBA) as an initiator for free radical polymerization [148]. Keratin was the selected copolymer for this application due to its prior uses for nerve repair and drug delivery, its biocompatibility and biodegradability. Keratin was combined with pNIPAAm to create a material capable of *in situ* gelation in irregularly shaped defects. The combined keratin-g-pNIPAAm hydrogel demonstrated fast *in vitro* iron adsorption (70% in 10 min). Furthermore, as an added iron-reducing functionality, the hydrogel was loaded with an iron chelator, deferoxamine mesylate (DFO), and shown to decrease the iron content in a rat model of intracerebral hemorrhage. Finally, the hydrogel was shown to biodegrade *in vivo* with an approximate 80% loss in volume over 28 days [148].

Drug delivery for neurodegenerative diseases

As with applications for treatment of spinal cord and brain injuries, pNIPAAm has been explored for delivering treatment to reduce damage associated with neurological disorders. An injectable pNIPAAm hydrogel was synthesized with a multifunctional poly(amidoamine) (PAA) crosslinker to make the gel biodegradable [60], which was demonstrated by 3 weeks of aqueous incubation resulting in a 50% loss in gel mass. This degradable pNIPAAm-based hydrogel was explored for the local and sustained release of the activin B as a potential therapy for PD. The hydrogel helped to prolong the otherwise short half-life of activin B while maintaining its bioactivity, as demonstrated by the promoted migration of bone marrow-derived mesenchymal stem cells *in vitro*. Furthermore, in a murine model of PD, the hydrogel resulted in significant increase in detectable drug concentration at 35 days compared to soluble injection as well as behavioral improvements (**Figure 7**) and increased density of nerve fibers compared to control groups.

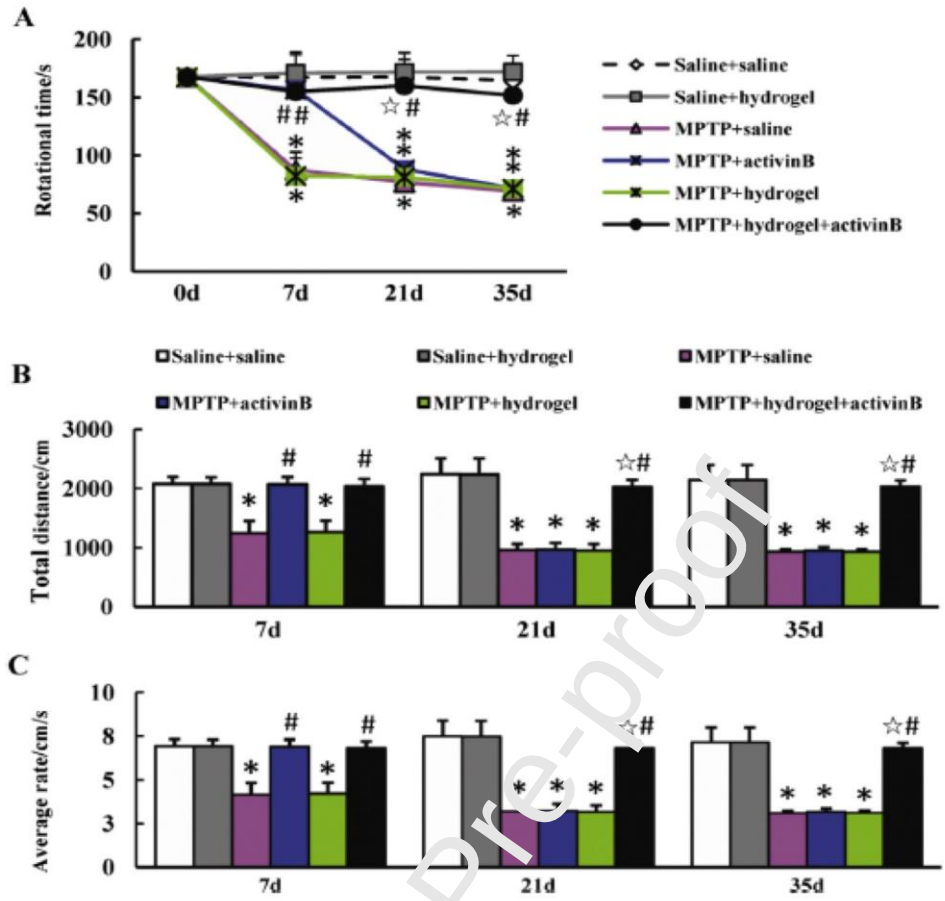


Figure 7: Activin B-loaded hydrogels improved the performance of PD model mice during behavioral tests. Behavioral tests were carried out. The experiment was divided into 6 groups: saline þ saline, saline þ hydrogel, MPTP þ saline, MPTP þ activin B, MPTP þ hydrogel and MPTP þ hydrogel þ activin B, and Each group contained 10C57 mice. A, Changes in the rotarod running times (s) for mice in each group 7, 21 and 35 days after surgery. B, Changes in the total running distances (cm) in open field tests for mice in each group 7, 21, and 35 days after surgery. C, Changes in the mean running speeds (cm/s) for mice in each group 7, 21, and 35 days after surgery. *, $p < 0.05$ compared with the saline þ saline treatment groups; #, $p < 0.05$ compared with the MPTP þ saline treatment groups; *, $p < 0.05$ compared with the MPTP þ activin B treatment group. Reprinted with permission from ref. [60].

While some activation of microglia and astrocyte cells was observed in hydrogel-treated animals, with the sustained release of activin B from the hydrogel this activation was reduced. Additionally, there was no detection of neuronal apoptosis in the striatum. Overall, the hydrogel improved the delivery of activin B, resulting in protection of neurons in the striatum, which is a key structure for the formation of motor memories and an important target for PD treatment [60].

6. Conclusions and Future Recommendation

In this review, thermoresponsive hydrogels that have been proposed for the treatment of CNS disorders are discussed, with particular emphasis on their advantages for direct therapeutic

delivery to the CNS and overcoming the limitations of the methods currently used in the clinic. The complex CNS architecture with its biological barriers represents a major challenge to deploying effective treatments. Clinically available strategies include the direct delivery of therapeutic molecules to the CNS via intrathecal delivery, convection enhanced delivery, and transient opening of the BBB, both with hyperosmotic solutions and focused ultrasound. However, often, these strategies induce the risk of significant tissue damage, local inflammatory reactions and infections. Thermoresponsive hydrogels are emerging as an appealing alternative drug delivery strategy that could realize sufficiently high drug concentrations at a biological target (such as the brain and spinal cord) in a minimally invasive fashion and limited systemic toxicity.

Thermoresponsive hydrogels have a number of key attributes that make them suitable for drug delivery to the CNS. As they are designed to be liquid at room temperature and progressively turn into gel at body temperature, they can be readily injected to create drug depots that conform with the surrounding tissue and release therapeutic payloads for several weeks. Also, thermoresponsive hydrogels can be designed to degrade or bioresorb for safe excretion or metabolization. Moreover, thermoresponsive hydrogels can encapsulate and release a variety of molecules, including anti-cancer and anti-inflammatory drugs, growth factors, exosomes, nanomedicines and cells, thus supporting combinatorial treatments.

A number of studies in this review highlight how the unique properties of thermoresponsive hydrogels have led to positive outcomes in a variety of CNS applications, including treatments for brain tumors, spinal cord and brain injuries, as well as neurodegenerative disorders. As an example, thermoresponsive hydrogels for GBM treatment have been appealing because of their ease of administration in the resection cavity after tumor removal, leading to a localized release of chemotherapeutics with reduced systemic toxicity. Moreover, even though further investigation is still required, thermoresponsive hydrogels have shown encouraging results in the field of GBM immunotherapy, which may drastically improve treatment for this invasive brain tumor.

The possibility to release both cells and growth factors via the administration of thermoresponsive hydrogels to the injured spinal cord or brain have shown new possibilities to guide neurological recovery and functional repair injuries, reducing the risk of permanent disabilities. Indeed, many studied have demonstrated that thermoresponsive hydrogels have shown a key role in improving cell survival after transplantation and promoting tissue regeneration with a consequent enhanced functional recovery. Moreover, thermoresponsive hydrogels have helped stabilize GFs thus increasing their cellular uptake, which may be favorable for the development of effective SCI therapies.

Importantly, studies that are reviewed herein highlight how thermoresponsive hydrogels can enhance drug bioavailability at the target site as well as reduce the frequency of administrations, thus helping overcome the drawbacks of traditional therapeutic agents commonly used to treat neurodegenerative disorders. In fact, the composition of the gel can be easily tuned to achieve suitable mucoadhesive properties that allow for nose to brain delivery. This delivery route may be particularly useful for the release of drugs to control the symptoms of AD and PD, in which less frequent administration regimens and consequent improved patient's adherence and compliance may improve therapeutic efficacy.

Despite all the positive preclinical research that has been shown with thermoresponsive hydrogels for CNS delivery, their effective use for the treatment of CNS disorders requires further optimization in drug loading and release, improved integration with the surrounding tissue and the development of predictive tool to efficiently manage the design space. In future work, loading and release could be optimized by adopting hierarchical drug delivery systems resulting from the mixture of molecular, nano and micro components within thermoresponsive hydrogels. In these hierarchical systems, therapeutic molecules would be dispersed within the hydrogel matrix as free molecules and encapsulated in nano/microparticles to limit the rapid release often occurring within the first hours of operation (burst release), extend drug deployment well beyond a few weeks, increase overall loading, and expand the portfolio of therapeutic molecules and medical applications. These hierarchically-structured thermoresponsive hydrogels could carry also temperature or mechanically sensitive vesicles for on command drug release. In addition, integration with the surrounding tissue is key in the development of thermoresponsive hydrogels for regenerative medicine. More sophisticated chemical conjugations and click chemistry approaches should be adopted to integrate moieties and ligands in the hydrogel matrix that stimulate cell migration, adhesion or proliferation as a function of the specific application. This would facilitate stem cell differentiation, neurogenesis, and axonal guidance to repair neuronal tissue and re-establish proper intracellular communication. Finally, computational tools combining biophysical-inspired models and artificial intelligence are expected to play a major role in the future development of 'smart' thermoresponsive hydrogels. The biophysical performance of these hydrogels depends on several governing parameters related to polymer type, including molecular weight, cross-linking degree, and blend; the drug type, its hydrophilicity or hydrophobicity; hierarchical organizations with molecules, nanoparticles and microparticles; and, eventually, the environmental conditions with diverse biological and mechanical stimuli.

In summary, thermoresponsive hydrogels are a promising technology for targeted therapeutic delivery to address existing clinical hurdles for drug delivery to the brain and spinal

cord. Furthermore, the development of 'smart' thermoresponsive hydrogels using the combination of a hierarchical design and predictive, computational tools is anticipated to revolutionize the field of drug delivery and regenerative medicine in the treatment and management of a variety of CNS disorders.

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Figure 1: Schematic representations of the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB).

Figure 2: Schematic representations for the healthy blood-brain barrier (BBB) and blood-brain tumor barrier (BBTB).

Figure 3: Schematic representation of conventional routes of drug administration for the treatment of CNS disorders: intracerebroventricular administration (ICV), intrathecal-lumbar injection (IT-L), convection enhanced delivery (CED), and focused ultrasound (FUS) combined with the infusion of microbubbles.

Figure 4: Representative images showing infiltration of astrocytes into the collagen-based hydrogels 10 days post seeding. The images were acquired under a 4x objective lens using a calcein AM viable cell stain and epifluorescence microscopy. Scale bar = 1000 μm. Dotted lines represent the interface. The images refer to the following groups: *Col (2 mg/ml)*: collagen at a concentration of 2 mg/ml; *Col + FGF primed cells*: collagen gel + astrocytes exposed to 150 ng/ml FGF-2 before the assay setup; *Col FGF Low* and *Col FGF High*: collagen gel loaded with 15 ng/ml and 150 ng/ml free FGF-2, respectively; *Col Gen (0.25 mM)*: collagen gel cross-linked with genipin 0.25 mM; *Col + Gen primed cells*: astrocytes exposed to 0.25 mM genipin for 24 h before the assay setup; *Col Gen FGF High*: collagen gel cross-linked with 0.25 mM genipin loaded with 150 ng/ml free FGF-2; *Col LMT PBS*: collagen gel + 0.1 mg/ml lipid microtubules loaded with PBS; *Col LMT FGF-low* and *Col LMT FGF-high*: collagen gel + 0.1 mg/ml lipid microtubules loaded with 0.11 mg/ml and 1 mg/ml FGF 2, respectively; *Col Gen LMT FGF Low* and *Col Gen LMT FGF-high*: collagen gel cross-linked with 0.25 mM genipin + 0.1 mg/ml lipid microtubules loaded with 0.11 mg/ml and 1 mg/ml FGF-2, respectively.
Reprinted with permission from ref. [94].

Figure 5: Effect of the collagen hydrogel on the host astrocytic response to GDNF-MSCs in vivo. 30,000 GDNF-MSCs were injected into each striatum either in control medium or in the collagen hydrogel, and the host astrocytic response to the cells was analyzed at Days 1, 4, 7 and 14 after transplantation. (A) A striatal astrocytic reaction to the implanted cells could be visualized using GFAP immunohistochemistry in the host striatum at each time-point examined. A reduction in the host astrocyte response to GDNF-MSCs is clearly visible in the hydrogel group compared to the control group (B) Post-hoc comparison of the group differences revealed that the collagen hydrogel significantly reduced the host astrocytic reaction to the GDNF-MSCs at the Day 1, Day 4 and Day 7 time-points. (C) Qualitative fluorescent staining for OX-42 and GFAP revealed that the astrocytes surrounded but did not infiltrate the GFP-MSC graft site.
Data are presented as mean ± S.E.M and were analyzed by 2-way ANOVA. **P < 0.01, ***P < 0.001 vs. Control GDNF-MSCs.
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Figure 6: In vivo inhibition of subcutaneous glioma xenograft growth by TGP alone, TGP loaded with DOX (TGP-dox), TGP-dox combined with DOX-loaded microspheres (TGP-dox+sphere-dox), and TGP-dox combined with DOX-loaded liposomes (TGP-dox+lipo-dox).
a) TGP-dox inhibited tumor growth for 14 days, followed by aggressive tumor growth until day 24. TGP-dox+sphere-dox inhibited the tumor growth until day 32, when the tumors started to increase. TGP-dox+lipo-dox inhibited tumor growth up to day 38. Values are presented as the mean

(n=5) ± standard deviation. Significant differences between tumor volumes were determined by analysis of variance (ANOVA,*p<0.01). Representative pictures of b) TGP-dox, c) TGP-dox+sphere-dox, and (d) TGP-dox+lipo-dox removed from mice at the end of the experiment. TGP-dox was colorless, suggesting that the DOX was completely released from the TGP; TGP-dox+sphere-dox presented light orange spots, suggesting the presence of DOX; TGP-dox+lipo-dox was bright orange in color, suggesting the retention of a large amount of DOX.
Reprinted with permission from ref. [136].

Figure 7: Activin B-loaded hydrogels improved the performance of PD model mice during behavioral tests. Behavioral tests were carried out. The experiment was divided into 6 groups: saline þ saline, saline þ hydrogel, MPTP þ saline, MPTP þ activin B, MPTP þ hydrogel and MPTP þ hydrogel þ activin B, and Each group contained 10C57 mice. A, Changes in the rotarod running times (s) for mice in each group 7, 21 and 35 days after surgery. B, Changes in the total running distances (cm) in open field tests for mice in each group 7, 21, and 35 days after surgery. C, Changes in the mean running speeds (cm/s) for mice in each group 7, 21, and 35 days after surgery. *, $p < 0.05$ compared with the saline þ saline treatment groups; #, $p < 0.05$ compared with the MPTP þ saline treatment groups; *, $p < 0.05$ compared with the MPTP þ activin B treatment group.
Reprinted with permission from ref. [60].

Highlights

- The use of injectable thermoresponsive hydrogels has received much attention as a possible approach to overcome the limitations of drug delivery to the central nervous system
- Thermoresponsive hydrogels have the unique property of being a free-flowing liquid at room temperature and turning into a viscous gel at body temperature
- Thermoresponsive hydrogels can be non-invasively administered virtually anywhere within the central nervous system without requiring any invasive surgery.
- Thermoresponsive hydrogels can be used as a local depot for prolonged and sustained release of therapeutic agents

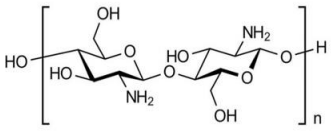
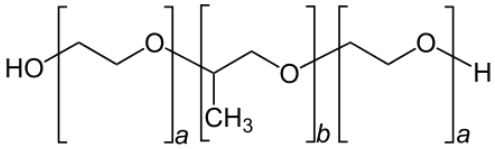
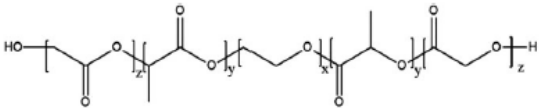
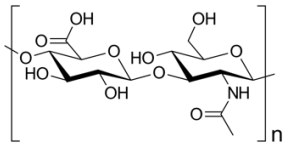
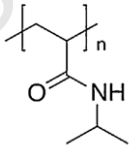
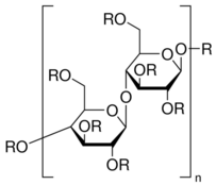
Natural Polymers	Synthetic Polymers
Chitosan 	**Poloxamer**  a: length of PEO b: length of PPO
Collagen 	**PLGA-PEG-PLGA**  x: repeating units of PEG y: lactide z: glycolide
Hyaluronan 	**pNIPAAm** 
Methylcellulose  R: H or CH_3	

Table 1: Chemical structures of natural and synthetic polymers used for the preparation of thermoresponsive hydrogels.

polymer	CNS Disorder	Sol-gel transition	Mechanical properties	Degradable	Delivery agent	Status of development
	Brain tumors	≥ 32 °C		Biodegradable	T-cells	*In vitro*
		34-37 °C		Biodegradable	Ellagic acid	*In vitro*
n-based	Spinal cord or brain injuries	37 °C			MSC	Mouse
		37 °C	G' = 200 Pa	Biodegradable	BMSC	Mouse
		32.6 °C		Biodegradable	Ferulic acid	*In vitro*
	Neurodegenerative disorders	30-35 °C		Biodegradable	Ibuprofen	*In vitro*
		37 °C	σ = 140-350 Pa		Ropinirole	Rat
	Spinal cord or brain injuries	37 °C	G' = 20-45 Pa	Biodegradable	MSC, NSC FGF-2	*In vitro*
n-based		Body temperature		Biodegradable	NT-3	Rat
	Neurodegenerative disorders	37 °C		Biodegradable	GDNF-MSCs Neurons	Rat
	Spinal cord or brain injuries	18-32 °C	ν = 10^0-10^6 Pa•s Shear thinning	Biodegradable		Rat
		37 °C		Biodegradable	EPO	Rat
				Biodegradable	EPO	Mouse
onan- ellulose- ed		37 °C		Biodegradable	EPO NPs EGF-PEG NPs	Mouse
				Biodegradable	cortically specified neuroepithelial stem cells	Rat
				Biodegradable	Peptide (KAFAK) and BDNF	Rat
		Physiological temperature		Biodegradable	CSa-PLGA MPs	Rat
			G' = 50-1000 Pa	Biodegradable	PLGA blank-MPs	Rat

Table 2: Biophysical features and biomedical applications of natural polymer-based thermoresponsive hydrogels
G' = storage modulus, ν = viscosity, σ = mucoadhesive force

Type of polymer	CNS Disorder	Sol-gel transition	Mechanical properties	Degradable	Delivery agent	Status of development	Ref.
PLGA-PEG	Brain tumors	Body temperature		Biodegradable	OncoGel™ (paclitaxel)	Clinical trials	[119,123-125]
	Spinal cord or brain injuries	30 °C			Atsttrin	Mouse	[126]
		Physiological temperature		Biodegradable	MFG-E8	Rat	[127]
pNIPAAm-based	Brain tumors	20 °C			Doxorubicin soluble liposomes or MPs	Mouse	[135,136]
					Camptothecin soluble and MP	Rat	[137-139]
					Vincristine	Rat	[138]
		28 °C		Non-biodegradable	Epirubicin, paclitaxel	Mouse	[140]
		32 °C		Biodegradable	Rhodamine, IgG	*In vitro*	[141]
	Spinal cord or brain injuries	30 °C	E = 1-4 kPa	Non-biodegradable	BDNF, NT-3	*In vitro*	[142]
		33 °C	G' = 25-50 kPa, G'' = 17-20 kPa	Non-biodegradable	BDNF, NT-3	Rat	[143]
				Non-biodegradable	BDNF	Rat	[144]
		31-32 °C	E = 50-250 kPa	Biodegradable	BDNF, NT-3	Rat	[145-147]
		37 °C	G' = 400 Pa	Biodegradable	DFO	Rat	[148]
	Neurodegenerative disorders	32 °C	$\nu = 10^0$-10^2 Pa•s	Biodegradable	Activin B	Mouse	[60]
Poloxamer-based	Spinal cord or brain injuries	37 °C		Biodegradable	GDNF	Rat	[55]
		37 °C	Strain amplitude = 6.5%		aFGF	Rat	[56]
		35-37 °C	ν = 7-16 Pa•s		FGF-2	Rat	[109-111]
		37 °C			NGF	Rat	[108]
	Neurodegenerative disorders	32-34 °C	0.1 Pa•s		Rivastigmine hydrogen tartrate NPs	*In vitro*	[61]
					Rivastigmin		[112]

					e tartrate		
		18-51 °C	σ = 380-1100 Pa	Biodegradable ("corrosion")	Geniposide	*In vitro*	[113]
		22-52 °C	σ = 148-540 Pa		Ropinirole	Mouse	[114]
		34 °C	ν = 1-1000 Pa•s		Amantadine hydrochloride	*In vitro*	[115]
		30-32 °C	σ = 100 Pa		Levodopa NP	Rat	[116]
		28-33 °C			Rasagiline mesylate	Rabbit, Rat	[117]

Table 3: Biophysical features and biomedical applications of synthetic polymer-based thermoresponsive hydrogels.

G' = storage modulus, G" = loss modulus, ν = viscosity, σ = mucoadhesive strength or force, E = compressive modulus