Mechanically Matching the Rheological Properties of Brain Tissue for Drug-Delivery in Human Glioblastoma Models

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Abstract

Peptide functionalized hyaluronic acid (HA\textsubscript{CF}) cross-linked by cucurbit[8]uril (CB[8]), a new class of drug-delivery reservoirs, is used to enable improved drug bioavailability for glioblastoma tumors in patient-derived xenograft (PDX) models. The mechanical and viscoelastic properties of native human and mouse tissues are measured over 8 hours via oscillatory rheology under physiological conditions. Treatment with drug-loaded hydrogels allowed for a significant survival impact of 45% (55.5 to 80.5 days). A relationship between the type of PDX tumor formed—a consequence of the heterogeneic nature of GB tumors—and changes in the initial survival is observed owing to greater local pressure from stiffer tumors. These biocompatible and tailorable materials warrant use as drug delivery reservoirs in PDX resection models, where the the mechanical properties can be readily adjusted to match the stiffness of local tissue and thus have potential to improve the survival of GB patients.

Keywords: glioblastoma, hydrogel, cucurbit[8]uril, drug-delivery, hyaluronic acid, rheology

1. Introduction

Glioblastoma (GB), the most common primary brain cancer in adults, has one of the lowest median survival rates for cancer of just 4.6 months when left untreated.\textsuperscript{1,2,3} Often diagnosed in the fifth or sixth decade of life, GB also results in one of the highest average years of life lost.\textsuperscript{4} The current standard of care regimen, the Stupp Protocol (SP), involves maximal surgical de-bulking followed by radiotherapy (30 fractions of 2 Gy) with concomitant and adjuvant alkylating chemotherapy.\textsuperscript{5} Ultimately, patients able to undergo and complete radical treatment for GB, still only experience a median survival of approximately 14 months, clearly demonstrating a significant need for new and improved treatment methodologies.

A promising approach to disease modification is to couple repurposed FDA approved chemotherapies with devices designed for local delivery, thus circumventing the blood-brain barrier (BBB) and its chemoselectivity with an aim of treating residual disease following surgical resection. This approach allows clinicians and scientists to utilize drugs that show promise in vitro or in vivo but fail to translate to the clinic when administered by standard oral or intravenous routes. Further benefits of a local delivery methodology lie in the potential for a drastic increase in drug bioavailability and the dramatic reduction in toxicity observed with systemically administered chemotherapy. Local delivery devices can provide controlled-release drug combinations immediately after surgical resection. In this study, we develop a functionalized hyaluronic acid hydrogel as a local delivery reservoir for the treatment of GB in patient derived orthotopic xenograft mouse models.

In a previous effort to realize these benefits, stiff polyanhydride wafers containing Carmustine (Gliadel\textsuperscript{R}) were approved by the Food and Drug Administration (FDA) and National Institute for Health and Clinical Excellence (NICE) as biomaterials for the local treatment of recurrent GB in 1996 and subsequently for primary high grade glioma in 2003.\textsuperscript{6,7,8} Their use in the clinic however, remains controversial owing to their unproven efficacy, and as such the wafers are not considered to be the current standard of care—SP.\textsuperscript{9} In fact, a recent clinical trial (NCT01310868) concluded that there was no survival benefit for patients receiving Gliadel\textsuperscript{R} following 5-aminolevulinic acid (5-ALA) fluorescence guided surgical resection versus those not receiving wafers (14.2 vs 14.3 months), when clinical factors including age, sex, tumor size, presence of residual fluorescent tissue, and surgical opening of the lateral ventricle were taken into account.\textsuperscript{9}
While patients receiving wafer implantation occasionally experience side effects such as seizures, edema, meningitis, and increased wound infections, it is thought that the major reason for the lack of enhanced clinical performance of Gliadel® wafers is its stiff matrix. [9, 10, 11, 12] The solid wafer affords poor apposition to the tumor cavity resulting in a suboptimal and uncontrolled drug-transfer effect. [13] Furthermore, a stiffness mismatch can result in a ‘foreign body reaction’ in the brain exacerbating the poor release profile due to formation of scar-like tissue. [14, 15]

Two recent reviews highlight advances in interactive materials, [16] lessons learned from current strategies to locally treat glioblastoma, and explores unmet clinical needs guiding the design of the next generation of local and injectable delivery devices. [17] A higher stiffness matrix does not necessarily lead to prolonged release. In fact, higher stiffness may exacerbate negative side effects. A local delivery device with timescale of release on the order of matrix degradation rate is required and clinicians, material and biomedical scientists must closely interact to ensure smooth, efficient clinical translation. Our efforts to generate clinically effective local delivery vehicles involve the use of hydrogels. In particular, hyaluronic acid (HA) hydrogels, which are widely used in medical cosmetics, have shown great promise in the field of cargo-delivery owing to their built-in biocompatibility. [18, 19, 20, 21]

We reported a hyaluronic acid polymeric backbone functionalized with the di-peptide residues cysteine-phenylalanine (HA_{CF}) that was physically cross-linked by the macrocyclic host molecule cucurbit[8]uril (CB[8]) in a 2:1 homoternary fashion. [18, 22] These materials were previously assessed to be biocompatible through in vitro and ex vivo experiments. [23] While we have previously studied the rheological properties of brain tumor tissue at 20 °C [24] and 37 °C [19], dehydration of the tissue over time limited the physiological relevance of the findings. Here, we report the first rheological data on human and mouse brain tumor tissue at physiological conditions, namely at 37 °C with immersion in pH 7.4 PBS buffer. Taken together, the purpose of this study was to assess our ability to mechanically match properties of brain tissue, and subsequently increase survival outcomes in human GB models.

2. Materials and Methods

All starting materials were purchased from Sigma Aldrich and used as received unless stated otherwise. Live cell imaging was performed with a Leica TSP8 Confocal Laser Scanning Microscope fitted with a full live cell chamber with temperature, CO2 and humidity control, a SuperZ Galvo Stage and Auto-Focus LAS AF. Fluorescent imaging of cell monolayers and tissue slices was performed with a Leitz DMRB Fluorescence microscope with Leica AS F software. Tissue samples were sliced to 15 μm with a Leica Cryotome CM3050 S. UV/vis spectroscopy was performed by using a Varian Cary 4000 UV-vis spectrophotometer, UV/vis release studies were performed by using a BMG Labtech CLARIOSTAR. 1H-NMR (500 MHz) and 13C-NMR (125 MHz) spectra were recorded in D2O, using a Bruker 500 MHz DCH Cryoprobe Spectrometer. Dialysis of the polymers was carried out by placing the reaction solutions into a dialysis tube (Spectrum Labs, Spectra/Por, standard grade regenerated cellulose dialysis membrane 6, MWCO 15,000 Daltons) which was subsequently submerged in specified aqueous solutions. The external solutions were stirred at room temperature and replaced periodically over a 72 h time period (ca. 4-5 times daily). The dialysed polymer solution was then transferred into a
round bottom flask, frozen in a dry ice/acetone bath and lyophilised.

2.1. FC-CF peptide

The methodology for the production of FC-CF peptide is from the previously published protocol from Rowland et al.[15] The peptide was collected as an off-white crystalline solid, 3,3′-disulfanediylybis(2-(2-amino-3-phenylpropanamido)propanoic acid) dihydrochloride. Yield: 2.643 g, 79 %;

1H-NMR (D$_2$O, 500 MHz) δ (ppm) = 7.43-7.25 (6H, m, Ar-H), 7.22-7.15 (4H, m, Ar-H), 4.65-4.55 (2H, dd, J = 8.5 Hz, 5.1 Hz, C-H), 4.26-4.18 (2H, dd, J = 7.0 Hz, 7.0 Hz, C-H), 3.25-3.07 (6H, m, HC-H) 2.95-2.85 (2H, dd, J = 14.4 Hz, 8.4 Hz, HC-H).

HRMS: calculated mass for [C$_{24}$H$_{31}$N$_4$O$_6$S$_2$]$^+$: 535.1685, observed mass: 535.1698

2.2. Functionalisation of hyaluronic acid with methacrylic anhydride

Methacrylate addition procedure utilised previously in modifying HA was adjusted.[15] Hyaluronic acid (1 g) was dissolved in 80 mL of pH 9.2 buffer (Breckland Scientific). The polymer solution was then cooled to 5 ºC in an ice/water bath and methacrylic anhydride (740 µL, 5.00 mmol) added drop-wise over a period of fifteen minutes. The reaction mixture was removed from the ice bath left to stir overnight. The reaction mixture was then transferred directly to dialysis tubing (MWCO 15 kDa) and dialysed against water for 5 days (water changed 3-5 times per day). The polysaccharide was then obtained by lyophilisation in yields upwards of 80 % and functionalisation with methacrylic anhydride observed by 1H NMR. Yield: 748 mg, 75% Degree of MA functionalisation: 10 %;

1H NMR: (D$_2$O, 500 MHz) δ (ppm) = 6.21-5.97 (1H, s, vinyl-H), 5.75-5.58 (1H, s, vinyl-H), 4.56-2.57 (36H, br, polysaccharide backbone), 2.14-1.69 (9H, s, -NCOCH$_3$ and 3H, s, allyl-H).

2.3. Michael addition of FC-CF to HA-MAs

The methodology for the production of FC-CF to HA-MAs was described.[15] Hyaluronic acid-methacrylate (HA-MA, 400 mg) was dissolved in PBS solution (100 mM, 100 mL) and degassed with nitrogen for 1 h in darkness as previously described.[23] Separately, FC-CF (930 mg, 1.55 mmol, ~10 molar equivalents to MA residues) was dissolved in water (5 mL) and triethylamine (1.28 mL, 9.24 mmol, 6.00 eq) added. The peptide precipitated on addition of the base and then re-dissolved. The solution was degassed with nitrogen for 15 min before the addition of DL-dithiothreitol (DTT, 235 mg, 1.53 mmol, 1 eq). The reaction mixture was stirred for 4 h and then injected into the degassed HA-MA solution and stirred continuously overnight. The solution was then transferred to dialysis tubing and dialysed against brine for 3 days and then water for 2 days. The functional polysaccharide was then isolated by lyophilisation, degree of functionalisation estimated by 1H NMR and the consumption of methacrylate observed to be 100 %. Yield: 348 mg, 87 %; Degree of CF functionalisation: 10 %;

1H NMR: (D$_2$O, 500 MHz) δ (ppm) = 7.47-7.17 (5H, m, aryl-H), 4.57-2.53 (65H, br, polysaccharide backbone), 2.16-1.73 (15H, s, -NCOCH$_3$), 1.24-1.04 (3H, s, CH$_3$).

2.4. Cucurbit[n]uril synthesis and separation

The methods used are from the synthesis and separation reported widely in the literature.[24][25]

2.5. Hydrogel formation and drug loading

Before constitution of the hydrogels, all components and equipment (stirrer bars, vials, etc.) were irradiated with UV-C (240nm) light for 2.5 h. Irradiation of hydrogels with 25 Gy yields hydrogels of lower moduli, this can be attributed to cleavage of the polymeric backbone, data not shown. HA-CF was dissolved in Sterile PBS 1X at a concentration of 20 mg mL$^{-1}$ (2 wt.%). CB[8] was added (approx. 5 mg mL$^{-1}$, 0.5 wt.% to yield a 0.5M equiv. to pendant -Phe groups) and the mixture stirred for 2 h until it expressed viscoelastic properties. At this point either doxorubicin hydrochloride (Cambridge Bioscience Ltd) or Gemcitabine (Cambridge Bioscience Ltd) or a combination was added to the hydrogel at a concentration of 1 mg mL$^{-1}$ (0.1 wt.%) and stirred until homogenous. For in vivo experimentation, IC$_{50}$ values vs. A25M and A25C cell lines, previous intra-cranial injections, discussions with NVS and neurosurgeons were used to inform the dosage of cargos.[23] The resulting concentrations of 0.5 mg mL$^{-1}$ doxorubicin and 0.35 mg mL gemcitabine were used within the hydrogels.

2.6. Physiological Oscillatory Rheology

Rheology was performed using a TA instruments DHR 2 rheometer fitted with an immersion cell. All measurements were taken at 37 ºC, while submerged in Sterile PBS 1X (Alfa Aesar). Dynamic oscillatory strain amplitude sweep measurements were conducted at a frequency of 1 rad s$^{-1}$. Dynamic oscillatory frequency sweep measurements were conducted at a 1% oscillation strain. Dynamic oscillatory time sweep measurements were conducted at 1 rad s$^{-1}$, 1% strain. All measurements were performed using a 8 mm parallel plate geometry with a gap of 1000 µm and analysed using TA Instruments TRIOS software, and plotted using Plot2. Further information can be found in the supporting information.

2.7. Derivation of Patient-Derived Cell Lines

Each GB specimen was anonymized, weighed, cut into small pieces and dissociated enzymatically using Accutase (Invitrogen, UK). HBSS Ca$^{2+}$/ Mg$^{2+}$ free was added before filtration. The suspension was filtered through a 40 µm strainer (Falcon, UK) to isolate single cells. For removal of red blood cell, the single cell suspension was
washed with filtered red cell lysis buffer consisting of NH₄Cl (8.3 g), KHCO₃ (1.0 g) and 5% of EDTA (1.8 mL) in double distilled (dd) water (1 L). Cells were rewarshed in HBSS Ca²⁺/Mg²⁺ free before quantification of live cells by trypan blue exclusion. Cells were seeded at standard density of 15,000 cells per cm² in defined SF medium in 37 °C, 5% CO₂ incubator. All primary cultures monitored daily for primary neurosphere formation. Primary neurosphere cultures were rewarshed and fed weekly for 2-6 weeks depending on the sample. After a variable priming period in which primary spheres size increased, spheres were harvested, washed once in HBSS Ca²⁺/Mg²⁺ free before being seeded without dissociation on ECM-coated flasks (Nunc, Thermo Scientific) to grow as primary monolayer cultures. As the primary monolayer approached confluence cells were dissociated by incubation with Accutase at room temperature and washed with HBSS Ca²⁺/Mg²⁺ free. The cell viability was assessed by trypan blue exclusion and cells reseded onto ECM-coated flasks at a density of 15,000 cells per cm² to generate the subsequent monolayers. Cells were grown in 96 well plates, sterilised coverslips in 24 well plates or in T25 and T75 flasks. Extracellular matrix gel (ECM) from Engelbreth Holm-Swarm mouse sarcoma (Sigma) was used as adhesive substrate after being diluted 1:10 in HBSS before coating the flasks.

2.8. Cell Culture

Cells were cultured under serum-free (SF) conditions. The SF media consisted of phenol-free Neurobasal-A (NBA) (Invitrogen, UK) with 20 mM L-glutamine, 1% v/v penicillin, streptomycin and fungicid (PSF) solution, 20 ng mL⁻¹ human epidermal growth factor (hEGF, Sigma, UK), 20 ng mL⁻¹ human fibroblast growth factor (hFGF, RD systems, UK), 2% v/v B27 (Invitrogen, UK) and 1% N₂ v/v (Invitrogen, UK). All cell cultures were grown in appropriate incubators at 37 °C in 5% CO₂.

2.9. Patient-Derived Xenograft Hydrogel Implants

Orthotopic implantation of patient-derived cells were performed as a single cell suspension (100,000 cells in 3 μL Matrigel) into female NOD-SCID mice aged 6–8 w. Cells were delivered using a 5 μL needle in a minimally traumatic technique at the following co-ordinates relative to bregma: anteroposterior (AP), +2 mm; lateral (L), +1 mm and dorsoventral (DV) 2 mm from the skull surface in accordance with the UK Animal (Scientific Procedures) Act 1986 and the Cambridge University Commission for Animal Health.Orthotopic injection of HA_C₁₀_F₁₀ hydrogels was performed 14 d post cell implantation. 5 μL of hydrogel was injected utilizing the same coordinates as above. To prepare a space for hydrogel injection, a pseudo-cavity was formed by lowering the needle to DV -2.5 mm and then withdrawing the needle to -2.0 mm prior to injection. All mice were maintained until they showed clinical symptoms and were sacrificed using approved Schedule One methods.

2.10. HA_C₁₀_F₁₀ hydrogel drug release

0.15 mL of HA_C₁₀_F₁₀ Hydrogel was loaded into Millicell Cell Culture Inserts (0.1 μm pore size). 1.0 mL of prewarmed PBS was loaded into the well of a 24-well plate so that the PBS and insert membrane creates an interface. The well plate was sealed, and placed within a 37 °C incubator throughout the experiment. To sample, the cell inserts were temporarily removed, and the plate absorbance recorded from 220 nm to 800 nm on a BMG Labtech CLARIOSTAR plus plate reader set at 37 °C. Data was analysed in BMG MARS software before plotting with Plot2.

3. Results and Discussion

To design materials that fulfill our design principles, we first analyzed the rheological properties of human GB tissue, patient-derived xenograft tissue and normal mouse brain tissue under physiological conditions. Human tumor tissue was characterized using 5-ALA owing to the tumors reduced ferrochelatase activity, causing an accumulation of protoporphyrin IX in the tumor cells which can be visualised with a fluorescent operative microscope. All tissues were maintained at 37 °C and immersed in PBS buffer (pH=7.4) throughout all experiments. Oscillatory time sweeps (1% strain, 0.1 rad/s, 37 °C) were performed following a pre-shear for 60 s (10% strain, 10 rad/s) (Figure 2A). Following the initial shear, the tissue recovered immediately (no increase in G’ or G” is observed before the first time point is measured). Both moduli remained linear for the duration of the 8 h measurement, indicating no dehydration or degradation occurred during this period, in contrast to previous reports when both occurred within 1 h. Recording mechanical data in an environment that more closely mimics the native area resulted in a dramatic improvement in measurement reproducibility and reliability. Results from our previous studies yielded a G’ range of 150 < G’ < 650 Pa, however measurement times were limited and tissue degradation was observed over time following tumour resection. The majority of 5-ALA positive human GB tissue measured was stiffer than 5-ALA negative tissue and normal mouse brain tissue. Tissue was characterized using 5-ALA owing to the tumors reduced ferrochelatase activity, causing an accumulation of protoporphyrin IX in the tumor cells which can be visualised with a fluorescent operative microscope. However, no dehydration or degradation occurred during this period, in contrast to previous reports when both occurred within 1 h. Recording mechanical data in an environment that more closely mimics the native area resulted in a dramatic improvement in measurement reproducibility and reliability. Results from our previous studies yielded a G’ range of 150 < G’ < 650 Pa, however measurement times were limited and tissue degradation was observed over time following tumour resection. The majority of 5-ALA positive human GB tissue measured was stiffer than 5-ALA negative tissue and ‘Normal’ brain tissue (G’ = 1185±4 Pa vs. 179 Pa±28 Pa vs. 240 Pa±22 Pa at 1% strain). This is attributed to the infiltrative cancer cells disrupting the normal extracellular matrix within the brain. Areas of the tumor (5-ALA positive) that were recorded as mechanically softer showed signs of necrosis, which accounted for the lowering in elastic moduli (G’ = 41 ±3 Pa) (Supporting Information Figure S4).

Two patient-derived xenograft (PDX) models, A25M and A25C were developed with tumor tissue resected from a 50-year old male with a left temporal GB (IDH-, MGMT+, MiB 33%, GFAP+, overall survival 468 d). The cell lines were derived from two distinct fragments within the same tumor. A25M was from tissue that was sampled at the
margin of the tumor, while A25C was sampled from the tumor core. PDX models were chosen to preserve complex cell-cell interactions and the tumor microenvironment, which are critical in order to more accurately recapitulate human disease mechanics.[15, 28] In addition, PDX models have shown advantages as a preclinical model for drug screening and biomarker development. The tumor formed from A25C cells in the right hemisphere of a mouse was less stiff than normal, healthy mouse brain tissue \( (G' = 89\pm1.9 \text{ Pa vs. } 338\pm8 \text{ Pa at 1\% strain}) \) (Figure 2) as the tumor that formed post-implant was found to be composed of widely dispersed cells.

In contrast, the A25M tumor formed a more nodular, focal density of cells with an elastic modulus \( (G') \) greater than the healthy tissue \( (G' = 518 \text{ Pa}\pm28 \text{ vs. } 338\pm14 \text{ Pa at 1\% strain}) \). The differing growth patterns observed from models derived from the same tumor demonstrated the intratumoral heterogeneity present in GB and further highlight the benefit of providing effective combinatorial therapeutics locally and immediately after surgery.

To mechanically match the hydrogel delivery reservoir to native brain tissue, moduli tailorability was built into the hydrogel design. First, the dynamic cross-linking density was altered. Functionalized HA\( _{CF(x)} \) hydrogels \( (x = 3\%, 10\% \text{ and } 20\%) \) were produced in PBS buffer by addition of CB[8]. These injectable hydrogels underwent dynamic oscillatory rheology measurements at 37 °C immersed in PBS (Figures 2C and S18). At 2 wt%, HA\( _{CF(10)} \) and HA\( _{CF(20)} \) materials with 0.5 M equivalents of CB[8] afforded gel materials \( (G' > G'') \), whereas the HA\( _{CF(3)} \) did not (Figure 2C and Supporting Information Figure S3) due to the decreased number of dynamic cross-links formed by the 2:1 homo-ternary complex. It is these CF:CB[8] complexes that physically hold the hyaluronic acid backbone together.

In oscillatory strain amplitude sweeps, the hydrogels presented broad linear viscoelastic (LVE) regions that stretched across 3 decades of oscillatory strains, while critical strain points were observed at 80% and 20% for the HA\( _{CF(10)} \) and HA\( _{CF(20)} \), respectively. This correlated with increased stiffness of the gel being attributed to the greater cross-linking density, but afforded gels that are less tough as a result of the dynamic cross-links dominating the gel process, whereas the polymer entanglements contribute to a greater degree in the HA\( _{CF(10)} \). HA\( _{CF(10)} \) concentration by weight can also be altered to modulate the stiffness of the hydrogel. However, the effect was less dramatic as we can increase the \( G' \) at 1\% strain from 10 Pa

Figure 2: Oscillatory rheological analysis at physiological conditions, 37 °C and submerged in PBS. A) Digital photograph of experimental rheology setup, with schematic diagram. B) Oscillatory time sweep at 1\% shear, 0.1 rad/s of HA\( _{CF(10)} \) 2WT%, normal mouse brain tissue, A25C mouse tumor, A25M mouse tumor and human GB tumor. C) Oscillatory rheological amplitude sweep of HA\( _{CF(x)} \) hydrogels at 1 rad/s at varying weight percentages. D) Oscillatory rheological amplitude sweep at 1 rad/s comparing human GB to mouse GB and normal brain tissue. Further rheological analysis can be found in the Supporting Information Figures S2-7.
to 100 Pa to 206 Pa with 1 wt%, 2 wt% and 4 wt% hydrogels. It was noted that HA<sub>CF</sub>(10), at 2 wt% provided the best mechanical match to the healthy mouse tissue, without being stiffer than the native mouse, normal or peripheral tissue human tissue samples. This rheological matching of our hydrogel to tissue is in stark contrast to the stiff copolymer matrix of Gliadel<sup>®</sup> (consisting of 1,3-bis(p-carboxyphenoxy)propane (CPP) and sebacic acid (SA) in a 20 to 80 molar ratio (p(CPP:SA; 20:80)) wafer, which was dried and compressed to form a pellet wafer). We postulated that our tissue-matched HA<sub>CF</sub>(10) hydrogels would provide superior drug-delivery performance and a reduction in the incidence and the severity of observed side effects.

Once the mechanical properties of the delivery vehicle had been tuned to match that of the normal mouse brain tissue, we endeavoured to assess the delivery profiles of the chosen HA<sub>CF</sub>(10) hydrogel through an <i>in vitro</i> hanging well experiment. The release of broad-spectrum chemotherapeutics doxorubicin (DOX) and gemcitabine (GEM) from the hydrogel at 37 °C were monitored via UV-absorbance at λ<sub>MAX;GEM</sub> = 275 nm and λ<sub>MAX;DOX</sub> = 487 nm. GEM is of particular interest in the treatment of GB, especially in combination therapies, having previously shown efficacy in <i>vitro</i>, however it lacks BBB penetrance and drug concentration at the target site. DOX has been shown to have efficacy against glioblastoma cells <i>in vitro</i> and <i>in vivo</i> when bypassing the BBB. DOX and GEM demonstrated an initial bolus release from the HA<sub>CF</sub>(10) hydrogel within the first 8 h establishing an immediate, effective dose in the local tissue. This was followed by a sustained, controlled release for up to 2 weeks (Supporting Information Figure S7). At the 2 week timepoint <i>in vitro</i>, 30% of the DOX and GEM have been released from the HA<sub>CF</sub>(10) hydrogel. It is unlikely that this exact release profile will be observed <i>in vivo</i> due to the complex flow profiles of cerebral spinal fluid within the brain and the body’s inherent degradation mechanism from hyaluronidase (HAase). However, we did not observe any erosion of the hydrogel over 8 h in the oscillatory rheology time sweep, suggesting that the initial bolus release in <i>vitro</i> will translate to the <i>in vivo</i> context to a reasonable degree (Figure 2A). The drug dosage was calculated using previous <i>in vitro</i> assays of A25M and A25C and the release data acquired from the HA<sub>CF</sub>(10) hydrogel (Figure S13).

In order to assess the <i>in vivo</i> suitability and biocompatibility of the HA<sub>CF</sub>(10) hydrogel and its applicability towards the clinic, a safety trial was first conducted. Female NODSCID mice (n=4 per group) and C57BL/6 mice (n=3 per group), aged 8 weeks were implanted (+2 mm AP, +1 mm ML, -2.5 mm DV, relative from bregma) with 5 µL of hydrogel, FITC-labelled hydrogel, CB[8] solution, or PBS. The mice were sacrificed after 21 d as no adverse events and no clinical symptoms were observed. Haematoxylin and Eosin (H&E) staining was undertaken to assess for frank necrosis, inflammatory features or immune cell infiltration surrounding the injection site. These features

![Figure 3](image-url)

**Figure 3:** A) Censored survival curve of mice with A25M or A25C tumor, and given treatment protocol (Controls, GelD and GelU). Censored survival curve for B) A25M or C) A25C tumor-bearing mice and right hemisphere sagittal sections tumour-bearing mouse brain tissue stained with Human specific Ki-67 antibody. D) Uncensored survival curve data for A25M/A25C cohorts, with surgery associated deaths included prior to 10 days. (P values are Log Rank relative to control. Not significant (ns), P<0.0502(***), P<0.0021(**), P<0.0002(***))
were not demonstrated in our samples. Clear injection sites were observed in all cases (Supporting Information Figure S14) and no inflammation was observed through histology.

To test the therapeutic value of the tailor able hydrogel in our models, we implanted NODSCID mice with A25C or A25M primary GB cells (see Supporting Information Section S4 for description of methodology). After allowing the tumors to grow for two weeks post-implant, the mice were randomized and underwent a second microscope-guided stereotactic surgery for intra-tumor hydrogel injection or a PBS injection (simulated study carried out in Supporting Information Figure S18). Surgical success was achieved in 100% of cases, with all mice recovering from the anaesthesia (n=33/33). Each PDX line underwent a combination of the following treatment arms; Gel_u (unloaded hydrogels), Gel_d (Gel_gem, Gel_dox, Gel_combined) or Control_u (saline injection)/(Drug concentrations can be found in the Materials and Methods). An overall survival advantage was observed in cohort treated with Gel_d (median survival = 80.5 d), compared to Control_u (median survival = 55.5 d), Gel_u group (median survival = 52.5 d) or no treatment group (median survival = 58 d, data not shown) (Figure 3A). This significant increase in survival for the Gel_d cohort (*p=0.04 compared to Control_u) can be attributed to the local release of the drug into the tumor, directly effecting the tumor cells due to the initial diffusion based release, presumably followed by a slow erosion of the hydrogel.[31]

As expected, the Gel_u cohort showed no overall impact on survival.

When comparing the two PDX models, clear differences in initial survival were observed. All mice recovered from the surgery and anaesthesia. However, 10 mice implanted with the A25M cell line suffered clinical symptoms requiring termination within 10 d of the intervention. These early symptomatic mice came from all treatment arms (Figure 3G), including the control groups. Therefore, it is unlikely that the symptoms were caused by the drugs used, or the hydrogel itself. When interrogating the data from A25C, these mice did not suffer a similar fate. Mice implanted with A25C not only survived the early post-intervention period, but also recapitulated the overall data, showing a clear survival advantage for those mice treated with drug loaded hydrogels (82.5 d vs. 54.5 d). As mentioned previously, these two cell lines lead to tumors with different growth patterns. The potential underlying cause of these early deaths in A25M mice becomes clear when looking at human specific Ki-67 stains of tumor-bearing mouse brains that underwent no treatment (Figure 3B,C). A25C tumor growth is extremely diffuse and infiltrative in nature, with tumor cells that spread anteriorly and posteriorly throughout the right hemisphere. It is proposed that the Gel_d treatment therefore was able to release the drug from the injection site in the brain, thus increasing the bioavailability of the drug and providing a survival benefit.

In contrast, A25M grow as large, nodular tumor masses that remain within the implant region. This was observed in rheological measurements, where the A25M tumors in mice were stiffer than native healthy brain tissue. We postulated that the injection of saline, unloaded or loaded hydrogels, or indeed any material, into the heavier tumor burden of the A25M exacerbates post-operative swelling. Coupled with the mass effect of the stiffened area this causes clinical symptoms requiring the mouse to be sacrificed. Therefore, these mice were excluded from further survival analysis since they did not survive the initial post-operative recovery period to be included in overall survival analysis. The A25M PDX model, therefore, provides the opportunity for tumor resection prior to hydrogel injection. By treating the resection cavity (Simulation in Supporting Information Figure S19) with our hydrogel, we would be able to both reflect the clinical scenario and mimic the mechanical properties of the “healthy” brain of the resection cavity to achieve greater tissue apposition and treatment efficacy.

4. Conclusions

This demonstrates the first report of oscillatory rheological mechanical testing of human and mouse brain tumor tissue at physiological conditions. This information was then used to guide the modulation of physically cross-linked hydrogels formed of hyaluronic acid in PDX mouse models to match that of the native tissue ensuring no mechanical mismatch occurs between the implant and the brain, reducing potential side-effects. Brain–stiffness matched hydrogels gave good drug bioavailability in these local delivery devices, resulting in increased survival statistics of up to 45%. However, in order to delineate the relationship between drug reservoir stiffness and bioavailability, further studies are required. While a significant survival advantage was gained in our PDX mouse models after drug-loaded hydrogel injections, local increased pressure from a stiffer tumor resulted in surgery-associated deaths in some cases. Therefore, future studies should include a GB tumor resection model to more accurately represent and assess true clinical translation.

5. Data Availability

Datasets related to this article can be found at XXXXXXX hosted at the Cambridge Data Repository.

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7. Tissue Ethics

Brain tumor tissue was collected from patients with GB using protocols compliant with the UK Human Tissue Act 2004 (HTA licence ref. 12315), approved by the Local Regions Ethics Committee (LREC ref. 04/Q0108/60) and in accordance with the Declaration of Helsinki. Informed consent was obtained from each patient. Human tissue for rheological analysis was taken under LREC 18/EE/0172.

8. References


