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Cryogel scaffolds: soft and easy to use tools for neural tissue culture

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The mammalian central nervous system (CNS) is highly complex, with a vast array of processes and interactions occurring in a dynamic and often transient manner. How these processes are combined to regulate our behavior remains poorly understood. This has in turn led to a lack of understanding of how these processes have gone awry in the many disorders of the nervous system. In order to address this, researchers need a controlled way to manipulate the nervous system in *in vitro* and *ex vivo* cultures, in both a specific area and for a specific period of time to start to pick apart these interactions. To date, this has been technically challenging, especially when modeling focal injury to the CNS or when working with human brain tissue.

Here, we suggest that biomaterials could help bridge this gap. One technical challenge of manipulating nervous system tissue is that it is inherently soft and susceptible to mechanical damage. The use of biomaterials such as conventional hydrogels, which match the mechanical properties of the CNS, could help overcome this issue, but these have so far been hindered by their mechanical weakness. For instance, even microscale hydrogels with a Young's modulus as high as 30 kPa, over three times stiffer than brain tissue (Eigel et al., 2021a), could not be mechanically removed from their template without breaking (Eigel et al., 2021b), suggesting that they may be difficult to handle, load and place onto the CNS tissue itself. However, a more recently developed class of biomaterial, cryogelated hydrogels – or cryogels, offer a convenient solution to this problem. They can be extremely soft, whilst remaining mechanically tough enough to be handled with forceps. These mechanical properties make them particularly well-suited for the local delivery of reagents to brain and spinal cord tissue. These properties are also a major reason why cryogels have already been used for a wide range of biological applications (Razavi et al., 2019). Herein we discuss the recent development of cryogel tools for CNS manipulation and give our perspective on the future directions of this technology.

Cryogels are similar to their hydrogel counterparts in that they are composed of a 3-dimensional hydrophilic network. However, an important difference is their macroporous structure, with comparatively large open pores surrounded by thin struts of hydrogel (**Figures 1A** and **2**). This structure is obtained by freezing the hydrogel precursor solution prior to (or during) crosslinking, forcing the polymer network to form around the ice crystals (Eigel et al., 2021a). The sponge-like morphology of cryogels makes them tough, due to the condensed polymer network found in the struts. However, macroscopically they are soft and can be substantially compressed (**Figure 1B**), reforming to their original size and shape once the compressive force is removed (Bencherif et al., 2012). This is an important characteristic that enables these cryogels to be inserted and then later removed from CNS tissue (Eigel et al., 2021b).

This mechanical toughness has enabled these macroscale cryogels to be easily used to deliver reagents to regional areas of tissue in culture and improve models of CNS injury. For example, cylinder-shaped cryogels were used to create a focal *ex vivo* model of demyelination (Eigel et al., 2019). These cryogels were dipped in a demyelinating agent, lysophosphatidylcholine, and then placed next to brain or spinal cord tissue slices in culture. As depicted in **Figure 2A**, this created a small region of demyelination surrounded by healthy tissue (Eigel et al., 2019). Aside from their ease of use, the cryogels also provided the important advantage of more accurately reproducing the patchy demyelination pathology observed in multiple sclerosis in humans (Eigel et al., 2019), in comparison to pre-existing "global demyelination" models (Zhang et al., 2011). This patchy demyelination allowed the role of the infiltrating glial cells, from the surrounding unaffected areas, in remyelination to be analyzed. Another advantage of this focal demyelination approach is that it can also be applied to create focal regions of demyelinated grey matter *in vivo* (Zoupi et al., 2021). This has allowed researchers to unpick mechanisms of demyelination *vs*. inflammation in neural degeneration, and highlight the selective vulnerability of specific neuronal types to degeneration following demyelination (Zoupi et al., 2021). The ability of the cryogels to improve the replication of patient pathology in these models highlights their potential to be further developed as a platform for analyzing therapeutics for remyelination.

The mechanical toughness of cryogels has also proved useful when working with human brain tissue cultures. Cryogels generated using 3D printed templates have allowed researchers to label specific regions of human fetal brain tissue slices with fluorescent dyes, enabling specific cells to be identified and followed in culture (Eigel et al., 2021b). They have also been used to transiently apply a neurotoxin to reversibly and repeatedly inhibit neuron activity in a spatiotemporal manner within a brain tissue slice (Eigel et al., 2021b). Human tissue cultures lack many of the genetic tools available in animal models, but we speculate that cryogels could also be used to deliver genetic material to this tissue, either through viral vectors or non-viral constructs. A notable advantage of the cryogels for such applications is that as they are typically formed within a template, meaning that they can be created to almost any size or shape whilst retaining their properties post-compression (Bencherif et al., 2012). This has allowed the formation of high aspect ratio (elongated) line-shaped cryogels, which, despite narrow widths down to 150 µm, are strong enough to be handled with forceps, loaded with reagents and placed onto brain tissue slices (**Figure 2B**) (Eigel et al., 2021b; Ucar et al., 2021). This would enable cryogels to be made to order and reproducibly target a specified area of the brain tissue slice. This characteristic, alongside their ability to be removed without damaging the tissue (Eigel et al., 2021b), gives them a major advantage over conventional hydrogels, as those formed from the equivalent monomers/solid content cannot be handled without breaking (Eigel et al., 2021b; **Figure 1C**). Overall, cryogels have shown promise for use as biocompatible tools to deliver a wide range of cargos to neural tissue cultures, which could include CRISPR/cas9 systems, therapeutics and even cells.

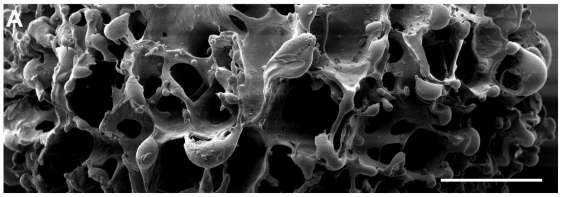
One characteristic of cryogels that makes them suitable tools for cell delivery is that the pore size of the scaffold can easily be adjusted by varying the freezing regime used (Eigel et al., 2021a). Slower freezing rates yield larger pores at sizes suitable for cell adherence and growth within the cryogel scaffold itself (**Figure 2D**) (Newland et al., 2020). Indeed, pores can be made sufficiently large enough to allow infiltration of the neural progenitor cells throughout the cryogel structure within the first three days of culture (Newland et al., 2020). When used as a 3D cell culture system, these cryogels allowed 28 days of neural progenitor cell culture without the formation of a necrotic core or spontaneous differentiation, two commonly occurring problems of 3D neurosphere cultures. In principle, this

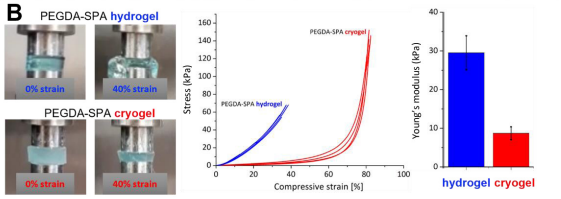
culture technique could be adapted to organoid cultures, potentially allowing organoids to retain a viable core as they grow.

Whilst many of the aforementioned techniques have largely revolved around forming models of disease and manipulating cultures, cryogels have also been investigated for experimental therapeutic uses. An early study by Béduer and colleagues clearly highlighted the potential that cryogel scaffolds have for improving cell transplantation (Béduer et al., 2015). Large neuronal networks could be grown on the cryogels, which, to prove injection feasibility, were compressed through a needle without damage or loss of viability (Béduer et al., 2015). Continuation of this work led to the formation of neurothreads, elongated cryogel cylinders within which dopaminergic neuron progenitor cells can be cultured and matured. Transplantation of neurothreads harboring mature dopamine neurons into the mouse striatum allowed robust survival and spread of the grafted neurons (Filippova et al., 2021).

The importance of this work for future transplantation protocols should not be underestimated. Studies have shown that the enzymatic removal and subsequent replating of mature dopamine neurons in culture causes extensive damage and cell death, with less than 25% of cells surviving (Adil et al., 2017; Filippova et al., 2021). This could be a major contributing factor to the poor survival rate of *in vitro* developed mature dopamine neurons that are transplanted into the brain. To negate the need for enzymatic removal of cells from their culture substrate, neurons can be directly grown onto cryogel neurothreads, and the cell-loaded cryogel can then be transplanted into the CNS tissue. In fact, using this approach resulted in a 20-fold reduction in the number of cells required to achieve a similar yield of viable cells from a traditional striatal graft (Filippova et al., 2021). We fully expect that future studies will show that implantation of cell-loaded cryogels will improve graft survival, maturation and even function of the grafted cells. This could be either through bypassing detachment-induced anoikis (programmed cell death upon loss of attachment) allowing more differentiated progenitors to be transplanted, and/or through continued provision of maturation cues such as growth factors from cryogels.

Heparin-containing cryogels have already been shown to control the release of pre-loaded growth factors such as nerve growth factor and glial cell line-derived neurotrophic factor (Newland et al., 2021). Furthermore, fully synthetic cryogels incorporating sulfonate groups to mimic the charged





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	Hydrogel	Cryogel
Nature of pores	Nano/micropores between polymer chains	Interconnected macropores (from micrometres to several hundred micrometres)
Shape	Highly variable depending on mould used	Highly variable depending on mould used Can retain original shape after substantial deformation/dehydration
Elastic modulus	Highly variable from soft to stiff depending on design	Highly variable from soft to stiff depending on design
Toughness	Generally weak but depends on design	Typically tough and able to resist a large compressive strain

Figure 1 | An overview of the properties of cryogels that make them useful biomaterials for neuroscience applications.
(A) Scanning electron micrograph showing the cryogel struts and large macropores (scale bar: 50 µm). Adapted with permission from Newland et al. (2020). (B) Mechanical testing analysis comparing hydrogels and cryogels with exactly the same monomer composition, showing that cryogels can withstand greater stress and strain before rupture, despite being about three times macroscopically softer than the equivalent hydrogels. Adapted with permission from Eigel et al. (2021). (C) General overview of physical characteristics that are typical of hydrogels and cryogels. PEGDA-SPA: Poly(ethylene glycol) diacrylate-co-3-sulfopropyl acrylate.

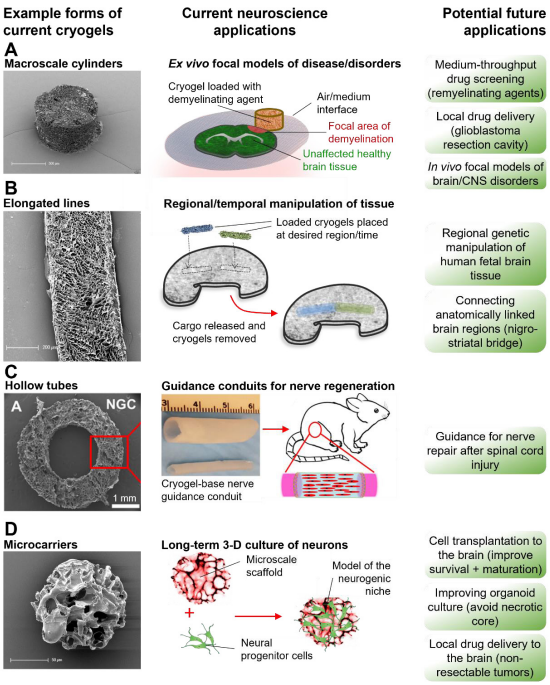

Figure 2 | A representation of different cryogel structures used for neuroscience applications in culture, with scanning electron microscope images of example cryogel forms, current uses, and potential future uses.
(A) Macroscale cylindrical cryogels have been used for modelling multiple sclerosis and hold potential for local drug delivery. (B) Elongated line-shaped cryogels have been used to deliver reagents to human fetal brain tissue, which could potentially be adapted to include genetic manipulation. (C) Hollow tube-shaped cryogels have been used as nerve guidance conduits which hold promise for both peripheral and central nervous system regeneration. (D) Microscale spherical cryogels (microcarriers) have recently been used as a 3D cell culture platform which could potentially be used for a wide range of *in vivo* applications. Reproduced with permission from Eigel et al. (2019) and Wu et al. (2019). CNS: Central nervous system; NGC: nerve guidance conduit.

nature of heparin, also electrostatically bind and release molecules for controlled delivery (Eigel et al., 2021b). Such developments pave the way for creating local implantable reagent delivery systems via highly reproducible cryogel production and the ability to fine-tune release rates, by adjusting the degree of sulfonation. Biodegradability of the cryogel carrier system is often thought to be an advantageous property for implantation. Whilst this may appear to have many benefits, degradation of the cryogel may change the local tissue environment in which it resides, potentially resulting in additional adverse host responses to the cryogel and its degradation products. The benefits of biodegradation would therefore need to outweigh those of a stable and well-tolerated carrier system that does not carry this risk.

The ability of cryogels to deliver reagents, proteins and cells makes them an attractive starting point for the design of soft implantable therapeutic drug delivery systems. For example, the resection cavity left after glioblastoma surgery could be filled with soft cryogel delivery systems, allowing repurposing of drugs that cannot pass the blood-brain barrier. Combining imaging of the tumor with 3D printing technology to create molds, cryogels of a particular size and shape could be prepared to approximately match that of the cavity. Furthermore, charged cryogels could be electrostatically loaded with a precise amount of oppositely charged chemotherapeutics (e.g., doxorubicin). Filling the resection cavity with this drug loaded cryogel could potentially achieve an even and sustained release of these therapeutics to all surfaces of the resection cavity. Alternatively, injectable microscale cryogels (such as those shown in **Figure 2D**) may be useful for local intratumoral delivery of therapeutics to non-resectable tumors, provided that they can still be reached by stereotactic injection. Cryogels, with their unique combination of high elasticity, high toughness and macroporosity, are well-suited to applications such as nerve guidance conduits, giving mechanical support to regenerating neurons without hindering fluid flow to these cells (**Figure 2C**) (Wu et al., 2019).

In summary, cryogel scaffolds have been used for a variety of applications in the field of neuroscience. This is clearly a field in its relative infancy, especially when compared with conventional hydrogels. Their key mechanical properties enable them to be easily handled, compressed or injected, yet still return to their original shape. Combined with their macroporous structure and versatility in their design and chemical composition, these unique characteristics make cryogels a highly promising new tool for the study of the CNS and its many related disorders. Soft and implantable cryogels also hold promise for affinity-based drug delivery systems, particularly where long-term release and tissue-matched mechanical properties are highly desirable.

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