Cryogel biomaterials for neuroscience applications

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Abstract

Biomaterials in the form of 3D polymeric scaffolds have been used to create structurally and functionally biomimetic constructs of nervous system tissue. Such constructs can be used to model defects and disease or can be used to supplement neuronal tissue regeneration and repair. One such group of biomaterial scaffolds are hydrogels, which have been widely investigated for cell/tissue culture and as cell or molecule delivery systems in the field of neurosciences. However, a subset of hydrogels called cryogels, have shown to possess several distinct structural advantages over conventional hydrogel networks. Their macroporous structure, created via the time and resource efficient fabrication process (cryogelation) not only allows mass fluid transport throughout the structure, but also creates a high surface area to volume ratio for cell growth or drug loading. In addition, the macroporous structure of cryogels is ideal for applications in the central nervous system as they are very soft and spongey, yet also robust, which makes them a user-friendly and reproducible tool to address neuroscience challenges. In this review, we aim to provide the neuroscience community, who may not be familiar with the fundamental concepts of cryogels, an accessible summary of the basic information that pertain to their use in the brain and nervous tissue. We hope that this review shall initiate creative ways that cryogels could be further adapted and employed to tackle unsolved neuroscience challenges.

Keywords

Biomaterials, cryogels, hydrogels, central nervous system, peripheral nervous system, neuroscience

Introduction

Biomaterials have been used widely in the field of tissue engineering and regenerative medicine for applications such as the immobilization of biomolecules such as collagen or RGD moieties (Cui et al., 2006; Li et al., 2008), drug carrier systems (Friess, 1998; Ruszczak and Friess, 2003), providing sustained delivery of therapeutic molecules (Cao et al., 2019; Silva et al., 2009) and as scaffold-based hosts for cells (Wang et al., 2011). In order to suit the intended application, they can take a variety of forms such as self-assembling peptides (Collier, 2008), electrospun nanofibers (Mehrasa et al., 2015) with the most prominent being hydrogels (Carballo-Molina and Velasco, 2015). A range of well-defined fabrication techniques has been employed to prepare a diverse array of biomaterial scaffolds, either of natural, synthetic or hybrid origin, with different structures and specific properties (Emerich et al., 1999; Gupta et al., 2006; Mittapalli et al., 2013). Biomaterials can be categorized as biodegradable or non-biodegradable, which depends on their intended use or fate after injection or implantation (Bhang et al., 2007; Ta et al., 2008).

For many applications, matching the mechanical properties of the target tissue is critical for correct cell function and successful integration and biocompatibility of the scaffold with the surrounding area (Liu et al., 2018; Seidlits et al., 2010). In this regard, the brain is extremely soft, with an elastic modulus that is 10 times lower than the liver and nearly 50 times lower than muscle (Discher et al., 2005). As with nearly all soft tissues, brain tissue also shows stress relaxation, with the shear modulus decreasing to a few hundred Pa in several seconds (Bilston et al., 1997; Millesi et al., 1995). The groups of Cheng et al. and Aurand et al. summed up literature data relating to the mechanical properties for the brain and spinal cord (**Table 1**) (Aurand et al., 2012; Cheng et al., 2008; Niemczyk et al., 2018). In general, the elastic modulus of the human brain varies throughout the regions and is dependent *inter alia* on gender, age and diseases. Magnetic resonance imaging studies determined brain storage moduli values for white matter to be around 2.7 kPa and grey matter to be around 3.1 kPa (Green et al., 2008). However, further studies that were performed with atomic force microscopy (AFM) have shown significant differences between the effective elastic moduli of white matter (294 Pa) and gray matter (454 Pa)(Christ et al., 2010). In addition, *in vivo* studies demonstrated

that brain tissue becomes softer with increasing age (Cheng et al., 2008; Niemczyk et al., 2018). It is therefore challenging to create materials that can mimic the structure and mechanical properties of native tissues.

Soft biomaterials (shear modulus: 0.1–4 kPa (Möllers et al., 2009; Wegst et al., 2010)) with the stiffness similar to the brain, have been used to differentiate neural progenitor cells into neuronal lineages *in vitro* (Brännvall et al., 2007). Additionally, glia cell differentiation was promoted on stiffer scaffold materials (7–10 kPa) (Tseng et al., 2015), while cells cultured on intermediate stiffness scaffolds (3.5 kPa) showed high levels of proliferation (Leipzig and Shoichet, 2009). It is thus possible to manipulate the cell lineage, proliferation, and growth of neural cell cultures by fine-tuning the mechanical properties of a scaffold.

Table 1: Overview of the mechanical properties of the human brain and spinal cord (Bartlett et al., 2016; Bilston and Thibault, 1995; Budday et al., 2017; Cheng et al., 2008; Niemczyk et al., 2018; Taylor and Miller, 2004; Weickenmeier et al., 2018)

	Shear modulus	Young's modulus	Storage modulus	Loss modulus
Brain	0.4-1.4 kPa (Budday et al., 2017)	3-10 kPa (Taylor and Miller, 2004)	1.18-2.22 kPa (Weickenmeier et al., 2018)	0.63-1.14 kPa (Weickenmeier et al., 2018)
Spinal cord	5-42 kPa (Bartlett et al., 2016)	1.02-1.37 MPa (Bilston and Thibault, 1995)	not available	not available

Hydrogels (**Figure 1**) are frequently used scaffold materials (Hejčl et al., 2009; Jon Goldberg and Kuhn, 2013; Lee and Mooney, 2001; O'Brien, 2011) which have many favorable properties for use in the central nervous system (CNS). Hydrogels are water-insoluble and exhibit very hydrophilic nanoporous 3D polymer networks (**Figure 1**) that can absorb large amounts of water (~30 % as a lower limit), while retaining a mechanically stable structure (Hoffman, 2012). Generally, they exhibit properties of being non-toxic/non-immunogenic with a high permeability for small molecules and nutrients such as O_2, allowing cell survival inside the scaffold (Buwalda et al., 2017; Hoare and Kohane, 2008; Hoffman, 2012; Ta et al., 2008).

A major advantage of using hydrogels lies in the ability to tune the mechanical properties, to those of the target tissue such as soft CNS tissue. This tuning can be performed via regulation of the crosslinking density (Leach et al., 2007) or the choice of polymer/monomer type (Spicer, 2020). In addition, hydrogels can be modified with adhesion ligands, such as fibronectin (Nuttelman et al., 2001) or RGD moieties to facilitate cell infiltration and neurite growth (Gunn et al., 2005; Hersel et al., 2003).

Hydrogels can also be designed to present a composition-dependent lower critical solution temperature (LCST) (Crompton et al., 2007; Stabenfeldt et al., 2006) or upper critical solution temperature (UCST) (Q. Wang et al., 2009). These abilities can be exploited to formulate hydrogel prepolymers to exist in a liquid state at room temperature while forming gels at body temperature *in situ*, allowing for minimally invasive delivery through small-gauge needles using MRI guidance (Gupta et al., 2006; Massensini et al., 2015).

A subclass of hydrogels, termed cryogels, can be created with a macroporous structure as shown in **Figure 1**. Cryogels can be a highly advantageous form of hydrogel for neuroscience applications as they can be mechanically very soft (Young's modulus: 0.3 kPa - 8.2 kPa (Bruns et al., 2018; Li et al., 2014; Liu et al., 2014; Welzel et al., 2014; Zeng et al., 2015)), while remaining remarkably robust (compressibility: 60% to 90% (Bencherif et al., 2012; Plieva et al., 2008)).
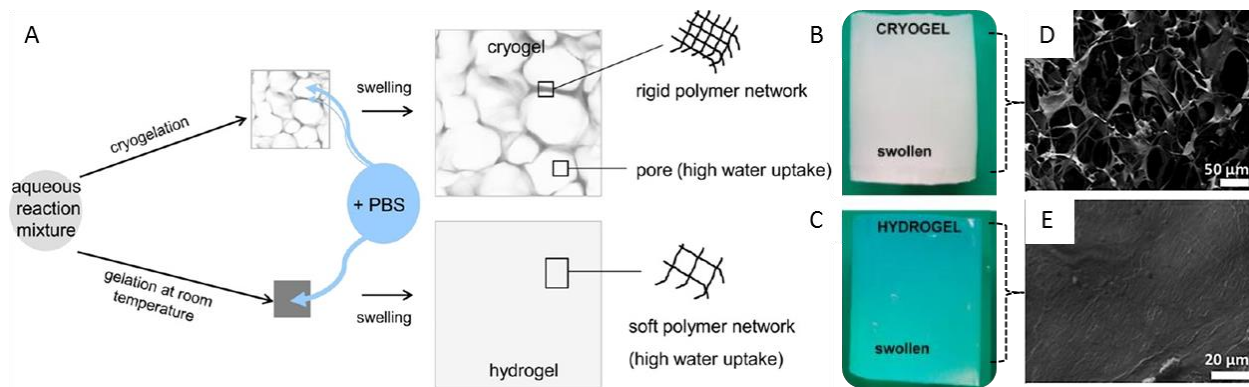
Figure 1: **Differences in structure between a hydrogel and cryogel.** (A) Comparison of formation, swelling and internal microstructure of cryogels (B) vs. hydrogels (C) (considering a given amount of aqueous reaction mixture, i.e., given the amount of building blocks). Scheme was reproduced with permission (Welzel et al., 2012). Photographs of cryogel (B) and hydrogel (C) adapted with permission (Ak et al., 2013). (D, E) Scanning electron microscope (SEM) images of cryogel and hydrogel topography adapted with permission (Bencherif et al., 2012).

The mechanical properties of cryogels affords them with a wide range of potential uses in the brain and nervous system (Béduer et al., 2015, 2014) (**Figure 2**). These include: i) tubular materials for nerve guidance conduits (NGCs) to facilitate tissue regeneration after injury (Wu et al., 2019), ii) cell-instructive hosting platforms to recapitulate the native environmental niche (Newland et al., 2020a), allowing cell development and protection during delivery for cell therapies (Béduer et al., 2015; Filippova et al., 2021), iii) spatiotemporal controlled focal delivery of biomolecules for cell and tissue manipulation (Schirmer et al., 2020), and iv) specific binding sites for complexing/isolation of undesirable substances from body fluids to prevent tissue damage (Baydemir et al., 2009). This review will give a brief introduction to cryogel synthesis techniques and how cryogels have been used in the field of neuroscience. It will also shed light on possible future directions of cryogel research, such as their use as self-healing (Lin and Hsu, 2020) or conductive scaffolds (Humpolíček et al., 2018) for applications on neural cells and tissue.
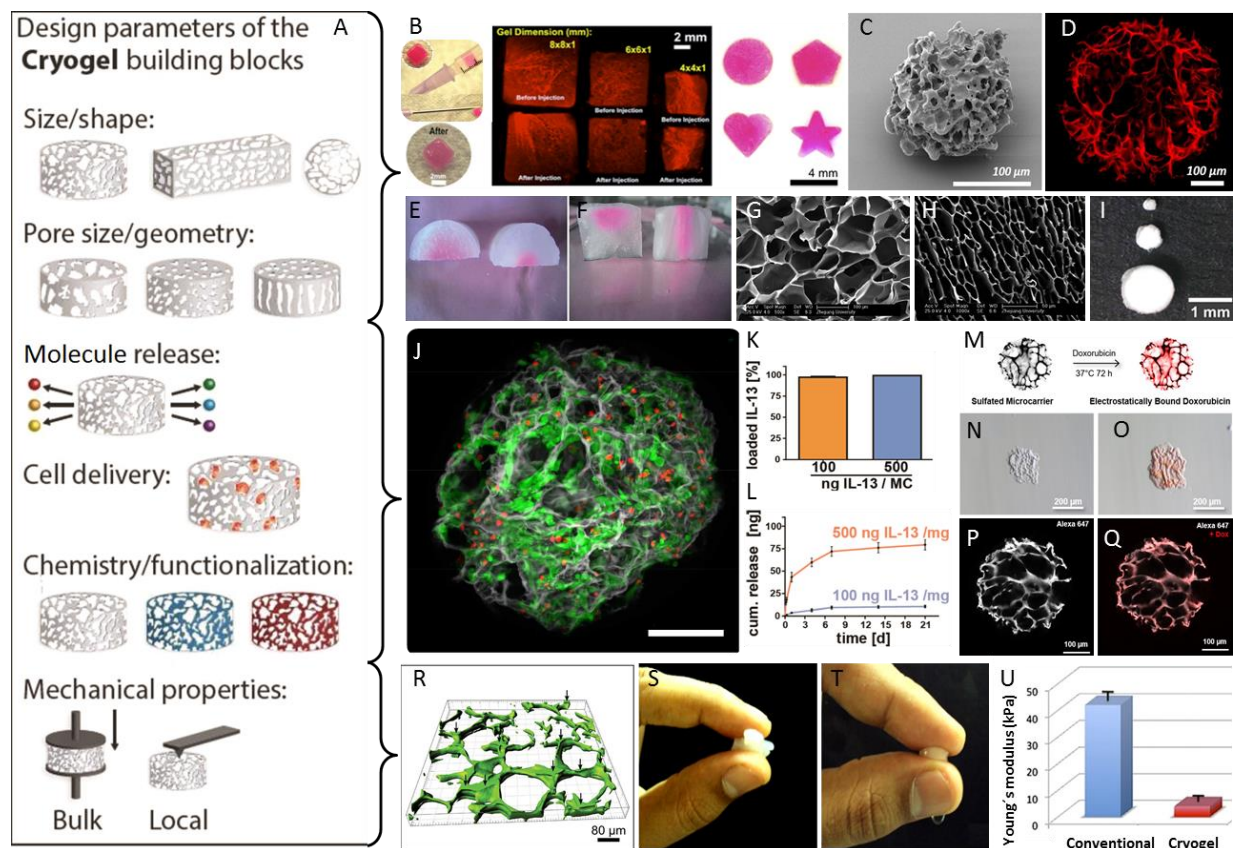

Figure 2: Cryogels are highly adaptable networks that can be tailored to their specific application. (A) Overview of different design parameters of cryogels that can be tuned by usage of different chemistries and processing techniques (adapted with permission (Kühn et al., 2020)). (B) Images

of methacrylated-alginate cryogels before and after injection through a 16-gauge needle, including fluorescent microscopy images of cryogels with different sizes (adapted with permission (Bencherif et al., 2012)). (C, D) SEM and confocal laser fluorescence microscopy image showing the structure of macroporous microcarriers in their dry and swollen state (reproduced with permission (Newland et al., 2020c)). (E-H) Diffusion of stained water through conventional cryogels (E & F (left)) in comparison to cryogels prepared by unidirectional freezing (E & F right) together with corresponding SEM images of a conventional cryogel (G) compared to one with more aligned pores created by the unidirectional freezing process (H) (adapted with permission (Wu et al., 2012)). (J) Representative image of live (green) and dead neural precursor cells (NPCs) (red) on microcarriers (white) at day 7 (reproduced with permission (Newland et al., 2020b)). (K-L) Heparin-based microcarriers load IL-13 (K) and sustain its release (L) (reproduced with permission (Schirmer et al., 2020)). (M-Q) Schematic depiction (M), brightfield microscopy images (N, O) and confocal laser fluorescence microscopy images (P, Q) of loading doxorubicin to sulfated microcarriers (reproduced and adapted with permission (Newland et al., 2020c)). (R) Representation of the atomic force microscopy (AFM)-nanoindentation measurement principle on cryogels (adapted with permission from (Welzel et al., 2014)). (S, T) A hydrogel compressed up to 50% (S) showed permanent fracture, while a cryogel (T) compressed up to 80% of its original length (adapted from (Tripathi and Melo, 2019)). (P) Young's moduli for alginate cryogels and conventional hydrogels (adapted from (Bencherif et al., 2012)).

An overview of cryogelation and the properties of cryogels

Cryogelation

Cryogels, like hydrogels, are three-dimensional hydrophilic networks, however, they typically exhibit an interconnected (open cell) macroporous structure (**Figure 1**) that is created by polymerizing a hydrogel precursor solution below the freezing temperature of the solvent (cryogelation/cryopolymerisation) (**Figure 3**). The formation of ice crystals, which act as a porogen, concentrates the precursor solution into a dense monomer, crosslinker and initiator-rich phase (non-frozen liquid microphase) in which the crosslinking itself takes place. After crosslinking, large, heterogeneous and interconnected pores are generated upon thawing, which are a replica of the linked ice crystals that were formed during the freezing process (**Figure 3**). The physicochemical and mechanical properties of cryogels can be controlled by changing the preparatory parameters of the cryogelation process (**Table 2**), e.g. polymerization temperature (processed freezing temperature), cooling rate, and the monomer/solvent type and concentration.
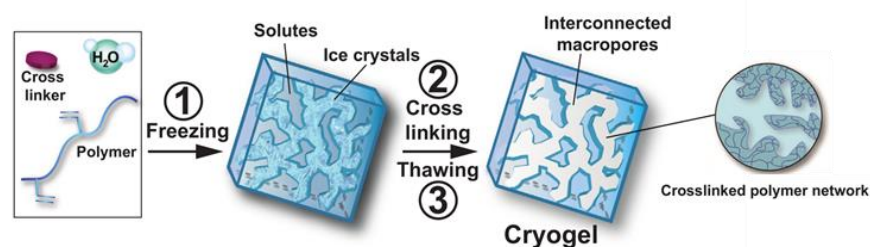

Figure 3: An overview of the cryogelation process. (1) For the formation of cryogels, a hydrogel precursor solution is frozen (T < 0°C). This process leads to a phase separation of the solvent (e.g., water) into a frozen phase (ice crystals) and a nonfrozen phase around ice crystals where the gel precursors (monomers, polymer, crosslinker, and initiator) are expelled. (2) Next, concentrated gel precursors are crosslinked around ice crystals (porogens). (3) Following cryogelation, thawed ice crystals give rise to a macroporous sponge-like hydrogel, known as a cryogel. Reproduced with permission (Eggermont et al., 2020).

Control of pore size, porosity and stiffness

The most effective method to modulate pore size, porosity and stiffness is by adjusting the temperature at which cryogelation occurs, as well as the type and concentration of the monomers (Kirsebom et al., 2009). The processed freezing temperature and accompanying cooling rate directly affects the amount of the non-frozen liquid microphase and the ice crystal sizes formed. This influence can be explained by the nucleation theory (**Figure 4A**) (Adapa et al., 2000; Cook and Hartel, 2010). For example, a high cooling rate and low freezing temperature will increase ice nucleation sites, which results in a large number of small solvent crystals and a smaller non-frozen liquid phase. Hence, the cryogel network will present smaller pores, but with tougher and denser pore walls (struts) (e.g. modulus of silk fibroin cryogels increased from 2 to 15 MPa as preparation temperature is decreased from −5 to −18 °C (Ak et al., 2013)) (**Figure 4D**, **Table 2**).

A similar significant effect can be achieved by increasing the monomer concentration or molecular weight (MW) of the pre-polymer (**Figure 4B**, **Table 2**). By contrast, lowering the monomer concentration or pre-polymer MW in the precursor solution have shown to increase ice crystal formation resulting in larger pores (**Figure 4B, D**). As an example, at a processing temperature of −18 °C, the pore diameter of silk fibroin cryogels increased from 10 ± 3 to 33 ± 10 µm as the concentration of the silk fibroin precursor solution decreased from 12.6 to 4.2% (Ak et al., 2013). Conversely, a higher pre-polymer concentration or MW can increase the cryogel stiffness. For instance, the Young's

modulus of PEG based cryogels with a monomer concentration of 5% and 7.5% w/v was within 3.4–7.0 kPa, but this increased to 15.4 ± 3.4 kPa when a 10% wt/v monomer concentration was used. (**Figure 4B**, **D**, **Table 2**) (Bruns et al., 2018). No effect of the cryogelation temperature or monomer concentration have been obtained on pore interconnectivity (Rezaeeyazdi et al., 2018).

Temperature quenching, which is the rapid cooling of the precursor solution, followed by a higher processing temperature, can also substantially affect the resulting pore size and stiffness (**Table 2**). In this case, an induced temporal supercooling produces a high amount of large ice crystals before cryopolymerisation, which leads to an increase in pores size but much thinner pore walls, resulting in a very low modulus (≈ 1 kPa) (**Figure 4C**, **Table 2**) (Bruns et al., 2018; Cook and Hartel, 2010). In summary, in order to obtain a soft network with large interconnected macropores, mild freezing temperatures and low cooling rates in combination with low monomer concentrations are suggested (**Table 2**).
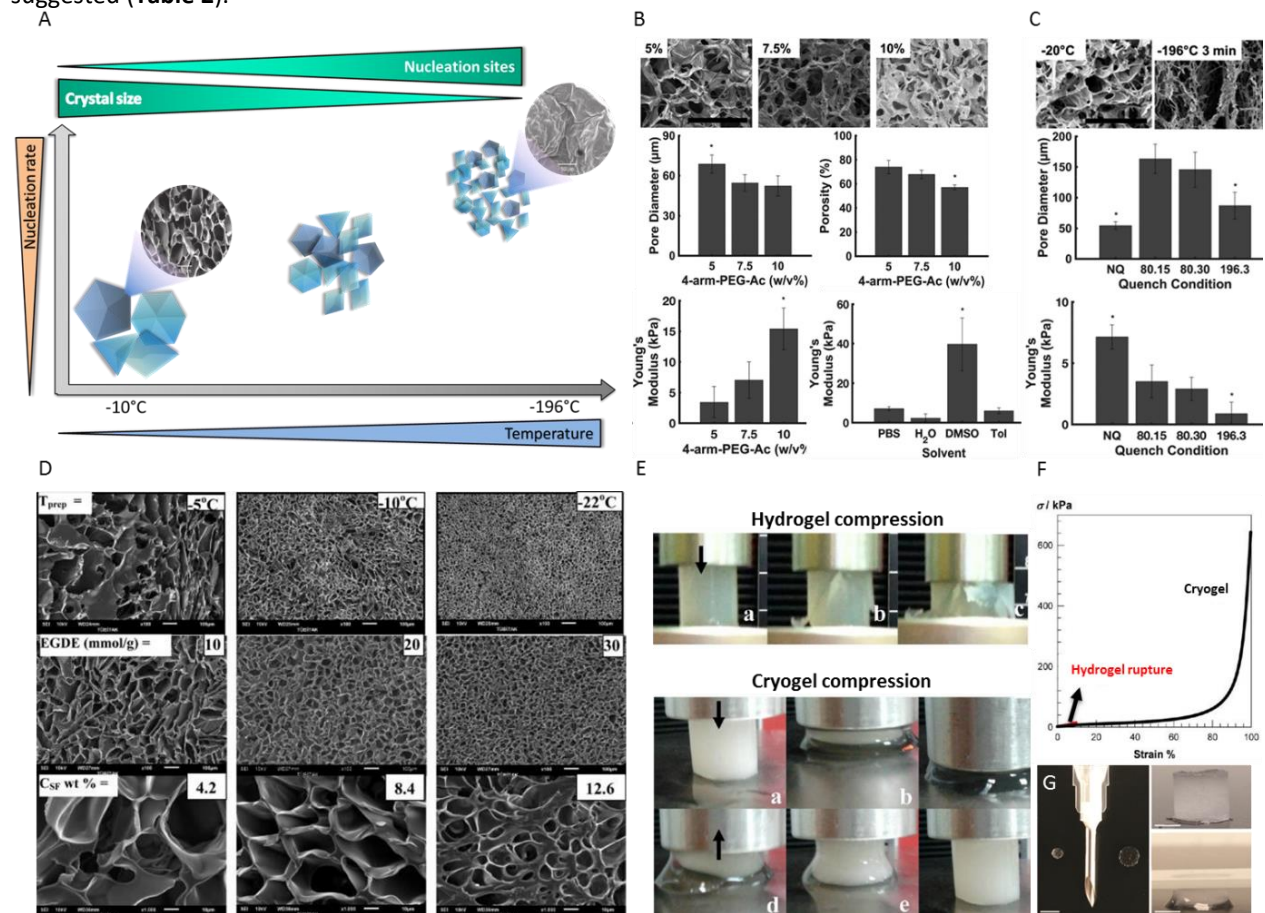

Figure 4: Cryogelation temperature and monomer concentration, effects the pore size and mechanical properties of cryogels. (A) Schematic representation of the ice nucleation process (inserted SEM images adapted with permission from (Kirsebom et al., 2009)). (B, C) Effect of monomer concentration, solvent and quenching on 4-arm PEG-acrylate cryogel properties including pore diameter, porosity and Young´s modulus. The scale bar represents 100 μm (reproduced with permission (Bruns et al., 2018)). (D) SEM images of cryogels formed at various temperatures, and monomer concentrations; scale bars = 100 μm (first and second rows) and 10 μm (bottom row) (reproduced with permission (Ak et al., 2013)). (E) Photographs of silk fibroin hydrogels (top) and cryogels (bottom) during the compression tests. (F) Stress–strain curves of these gel samples as the dependence of the nominal stress σ on the degree of compression (adapted with permission (Ak et al., 2013)). (G) Photographs of 1.5 and 3 mm diameter cryogels next to a 16G needle, including cryogels before compression (top right) and after (bottom right) compression (scale bars = 3 mm (reproduced with permission (Bruns et al., 2018))).

Structural benefits of cryogels

In comparison to other biomaterial systems, some major advantages of cryogels include: i) economic and resource efficient fabrication (Lozinsky et al., 2003; Plieva et al., 2011) ii) simple fine-tuning of morphological and mechanical properties such as porosity, pore geometry, stiffness and toughness (Henderson et al., 2013; Okay and Lozinsky, 2014) (**Table 2**) iii) diverse palette of physico-chemical opportunities for functionalisations such as conductivity,

thermally responsiveness (Deng et al., 2018), self-healing (Liu et al., 2018; Nadgorny et al., 2018), degradability (Koshy et al., 2014) that can give novel biological functions.

Macroporosity – The interconnected macropores (e.g. 92% connectivity (Bencherif et al., 2012)) of cryogels support fluid flow and make diffusion of biomolecules/nutrients possible (e.g. water or O_2) (Moo-Young, 2019; Plieva et al., 2005; Shiekh et al., 2018). This can be essential when considering applications such as tissue regeneration after focal cerebral ischemia (Qi et al., 2017). The high surface area available of the porous network can also be exploited to either enable cell infiltration and growth via adhesive ligands or to bind specifically/selectively to signaling molecules (Newland et al., 2015; Saparov et al., 2019). As a result, cryogels can be used as a delivery vehicle for cell and drug delivery applications within tissue regeneration, transplantation and manipulation (Bakhshpour et al., 2019; Hixon et al., 2017; Memic et al., 2019). Another example of utilization of the macroporous nature comes from the incorporation of conductive elements, such as polyaniline or polypyrrole (PPy), for the modulation and stimulation of the electrically excitable neural tissue (Deng et al., 2018; Vishnoi and Kumar, 2013a).

Table 2: Summary of the effects of preparatory conditions on cryogel formation. The amount of arrows signifies the parameter impact: ↑ 10–25% change, ↑↑ 25–50% change, ↑↑↑ 50–75% change, ↑↑↑↑ 100% change and (X) 0% change.

Parameter	Effect on cryogel architecture		
	Pore size (µm)	**Porosity (%)**	**Stiffness**
Initial monomer concentration low → high wt% (Bruns et al., 2018; Kirsebom et al., 2009; Okay and Lozinsky, 2014)	⬇⬇	⬇⬇	⬆⬆⬆⬆
Temperature gradient -10 °C → -196°C (Kirsebom et al., 2009; Rezaeeyazdi et al., 2018)	⬇⬇⬇⬇	⬆⬆⬆⬆	⬆⬆⬆⬆
Temperature quenching (Bruns et al., 2018; Cook and Hartel, 2010)	⬆⬆⬆⬆	⬆	⬇⬇⬇
Pre-polymer MW low → high (Lin et al., 2011; Memic et al., 2019; Wu et al., 2012)	⬇⬇⬇	⬇⬇	⬆⬆⬆
Solute concentration low → high (Bruns et al., 2018; Jiang et al., 2019; Kirsebom et al., 2010)	⬇⬇⬇⬇	⬆⬆⬆⬆	⊗

Stiffness – The elastic sponge-like network structure provides cryogels with outstanding mechanical stability that makes them capable of maintaining their architectural integrity (integrity rate of 98.7 ± 2.2% (Liu et al., 2014)) after being exposed to high deformations or compressions (90 % or more compression strain (Bencherif et al., 2012)). Additionally, cryogels also exhibit shape-memory properties as, once the mechanical load is released, the network returns to its original undeformed shape (**Figure 4E, F**). Cryogels can therefore be formulated for administration through a small needle without being destroyed (**Figure 5A-C**, **F-G**).

A recent study demonstrated the injection of various cryogel sizes (1.5, 2.0, 2.5, and 3.0 mm) through a 16G needle (Bruns et al., 2018). In this, the 1.5 mm cryogel must be compressed to 63 % of its original size, while the 3.0 mm cryogel must be compressed to 16% of its original size to be able to pass through the needle (Bruns et al., 2018). The combination of shape-memory properties and high mechanical strength makes cryogels a feasible scaffold vehicle for minimally invasive delivery and protection of cells (Li et al., 2014; Liu et al., 2014).

Cryogels vs. conventional hydrogels
In comparison to cryogels, conventional hydrogels intrinsically exhibit drawbacks for some applications. For example, they can dehydrate quickly and lose their shape when they are not permanently exposed or covered with aqueous solution (Ahmed, 2015; Hoffman, 2012). Also, their mechanical stability and integrity is typically low (e.g. microhydrogels (submicro- or micro-engineered (0.1-100μm) hydrogel (Kawaguchi, 2014)) could only withstand 20% strain before mechanical fracture whereas microcryogels exhibited 70% strain without destruction (Liu et al., 2014)) (**Figure 5E**). Furthermore, the mechanical stability is also highly affected by the water content, which can make hydrogels difficult to handle/move (Hoffman, 2012; Omidian and Park, 2012).
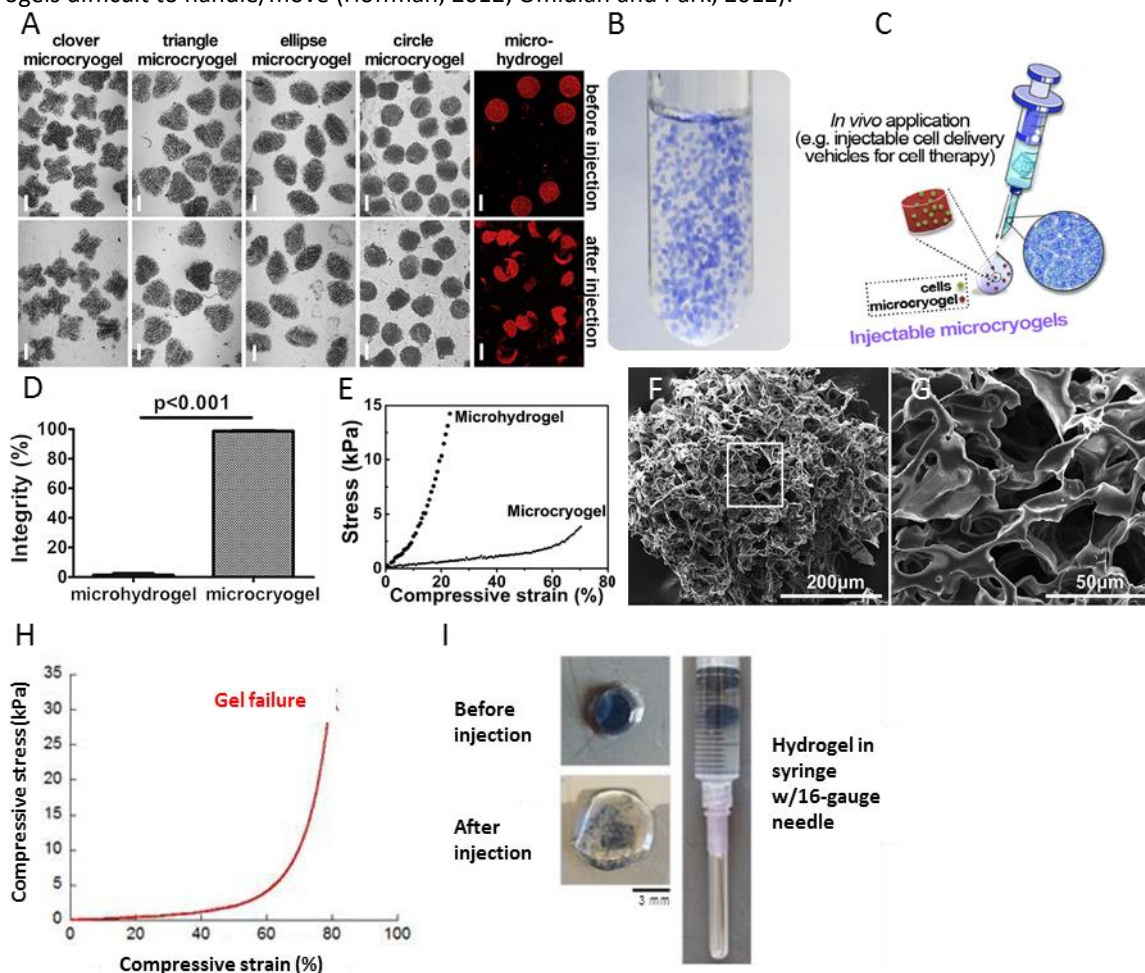

Figure 5: **Injectability and mechanical robustness of cryogels.**(A) Microscopic images of the microhydrogels (fluorescently stained by Nile red for enhanced visualization) and the microcryogels with different shapes before and after injection (scale bar = 500 μm). (B) Photograph of the microcryogels in suspension. (C) Schematic illustration of the injectable microcryogels. (D) The integrity statistics of microhydrogels vs. microcryogels after injection. (E) Strain–stress curves for microhydrogels and microcryogels subjected to compression tests. (F, G) SEM images of the microcryogels showing interconnected and macroporous structures. Reproduced and rearranged with permission from (Eggermont et al., 2020; Liu et al., 2014). (H) Compressive stress vs. strain of for a PEDOT/pAAc-PEGDM hydrogel, until failure. (I) Photographs of a 3 x 1.25 mm cylindrical nanoporous PEDOT/pAAc hydrogel before and after injection through a 16-guage needle, showing that the gel cannot survive the strains associated with injection. Reproduced with permission (Ghatee et al., 2019).

When comparing hydrogels versus cryogels, both based on methacrylated alginate, the hydrogels exhibit a higher Young's modulus (42 ± 2 kPa) in comparison to the cryogels (4 ± 2 kPa) (Bencherif et al., 2012). The lower Young's

modulus of the cryogels makes them more suitable for applications involving soft tissue. Additionally, this makes it unfeasible to administer pre-formed, crosslinked hydrogels through a fine needle, as they would lose their structure after injection (**Figure 5D, H, I**). Furthermore, although hydrogels are porous, without macropores they can hinder cell penetration (Vogler et al., 2018) or diffusion of molecules (oxygen, nutrients or waste) throughout the scaffold structure (Eggermont et al., 2020). In contrast to hydrogels, cryogels offer several advantages: i) enhanced user-friendly handling due to their mechanical robustness and sponge-like nature ii) ease of storage prior to use, and iii) autoclavable sterilization is possible (Villard et al., 2019). Specifically, unlike cryogels which can be autoclaved in their dehydrated state, conventional hydrogels need constant hydration to maintain network stability and cannot sustain autoclaving as the thermal sterilization process induces an irreversible and destructive network shrinkage (Rizwan et al., 2020; Villard et al., 2019).

Cryogel composition - Natural, synthetic or both

As **Figure 6** shows, a variety of starting materials have been used to form cryogels; these can broadly be categorized as follows. Natural biopolymers such as collagen, gelatin, chitosan, or entirely synthetic polymers including PEG, pHEMA or polyaniline (Henderson et al., 2013; Memic et al., 2019; Tripathi and Melo, 2019). Biohybrid combines both, such as PEG and heparin or gelatin (Sharma et al., 2015; Welzel et al., 2012). The majority of cryogel networks are covalently crosslinked via free radical polymerization, carbodiimide chemistry or Diels-Alder reaction, due to higher reproducibility and enhanced mechanical stability than that achieved by physical crosslinking (Memic et al., 2019). The cryogel can be designed to suit its intended application such as by use of synthetic PVA for bioseparation, natural gelatin as 3D cell culture material or biohybrid PEG-co-heparin copolymer for delivery of specific growth factors (Memic et al., 2019). If cell delivery is required, collagen, chitosan, gelatin or synthetic PEG functionalized with RGD/laminin motifs can be applied (Filippova et al., 2021; Huynh et al., 2019). In that regard, Li et al. evaluated the use of a biopolymeric cryogel made of chitosan-co-gelatin for transplanting bone marrow mesenchymal stem cells (BMSCs) for healing the defective spinal cord of a fetal rat model of spina bifida aperta (Li et al., 2016). The BMSC seeded cryogel promoted cell viability, growth and secretion of neuronal markers and neural stem cell markers in the malformed spinal cord, which showed a significant impact on the regeneration and repair of the defective region *in vivo* (Li et al., 2016).
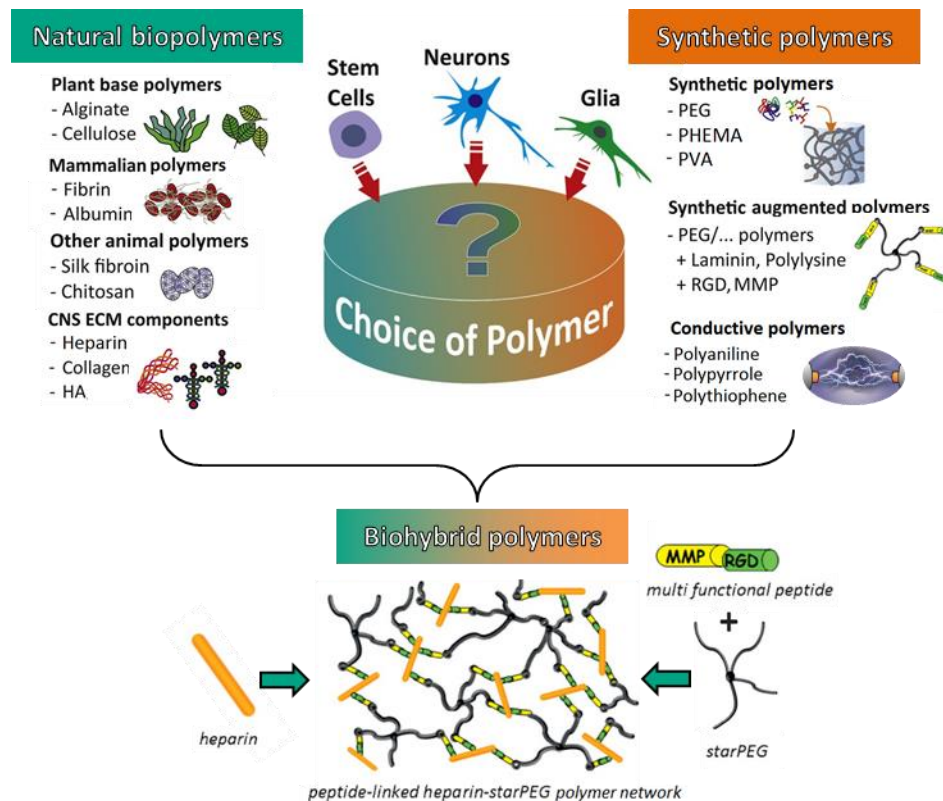
Figure 6: A variety of monomer/polymer compositions can be used for cryogel formation. Scheme of different sources of polymer used in cryogel preparations for applications in neurosciences. HA: hyaluronic acid; PEG: poly(ethylene glycol); PHEMA: poly(2-hydroxyethyl methacrylate); PVA: polyvinyl alcohol; RGD: Arg-Gly-Asp; MMP: matrix metalloproteinase. Adapted with permission from (George et al., 2020). Schematic view of the formation of a starPEG-heparin network with bifunctional peptide-linkers was adapted with permission (Tsurkan et al., 2010). Graphic displaying electrical conductivity adapted with permission from (Talebian et al., 2019)

In addition, another more recent approach showed the development and use of PEG-heparin copolymer cryogels for islet encapsulation, where cell-instructive properties were imparted by covalently modifying the heparin component post-cryogelation using adhesion ligands (RGD) (Borg et al., 2016). When drug delivery is desired, biomolecules such as heparin or collagen have been used to control the release of growth factors (Saparov et al., 2019; Saraswathy et al., 2020). As an example, Cai et al. reported the creation of monolithic cryogel columns comprised of the synthetic poly(2-hydroxyethyl methacrylate) (pHEMA) component and the methacrylated biopolymer heparin (Cai et al., 2019). Heparin functioned herein as a synergistic element to release basic fibroblast growth factor (bFGF) which contributes to the promotion of fibroblast proliferation, neovascularization and nerve regeneration. To add conductivity to a cryogel, for tissue stimulation/manipulation, electrically conductive polymers, such as polypyrrole (PPy), can be blended with the existing scaffold (Ghasemi-Mobarakeh et al., 2011). Vishnoi and Kumar have shown an approach for the synthesis of electrically conductive cryogels made of chitosan and gelatin blended with PPy, a heterocyclic conductive polymer (Vishnoi and Kumar, 2013a). They applied this scaffold to a neural cell line and demonstrated that electrical stimulation increased the cell's proliferation rate.

Applications of cryogels in neuroscience

Nerve guidance conduits

Damage to the neurons of the PNS or CNS can occur for several reasons, including a thermal, chemical, mechanical or ischemic trauma (Mietto et al., 2015). Depending on the trauma site, such injuries can cause dramatic negative impacts on cognitive, motor and psychological abilities of the patient, ultimately reducing their quality of life (Vardi and Merrick, 2008). Currently, the "gold-standard" treatment for peripheral nerve injuries are autologous nerve

grafts for bridging gaps larger than a centimeter (Cinteza et al., 2015; Scheib and Höke, 2013). However, this method is associated with significant limitations including donor-site morbidity, mismatched graft size, fibrotic scarring and sensory issues combined with vascular complications (Aigner et al., 2020; Daly et al., 2012; Huang and Huang, 2006).

Nerve guidance conduits (NGCs) (**Figure 7**), which are architecturally based on tubular structures with an empty lumen (**Figure 7B**) or intraluminal fillings, present a promising and attractive approach to replace natural nerve autografts (Huang and Huang, 2006; Yao et al., 2013). NGCs hold advantages in terms of adaptability both in terms of physicochemical composition and the use of different filling materials (**Figure 7A**) (Amani et al., 2019), including the incorporation of neurotropic factors, genetic material, extracellular matrix (ECM) components, cells, microchannels and fibers (Huang and Huang, 2006). In addition, their structural properties allow them to inhibit fibroblast ingrowth at the repair site, whilst providing cues for elongating axons bridging and regenerating the neural defect (Daly et al., 2012; Wu et al., 2019).
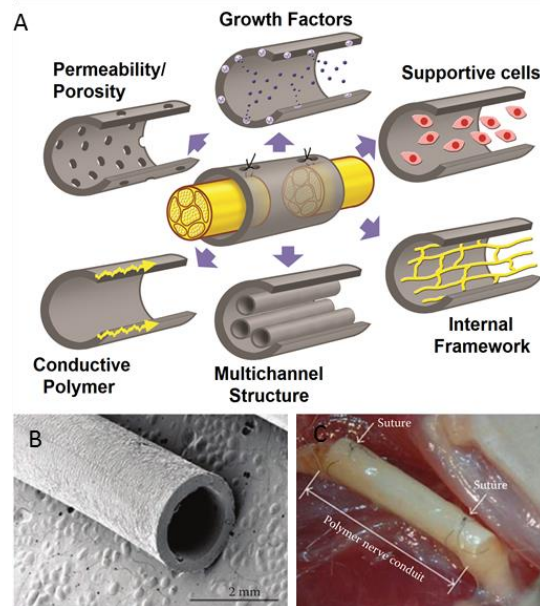

Figure 7: **Nerve guidance conduits can be created with different architectures and functional properties for promoting peripheral nerve repair.** (A) Modifications to the single lumen nerve tube for peripheral nerve repair (reproduced and modified from (de Ruiter et al., 2009)). (B, C) Cross-linked poly (caprolactone) fumarate polymer nerve conduits for nerve regeneration in rat sciatic nerve (B) before and (C) after implantation (used with permission from (S. Wang et al., 2009)).

One of the major drawbacks of synthetic NGCs is their high mechanical rigidity and low flexibility. For example, NGCs made of synthetic poly (l-lactic acid) (PLLA) based polymers possess a tensile strength range within 64.3–69.8 MPa (Nectow et al., 2012), which is well above the tensile strength of peripheral nerves *in situ* (11.7 ± 0.7 MPa) (Rydevik et al., 1990). In comparison, natural polymers are generally softer (e.g. collagen tensile strength of 25.4 kPa (Ryan et al., 2017)). However, the high mechanical stresses of the surrounding tissue, coupled with a high collagen degradation rate *in vivo* (Ryan et al., 2017), can result in compromised nerve regeneration via a collapsed guidance structure (Chiono and Tonda-Turo, 2015). Creating conduits to withstand such forces can require increased tube wall-thickness and decreased porosity which raises the issue of permeability limitations for nutrients and cells (Chiono and Tonda-Turo, 2015). It has been shown that wall-thicknesses exceeding 0.81 mm substantially reduces axonal growth and regeneration (Pyatin et al., 2017). However, whilst increasing porosity and decreasing the wall-thickness improves the nutrient, oxygen and cell influx, they in turn negatively affect the mechanical guide (Chiono and Tonda-Turo, 2015; Nectow et al., 2012). To overcome such difficulties, NGC material comprised of cryogels (**Figure 8**, **9**) have become an attractive alternative to traditional conduits, as they can be soft yet mechanically tough and still allow nutrient flow, thus showing great potential to improve peripheral nerve regeneration (Carvalho et al., 2019; Razavi et al., 2019). Singh et al. described the development of a polyurethane based NGC with an aligned gelatin-co-chitosan cryogel filling to enhance peripheral nerve regeneration (Singh et al., 2019). The material was tested on neonatal dorsal root ganglion explants and Schwann cells *in vitro*, which showed growth and migration along the aligned pores forming "Bands of Bungner"-like structures.
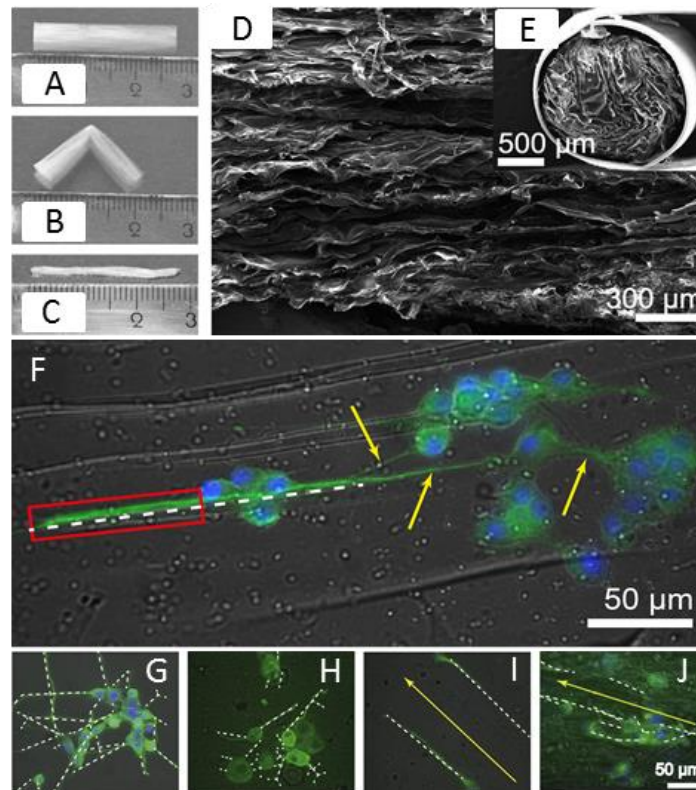
Figure 8: Cryogels have been incorporated into nerve guidance conduits. (A) Photograph of a collagen cryogel stored in PBS; (B) Bending of the collagen cryogel in PBS resulted in a sharp bending edge indicating its anisotropic character; and (C) PBS removal from a collagen cryogel yielded a significant volume reduction. (D) SEM image of a freeze-dried collagen cryogel and (E) its cross-section in the chitosan tube. (F) PC-12 cells on a collagen cryogel forming bundle-like structures (red box) compared to single neurites (yellow arrows). The bundle-like structure is aligned with the collagen cryogel (dashed line). (G-J) Analysis of neurite lengths grown on the materials used in this study. Neurites are marked with white lines to highlight their direction of outgrowth. (G) On a collagen film (positive control) and (H) on an RGD film, neurites grew isotropically, whereas (I) an anisotropic non-woven mat and (J) an anisotropic collagen cryogel allowed guidance of neurites into longitudinal direction. Yellow arrows indicate the orientation of anisotropic structures observed in the non-woven mat and in the collagen cryogel. Adapted and used with permission (Aigner et al., 2020).

In another study, a PPy based NGC with the same cryogel filling was designed (Vishnoi et al., 2019). Chitosan and gelatin were chosen to mimic the natural ECM environment, contributed synergistically with mechanical strength/flexibility and a positive charge (chitosan) and RGD motifs (gelatin) to improve cell adherence and proliferation (Lee et al., 2020; Vishnoi et al., 2019). Additionally, PPy functioned as the electrical conducting component in the scaffold, which have shown to actively stimulate cell growth and proliferation (Vishnoi and Kumar, 2013a). The cryogel filled NGC was applied *in vivo* in rats and in an *in vitro* scaffold culture system (Vishnoi et al., 2019). Successful regeneration and cellular reconstruction of a critically injured rat sciatic nerve was demonstrated, as determined by electrophysiological tests. Moreover, no adverse immune reaction, necrosis or tumorgenesis were present. In the scaffold culture system, the cryogel matrix mimicked the *in vivo* ECM environment of bone marrow stem cells (BMSCs), which resulted in a differentiation of them into a neural lineage. The successful differentiation was proven by the presence of neurotransmitters and neuronal markers.

Aigner and co-workers developed chitosan based self-rolling NGCs and investigated different inner tube modifications, including a crosslinked anisotropic collagen cryogel network as the luminal filling material (**Figure 8**) (Aigner et al., 2020). The cryogel matrix provided binding sites and biomechanical support for neuronal PC-12 cells. The moist cryogel tensile strength, maximum strain, and Young's modulus was: 0.15 ± 0.04 MPa, $70 \pm 7\%$, 0.22 ± 0.04 MPa respectively, in comparison, values for peripheral nerve tissue are: 0.15 ± 0.3 MPa for Young's modulus and $38.5 \pm 2\%$ maximum tensile strain (Aigner et al., 2020). The PC-12 cells differentiated inside the NGCs and formed long and parallel neurites along the lamellar guiding structures; an important feature for nerve repair.
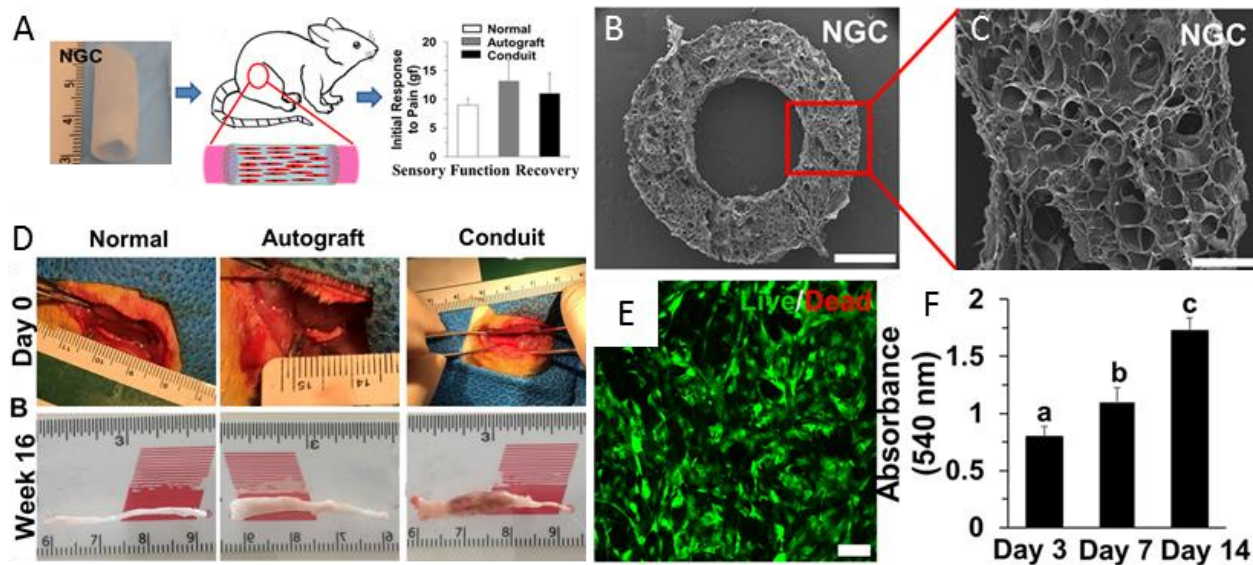
Figure 9: **Cryogel nerve guidance conduits for sciatic nerve repair.** (A) Scheme represents the use of cryogel-based guidance conduit for regeneration of sciatic nerve in rats. (B, C) SEM images of the cryogel NGC. (D) Repair of a rat sciatic nerve lesion with a length of 10 mm bridged with the autograft and the cryogel-based NGC. The normal nerve on the contralateral side was employed as the control. (E) Representative image showing dead (red) and live (green) rabbit Schwann cells cultured on the cryogels for 14 days; scale bar = 100 µm. (F) Quantification of rabbit Schwann cell proliferation at days 3, 7, and 14 of culture on the cryogels. Adapted and rearranged with permission from (Wu et al., 2019)

Whilst in the above cases, cryogels have been used as fillings for NGCs, recent studies by Wu et al. (Wu et al., 2019) and Tao et al. (Tao et al., 2017) demonstrated the engineering of a guidance conduit made from a cryogel scaffold. In the study of Wu et al., the cryogel-based NGC (**Figure 9A-C**) is composed of a biohybrid network of methacrylated gelatin, methacrylated hyaluronic acid and 4arm poly(ethylene glycol) acrylate (PEG-Ac). This NGC presented a favorable microenvironment for rabbit Schwann cells (**Figure 9E**, **F**) and the cryogel supported axon regeneration and remyelination of a transected sciatic nerve (**Figure 9A**, **D**) whereby the rat partly recovered its sensory function. Tao et al. used gelatin as the basis for the entire cryogel conduit (Tao et al., 2017). The cryogel conduit was also utilized in a rat sciatic nerve transection model and the cryogel was evaluated on its feasibility for nerve regeneration after neurorrhaphy. The results indicated a beneficial effect on peripheral nerve recovery after neurorrhaphy, as shown by electrophysiological experiments and the static sciatic index.

Cell delivery and transplantation for neural regeneration

Neurological disorders are diverse in their cause and progression. Most of them lack sufficient therapies because, in comparison to most other tissues and organs, regeneration of the affected neurons is a complex and regionally constrained process (Garg, 2017). Cell-based therapeutic approaches hold great promise for regeneration and restoration of diseased or damaged neural tissues (Jain, 2009; Yasuhara et al., 2020). However, cell delivery methods raise some problems, e.g. cells incurring mechanical damage by the injectable administration route, which leads to a high cell loss (Amer et al., 2015). Moreover, lifting cells from an adherent surface, and inappropriate cell-cell interactions contribute further to poor cell survival and reduced therapeutic effect (Adil et al., 2017).
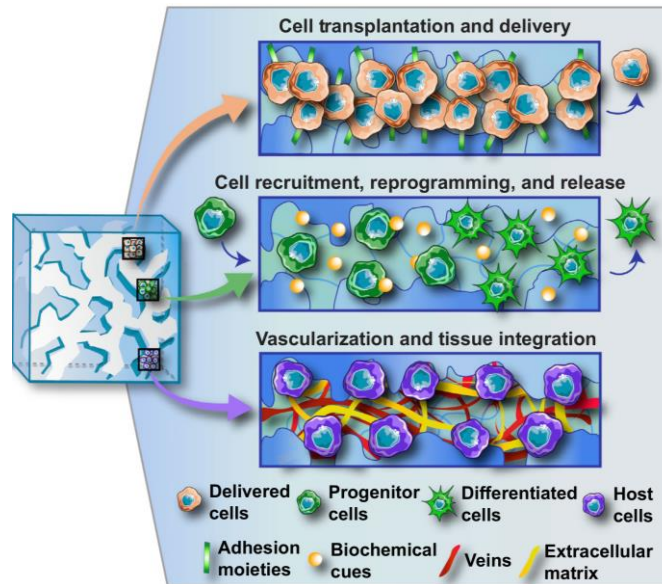
Figure 10: **Cryogels are suitable polymer network system for cell delivery and can create a confined cell-instructive niche for various biomedical applications.** The interconnected macropores of cryogels confer an environment that allows cell attachment and protection during injection and transplantation. Additionally, the open macroporous structure facilitates cell-instructive properties, which promote recruitment and trafficking of cells. The inherent properties of cryogels promote neovascularization, stimulate native extracellular matrix formation, and facilitate tissue integration. Reproduced with permission (Eggermont et al., 2020).

To overcome such issues, different scaffold materials have been developed to protect cells from damage upon delivery and from harmful *in vivo* microenvironments (Hoban et al., 2013). To this end, microscale cryogels have been examined for neural cell adhesion, injectability and delivery (**Figure 10**) (Eggermont et al., 2020; Hixon et al., 2017; Memic et al., 2019; Razavi et al., 2019). Beduer et al. developed injectable laminin and poly-l-ornithine (PLO) coated cryogels based on alginate and carboxymethyl cellulose for minimally invasive delivery (Béduer et al., 2015). The cryogels were infiltrated with primary mouse cortical neurons or human neuroblastoma SH-SY5Y cells. Due to the compressible sponge-like nature of the network these cryogels had both a high local Young's modulus in the struts (4.2 MPa) and a low macroscopic (whole cryogel) Young's modulus (0.6 kPa). This property allowed not only injectability (compression through the needle), but also maintained cell adhesion to the struts, protecting the neuronal network during injections (**Figure 11**).
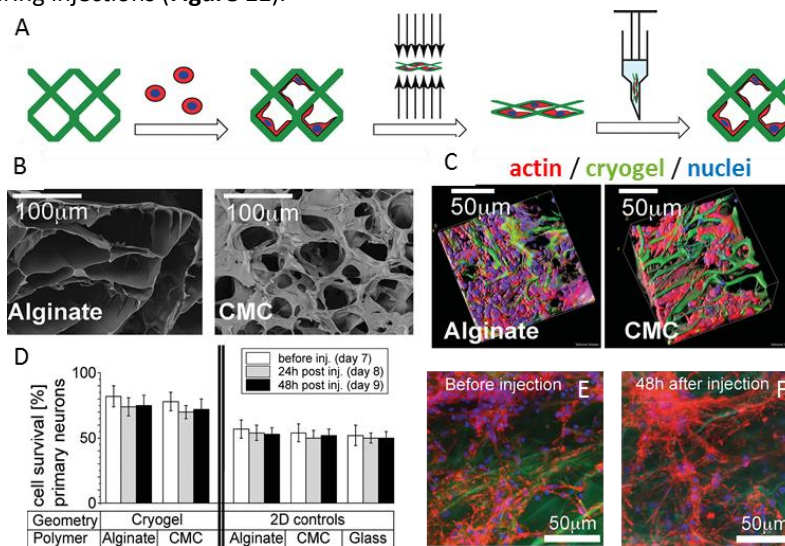
Figure 11: **Structure, neuronal cell adhesion, and injection principle for alginate and carboxymethyl-cellulose (CMC) cryogels.** (A) Scheme of cryogels protecting adherent neuronal cells at the injection site. During the injection, the cryogel is partially dehydrated, losing up to 90% of its volume without cell damage, and can be passed through an injection device such as a syringe before swelling back to its original volume. (B) SEM images of alginate (3%) and CMC (1%) cryogels. (C) Confocal micrographs of the human neuronal cell line SH-SY5Y growing throughout the cryogel. (D) Quantification of cell viability for mouse primary neurons before, 24 and 48 h after injection through a syringe. The viabilities for the 2D

controls are taken at the same time points, but without the injection procedure. (E, F) Representative images of mouse primary neurons on a cryogel before and 48 h after injection. Reproduced and rearranged with permission (Béduer et al., 2015)

Newland et al. fabricated spherical 300 μm diameter biohybrid starPEG-co-heparin cryogel microcarriers, which retained their original shape without any destruction when injected through a 27G needle, while maintaining PC-12 cell viability (**Figure 12**) (Newland et al., 2015).
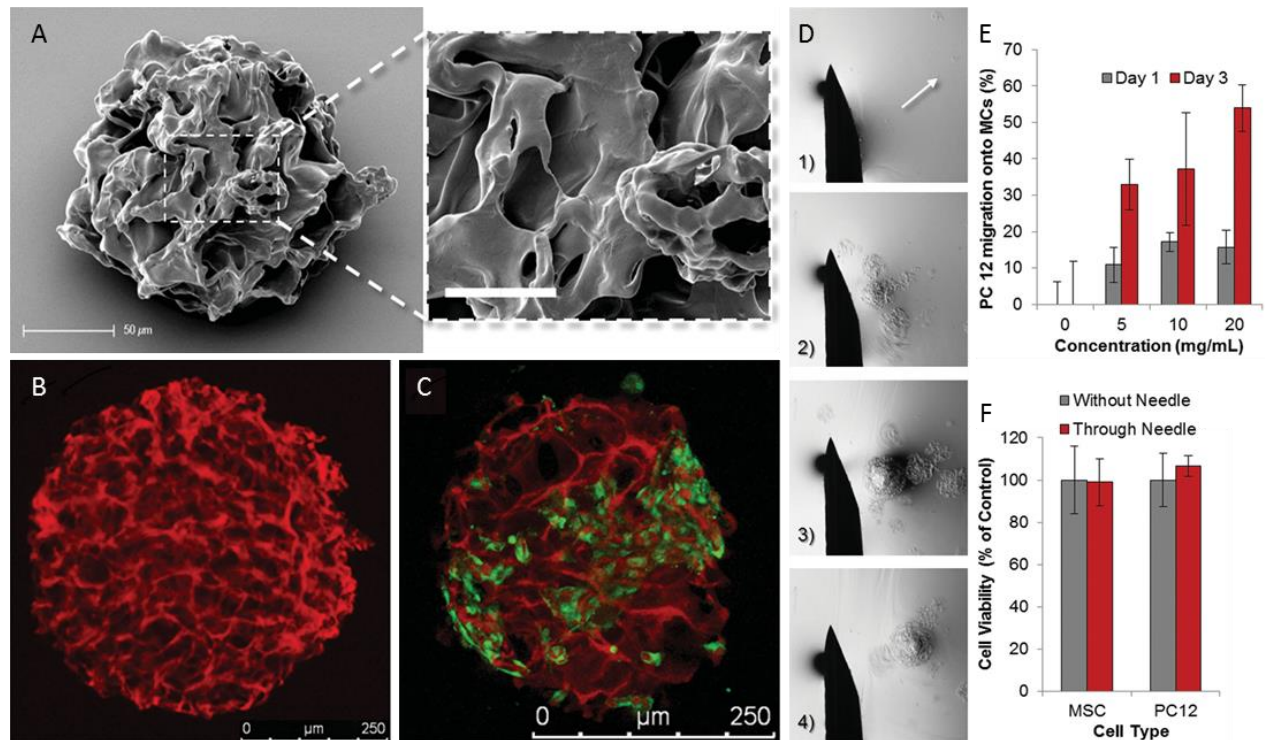

Figure 12: **Microscale spherical cryogels (microcarriers) for cell delivery.** (A) SEM image of a dry microcarrier. (B, C) Representative confocal fluorescence microscopy images of Alexa 647 labeled, hydrated cryogel microcarriers (red) infiltrated with GFP positive mesenchymal stem cell (rMSC) (green). (D) Visualized injection through a 27-gauge needle by light microscopy. (E) Quantification of rMSC and PC12 migration onto the cryogel microcarriers as a function of microcarrier concentration and time. (F) Injection of cryogels through a 27-gauge needle was shown to have no detrimental effect on cell survival. Adapted and rearranged with permission (Newland et al., 2015)

In another study, laminin enriched gelatin and dextran cryogels were designed and tested for implantation into the rat brain (Jurga et al., 2011). The results showed that the cryogels did not induce excess inflammation or scarring by glial cells. Moreover, the laminin attracted the infiltration and migration of neuroblasts throughout the network, which is a valuable property for supporting endogenous regeneration of damaged brain areas and lost circuits. In addition, they also documented a significantly increased degradation rate of gelatin cryogels when synthesized with a low amount of crosslinker (0.1 % glutaraldehyde). This resulted in a fast accumulation of decomposition products, which had toxic effects for cells and tissue at implant site. Such studies show that there can be a biocompatibility balance between biodegradable and non-degradable biomaterials, as exemplified with PEG based hydrogels (Bjugstad et al., 2010).

Filippova et al. employed a cryogel approach to leverage mature dopaminergic neurons for transplantation into the brain for improving Parkinson´s disease cell therapy (Filippova et al., 2021). There appears to currently be a trade-off between using immature dopamine progenitors (less control over fate, but better graft viability) or more mature dopamine progenitors (more differentiated, but low graft survival). Culturing the progenitors on a scaffold, thus removing the need for passaging cells prior to transplantation, may be a route to better graft viability (Adil et al., 2017), of particular importance for cell replacement therapies with more mature neuron populations. In this case, carboxymethyl cellulose was the basis for the cryogel scaffold, processed in the shape of microcylinders and additionally surface modified by laminin, collagen IV, fibronectin and Matrigel matrix (Filippova et al., 2021). Cryogels, which possessed laminin or Matrigel, allowed the most neural adhesion and neurite development. The results

showed an overall very high biocompatibility of the scaffold to neuronal cell types, while the material was injectable. The implantation of the cryogel into the mouse brain promoted cell survival of mature neurons, which had been differentiated from neural progenitor cells, thus providing a feasible neural network transplant.

Cell culture

Cell culture systems allow researchers to mimic key features of the *in vivo* situation in order to unravel the complex cell-to-cell and cell-to-ECM interactions within their surrounding ECM microenvironmental niche (Lin and Iacovitti, 2015). A better knowledge and more realistic recapitulation of cell niches holds potential for further understanding its relevance in cell growth and differentiation and their use for endogenous repair and potential regeneration at damaged tissue sites (Ehret et al., 2015; Lin and Iacovitti, 2015). In the case of neural cells, active neurogenesis arises in only two distinct regions of the brain, known as the stem cell niche (the subventricular zone and dentate gyrus). The generated stem cells and neural progenitor cells (NPC) possess high regenerative capacity, which is great for exploring potential mechanisms of neural development (Lin and Iacovitti, 2015; Ming and Song, 2011). The use of conventional 2D culture or 3D neurosphere culture approaches has not been expedient for adequately reproducing their niche. Both methods represent substantial drawbacks of either a lack of cell-to-cell contacts (2D) or oxygen/nutrient gradients (neurosphere) leading to population heterogeneity combined with spontaneous differentiation (Ehret et al., 2015; Jensen and Parmar, 2006). Consequently, there is an increasing demand for sufficient 3D cell culture systems, predominantly scaffold materials, which can overcome these drawbacks (Bhat and Kumar, 2013).

Cryogels have emerged as a promising tool for 3D cell culture systems as they showed the ability to closely mimic the native ECM (**Figure 10**) (Mahumane et al., 2018; Memic et al., 2019). In this context, Newland et al. recently reported the development of a static and dynamic 3D *in vitro* NPC culture platform using starPEG-co-heparin cryogel spheres modified with RGD motifs (Newland et al., 2020a, 2020b) (**Figure 13**). The macroporous nature of the cryogel microcarrier scaffold allowed nutrient flow throughout the scaffold without the formation of a necrotic core (**Figure 13D, E**). Thus, the culture of multipotent NPCs could be continued for 28 days with little spontaneous differentiation (**Figure 13F, G**). Furthermore, on withdrawal of growth factors (differentiation conditions) a greater number of neurons could be achieved than by 2D culture methods (**Figure 13 H-J**).
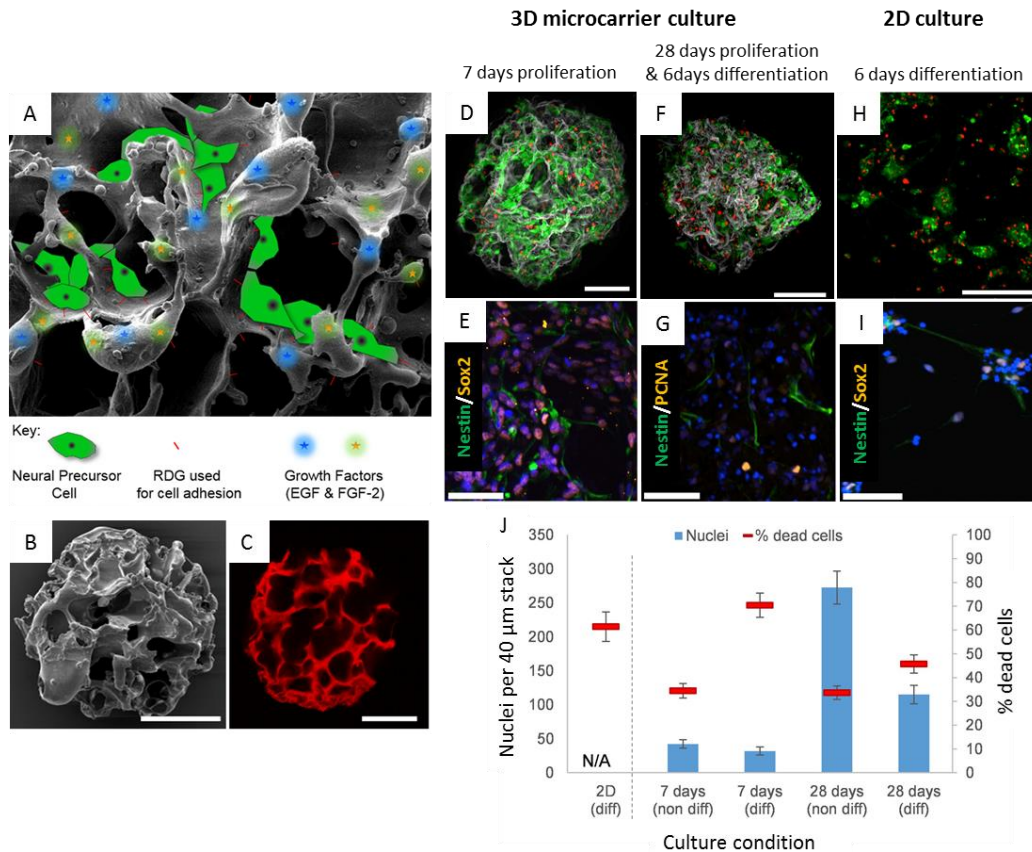
Figure 13: **Cryogel biomaterials for 3D cell culture.** (A) Schematic depiction of NPCs cultured on microcarriers comprised of poly(ethylene glycol), heparin and the RGD peptide. (B,) SEM (dry) and (C) confocal laser scanning microscopy (hydrated) image of microcarrier (scale bars represent 50 µm). (D-I) Representative images of microcarrier NPC culture performed for 7 and 28 days either with or without 6 subsequent days of differentiation. (H, I) For comparison, 2D NPC culture was grown to 80% confluency, differentiated for 6 days and stained. All scale bars mark 50 µm. (D-H) Green staining indicates living cells (calcein AM) and red staining indicate dead cells (propidium iodide). (E-I) Green staining indicates cells positive for nestin, orange is either Sox2 (E, I) or PCNA (G), and blue represents cell nuclei (Hoechst). (J) Quantification of the number of nuclei and percentage of dead cells cultured in proliferation conditions (non diff) or differentiated (diff) for 6 days after different culture periods (until 80% confluency in the 2D case) showing a large number of NPCs can be cultured and differentiated on the microcarriers. Adapted and rearranged with permission (Newland et al., 2020b).

Whilst the above mentioned microcarriers used RGD motifs for cell adhesion, a variety of other functionalization strategies have been exploited for cell culture in cryogels. Recombinant N-cadherin extracellular domains conjugated to alginate cryogels with uniaxially aligned microchannels recapitulated the role of cell-cell adhesion and guided the formation of neural networks in cortical cells (Vega et al., 2016). To increase the proliferation rate of cells, alpha-ketoglutarate (α-KG) loaded gelatin and chitosan microspheres have been added into a polypyrrole based cryogel. α-KG was shown to increase proliferation rates of cells by reducing the toxic effect of ammonia (Nedergaard et al., 2002), allowing neuro-2a cells to extended their proliferation rate over a longer time (Vishnoi and Kumar, 2013b). Functionalization of cryogels with laminin allowed human umbilical cord blood-derived stem cells (CBSCs) to be differentiated into artificial neural tissue when co-cultured with certain growth factors (Jurga et al., 2011). Interestingly, these studies showed that a pore size distribution of 20-160 µm was a well-suited range for cells forming a neural network structure. The pores were small enough to support cell-to-cell contacts and large enough to facilitate mass transport and neuroendothelial cell infiltration (Jurga et al., 2011; Sarnowska et al., 2013). In smaller pores, the CBSCs remained undifferentiated, (without expressing MAP2, Nestin, or GFAP) demonstrating the importance of pore size optimization for cell differentiation in porous scaffolds.

More recently, cryogels with dynamic self-healing properties and the ability to be fabricated by 3D printing were developed (Lin and Hsu, 2020; Nadgorny et al., 2018). This opens doors to a variety of additional cell culture applications within neurosciences. As Lin et al. impressively demonstrated, injectable cryogels with self-healing and

biodegradable qualities allowed good cell proliferation (571%) of mouse neural stem cells for 7 days of culture (Lin and Hsu, 2020).

Ex-vivo tissue manipulation

Tissue slice culture, where sections of living matter can be maintained *ex vivo*, can be exploited for a range of research uses. To a certain extent, they resemble the *in vivo* situation by containing the various cell types of the respective organ tissue and preserving most of the native cellular microenvironment, including the complex cell-cell connections (de Hoyos-Vega et al., 2020; Gibbons and Dragunow, 2010). In that regard, brain tissue slices have allowed exploration of the mechanism of neurogenesis, but have also allowed investigation into regenerative and degenerating processes of certain diseases in order to identify new therapeutics (Cho et al., 2007). However, one notable drawback of working with such *ex vivo* brain slice model systems has been the lack of precision tools to reproducibly monitor, mark and manipulate specific tissue areas using pharmacological or genetical factors (Ting et al., 2018).

Recently, cryogels are being considered to have great potential as highly versatile tools for site-specific manipulation of cells and tissue (Eggermont et al., 2020; Memic et al., 2019; Tripathi and Melo, 2019). Previously, Jurga et al. described the use of laminin-containing cryogels in a hippocampal organotypic slice culture (Jurga et al., 2011). The results showed intensive attachment and migration of neuroblasts throughout the entire network structure, whereas glial cells were found to be constrained to the scaffold periphery not inducing any scar formation. More recently, cryogel scaffolds composed of poly(ethylene glycol) diacrylate (PEGDA) have been used to create an *ex vivo* model of demyelination (Eigel et al., 2019) (**Figure 14**). The cryogels were loaded with a demyelinating agent lysophosphatidylcholine (LPC) and applied next to mouse tissue slices of the cerebral cortex and spinal cord in order to create distinct focal areas of demyelination (Eigel et al., 2019). This study showed that cryogels provide a tool to successfully recapitulate focal lesions as they appear in multiple sclerosis pathology. This improvement over non-focal "global" demyelination of the slice is therefore of importance for further investigating future pro-remyelinating therapeutics. A very recent study performed by Zoupi et al. even showed the successful application of such PEGDA based cryogel tools in an *in vivo* mouse model to provide a better understanding of the role of demyelination in neuron degeneration. By using the cylindrical PEGDA cryogels, with adjusted diameters (2 mm x 0.5 mm), the group achieved a novel LPC toxin induced model of focal subpial cortical demyelination in mice (Zoupi et al., 2021).
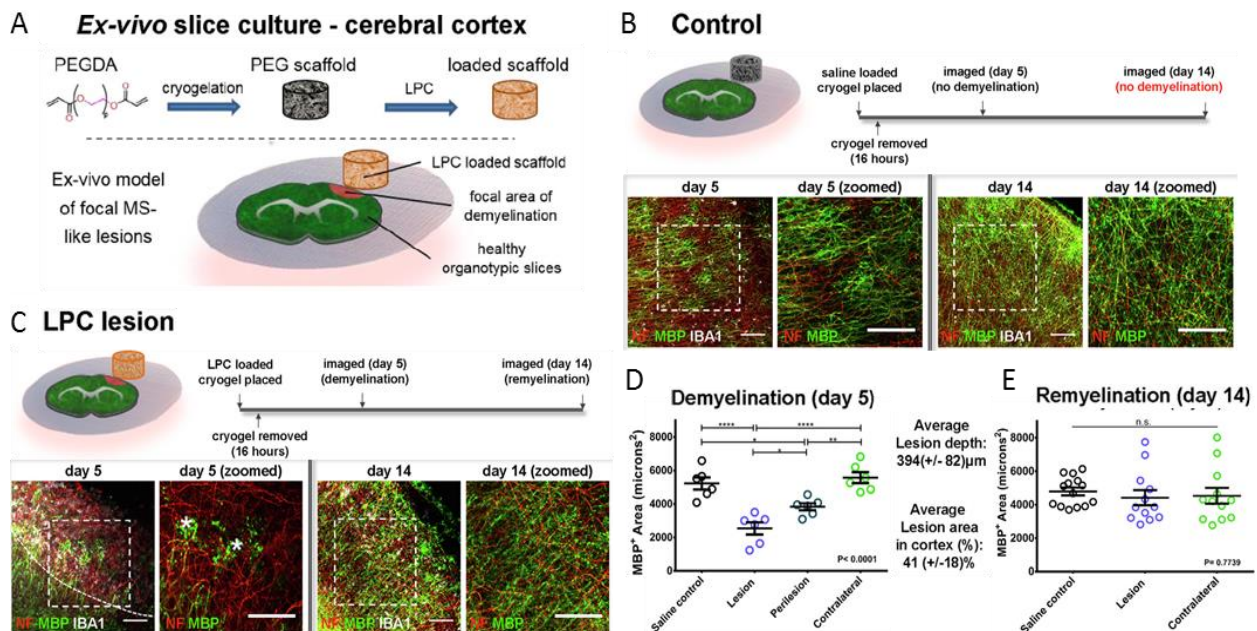
Figure 14: **Cryogel scaffolds for focal manipulation of *ex vivo* slice cultures.** (A) Schematic depiction of the ex-vivo brain slice model for focal demyelination to mimic Multiple Sclerosis (MS) pathology. (B) Schematic representation of a saline loaded cryogel (control condition) placed next to a cortical brain slice with corresponding timeline of study procedure. Immunohistochemistry of coronal sections for myelin basic protein (MBP) (green), neurofilament (NF) (red), and ionized calcium binding adaptor molecule 1 (IBA1) (white) for brain slices cultured in the control condition. Higher magnification images (zoomed) are taken from the regions marked by the dashed box, with IBA1 omitted for clarity. No demyelination

occurs in this condition (scale bars represent 100 μm). (C) Schematic representation of a cryogel loaded with the demyelinating agent lysophosphatidylcholine (LPC) (test condition) placed next to a cortical brain slice with corresponding timeline. Micrographs taken at day 5 show extensive demyelination (lesion border shown by dashed line) with myelin debris marked with an asterisk in the zoomed images. By day 14 the presence of increased numbers of myelinated axons is clear and IBA1 microglial cells are more evenly distributed, showing that spontaneous remyelination has occurred (scale bars represent 100 μm). (D) Quantification of the extent of demyelination at day 5. The area of MBP+ signal was measured and the lesion area shows a significant reduction of MBP signalling compared to the saline control, the perilesion and contralateral hemisphere. (E) Quantification of the extent of remyelination at day 14. The area of MBP+ signal was measured. The lesion area shows similar MBP signaling when compared to the saline control and the contralateral hemisphere showing that spontaneous remyelination has occurred. Adapted and rearranged with permission (Eigel et al., 2019).

Additionally, microscale PEGDA cryogels containing negatively charged sulfonated moieties have recently been developed as an easy-to-use tool for manipulating *ex vivo* culture, because they enabled a precise and regionally constrained delivery of probes (fluorescent dyes, tetrodotoxin or a growth factor) to specific tissue regions without causing damage (Eigel et al., 2021; Ucar et al., 2020).

Local delivery to the CNS

The most important factor limiting the delivery of therapeutic molecules to the CNS is the blood–brain barrier (BBB) (Pardridge, 2007). In the healthy CNS, the BBB functions as a protecting barrier, which restricts the transit of certain molecular compounds from the blood to the brain. In general, pharmaceutical strategies for the CNS are typically limited to lipid-soluble small molecule drugs with a molecular weight (MW) <400 Da that cross the BBB via lipid-mediated free diffusion (Pardridge, 2007). Local delivery to the CNS, via direct injection into the brain or spinal cord, offers a means to bypass the BBB, opening avenues for delivering drugs, genetic material or proteins that would otherwise not reach the region of interest. One example reaching clinical trial in patients with Parkinson's disease has been the direct monthly infusion of glial cell line-derived neurotrophic factor (GDNF) (Whone et al., 2019). Drug delivery devices, which mediate a sustained release of the therapeutic molecules, may reduce the number of repeat administrations (or negate their need) (Newland et al., 2016).

Injectable cryogels, with their high surface area to volume ratio, offer a means of loading high amounts of drug for delivery to the brain parenchyma. Newland et al, employed spherical, heparin-containing cryogels to bind and release growth factors (Newland et al., 2015) and cytokines (Schirmer et al., 2020) (**Figure 15**). The highly sulfated heparin molecules within the cryogel structure allowed electrostatic binding of GDNF, nerve growth factor and interleukin-13, with slow protein release without an initial burst (Newland et al., 2015; Schirmer et al., 2020). These materials are in the early stage of development, but Schirmer et al. have shown that intrastriatal injection of IL-13 loaded microcarriers results in a phenotypic switch of monocytes to a pro-regenerative (M2 − like) phenotype showing potential for immunomodulation in the CNS.
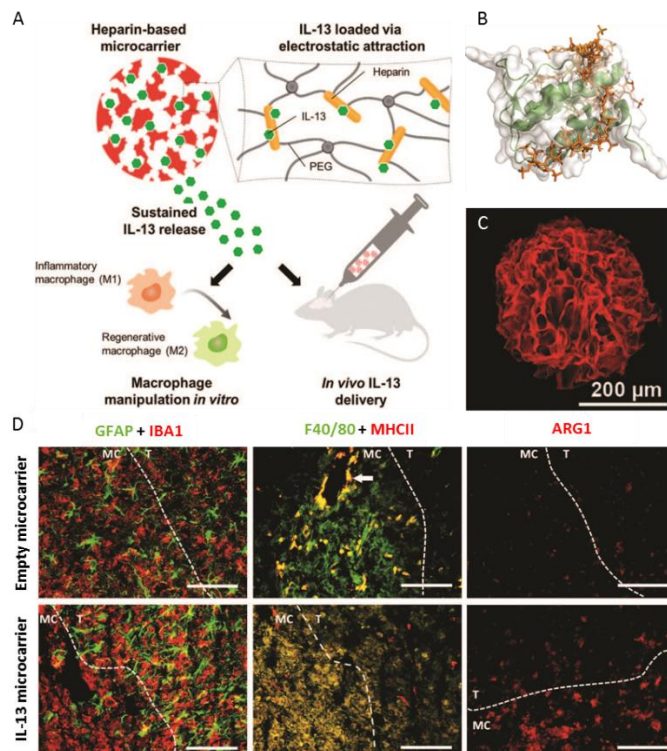
Figure 15: **Cryogel microcarriers as local drug delivery devices.** (A) Schematic overview of the experimental approach for using heparin based microcarriers to load and release of IL-13 for phenotypic manipulation of macrophages/microglia. (B) Representative image of the *in silico* modeling of the electrostatic interaction of IL-13 (green) with the heparin biopolymer (orange). (C) Representative confocal microscopy image of a hydrated ATTO 647 labeled microcarrier. (D) As with any needlestick injury, the injection of empty microcarriers was associated with GFAP+ astrocytes and IBA1+ monocytes, but no ARG1 (M2 polarization) (top panel). IL-13 loaded microcarriers (bottom panel), however, gave rise to robust overexpression of ARG1 showing their ability to modulate the host response post-injection, with double positive staining of F40/80 and MHCII indicating the infiltration of peripheral macrophages. Microcarrier injection area is outlined with a dashed line where MC = microcarrier, and T = brain tissue. The white arrow indicates the location of a blood vessel, scale bars = 100 μm. Adapted and rearranged with permission (Schirmer et al., 2020).

Bioelectric stimulation

Electrical stimulation of neural cells has been demonstrated to enhance their growth and differentiation (Ghasemi-Mobarakeh et al., 2011). Moreover, it was observed that such stimulation could significantly improve the regeneration and sprouting of injured axons *in vitro* and *in vivo* (Goganau et al., 2018). In order to better represent the native biological environment, scaffolds have been created with electrical conducting properties (Hardy et al., 2013). Vishnoi and Kumar have synthesized conducting cryogels using chitosan, gelatin and PPy (**Figure 16A-C**) (Vishnoi and Kumar, 2013a). Scaffold synthesis via the cryogelation process allowed the use of a comparatively small amount of polypyrrole, due to the process of cryoconcentration. Additionally, although PPy is typically brittle in nature, good mechanical properties can be achieved when blended with chitosan and gelatin. The group tested a variety of different voltages for different durations, which resulted in enhanced proliferation of neuro-2a and cardiac muscle cells C2C12 cultured on the cryogel.
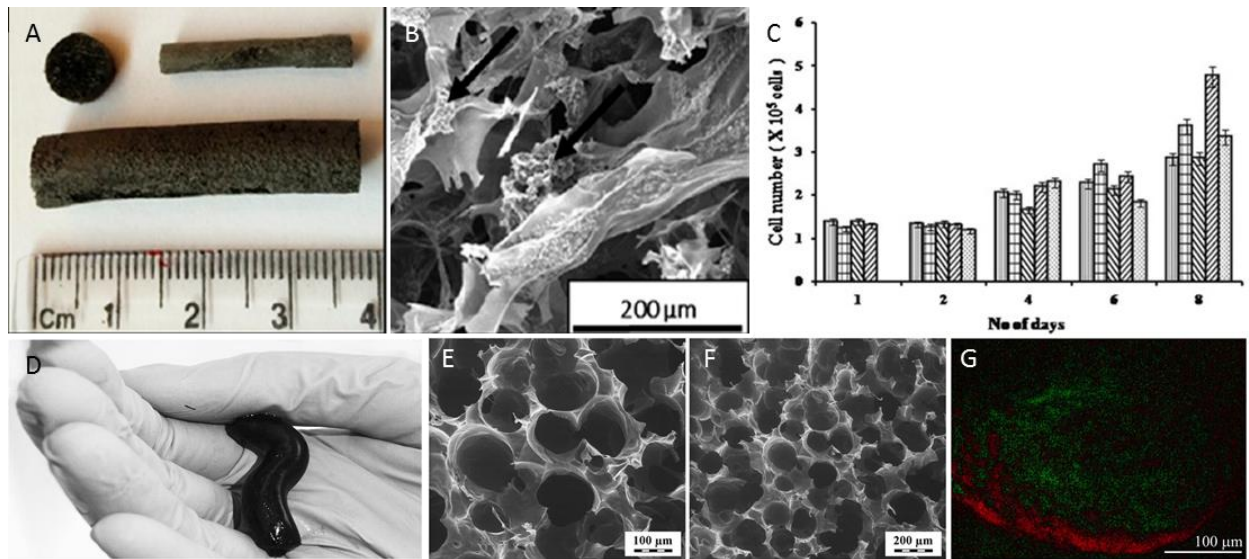
Figure 16: **Conductive polymer cryogels for bioelectric stimulation.** (A) Representative photograph of chitosan-gelatin-polypyrrole cryogels. (B) SEM image of the synthesized cryogels showing interconnected porous architecture and the presence of incorporated PPy (black arrows) (adapted with permission (Vishnoi et al., 2019)). (C) Voltage optimization for Neuro2a cells (square with vertical fill); 30 mV: (square with horizontal fill) 50 mV: (square with upper left to lower right fill) 70 mV: (square with upper right to lower left fill) 100 mV: (square with dots fill) control. As compared to the control, 100 mV showed better proliferation rate (adapted with permission (Vishnoi and Kumar, 2013a). (D) Polyaniline cryogel mimicking the properties of native tissue. Polyaniline is green in transparent thin films but appears black in thick layers or in powder form. (E, F) SEM images of the polyaniline cryogel. (G) Beating foci within spontaneous differentiating embryonic stem cells on polyaniline cryogel. Red spots correspond to the movement of cells to the right Green spots indicate no difference between the images. Reproduced and rearranged with permission (Humpolíček et al., 2018).

Macroporous PEDOT-PSS (poly(3,4-ethylenedioxythiophene) polystyrene sulfonate) based conducting cryogels, exploited ice segregation-induced self-assembly when frozen unidirectionally using liquid nitrogen (Zhang et al., 2011). This process introduced interesting features such as longitudinal pores, which can stimulate directional neural cell growth. A further example of a intrinsically electrical conductive cryogel network was demonstrated by Humpolicek et al. in which the scaffold was synthesized from polyaniline blended with polyvinyl alcohol (PVA) (Humpolíček et al., 2018) (**Figure 16D-F**). These cryogels facilitated the formation of beating foci within spontaneously differentiating embryonic stem cells, as well as the adhesion and growth of neural progenitor cells (**Figure 16G**). Studies such as these prove the concept that conductive cryogels can be used to guide neural cell growth and be used to electrically simulate cells, paving the way for new soft yet flexible/stretchable bioelectronic implants for the nervous system. Beyond the development of new bioelectric cryogels materials, the use of fabrication technologies for material patterning that are based on stereolithography (3D printing) or photolithography can be additionally employed. Such methods can facilitate a customized and on-demand engineering of soft biocompatible cryogel networks that can be used for an effective integration into bioelectronic interfaces (neural implants) (Akbar et al., 2020).

Future perspectives

Over the past decade, advances in biomaterial development have influenced our fundamental understanding of the nervous system and have generated new methods and therapeutic approaches for its regeneration and repair (Khaing et al., 2014; Liu and Hsu, 2020; McMurtrey, 2015). Cryogels are a promising biomaterial candidate for such neuroscience applications (Eigel et al., 2019; Kumar, 2016; Memic et al., 2019). Their macroporous nature and tunable structural properties can be advantageous for applications such as cell-instructive culture platforms, spatiotemporal controlled drug delivery, cell carriers and nerve guidance conduits. These are just a few prominent examples, which have been successfully utilized to replicate or repair neural system components.

Many of these examples are in the early stages of development. The arising culture model of cerebral organoids can recapitulate the developmental profile of the brain up to 24 weeks after conception (Chiaradia and Lancaster, 2020). However, the formation of a necrotic core in the organoid due to hypoxia leads to undesirable heterogeneity in the

tissue composition and morphology. Cryogels, due to their physico-chemically adjustable, soft and macroporous network structure, can be developed with intrinsic oxygen-generating scaffold properties (Razavi et al., 2020; Shiekh et al., 2018), which can serve as a continuous source to supply oxygen or nutrients (e.g., glucose) (Behl et al., 2020; Uygun et al., 2015) that may prevent the hypoxia-induced damage at the organoid core. Consequently, this could retain cell viability and/or facilitate cell growth and moreover, increase the homogeneity of organoid culture by the possibility of introducing additional exogeneous patterning growth factors (Newland et al., 2020b). Therefore, looking ahead, the use of cryogel technologies could likely impact the field of 3D cell and organoid culture, not just for CNS applications, but a wide range of tissue types. The macroporous structure of cryogels could allow a better nutrient flow to the core of the developing cell organoid mass which may delay the size-limiting hypoxic/necrotic core formation that currently hinders spheroid and organoid development (Qian et al., 2019).

Since metal ions have been linked to triggering the pathophysiology of neurodegenerative diseases (Altmann et al., 1989; Yegambaram et al., 2015), cryogels may play a future role in sequestering such substances from the peripheral blood. The high surface area to volume ratio make them well suited to such applications, and early studies have shown that toxin and waste removal could be possible (Baydemir et al., 2009; Demircelik et al., 2009; Gun'ko et al., 2013). In addition, cryogelation provides a chance for effective molecular imprinting. The technique of molecular imprinting is used to confer specific molecular recognition properties to a synthetic polymer network by including template molecules into its synthetic pathway, which interact during cryogelation with the monomer (Wang et al., 2015). When cryogelation is completed, the template molecule can be removed, which results in a chemically, and a sterically complementary imprint within the cryogel network. Thus, the cryogelation process allows a powerful method for embedding at sites for high specific binding of proteins or ionic interactions, which made it possible to sequestrate metal ions such as aluminum, iron and copper or catabolic compounds such as bilirubin from the blood plasma using cryogels (Baydemir et al., 2009; Demircelik et al., 2009).

Another potential use of cryogels could be as advanced delivery systems that can provide exogenous nutrient supply to support grafts at post-implantation sites. Recent studies have hypothesized that nutrient supply, especially glucose, may be critical to cell survival post-transplantation (Deschepper et al., 2013; Moya et al., 2018), with neurons being vulnerable to conditions of oxygen-glucose deprivation (OGD) (Wohnsland et al., 2010). Cryogels offer a very attractive option, as their high surface area and adjustable porous architecture, combined with a chemically amendable microstructure, can support the possibility for encapsulation and supply of molecules (e.g. oxygen (Razavi et al., 2020) or glucose (Gutiérrez et al., 2007)) to maintain essential metabolic functions and an efficient exchange of fluid flow (Hedström et al., 2008). For instance, cryogels could potentially be fabricated with phenylboronic acid based moieties, which have previously been exploited for a pH-regulated entrapment and controlled release of glucose (Ma and Shi, 2014; Wang et al., 2019), while the high pore surface area of the cryogel network would provide a large space for glucose loading.

The soft and compressible nature of cryogels, make them well suited to use as implantable local drug delivery devices. The prognosis for patients with brain cancers such glioblastoma multiforme is so poor that there is a strong rationale for developing better therapeutic strategies, including local delivery devices (Bastiancich et al., 2016). Whilst one such system, implantable carmustine-loaded wafers (Gliadel), has failed to greatly impact on glioblastoma multiforme patient survival (Chowdhary et al., 2015), a meta-analysis of pre-clinical data showed local delivery of the chemotherapeutic temozolomide out-performed systemic delivery (Hirst et al., 2013). In the case of Gliadel, mechanical mismatch between the stiff wafer and soft brain tissue, and the poor release profile from the wafers (Tabet et al., 2019), are downsides which cryogel scaffolds could readily address. For example, Newland et al. fabricated PEG-co-heparin cryogels for the local administration of doxorubicin adjacent to orthotopic breast cancers in a mouse model (Newland et al., 2020c). The group observed a sustained drug release over 42 days without an initial burst, which reduced the tumor growth and the metastasis rate. Microscale, injectable cryogel microcarriers such as these could potentially be used in non-resectable brain tumors. Operable tumors would require larger cryogels to be placed in the resection cavity. These may be an attractive option for surgeons as they are soft, compressible (space-filling), yet mechanically robust for ease of handling. Gamma knife radiation (GKR) surgery is

being used as an effective non-invasive radiochemotherapy for the treatment of unresectable brain tumors. However, this treatment is also associated with severe side effects of brain tissue necrosis and cerebral edema due to high doses of radiation (Inoue, 2006). The option to use cryogels to overcome the downside of GRK could provide the opportunity to either reduce the cerebral edema using the highly swellable polymer network as an osmotic transport device or to provide a local delivery of anti-inflammatory substances against necrosis.

Whilst forecasting the future uses of a material is clearly prone to stochastic events, we have tried to give a few areas where the unique properties of cryogels may make them suitable for a particular neuroscience application.

Conclusion and remarks

The CNS consists of soft heterogeneous tissue and is diverse regarding cell populations. Biomaterials designed to model or regenerate the CNS therefore need to mimic such tissue with mechanical properties similar to the CNS, in order to support the development and recovery of neural network functions. Cryogels are a versatile macroporous class of biomaterials, which exhibit many interesting features for applications within neurosciences. By simply controlling fabrication parameters (e.g. monomer/polymer concentration, temperature, solute type and cooling rate) their architecture can be customized and physiochemically fine-tuned for their intended use. The high pore connectivity enables liquid flow through the structure, and the large surface area of the pore walls enables various binding capacities for bioactive substances. Due to their soft yet robust mechanical characteristics, cryogels can be created in different shapes and sizes, and retain this shape after rehydration/injection etc., making them easy to store and handle. This can be exploited for uses as minimally invasive delivery vehicles for both cells/tissue and spatiotemporal release of therapeutic agents to support the recovery of damaged CNS tissue. In conclusion, cryogels are very adaptable biomaterials that can be utilized in a wide range of neuroscience applications, spanning from nerve guidance conduits for mediating axonal recovery to cell/drug carriers for transplantation/local delivery at the appropriate site.

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