

Short communication

Injection-free, spatially controlled delivery of glioblastoma cells to human brain slice cultures using soft cryogel biomaterials

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ABSTRACT

Organotypic brain slice culture has emerged as a valuable *ex vivo* system for studying brain development, function, and disease in a multicellular tissue context. More recently, this has extended to the use of human tissue derived from fetal, post-mortem, or discarded surgical specimens. In glioblastoma research, focal delivery of exogenous cells to brain slices is often technically challenging, particularly when spatial control is required. Here, we present a simple and experimentally accessible method for localized cell delivery to human brain slice cultures using soft donut-shaped cryogel biomaterials. Stereolithography-based 3D printed templates were used to generate well-defined cryogels by sub-zero polymerization of poly(ethylene glycol) diacrylate and 3-sulfo-propyl acrylate. The resulting macroporous scaffolds were soft, easy to handle, and could be positioned directly on top of human brain slices prior to loading brain tumor cells into the central lumen. This design enabled focal confinement of the administered cells while permitting invasion into the underlying tissue. The ability to tailor cryogel dimensions, together with the straightforward workflow, makes this approach a versatile platform for spatially controlled cell delivery to brain slice cultures. Under the conditions tested, cryogel placement was not associated with an increase in local GFAP (Glial Fibrillary Acidic Protein) signal, supporting compatibility with the tissue during culture.

1. Introduction

The *ex vivo* culture of brain or spinal cord tissue, has become a prominent research tool in modelling disease and treatment therapeutics [1]. By sectioning tissue into multiple thin slices, more samples per rodent brain can often be utilized, allowing more experiments per animal and reducing the need for live animal experimentation [2]. Although not without physical damage during cutting, much of the complexity of the

brain tissue can be preserved and studied, allowing extensive modelling of neurodegenerative diseases which are challenging to achieve with standard *in vitro* cell culture models [3]. For example, rodent brain *ex vivo* slices have already been utilized to understand diseases such as Alzheimer's disease [4,5], Parkinson's disease [6,7], traumatic brain injury [8], multiple sclerosis [9,10], and brain cancers *etc.* [11]. Human fetal tissue has also been studied, particularly for enhancing our understanding of human developmental biology [12].

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Recently, 'access tissue' from patients with epilepsy or brain tumors, presents another useful resource. Tissue taken from the point of entry towards the surgical resection of epileptogenic, or cancerous tumor tissue, can be cultured and studied either as dissociated cell matter, or as slice cultures [13–15]. In some cases, the possibility to obtain both predominantly non-malignant resected tissue, and disease affected matter from the same patient has allowed a myriad of omics analyses to improve our understanding of disease progression [13,16].

Glioblastoma (GBM), the most aggressive and lethal brain cancer, is an area of significant unmet therapeutic need regarding effective treatment therapeutics, which have not had a transformative impact over the past three decades. The standard treatment of care involves surgical resection (where possible/practical), followed by a combination of radiation therapy, and temozolomide chemotherapy [17]. However, the prognosis remains poor [18], with little clinical impact. Median survival post-diagnosis has recently been reported to be approximately 17 months in large scale clinical trials with selected patient populations, and the five-year survival rate is below 10% [18,19]. Therefore, extending survival and improving quality of life remain paramount; however, achieving this relies on the development of new models that more accurately recapitulate the disease.

Such models are essential for testing novel therapeutics, which currently show limited efficacy due to extensive intratumoral and interpatient heterogeneity, resulting in inconsistent treatment responses [20,21]. For instance, many studies use long established cell lines such as U87-MG cells to assess therapeutic efficacy, yet these cells fail to mimic hallmark features that many GBMs possess [22,23], such as high infiltration and invasion, and thus offers limited translational value in drug development in the context of such complex diversity in tumor response.

Human organotypic brain slice cultures offer a particularly valuable platform for modelling glioblastoma in a setting that retains native tissue architecture and cellular complexity [24–26]. A persistent experimental challenge, however, is the controlled introduction of exogenous tumor cells into a defined region of the slice. Surface seeding is straightforward but often provides limited spatial control [27], whereas microinjection enables focal delivery but typically requires dedicated equipment and technical expertise [13]. Accordingly, there remains a need for complementary delivery strategies that are spatially defined, experimentally accessible, and straightforward to implement across laboratories.

To address this need, herein we present a soft cryogel-based method for localized cell delivery to human brain slices without the need for injection apparatus. By placing a donut-shaped biomaterial on the tissue surface and loading cells into its central lumen, the transplanted cells can be spatially restricted to a defined region of the slice. We hypothesized that the soft yet robust properties of cryogelated hydrogels would provide favorable mechanical compatibility with the tissue while remaining easy to handle with standard laboratory tools such as forceps. In addition, the macroporous sponge-like architecture of the cryogels enables dehydration and rehydration without loss of form, which is advantageous for sterilization, storage, and routine laboratory use. Here, we describe the design and synthesis of the donut-shaped cryogel biomaterial and demonstrate its use as a versatile and user-friendly platform for spatially controlled delivery of glioblastoma cells onto human brain slice cultures.

2. Materials and methods

All reagents were used as received from Merck unless otherwise stated.

2.1. Template design and production

The templates for creating the cryogels were designed in two parts, an upper part to define the donut diameter and a lower part to define the

lumen diameter. Two different sized templates were made with the following internal mold dimensions: 2.5 mm diameter with a lumen of 1 mm diameter, or the smaller version was 2 mm diameter with a lumen of 0.8 mm. Both versions had an internal mold height of 1 mm. These were created in Autodesk Inventor and exported as a .STL file in high resolution. Both parts for each template were printed in the X-Y orientation using an ASIGA MAX-27 3D printer with Asiga PlasWhite V2 resin. Prints were removed from the print head, washed in isopropanol three times, and post-cured with UV light (DR-301C, Asiga) for 30 min. The two components of the template were plasma treated with low-pressure air plasma (PDC-003-CE, Harrick Plasma, connected to a CRVpro6 vacuum pump, Welch) for 2 min prior to assembly and filling with the precursor solution containing monomers, to make the surfaces more hydrophilic and prevent any adhesion of the cryogels to the template.

2.2. Cryogel synthesis and purification

Donut-shaped cryogels were created by filling the 3D printed template with an aqueous solution containing the monomers 3-sulfopropyl acrylate (SPA) (potassium salt) and poly(ethylene glycol) diacrylate (PEGDA) (M_w 700 g/mol) at a 1:1 M ratio, with 2-hydroxy-2-methylpropiophenone (HMPP) as a photoinitiator (PI) at a PI: total monomer ratio of 1:35. The solid content of this precursor solution was 10% wt/vol with MilliQ (Merck Millipore) filtered water. For fluorescently labelled cryogels, iFluor® 647 Maleimide was added to the monomer solution (0.05% v/v from a 10 mg/mL stock solution). The templates were placed at -20°C for 30 min for ice crystal formation, before being irradiated with UV light (Kohler UV hand lamp, 365 nm (2 tubes of 15 W)) for 2 min whilst still at -20°C . The templates were brought to room temperature, and the upper part was removed from the lower part, with the donut-shaped cryogels being removed manually into 20 mL of ethanol. Washing/purification consisted of removing and replacing the ethanol three times, before being dried in a vacuum oven (Gallenkamp) at room temperature at reduced pressure.

2.3. Characterization of the cryogel scaffolds

An EVOS imaging system (M7000, Thermo Fisher) was used to observe the swelling degree of the cryogels. Dehydrated cryogels were put into clear bottomed 96-well plates (Corning Inc., costar®, 3595) and bright field images were taken. To image the cryogels after rehydration, 50 μL of phosphate buffered saline (PBS) was added into each well. The exported images were merged bright field images and CY5 fluorescent light source images together. These were tiled from 12 individual images (3×4 , $4 \times$ objective). ImageJ was used to analyze the size of cryogels in their dry and hydrated state.

Confocal microscopy was performed using a Leica SP5 laser scanning confocal microscope using a HeNe laser for excitation at 633 nm. Four samples of each size of donut-shaped cryogel were hydrated in PBS and imaged using a $10\times$ air objective lens with a $10\ \mu\text{m}$ z-stack (interval distance of $2\ \mu\text{m}$) to obtain a projected average image representing a small section through the cryogel structure. The pore size was calculated from these 2D projected average images from four samples, with four areas being imaged at random for each cryogel. Each selected pore was measured manually (in ImageJ) in both the X and Y directions (*i.e.* perpendicular measurements for each pore) with the average value representing the diameter of that particular pore. 1000 pores were measured from the 16 images, with data plotted *via* a distribution plot.

For porosity analysis, confocal images were acquired using an Andor Dragonfly spinning-disk confocal microscope (Oxford Instruments, Abingdon, UK) equipped with a $10\times/0.45$ NA objective and $2\times$ camera magnification. A total of 12 independent regions of interest (ROIs) were analyzed, comprising four ROIs from each of three independent samples. Corresponding z-stacks were recorded with a range of $50\ \mu\text{m}$ and a z-step size of $1\ \mu\text{m}$.

Three-dimensional image reconstruction and analysis were

performed using Imaris (version 11.0.1; Oxford Instruments, Abingdon, UK). To determine the cryogel volume (volume of the pore walls/of the polymer scaffold), the acquired z-stacks of each region of interest (ROI) were reconstructed in three dimensions. The polymer scaffold was subsequently segmented using the Surface function in the image analysis software Imaris. The intensity threshold was adjusted to ensure that the polymer scaffold was captured as completely as possible.

Then, the porosity ϕ of the cryogels was calculated based on the ratio of the cryogel volume to the total volume of the analyzed stack according to the following equation:

$$\phi = 100\% - \frac{\text{Cryogel Volume}}{\text{Total Volume}} \times 100$$

The total volume of the analyzed image stack was calculated from the x- and y-dimensions of the field of view and the height of the acquired z-stack. During image acquisition, care was taken to image only regions in which the cryogel was completely contained within the field of view, thereby avoiding errors caused by truncated structures. The reported values represent the mean of all measurements.

To visualize the surface topography and macroporous nature of the cryogels, they were dried in a vacuum (VacuTherm, Thermo Scientific) before being placed onto the carbon film attached to a scanning electron microscope (SEM) stub. Samples were sputter coated with gold using a DSR1 Desk Sputter Coater (VacCoat) before being imaged with a Tescan Vega SEM at 5 kV.

2.4. Chemical characterization of the cryogels

Fourier-transform infrared (FTIR) spectroscopy was carried out using an IR Spirit with the QATR-S accessory (SHIMADZU). This was used to analyze whether complete crosslinking of the polymer structure had occurred with incorporation of the sulfonate groups in the cryogels. Purified cryogels, which had been washed three times for 10 min each in 1 mL of ethanol were analyzed in comparison to the SPA and PEGDA monomers.

2.5. Analysis of the mechanical properties of the cryogels

Uniaxial compression testing was performed on the 500 N Zwicki Bench Top Portable Material Testing Instrument (Zwick-Roell). Data was analyzed with the TestXpert II software package. A gradual increase in compression force to a maximum of 2.5 N was applied upon cryogel samples created in a 15 mm diameter $\times$ 5 mm high cylindrical mold, with all other parameters kept the same as the donut shaped cryogels. The stress-strain curves were calculated from the height of the sample, diameter of the probe (16 mm), and the degree of force *versus* the degree of sample deformation. The Young's elastic modulus values were calculated from the slope of the linear portion of the stress strain plots (typically ranging from 5 to 20% strain), and an average determined from $n = 4$ specimens for each sample type.

2.6. Brain slice culture

Organotypic slice cultures were prepared from cortical access tissue as previously published by Ravi et al., with further details given in the supporting information [13]. Permission to use human brain tissue was obtained from the local ethics committee of the University of Freiburg (Approval 100020/09 and 5565/15). Human brain tissue specimens were obtained with informed consent under a Declaration of Helsinki, as requested by the local ethics committee (No. 187/04).

2.7. GBM cells and cryogel-mediated transplantation

Patient-derived glioblastoma cells, fluorescently labelled with a lentivirus (rLV.EF1.ZsGreen1-9; Takara-Bio Inc.;0040VCT), were cultured in 89% MEM, 10% FCS, 1% penicillin/streptomycin medium

(cell culture medium (CCM)) as described previously [15]. Donut-shaped cryogels (created using the 2.5 mm diameter template) were soaked in 70% ethanol for 20 min, then thoroughly washed in CCM to remove the ethanol. After rinsing, the cryogels were placed on a sterile dry dish to remove excess liquid. Using tweezers, the cryogels were placed on the brain slice at the interface between gray and white matter. A 2 μ L drop of GBM cell suspension (40,000 cells/ μ L) was carefully added to the cryogel lumen. An EVOS 7000 microscope (Invitrogen™ EVOS™ cell imaging system (EVOS)) was used to observe the grafted GBM cells, and the prepared sections were then cultured for 1 and 5 days in a humidified incubator at 37 °C and 5% CO₂ until being imaged.

2.8. Immunofluorescence staining

Slices were transferred into plastic molds using Pasteur pipets and the remaining medium was pipetted off. Then the slices were covered with OCT, frozen using dry ice, and stored at -80 °C until further use. To section the OCT blocks, they were first acclimated to the -20 °C environment of the cryotome and then glued onto the sectioning block side-ways for vertical sectioning using OCT. Vertical OCT sections were cut at a thickness of 30 μ m and groupwise (4–6) mounted. The location of the cuts was chosen to maximize the amount of tissue per slice and to pass through the region of tissue where the cryogel was located. For immunofluorescence staining of astrocytes (via glial fibrillary acidic protein (GFAP)), slices were first fixed using 4% paraformaldehyde (10 min at room temperature (RT)) and subsequently washed with PBS.

Permeabilization ensued using 20% methanol (2–3 min, RT) and then the sections were washed two more times with PBS. After that the sections were covered with 500 μ L of 1% Triton in PBS per section and incubated for 1 h at RT. Post incubation, they were washed twice with PBS. Next, slides were covered with 500 μ L of 1% Triton, 20% BSA-PBS and incubated for 1 h at RT. After this, the sections were covered by 500 μ L of 1% Triton, 5% BSA-PBS, containing the primary antibody, anti-GFAP (chicken, ab4674, Abcam) and incubated for 1 h (RT). Then the slides were washed twice more using PBS, followed by an incubation with 1% Triton, 5% BSA-PBS, containing the secondary antibody (goat anti-Chicken IgY (H + L) Alexa Fluor™ 647, 1:500, A-21449, Thermo Fisher Scientific) for 40 min. Then the slides were washed two times with PBS, incubated for 5 min with 1:1000 DAPI, and washed once in MilliQ water. Finally, one drop of fluoro mounting medium was added and the sections were covered using a coverslip glued in place using nail polish. Post immunofluorescence staining, slides were stored at 4 °C and protected from light. Single-plane images of slice culture experiments were taken with the EVOS imaging system, whilst images of immunofluorescence stainings were taken with a Mica microscope (Leica Microsystems).

2.9. In vitro cell culture

Normal human astrocytes (ScienCell Research Laboratories) were cultured in Astrocyte Basal Medium supplemented with 2% (v/v) fetal bovine serum, penicillin/streptomycin, and Astrocyte Growth Supplement according to the manufacturer's instructions. Cells were maintained at 37 °C in a humidified incubator with 5% CO₂ until they reached 80–90% confluency. Cells were then detached using trypsin, harvested, and seeded into poly-L-lysine-coated 96-well plates at a density of 2500 cells per well. Following a 24 h incubation to allow cell attachment, sterile, dry cryogels were placed into the wells. Cells were incubated with the cryogels for 1, 3, or 7 days. At each time point, 10 μ L of PrestoBlue® reagent (Invitrogen) was added to each well, and the plates were incubated for a further 2 h at 37 °C. The culture medium was then transferred to a fresh 96-well plate, and fluorescence was measured using a Tecan microplate reader with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Fluorescence values were background-corrected and normalised to untreated control cells to determine the percentage cell viability.

To generate defined focal regions of GBM cells *in vitro*, cryogels were used to spatially localise cells. Glass-bottom well plates were coated with Matrigel before cryogel placement. GBM cells were seeded into the cryogel lumen and imaged immediately (0 h) using brightfield and GFP fluorescence. Following a 2 h incubation, culture medium was added and cells were imaged at defined time points up to 72 h. Cryogels were then removed, and the underlying regions were imaged to assess cell proliferation and migration.

2.10. Statistical analysis

Throughout this manuscript, all data were processed, analyzed, and plotted using GraphPad Prism software (Version 10). Fluorescence optical density of stained *ex vivo* tissue slices (DAPI, GBM, or GFAP) was quantified using the open-source software ImageJ (Fiji). Individual fluorescence channels were first isolated, and a region of interest (ROI) measuring approximately $1000\ \mu\text{m} \times 300\ \mu\text{m}$ was placed over areas corresponding to the tissue region located underneath the cryogel scaffold, the scaffold lumen, or regions of tissue directly in contact with the scaffold *versus* deeper regions not in contact. When necessary, the ROI edges were adjusted to account for tissue curvature and to avoid including non-tissue (“dead space”) areas. Mean gray values were measured within each ROI and averaged across three independent tissue samples. The depth of GBM cell infiltration was quantified by measuring the maximum extent of GBM marker diffusion at representative points across each slice.

3. Results and discussion

The ability of glioblastoma cells to migrate and invade the surrounding brain parenchyma is a key factor affecting tumor recurrence, as maximal safe surgical resection inevitably leaves malignant cells behind. Therefore, a better understanding of brain tumor cell migration and invasion mechanisms may shed light on druggable targets and potential future therapeutics for repurposing [28]. Means of analyzing tumor cell migration, have varied widely, from straight-forward scratch assays in well plates [29], to injection of tumor cells into mice brains with subsequent removal of the brain for *ex vivo* slice culture/imaging [22]. The use of discarded surgical tissue from human brains is an exciting advancement to allow visualization of tumor migration. For instance, Ravi et al. recently showed that placing a 1 μL suspension of brain tumor cells onto human organotypic brain slice culture, allowed visualization of individual tumor cells along blood vessels and their increased migration rate in hypoxic conditions [30]. However, no low magnification analysis was performed for analysis of global migration and the overall spread of the tumor bulk through the tissue. Indeed, we have previously shown pipetting of reagents directly on top of human brain tissue in culture to be very haphazard in nature, with no means to control positioning or surface area affected [31].

Beyond this, there are very limited means by which to locally intervene on *ex vivo* slices for development of culture models, and as such we developed cylindrical scaffolds with a lumen to act as a port for accurate addition of cells to tissue in a defined surface region. Cryogelation was adopted as a strategy for scaffold synthesis, as the resulting macroporous structure is approximately three times softer than the chemically equivalent hydrogel [31]. However, cryo-concentration of the polymer network elicits greater strength and a more robust nature for manual handling with forceps than the equivalent hydrogel. This combination of soft, yet robust, mechanical properties is key to their use as an *ex vivo* tool [32–34]. The PEGDA/SPA copolymer network formed herein rendered a soft ($\sim 5\ \text{kPa}$) scaffold which could be easily handled in the dry or hydrated state and placed onto tissue in culture.

3.1. Template-based synthesis for reproducible production of donut-shaped cryogels

In order to fabricate a precise localized region of application for GBM cell delivery, we used stereolithography template assisted synthesis. Templates were designed using computer aided design, with two interlocking components, which when brought together would form a mold in which the cryogels were synthesized (Supplementary Fig. S1). By having projections in the lower part of the template, a lumen in each cryogel could be formed, with the outer diameter of the cryogel being governed by the top part of the template. The monomer precursor solution was pipetted slowly into the filling wells until level across all apertures ensuring uniformity of cryogel dimensions (Fig. 1a). The two-part template system also allowed ease of removal from the mold post-synthesis, as the upper part could simply be removed and added to excess ethanol where the cryogels would wash from the template and float freely.

We have previously shown that biomaterials composed solely of poly (ethylene glycol) adhere to tissue in culture [31]. This impedes the users' ability to remove the cryogel from the brain tissue without affecting its structural integrity or inducing a glial response. The subsequent addition of negatively charged monomers to the polymer structure allowed ease of removal from tissue and ease of repeat administration without cytotoxic effects [31]. Therefore, herein we used SPA as a sulfonate-containing negatively charged monomer, which was crosslinked with PEGDA at a 50% molar ratio as shown in Fig. 1.

Polymerization at sub-zero degree Celsius temperature was initiated using ultra-violet light and HMPP as a photoinitiator, with the incorporation of SPA into the cryogel structure being confirmed by FTIR spectroscopy, as shown by the inclusion of stretch peaks in the cryogel spectrum for S=O and S–O at 1170 and $1040\ \text{cm}^{-1}$, respectively (Supplementary Fig. S2). Furthermore, elimination of unreacted toxic monomer species from structure was confirmed by the absence of C=C stretch peaks at $1635\ \text{cm}^{-1}$ or vinyl C–H bend peaks (807 and $980\ \text{cm}^{-1}$). The dried cryogels were visualized by scanning electron microscopy (Fig. 1b and c) which revealed the macroporosity of the scaffold, and the defined ‘donut’ morphology. Interestingly, images of the cryogel on its side revealed small ribs around its structure (Fig. 1c) which is likely attributed to the $50\ \mu\text{m}$ z-plane step size used during printing of the templates. This templated-based synthetic strategy allows simple adjustment of the cryogel dimensions to suit the *in vitro* experimental model system, as exemplified by the smaller donut dimensions (2 mm diameter, 0.8 mm diameter lumen) as shown in Supplementary Fig. S3.

3.2. Cryogelation yields a soft, yet robust, macroporous structure

Owing to their interconnected macroporous architecture, the cryogels exhibit a sponge-like behavior, swelling upon rehydration to facilitate water uptake (Fig. 2a). When the cryogel polymer network is hydrated, they swell to 1.2 times the size of the template (Supplementary Fig. S4). Cryogels created using the smaller template (2 mm diameter), swelled to an average diameter of $2.46\ \text{mm}$ ($\pm 0.02\ \text{mm}$), whilst those formed in the larger template (2.5 mm diameter) reached $3\ \text{mm}$ ($\pm 0.04\ \text{mm}$) (Fig. 2b). It should be noted that the cryogels have a total swelling degree, from dry to hydrated in PBS, of 1.6 (i.e. they are smaller than the template diameter when dry (also shown in Supplementary Fig. S4). Furthermore, reproducibility between various synthetic batches was confirmed by the consistent cryogel dimensions (Fig. 2b), and mechanical testing data (Fig. 2c). Importantly, the cryogels were found to have a low Young's modulus ($5.14 \pm 1.1\ \text{kPa}$) (Fig. 2c) in a range matching that of human brain tissue ($1\text{--}50\ \text{kPa}$ depending on measurement technique) [35–38], but could also be easily handled with forceps in both the dry and hydrated state, demonstrating their superior utility for a convenient and user-friendly platform in brain tissue related culture model development (Fig. 2d). Moreover, although the macroporous structure is clearly observable by light microscopy

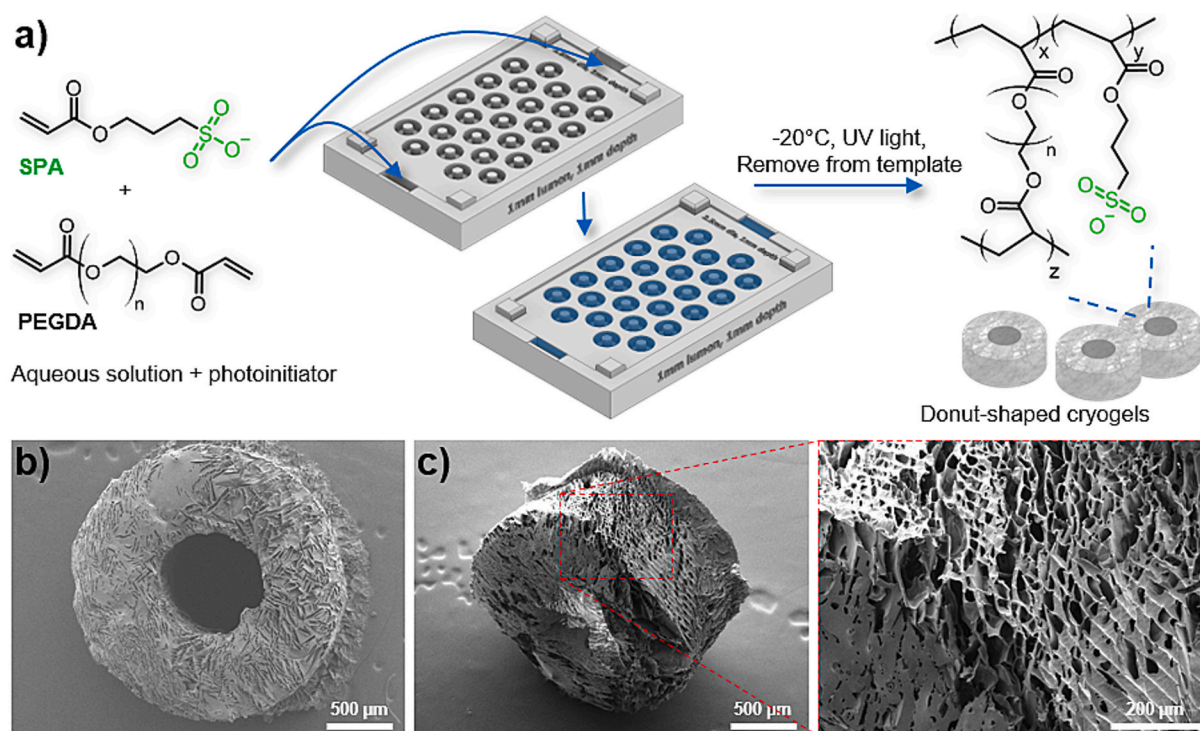


Fig. 1. Reproducible production of donut-shaped cryogels *via* template-based synthesis. A schematic diagram depicting cryogel synthesis from 3-sulfopropyl acrylate (SPA) and poly(ethylene glycol) diacrylate (PEGDA) in two-part, 3D printed molds. Scanning electron microscope (SEM) images of the donut-shaped cryogels in their dehydrated state, from above (b) and from the side (c) with higher magnification, showing the macroporous structure of the polymer material.

(Fig. S5), confocal microscopy of iFluor 647-labelled cryogels (Fig. 2e and Supplementary Fig. S6) enabled a more precise characterization of the pore architecture, revealing a median pore width of 75 μm with a

broad standard deviation of $\pm 45 \mu\text{m}$ (Fig. 2f). The use of spinning-disk confocal microscopy revealed an estimated porosity of approximately 64% (Supplementary Table S1) following analysis of reconstructed z-

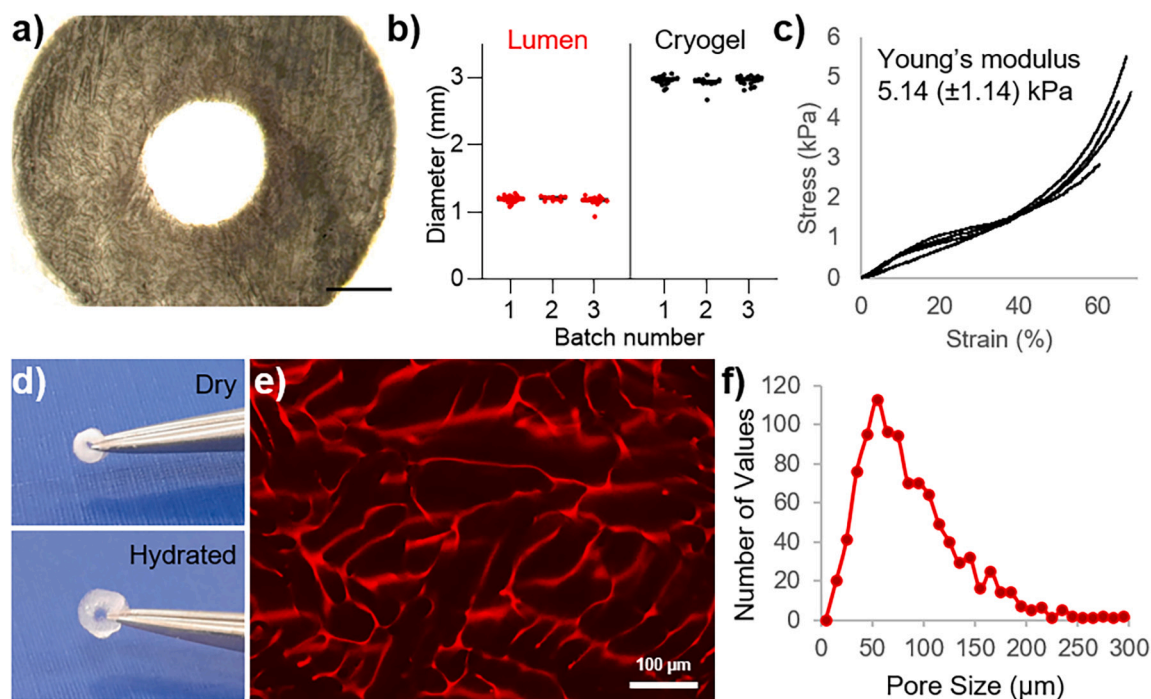


Fig. 2. Characterization of the donut-shaped cryogels. a) A light microscope image of the cryogel when hydrated in PBS (scale bar = 0.5 mm). b) Average diameters of the cryogels and their lumens for those produced in the 2.5 mm diameter template, across three different batches produced. c) Stress-strain curves of four cryogels undergoing uniaxial compression showing the relatively linear deformation up to ~40% of their original size, with a Young's modulus of $5.14 \pm 1.1 \text{ kPa}$. d) Despite their soft nature, the cryogels can be easily handled with forceps. e) Projected average confocal microscope image from a $10 \mu\text{m}$ z-stack, showing the macroporous hydrated cryogel structure, with subsequent analysis (f) of the diameter of 1000 pores being shown as a distribution plot.

stack images, suggesting a well interconnected porous architecture. While this macroporosity primarily contributes to the mechanical resilience and “sponge-like” behavior of the cryogel, it also facilitates adequate oxygen and nutrient flow, ensuring supply to the underlying *ex vivo* tissue slice remains unhindered. Notably, attempts to fabricate a conventional hydrogel analogue using the same 10% w/v monomer formulation were unsuccessful, likely due to the absence of cryo-concentration during polymerization; increasing the monomer concentration to 20% w/v enabled hydrogel formation (Supplementary Fig. S7), but the resulting constructs were markedly stiffer and more brittle (Supplementary Video 1) than the corresponding cryogels, highlighting the value of cryogelation in producing mechanically resilient scaffolds with superior elasticity, shape recovery, and handling

characteristics.

3.3. Application of donut-shaped cryogels to human brain slice cultures enables spatially controlled glioblastoma cell delivery

The donut-shaped cryogels were subsequently applied to *ex vivo* human brain slices (300 μ m thick) prepared from cortical access tissue obtained from three patients, as shown schematically in Fig. 3a. By placing the cryogels directly on top of the slice prior to loading fluorescent GBM cells into the central lumen, a clearly defined region of grafted cells was established, with strong fluorescence intensity maintained at both 1 day and 5 days post-transplantation (Fig. 3b). Small areas of cellular infiltration were also visible in the horizontal images, as

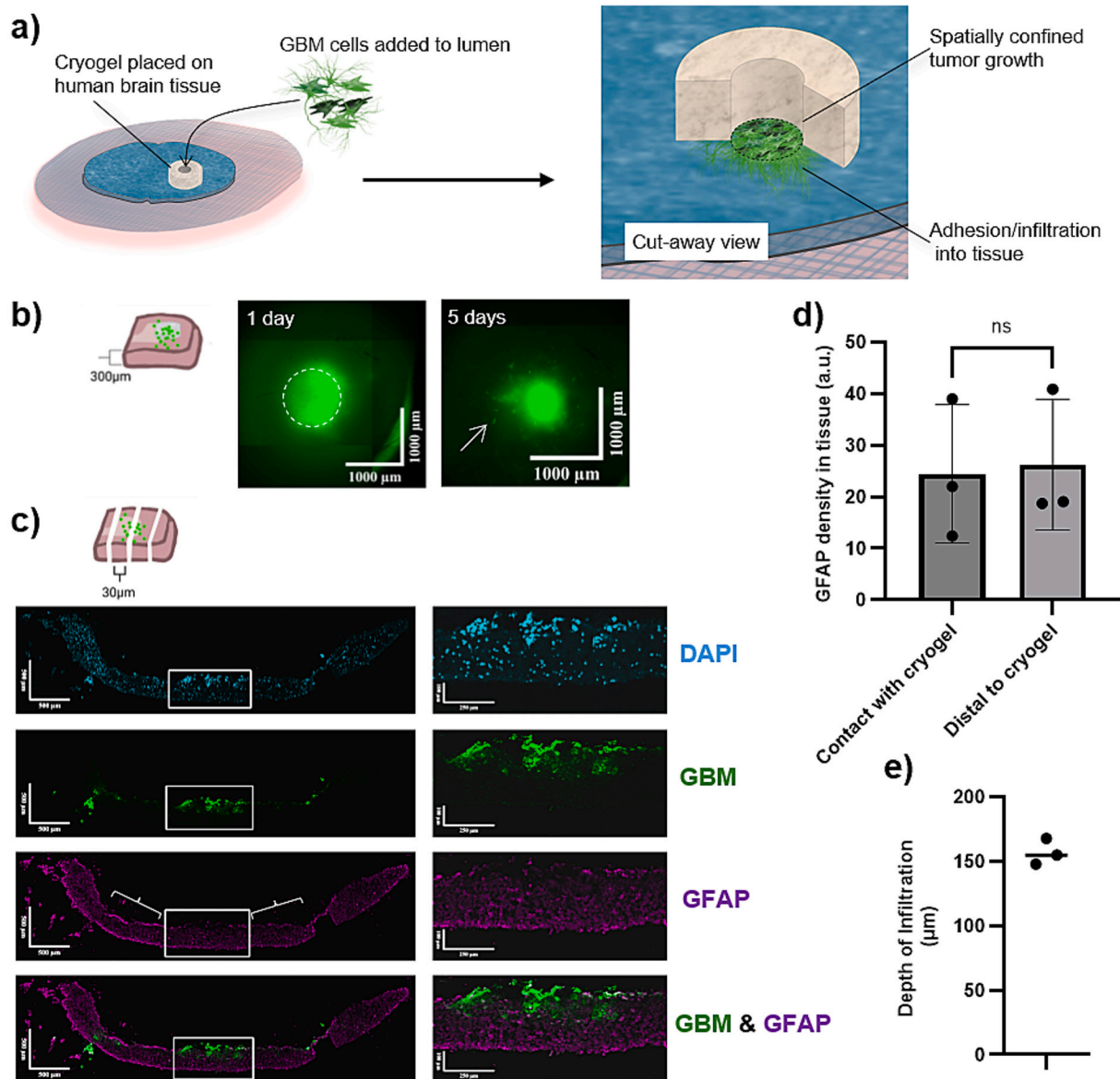


Fig. 3. Cryogel-mediated glioblastoma cell delivery to human brain slice cultures.

(a) Schematic diagram showing placement of the donut-shaped cryogel on cortical access tissue derived from epilepsy patient donors, followed by loading of 40,000 patient-derived GFP+ glioblastoma cells into the lumen. (b) Representative widefield fluorescence images showing formation of a spatially confined glioblastoma cell bulk within the cryogel lumen (white dotted outline), with focal invasion into the underlying tissue by day 5 (white arrow).

(c) Representative immunofluorescence images of vertical tissue sections 5 days after transplantation, demonstrating approximately 1 mm spatial confinement of glioblastoma cells together with invasion into the tissue. The cryogel lumen is indicated by the boxed region, with higher-magnification views shown.

(d) Quantification of GFAP signal intensity in tissue regions in contact with the cryogel compared with regions without contact, showing no detectable increase in local GFAP signal under the tested conditions ($n = 3$, error bars represent $\pm$ SEM, ns = not significant; unpaired t-test with Welch's correction).

(e) Quantification of maximum GFP+ GBM cell infiltration depth into the tissue, with an average depth of 157 ± 10 μ m.

indicated by the white arrow in Fig. 3b, demonstrating that the patient-derived glioblastoma cells retained their characteristic invasive behavior.

To further assess the compatibility of the cryogel with the tissue, vertically sectioned slices were stained for GFAP immunoreactivity and examined for evidence of a local astrocytic response (Fig. 3c, d and Supplementary Fig. S8). GFAP upregulation is widely used as an indicator of reactive astrocytes following tissue perturbation [39–41]. In the present study, cryogel placement for the duration of the culture period was not associated with an increase in local GFAP signal intensity, indicating no detectable increase in astrocyte response under the tested conditions (Fig. 3d). Moreover, incubation of the cryogels with human astrocytes for 1, 3 or 7 days revealed that the materials were well tolerated and had minimal impact on the overall cell viability (Supplementary Fig. S9). Furthermore, analysis of the supernatant of human brain slices for lactose dehydrogenase showed the cryogels did not induce cell membrane damage (Supplementary Fig. S10). Additionally, immunostaining for IBA1 (microglial activity), GFAP (astrocyte reactivity), and NEUN (neuronal activity) following incubation of the cryogels with *ex vivo* human brain slices revealed minimal differences across the entire slice when assessed from a top-down view (Supplementary Fig. S10). These findings support the suitability of the cryogel as an experimental interface for localized cell delivery in organotypic human slice cultures.

The grafted GBM cells remained predominantly confined within the boundaries of the cryogel lumen, indicating that the scaffold effectively limited cell suspension spread across the cryogel–tissue slice interface and enabled formation of a spatially defined tumor bulk. At the same time, fluorescence imaging showed that glioma cells migrated vertically into the underlying tissue, reaching measurable depths within the slice. This behavior was consistently observed across all three human slice cultures (Supplementary Fig. S11), with an average maximum infiltration depth of $157 \pm 10 \mu\text{m}$ (Fig. 3e). Together, these data show that the cryogel platform supports both focal cell placement and subsequent invasion into native human brain tissue.

Taken together, these findings establish donut-shaped cryogels as a simple and accessible method for spatially controlled glioblastoma cell delivery to human brain slice cultures. The platform enables focal placement of tumor cells without injection apparatus, supports formation of a spatially confined tumor bulk, and permits invasion into the underlying tissue. Moreover, in addition to *ex vivo* models, we demonstrated that the cryogels can be used to establish spatially defined focal regions of tumor cells *in vitro* by seeding cells within the lumen of cryogels positioned on Matrigel-coated culture wells (Supplementary Fig. S12). This further supports the potential utility of the cryogels across both *in vitro* and *ex vivo* applications, providing a versatile platform for studying tumor cell behavior, including migration and invasion. Beyond glioblastoma research, the same design principle may also be applicable to other *ex vivo* settings in which localized delivery of cells or biological agents to fragile tissue surfaces is required.

4. Conclusion

In this study, we developed soft donut-shaped cryogel biomaterials as a straightforward method for spatially controlled delivery of glioblastoma cells to human brain slice cultures. The cryogels were easy to handle, could be positioned reproducibly on the tissue surface, and enabled focal confinement of delivered cells without the need for injection apparatus. Under the conditions tested, cryogel placement was not associated with an increase in local GFAP signal, supporting compatibility with the tissue during culture. Glioblastoma cells loaded into the cryogel lumen remained spatially defined while invading into the underlying slice, demonstrating the utility of this platform for constructing experimentally tractable *ex vivo* glioblastoma models. These findings establish cryogel-guided cell delivery as an accessible and adaptable approach for building spatially defined human brain slice

models and broaden the methodological toolbox for *ex vivo* tissue-based research.

CRedit authorship contribution statement

Sophie Hill: Writing – review & editing, Visualization, Investigation, Formal analysis. **Aso Omer Mohammed:** Investigation. **Laurin Ganser:** Visualization, Investigation. **Sabarni Sarker:** Visualization, Investigation. **Yu Wang:** Investigation, Formal analysis. **Pakiza Mammadsalakhova:** Investigation. **Mohammad Al Shhab:** Investigation. **Nicole Fertala:** Formal analysis, Investigation, Writing – review & editing. **Petra B. Welzel:** Formal analysis, Investigation, Writing – review & editing. **Carsten Werner:** Resources, Supervision. **Simon Behringer:** Resources. **Julia M. Nakagawa:** Resources. **Ioannis Vasilikos:** Resources. **Juergen Grauvogel:** Resources. **Juergen Beck:** Resources. **Kevin Joseph:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Vidhya Ravi:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization. **Ben Newland:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioadv.2026.215226>.

Data availability

Data will be made available on request.

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