pubs.acs.org/Biomac

Article

Inverse Electron-Demand Diels–Alder Methylcellulose Hydrogels Enable the Co-delivery of Chondroitinase ABC and Neural Progenitor Cells

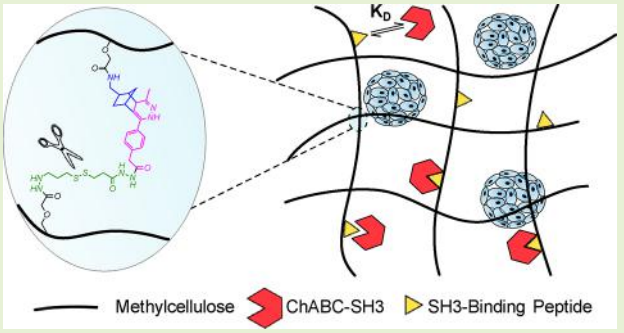
Vianney Delplace,[‖] Andrew J. Pickering,[‖] Marian H. Hettiaratchi, Spencer Zhao, Tove Kivijärvi, and Molly S. Shoichet*

Cite This: https://dx.doi.org/10.1021/acs.biomac.0c00357

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: A hydrogel that can deliver both proteins and cells enables the local microenvironment of transplanted cells to be manipulated with a single injection. Toward this goal, we designed a hydrogel suitable for the co-delivery of neural stem cells and chondroitinase ABC (ChABC), a potent enzyme that degrades the glial scar that forms after central nervous system (CNS) injury. We leveraged the inverse electron-demand Diels–Alder reaction between norbornene and methylphenyltetrazine to form rapidly gelling (<15 min) crosslinked methylcellulose (MC) hydrogels at physiological temperature and pH, with Young's modulus similar to that of brain tissue (1–3 kPa), and degradable, disulfide-containing crosslinkers. To achieve tunable, affinity-controlled release of a ChABC-Src homology 3 (SH3) fusion protein, we immobilized norbornene-functionalized SH3-binding peptides onto MC-methylphenyltetrazine and observed release of bioactive ChABC-SH3 over 4 days. We confirmed cytocompatibility by evaluating neural progenitor cell survival and proliferation. The combined encapsulation of neural stem cells and chondroitinase ABC from one hydrogel lays the framework for future in vivo studies to treat CNS injuries.



1. INTRODUCTION

Fast gelling, injectable hydrogels are compelling for local cell and biomolecule delivery where the latter can be used to manipulate the environment into which cells are transplanted, thereby improving cell viability and tissue integration.[1] Although tuning hydrogel degradation and crosslink density can be used to control protein release,[2] such strategies often require high polymer concentrations or stiff nanoporous structures, which are incompatible with both soft central nervous system (CNS) tissues and cell encapsulation.[3,4]

Affinity-controlled release strategies, which exploit the interactions between the therapeutic and the material itself, overcome many of the limitations of traditional drug delivery systems.[5,6] They are especially promising for the delivery of fragile proteins, as they eliminate harsh processing conditions that can impact protein bioactivity, such as covalent conjugation of the protein to the material or encapsulation in polymeric particles.[6,7] Furthermore, affinity-controlled delivery enables protein release kinetics to be tuned without relying on hydrogel crosslink density, which can impact cell survival and function.[8−10]

The co-delivery of enzymes or neurotrophic factors with cells can enhance the regenerative capacity of transplanted cells in the CNS.[11−13] Chondroitinase ABC (ChABC) is a potent enzyme that can facilitate better host tissue integration of transplanted cells by degrading chondroitin sulfate proteoglycans in the glial scar, which creates an inhibitory microenvironment following injury to the CNS.[13−15] For example, Fouad and co-workers demonstrated that continuous intraparenchymal infusion of ChABC decreased chondroitin sulfate levels after spinal cord injury in rats, leading to improved axonal growth through Schwann cell seeded guidance channels;[16] yet, this strategy required a mini-pump, which is inherently prone to infection. Replacing the mini-pump with a hydrogel delivery vehicle, ChABC and neuroepithelial cells were delivered with two separate hydrogels to the injured spinal cord and resulted in an increased number of neurons compared to treatment with cells alone.[13] Similarly, the co-delivery of ChABC and oligodendrogenic neural progenitor cells resulted in improved cell survival, integration, and behavioral recovery.[15] However, separate hydrogels were

Received: March 9, 2020
Revised: April 7, 2020
Published: April 10, 2020

required for ChABC and cell delivery, significantly complicating the procedure.

A single hydrogel capable of delivering both bioactive ChABC and viable neural cells should enhance the efficacy of the cell therapy by modulating the local environment surrounding the transplanted cells while simplifying the surgical procedure and limiting invasiveness with a single injection. Methylcellulose (MC) hydrogels are ideal for ChABC delivery because MC is resistant to ChABC degradation and has hydroxyl groups that can be functionalized for affinity ligand immobilization.[17] Moreover, as a naturally derived polymer, MC can be used for cell encapsulation with minimal toxicity to encapsulated cells.[13,15]

MC spontaneously gels with increased temperature; however, MC thermogels often require high polymer concentrations (typically 5% w/v), high temperatures, or high salt concentrations,[18−20] making mixing cells into the gels difficult. To tune the mechanical properties of MC thermogels, several polymer blends have been studied, including polyvinyl alcohol,[21] poly(ethylene glycol) (PEG),[22] poloxamer,[23] or hyaluronic acid (HA).[24−26] Of these combinations, only the physical blend of HA and MC allowed simultaneous cell delivery and affinity-controlled protein release,[27] but since ChABC rapidly degrades HA, this gel could not be pursued herein.[28] MC has been chemically crosslinked using either redox/UV-sensitive methacrylates[29−31] or PEG via thiol-maleimide chemistry.[32] The latter was used to study protein affinity-controlled release for spinal cord repair;[33] yet, the high MC content (5% w/v) is too viscous for cell encapsulation.

Inverse electron-demand Diels−Alder (IEDDA) "click" reactions are attractive tools for hydrogel synthesis as they are efficient under physiological pH and temperature and are considered as bio-orthogonal.[34,35] IEDDA crosslinking typically involves reactions between phenyltetrazine derivatives and strained cycloalkenes (e.g., norbornene, *trans*-cyclooctene). It has been shown to be cytocompatible and useful for peptide photoimmobilization.[36,37] However, covalent IEDDA crosslinks are not degradable, requiring further macromolecular design.[38]

We designed a bio-orthogonal and degradable IEDDA crosslinking strategy that enabled the formation of low-polymer-content MC hydrogels, composed of MC-norbornene and MC-methylphenyltetrazine containing disulfide bonds (Figure 1). We systematically varied the concentration and ratio of the two MC components to minimize gelation time and match the Young's modulus of the hydrogel to that of brain tissue. We confirmed the degradability of the chemically crosslinked MC gel for use as a resorbable delivery vehicle. Using an affinity release strategy based on the interaction between the Src homology 3 (SH3) domain and its peptide binding partners,[6,17,20,33] we demonstrated the controlled release of bioactive ChABC-SH3 fusion protein from IEDDA MC hydrogels containing immobilized SH3 binding peptides. We found good correlation between the experimental data and a mathematically predicted release profile.[39] Using the same peptide-modified, degradable MC hydrogel, we successfully controlled the release of ChABC-SH3 and encapsulated mouse neural progenitor cells, thereby demonstrating a proof of concept in vitro and paving the way for their co-delivery in vivo.

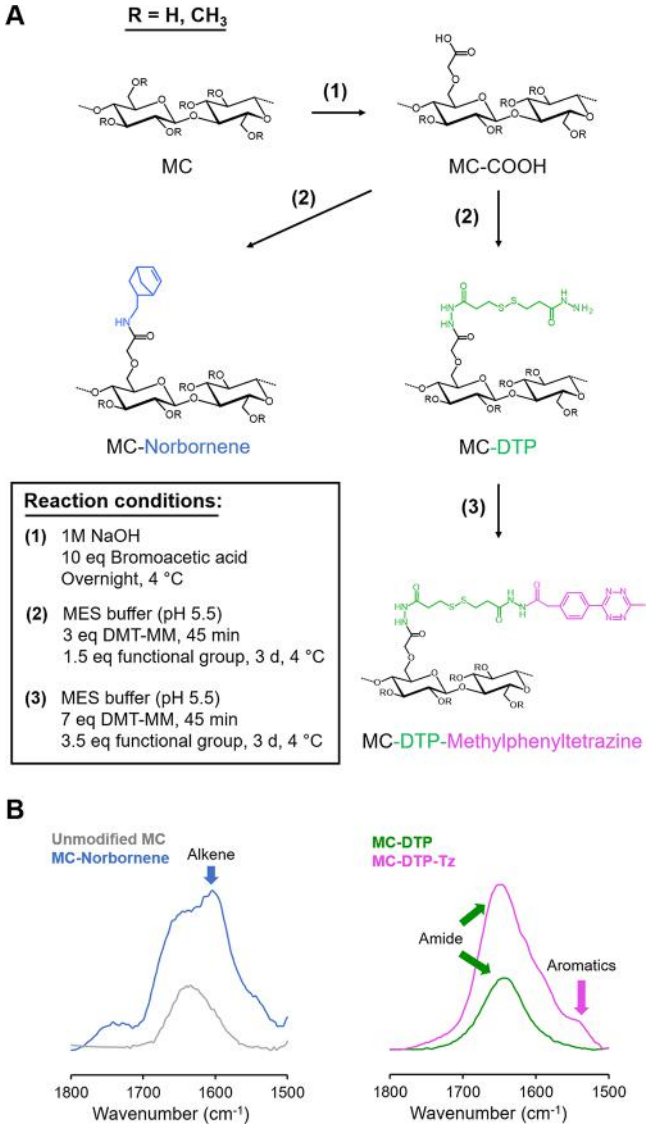


Figure 1. Synthesis of inverse electron-demand Diels−Alder (IEDDA) degradable MC gel components. (A) Schematic of the synthetic pathway of IEDDA MC gel components: (1) MC is modified with carboxylic acid groups (MC-COOH) via Williamson ether synthesis; (2) either norbornene or a disulfide-containing linker, namely, 3,3′-dithiodipropionic acid dihydrazide (DTP), is immobilized onto MC-COOH via amidation to obtain MC-norbornene (MC-Norb) and MC-DTP, respectively; (3) methylphenyltetrazine is reacted with MC-DTP via amidation to produce redox-cleavable MC-DTP-Tz. (B) FTIR measurements demonstrated the successful synthesis of MC-Norb, MC-Tz, and MC-DTP-Tz.

2. MATERIALS AND METHODS

2.1. Materials. Methylcellulose (300 kg/mol) was purchased from Shin Etsu (Tokyo, Japan). 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) and 5-norbornene-2-methylamine (a mixture of isomers) were purchased from TCI Chemicals (Portland, OR, USA). Methylphenyltetrazine-amine, HCl salt, and methylphenyltetrazine acid were purchased from Click Chemistry Tools (Scottsdale, AZ, USA). A Pierce Biotin Quantification Kit was purchased from Thermo Fisher Scientific (Mississauga, ON, Canada). A RealTime-Glo MT Cell Viability Assay was purchased from Promega (Madison, WI, USA). 3,3′-Dithiodipropionic acid dihydrazide (DTP) and methylcellulose-carboxylic acid (MC-COOH) were synthesized as previously reported.[40,41] Dulbecco's phosphate-buffered saline (without calcium and magnesium)

https://dx.doi.org/10.1021/acs.biomac.0c00357
Biomacromolecules XXXX, XXX, XXX−XXX

(PBS) was purchased from Wisent, Inc. All buffers were made with deionized water. All other solvents and reagents were purchased from Sigma-Aldrich and used as received.

2.2. Synthesis and Characterization of Hydrogel Components. *2.2.1. Methylcellulose-Norbornene and Methycellulose-Methylphenyltetrazine.* MC-COOH (100 mg) was dissolved in 0.1 M MES buffer (pH 5.5) (20 mL). DMT-MM (0.125 g, 3 equiv.) was added and allowed to react for 45 min to activate the carboxylic acid groups. 5-Norbornene-2-methylamine (77 μL, 1.5 equiv.) or methylphenyltetrazine-amine (HCl salt, 0.125 g, 1.5 equiv.) was added to the activated MC-COOH solution and allowed to react for 3 days under stirring and at 4 °C. The solution was dialyzed (MWCO 12−14 kDa, Spectrum Labs) against 0.1 M PBS for 1 day followed by deionized water for 2 days. The solution was filter-sterilized, lyophilized, and stored at 4 °C. The products were characterized by FTIR. The methylphenyltetrazine substitution was determined by spectrophotometry (wavelength 525 nm) using a methylphenyltetrazine standard curve. The degree of substitution of MC was quantified by ^{1}H NMR (Agilent DD2-500 spectrometer) using maleic acid (TraceCERT) as an internal standard in deuterium oxide (D_2O). The substitution of MC-norbornene was calculated by comparing the integral area of the norbornene olefin −CH proton (6.03 ppm, brs, 1H) to the olefin −CH protons of maleic acid (6.38 ppm, s, 2H). As a second method of determining norbornene substitution, MC-norbornene was reacted with biotin-PEG4-methyltetrazine (1 mL, 10 equiv.). The solution was dialyzed (MWCO 12−14 kDa, Spectrum Labs) against 0.1 M PBS for 2 days followed by deionized water for 2 days. The solution was filter-sterilized, lyophilized, and stored at 4 °C. The biotin content was analyzed using a Pierce Biotin Quantification Kit (Thermo Fisher Scientific, Mississauga, ON, Canada).

2.2.1.1. Methylcellulose-DTP-Methylphenyltetrazine. MC-COOH (250 mg) was dissolved in 0.1 M MES buffer (pH 5.5, 100 mL). DMT-MM (0.850 g, 3 equiv.) was added and allowed to react for 45 min to activate the carboxylic acid groups. 3,3′-Dithiodipropionic acid dihydrazide (DTP) (0.375 g, 1.5 equiv.) was added to the activated MC-COOH solution and allowed to react for 3 days under stirring and at 4 °C. The solution was dialyzed (MWCO 12−14 kDa, Spectrum Labs) against 0.1 M NaCl for 2 days followed by deionized water for 2 days. The solution was lyophilized and stored at 4 °C. The product was characterized by FTIR. Lyophilized MC-DTP (50 mg) was dissolved in 0.1 M MES buffer (pH 5.5) (10 mL). Methylphenyltetrazine acid (0.086 g, 3.5 equiv.) was dissolved in a mixture of 0.1 M MES buffer (10 mL) and DMSO (3 mL). DMT-MM (0.208 g, 7 equiv.) was added and allowed to react for 45 min. The activated solution was then added to the MC-DTP solution and allowed to react for 3 days under stirring and at 4 °C. The solution was dialyzed (MWCO 12−14 kDa, Spectrum Labs) at 4 °C against 0.1 M PBS with 2% DMSO for 1 day followed by deionized water for 2 days. The solution was filter-sterilized, lyophilized, and stored at 4 °C. The product was characterized by FTIR, and the degree of substitution was determined by spectrophotometry (wavelength 525 nm) using a methylphenyltetrazine standard curve.

2.2.2. SH3 Binding Peptide-Norbornene. A modified Src homology 3 (SH3) binding peptide (sequence: GGGK'KPPVVKKPHYLS, K_D = 2.7 × 10^{-5} M, K′ = *N*-ε-(5-carboxyfluorescein)-L-lysine) was synthesized using Fmoc-Ser(tBu)-Wang Resin and automated solid-phase peptide synthesis (Liberty Blue Automated Microwave Peptide Synthesizer). To functionalize the peptide with norbornene on the N-terminus, 2.25 equiv. of 5-norbornene-2-carboxylic acid was first activated using 4.5 equiv. of *N*,*N*′-diisopropylcarbodiimide for 20 min at room temperature in 1.25 mL of dichloromethane. The solution was then added to the resin-bound peptide in 1.25 mL of *N*-methyl-2-pyrrolidone and stirred at room temperature overnight. The peptide was purified as previously described.[42] Briefly, the norbornene-modified peptide was cleaved off the resin using trifluoroacetic acid (TFA), precipitated dropwise into cold diethyl ether, and purified using high-performance liquid chromatography; peptide fractions were confirmed using electrospray ionization mass spectroscopy. Acetonitrile was evaporated, and peptide solutions were lyophilized and stored at −20 °C.

To prepare MC-Tz-peptide and MC-DTP-Tz-peptide, MC-Tz or MC-DTP-Tz was dissolved in deionized water at 10 mg/mL, and the norbornene-modified SH3 binding peptide was added at a final concentration of 51.7 μg/mL (0.71 equiv.). The mixture was speedmixed (SpeedMixer DAC 150 FV2; FlackTek Inc., Landrum, USA) horizontally at 14000 *g* for 15 s and placed on an orbital shaker at room temperature overnight. The solution was then dialyzed (MWCO 12−14 kDa, Spectrum Labs) for 2 days in 0.1 M PBS followed by 2 days in deionized water, filter-sterilized through a 0.22 μm syringe filter, lyophilized, and stored at −80 °C. The peptide content was evaluated by amino acid analysis.

2.3. Synthesis of IEDDA Methylcellulose Hydrogels. MC hydrogel (1% (w/v) polymer content, 2:1 MC-DTP-Tz to MC-Norb ratio) was synthesized as follows: in a 2 mL Eppendorf tube, MC-Norb (10 mg) was dissolved in PBS (1 mL) and speedmixed and centrifuged for 10 s at maximum speed (14000 rpm). A similar procedure was used to dissolve MC-Tz. In a 1.5 mL Eppendorf tube, the two solutions were mixed in a 2:1 volume ratio of MC-Tz to MC-Norb by pipetting up and down until the appearance was uniform and incubated at 37 °C for gelation to occur. For the synthesis of nondegradable gels, a similar procedure was applied, using MC-Tz instead of MC-DTP-Tz.

2.4. Rheological Characterization. Gelation time and viscosity data were collected using a TA Discovery HR-3 rheometer (TA Instruments, New Castle, DE) equipped either with a 40 mm 1° aluminum cone for gelation time or a 20 mm steel parallel plate for viscosity measurements. A Peltier plate element was used for temperature control, and a solvent trap was used with the aluminum cone to minimize solvent evaporation during measurements.

For gelation time measurements, MC solutions for hydrogel synthesis were prepared as described above. Gels were first equilibrated for 30 s at 37 °C. Then shear storage modulus (G') and shear loss modulus (G'') were measured as a function of time at 0.1 Hz (angular frequency) and under 1% strain. Gelation time was determined as the crossing point of G' and G''.

For viscosity measurements, individual MC-Norb and MC-Tz solutions were prepared as described above. Shear stress was measured over shear strains ranging from 0.1 to 10000 s^{-1} at room temperatue, and the viscosity of each component was determined as the ratio between shear stress and shear strain.

2.5. Young's Modulus Measurements. 100 μL MC-Norb and MC-Tz/MC-DTP-Tz solutions for hydrogel synthesis were prepared as described above in a 16 well chamber slide (Nunc LabTek Chamber Slide, Thermo Fisher Scientific) and incubated at 37 °C overnight for complete gelation. The chamber slide bottom was detached to remove the gels from the wells. The Young's modulus was measured using a mechanical testing machine (Mach-1 Micromechanical System, Biomomentum) following a previously reported procedure.[43]

2.6. Swelling/Stability Analysis. 100 μL aliquots of MC-Norb and MC-Tz solutions for hydrogel synthesis were prepared as described above in preweighed 2 mL Eppendorf tubes and incubated overnight at 37 °C for complete gelation. Swelling and stability were then monitored in warm PBS (37 °C) following a reported procedure.[43] The mass ratio was determined as the ratio of hydrogel mass at the time point to the initial mass. For the degradation study, a similar procedure was applied using glutathione solutions, freshly prepared in PBS at various concentrations (0.1, 1, and 5 mM), instead of PBS alone.

2.7. Transparency. 100 μL aliquots of each hydrogel formulation were transferred to a clear 96 well plate (Greiner Bio-One) and incubated at 37 °C. The absorbance of the MC hydrogel was measured between 200 and 800 nm (TECAN plate reader, Infinite M200 Pro) at different time intervals (0, 1, 2, 3, 4, 6, 8, and 24 h), from which transmittance was calculated as follows: %transmittance = $10^{(2\ -\ \text{absorbance})}$.

2.8. Chondroitinase ABC-SH3 Expression and Purification. A ChABC-SH3 fusion protein of chondroitinase ABC and Src homology 3 (ChABC-SH3) was expressed and purified as previously described.[44] Briefly, the gene for ChABC-SH3 was inserted into a

https://dx.doi.org/10.1021/acs.biomac.0c00357
Biomacromolecules XXXX, XXX, XXX−XXX

pET28b + plasmid with kanamycin resistance (Genscript, Piscataway, NJ) such that the sequence reads from the N-terminus to the C-terminus: hexahistidine tag, SH3 domain, flexible linker, ChABC, and FLAG tag (DYKDDDDK). Plasmids were transformed into NiCo21 (DE3) *Escherichia coli* cells (New England Biolabs, Ipswich, MA) for protein expression.

Large-scale production and purification of ChABC-SH3 were performed as previously described.[44] Briefly, 1.8 L Terrific Broth cultures were grown at 37 °C, and protein expression was induced after reaching an optical density of 0.8 (600 nm) with isopropyl β-D-1-thiogalactopyranoside (IPTG, 1.8 μg/mL) for 18 h at 22 °C. Cultures were centrifuged, and cells were lysed using probe sonication. Soluble fractions were incubated with nickel-nitrilotriacetic acid (Ni-NTA) resin to allow the his-tagged ChABC-SH3 to bind to the resin. The cell lysate was poured through a gravity filtration column, washed with 10 × 10 mL of wash buffer (50 mM Tris pH 7.5, 500 mM NaCl, 30 mM imidazole), eluted using a high concentration of imidazole (40 mM Tris pH 7.5, 500 mM NaCl, 250 mM imidazole), and concentrated to 1−2 mL with a 10 kDa cutoff Vivaspin 20 centrifugal concentrator (Sartorius, Gottingen, Germany). The protein was further purified using size exclusion chromatography (Hi-load 16/600 Superdex 200 column, AKTA Purifier 10, GE Healthcare Life Sciences, Budapest, Hungary), concentrated, and stored in 20 μL aliquots at −80 °C.

2.9. Controlled Release of Chondroitinase ABC-SH3. To prepare gels for ChABC-SH3 release, MC-Norb, MC-DTP-Tz, and MC-DTP-Tz-peptide were dissolved in artificial cerebrospinal fluid (aCSF: 149 mM NaCl, 3 mM KCl, 0.8 mM $MgCl_2$, 1.5 mM Na_2HPO_4, 0.2 mM NaH_2PO_4) at a MC concentration of 1% (w/v), with the amount of MC-DTP-Tz-peptide based on a 50 or 100 molar excess of peptide to protein. 20 μg of ChABC-SH3 was mixed into each 100 μL gel, and solutions were incubated at 37 °C for 1 h for gelation to occur. For the synthesis of nondegradable gels, MC-Tz and MC-Tz-peptide were used instead of MC-DTP-Tz and MC-DTP-Tz-peptide.

Protein release was evaluated as previously described, with minor modifications.[44] Briefly, 400 μL of filter-sterilized aCSF containing 0.1% (w/v) bovine serum albumin and protease inhibitor tablets (1 tablet/10 mL, Roche, Basel, Switzerland) were added on top of the 100 μL gels in a 2 mL Eppendorf tube. The hydrogels and aCSF were incubated on an orbital shaker at 37 °C, along with a solution of ChABC-SH3 (2 μg/mL in aCSF) as a soluble protein control. Supernatants were collected and replaced with fresh aCSF at several time points over 7 days. After the last time point, the gels were digested at 37 °C overnight with 35 U/mL cellulase in 400 μL of aCSF. ChABC-SH3 concentration was measured using a custom enzyme-linked immunosorbent assay (ELISA),[17] and the ChABC-SH3 concentration was determined by a standard curve for each time point prepared from the soluble protein control and normalized to the no-protein control gel. Peptide-norbornene release was quantified by measuring the fluorescence of the supernatant (excitation 492 nm; emission 517 nm).

2.10. Mathematical Modeling of Protein Release. The cumulative release of ChABC-SH3 over 7 days was estimated using a previously developed mathematical model of SH3-based affinity release.[39] The experimental setup satisfies the conditions of the controlled release regime (Supporting Information), in which diffusion is the rate-determining step and a small proportion of peptides are complexed initially. The cumulative release is a function of a dimensionless time t^*

$$t^* = t/\frac{L^2}{D}\left(1 + \frac{C_{pep,T}}{K_D}\right) \quad (1)$$

where L (m) is the thickness of the gel, D (m^2/s) is the diffusion coefficient of ChABC-SH3, $C_{pep,T}$ (mol/L) is the total concentration of SH3 binding peptide in the gel, and K_D (mol/L) is the dissociation constant between the peptide and the SH3. The dimensionless time t^* for a given experimental setup can then be related to the cumulative protein release using the previously developed master curve. All parameters used in calculations based on the experimental setup are shown in Table S2, with results summarized in Table S3.

2.11. ChABC-SH3 Activity Assay. The enzymatic activity of ChABC-SH3 was evaluated via the degradation of chondroitin sulfate A as previously described.[44] Briefly, 50 μL of ChABC-SH3 released from the hydrogels was mixed with 50 μL of 18 mg/mL chondroitin sulfate A in a UV-Star microplate (Greiner Bio-One, Monroe, NC) and immediately read on a plate reader (Tecan Infinite M200 Pro) at 232 nm every 20 s for 20 min, and the slope of the linear relationship between absorbance and time was used to calculate the kinetic activity of ChABC-SH3. ChABC-SH3 concentrations were determined using the ELISA described above.

2.12. Neural Progenitor Cell Isolation and Culture. Neural progenitor cells were isolated from the brains of adult female mice (Charles River Laboratories) as previously described[45,46] and with approval from the Animal Care Committee at the University of Toronto. Briefly, the subependyma of the forebrain lateral ventricle was enzymatically digested using 1.33 mg/mL trypsin, 0.67 mg/mL hyaluronidase, and 0.2 mg/mL kynurenic acid for 45 min at 37 °C with gentle agitation. The cells obtained were centrifuged and resuspended in a serum-free medium (0.6% glucose, 5 mM HEPES, 3 mM $NaHCO_3$, 2 mM L-glutamine, 25 mg/mL insulin, 100 mg/mL transferrin, 20 nM progesterone, 60 mM putrescine, and 30 nM sodium selenite in DMEM/F12) containing 1 mg/mL trypsin inhibitor, 20 ng/mL epidermal growth factor, 10 ng/mL fibroblast growth factor, and 2 μg/mL heparin. Cells were seeded at an initial density of 10000 cells/mL and cultured in suspension as neurospheres for 1 week prior to encapsulation in hydrogels. Cell viability was determined using trypan blue exclusion.

2.13. Neural Progenitor Cell Encapsulation and Culture in IEDDA Hydrogels. Neurospheres grown in suspension were mechanically dissociated into single cells and aggregated using a forced aggregation technique in 400 μm AggreWells (StemCell Technologies) to create neurospheres with a uniform initial diameter of approximately 100 μm, with approximately 250 cells per neurosphere. IEDDA MC hydrogels for cell encapsulation were prepared as described above to achieve a final MC concentration of 1% (w/v) in a serum-free growth medium. ChABC-SH3 (200 μg/mL), SH3 binding peptide (50× molar excess), and neurospheres (approximately 200 neurospheres per 50 μL of solution) were added to the MC solution and gently mixed with a pipette. 50 μL gels were prepared on a 96 well tissue culture polystyrene plate and incubated at 37 °C for 30 min for gelation to occur, after which 100 μL of medium was added on top of the gels. For the tissue culture polystyrene (TCPS) control, approximately 200 neurospheres were suspended in 150 μL of medium and added to each well. The medium was replaced on days 3 and 5. For live/dead staining, 30 μL of PBS containing Hoechst (4 μg/mL), Calcein AM (2 μM), and ethidium homodimer (4 μM) was added to gels and incubated at 37 °C for 30 min. 10 min prior to imaging, 15 μL of the same staining solution was added to TCPS controls and incubated at 37 °C for 10 min. Neurospheres were imaged using an Olympus Fluoview FV1000 confocal microscope with *xy* scans captured in 30 μm intervals in the *z* direction. Neurosphere number and diameter were quantified using a GelCount instrument with an imaging resolution of 2400 dpi. Cell viability was determined using a RealTime-Glo MT Cell Viability Assay (Promega) following the manufacturer's protocol. NanoLuc luciferase and the cell-permeable pro-substrate were added to each gel and TCPS control. RealTime-Glo solution was also added to empty wells and cell-free gels as blanks. Luminescence was measured on a plate reader (Tecan Infinite M200 Pro) with an integration time of 1000 ms. Blank readings were subtracted from each well, and the luminescence was normalized to the initial value, which was recorded on day 1 to allow the pro-substrate to diffuse throughout the hydrogels and neurospheres.

2.14. Statistical Analysis. All statistical analyses were performed using GraphPad Prism version 7 (GraphPad Software, San Diego, CA). Differences between groups were assessed by Student's *t* test, one-way, or two-way ANOVA with Tukey's or Dunnet's post-hoc test,

https://dx.doi.org/10.1021/acs.biomac.0c00357
Biomacromolecules XXXX, XXX, XXX−XXX

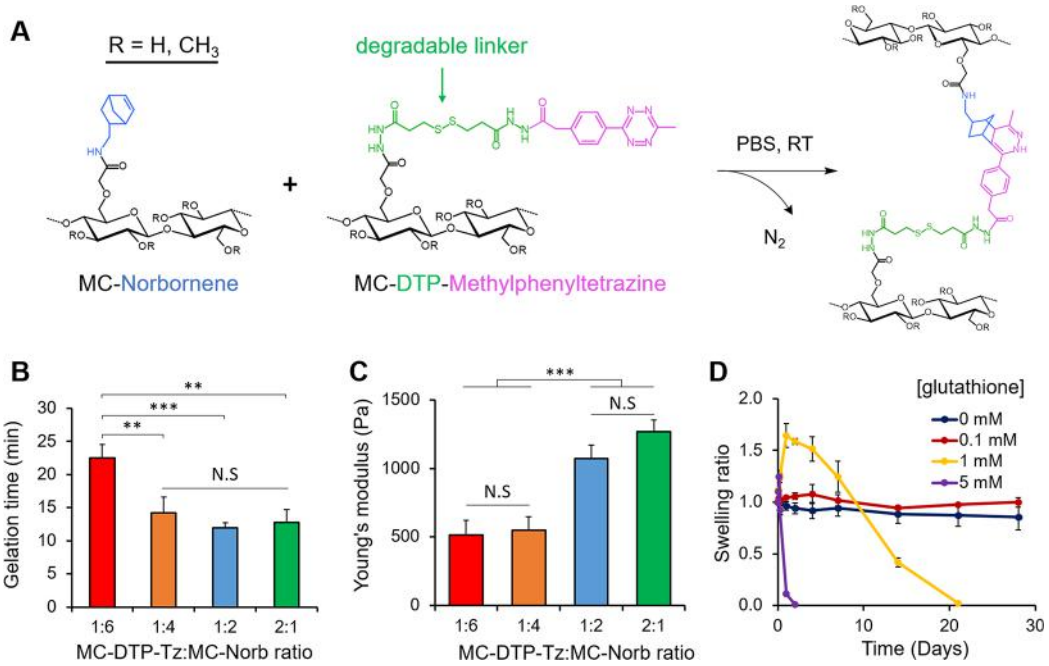


Figure 2. Design of a biodegradable inverse electron-demand Diels–Alder (IEDDA) MC hydrogel for the co-encapsulation of cells and proteins. Disulfide-containing IEDDA MC gels form within 12 min under physiological pH and temperature, with stiffnesses matching that of brain tissue, and biodegradation profiles suitable for protein and cell delivery. (A) Schematic of the synthesis of biodegradable IEDDA MC hydrogels where MC-norbornene (MC-Norb) reacts with disulfide-containing MC-tetrazine (MC-DTP-Tz). Covalently crosslinked hydrogels form upon simple mixing of the two components under physiological conditions. (B) Average gelation time of biodegradable IEDDA MC hydrogel formulations at a fixed total MC concentration of 1% (w/v) and different MC-DTP-Tz:MC-Norb ratios (1:6, 1:4, 1:2, and 2:1) as measured by rheometry (n = 3; mean ± SD). (C) Evaluation of the Young's modulus of biodegradable IEDDA MC hydrogels at a fixed total MC concentration of 1% (w/v) and different MC-DTP-Tz:MC-Norb ratios (1:6, 1:4, 1:2, and 2:1) (n = 3; mean ± SD). (D) Biodegradability of disulfide-containing, IEDDA MC hydrogels as a function of glutathione concentration (0, 0.1, 1, and 5 mM) (n = 3; mean ± SD). Statistical significance was determined using one-way ANOVA with Tukey's post-hoc test: N.S. = not significant, $^{**}p < 0.01$, $^{***}p < 0.001$.

when applicable. P values are represented as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

3. RESULTS AND DISCUSSION

3.1. Synthesis of IEDDA Methylcellulose Gel Components. To design a bioresorbable IEDDA MC hydrogel, we crosslinked MC-norbornene with a cleavable MC-tetrazine. MC-norbornene (MC-Norb) was synthesized by first modifying MC with carboxylic acid groups (MC-COOH) via Williamson ether synthesis and then immobilizing 5-norbornene-2-methylamine via amidation (Figure 1A). To synthesize cleavable MC-tetrazine, a biodegradable disulfide-containing linker, 3,3′-dithiodipropionic acid dihydrazide (DTP), was first reacted with MC-COOH followed by methylphenyltetrazine acid immobilization. The disulfide bond of the MC-DTP-Tz component is expected to degrade in the presence of naturally occurring thiols and under reducing conditions,[47−49] allowing encapsulated cells to integrate into the surrounding tissue and obviating the need for hydrogel removal. Using FTIR, we confirmed the successful grafting of norbornene (amide peak, 1660 cm^{-1}; alkene peak, 1600 cm^{-1}) and the sequential grafting of DTP (amide peak, 1660 cm^{-1}) and methylphenyltetrazine (amide peak, 1660 cm^{-1}; ring azo peak, 1575 cm^{-1}; aromatic peak, 1540 cm^{-1}) onto MC (Figure 1B). As tetrazine derivatives are pink, methylphenyltetrazine substitution could be directly quantified by UV–vis spectrophotometry (absorbance at 525 nm), demonstrating the successful methylphenyltetrazine grafting of 138 ± 42 μmol/g (approximately 2.8% substitution). A nondegradable MC-tetrazine (MC-Tz), without DTP, was also synthesized, having a methylphenyltetrazine substitution of 190 ± 17 μmol/g (approximately 3.8% substitution), which is consistent with previously reported MC substitution values.[41] The lower substitution obtained for MC-DTP-Tz vs MC-Tz is likely due to either the additional synthetic step or the limited solubility of the acid-modified methylphenyltetrazine reagent used only in the MC-DTP-Tz synthesis. The degree of substitution of MC-norbornene was measured by ^{1}H NMR and was determined to be 82 ± 27 μmol/g (approximately 1.6% substitution) (Figure S1). The lower substitution of norbornene than methylphenyltetrazine under similar reaction conditions has been previously observed with other polymers[43] and may result from steric effects and a difference in reactivity between the endo-exo norbornene isomers.

3.2. Physicochemical Properties of a Bioresorbable IEDDA Methylcellulose Hydrogel. We successfully synthesized IEDDA MC hydrogels by simply mixing MC-Norb and MC-DTP-Tz under physiological pH and temperature (Figure 2A). Given the ultimate goal of delivery to the brain and spinal cord where ChABC can degrade the glial scar that forms after injury, we aimed to design gels with low polymer content (≤1% (w/v) of MC), low modulus (1–2 kPa), and controlled rates of gelation (≤15 min). Using 1% (w/v) MC, we investigated the effect of the MC-DTP-Tz:MC-Norb ratio on gelation time (Figure 2B) and observed faster gelation times of 22.5 ± 2.0 and 11.9 ± 0.8 min with increased MC-DTP-Tz:MC-Norb ratios of 1:6 to 1:2, respectively. Further increasing the MC-DTP-Tz:MC-Norb ratio did not significantly change the gelation time of the resorbable MC gels,

https://dx.doi.org/10.1021/acs.biomac.0c00357
Biomacromolecules XXXX, XXX, XXX−XXX

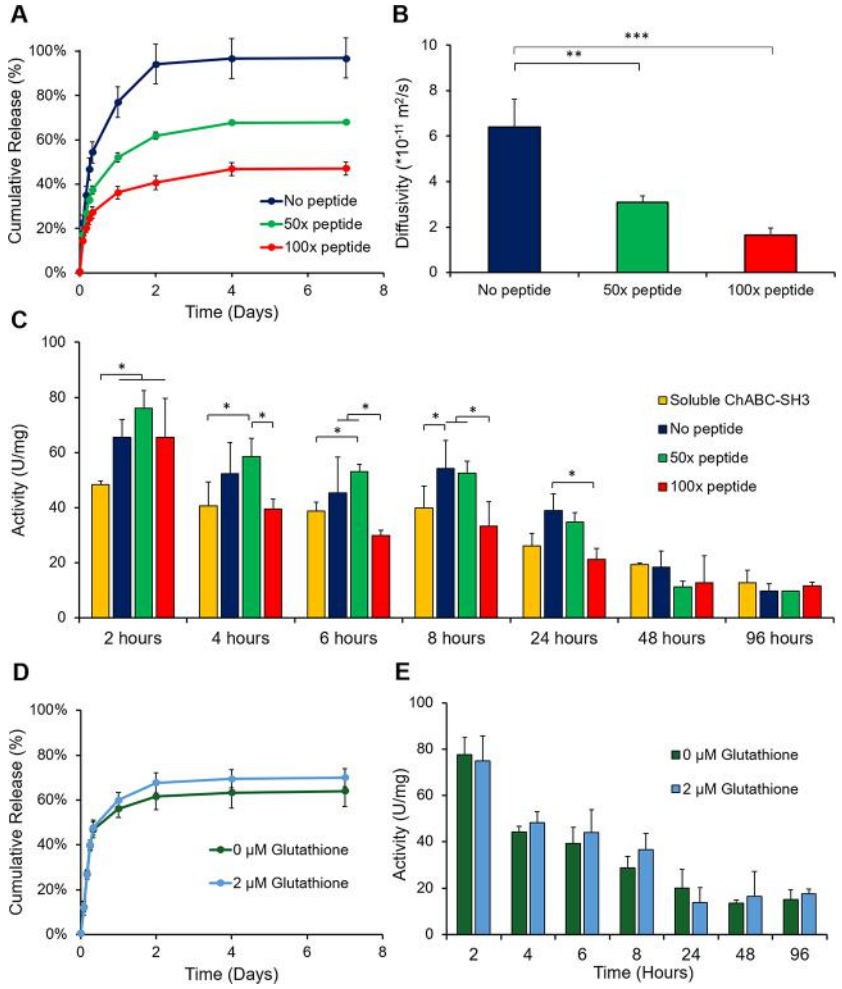


Figure 3. Affinity-controlled release of ChABC-SH3 from IEDDA MC hydrogels with SH3 binding peptides. (A) Cumulative release of ChABC-SH3 from 1% (w/v) IEDDA MC hydrogels (MC-Tz:MC-Norb ratio of 2:1) with SH3 binding peptide molar excess values (relative to SH3 protein) of 0 (no peptide), 50, and 100. (B) Effective protein diffusivity through IEDDA MC hydrogels calculated using the short-time approximation for Fickian diffusion from a thin polymer slab. (C) Specific activity of soluble ChABC-SH3 incubated in artificial cerebrospinal fluid at 37 °C or released into aCSF from MC hydrogels with 0 (no peptide), 50, and 100 molar excess peptide to protein. (D) Cumulative release of ChABC-SH3 from degradable 1% (w/v) IEDDA MC hydrogels (MC-DTP-Tz:MC-Norb ratio of 2:1) with a 50 times excess of SH3 binding peptide to ChABC-SH3, with or without a physiological glutathione concentration (2 μM). (E) Specific activity of ChABC-SH3 released from degradable IEDDA MC hydrogels with or without glutathione. (n = 3; mean ± SD). Statistical significance was determined using one-way ANOVA with Tukey's post-hoc test for (B) and two-way ANOVA with Tukey's post-doc test for (C): *p < 0.05, **p < 0.01, ***p < 0.001.

which plateaued at approximately 12 min. A simlar trend was observed for nondegradable gels (Figure S2A), with gelation times that plateaued at 5 min (Figure S2B), reflecting the higher degree of substitution of the MC-Tz than that of the cleavable MC-DTP-Tz (138 ± 42 vs 191 ± 17 μmol/g) and thus the higher crosslink density. Notwithstanding this difference, resorbable IEDDA MC gels that gel in 12 min are practical for cell and protein co-encapsulation, providing a sufficient time for mixing and injection prior to gelation.

We investigated the effect of the MC-DTP-Tz:MC-Norb ratio on the stiffness of IEDDA 1% (w/v) MC hydrogels. Increasing the MC-DTP-Tz:MC-Norb ratio from 1:6 to 1:2 led to a significant increase in stiffness from 0.51 ± 0.11 to 1.08 ± 0.10 kPa (Figure 2C). However, further increasing this ratio to 2:1, with excess methylphenyltetrazine, did not significantly increase the Young's modulus of IEDDA MC gels, similar to what was observed in gelation time by rheology and likely because norbornene was the limiting reagent. A similar plateau in Young's modulus was observed when increasing the MC-Tz:MC-Norb ratio from 1:6 to 1:2 for nondegradable gels (Figure S2C) and is consistent with the plateau observed in other crosslinked hydrogels.[50] Importantly, the stiffness of optimized IEDDA 1% (w/v) MC hydrogels with MC-DTP-Tz:MC-Norb ratios of either 1:2 or 2:1 matches that of human brain tissue, which is between 1 and 3 kPa.[51−53] A transmittance study (peak at λ = 525 nm) confirmed the consumption of methylphenyltetrazine during gelation (Figure S3), leading to degradable and nondegradable materials that are highly transparent over the entire visible-light spectrum, with the color after 24 h varying from colorless (transmittance >0.97) to faintly pink (transmittance >0.91) depending on both the degree of Tz substitution and the Tz:Norb ratio. The compositions and physicochemical characteristics of the degradable and nondegradable formulations tested are summarized in Table S1.

To test whether disulfide-containing IEDDA MC gels are degradable, we immersed them in glutathione (at 0.1, 1, or 5 mM), a naturally occurring reducing agent; these hydrogels

https://dx.doi.org/10.1021/acs.biomac.0c00357
Biomacromolecules XXXX, XXX, XXX−XXX

degraded at a rate dependent on glutathione concentration (Figure 2D). In contrast, we did not observe degradation when these gels were incubated in PBS or when nondegradable IEDDA MC gels were immersed in glutathione (Figure S2D); in these conditions, both gels exhibited minimal swelling and long-term stability. It should be noted that the glutathione concentrations tested herein correspond to accelerated degradation conditions as these concentrations are orders of magnitude higher than those typically found in the brain.[54] While more work will be necessary to determine the biodegradation rate of IEDDA MC gels in the brain, similar disulfide-crosslinked hydrogels of PEG were shown to successfully degrade within 4 weeks in vivo.[50] As the CNS is highly dependent on redox reactions involving molecules that can cleave disulfides, such as glutathione,[55] our disulfide-containing MC gels should be useful as biodegradable carriers for drug and cell delivery to the CNS.

3.3. Affinity-Controlled Release of Chondroitinase ABC. To investigate the affinity-controlled release of the fusion protein ChABC-SH3 from IEDDA MC hydrogels, we studied both degradable and nondegradable 1% (w/v) MC hydrogels using a MC-Tz:MC-Norb ratio of 2:1, i.e., excess methylphenyltetrazine, to enable the pre-grafting of norbornene-modified SH3-binding peptides to MC-Tz without altering the physicochemical properties of the gels. Peptide-modified MC-Tz was synthesized by IEDDA coupling of MC-Tz (or MC-DTP-Tz) and norbornene-functionalized SH3-binding peptide, which resulted in 23.8 ± 4.6 μmol peptide/g MC-Tz and 15.6 ± 2.7 μmol peptide/g MC-DTP-Tz, as determined by amino acid analysis. The calculated peptide substitutions accounted for approximately 12% of the available methylphenyltetrazine groups on both MC-Tz and MC-DTP-Tz, leaving excess methylphenyltetrazine groups to react with MC-norbonene. This allowed us to synthesize IEDDA MC hydrogels using MC-Tz-peptide or MC-DTP-Tz-peptide, with similar stiffness to gels without peptides (Figure S4). Using a fluorescently labeled, norbornene-functionalized SH3-binding peptide, we further confirmed successful peptide immobilization into the gels as a negligible amount (2.3 ± 0.7%) of peptide was washed out of the gel during release studies.

To test the effect of SH3 binding peptide concentration on ChABC-SH3 release independent of material degradation, we first prepared nondegradable IEDDA MC gels with no, 50, or 100 times molar excess peptide. ChABC-SH3 release appeared to reach a plateau after 2 days in hydrogels without peptides; however, with 50 and 100 times excess peptides, ChABC-SH3 release was extended to 4 days (Figure 3A). To compare protein release rates, we calculated the effective diffusivity using the short-time approximation for unidirectional diffusion from a thin polymer slab (Figure 3B).[56,57] Effective protein diffusivity was significantly reduced from 6.2 ± 1.94 × 10^{-11} m^2/s in gels without binding peptides to 2.91 ± 0.47 × 10^{-11} and 1.91 ± 0.52 × 10^{-11} m^2/s for the hydrogels with 50 times excesss and 100 times excess peptides, respectively, confirming controlled release.

The SH3 affinity-based release strategy used herein has been used to control the release of various other proteins from hyaluronic acid and methylcellulose-based gels, including fibroblast growth factor 2-SH3 (FGF2-SH3) and ciliary neurotrophic factor-SH3 (CNTF-SH3).[6,20] Interestingly, despite the lower polymer concentration and crosslink density of IEDDA MC gels than those of previous MC gels designed for affinity release,[6,17,27,32] these results are in close agreement with a previously described mathematical model of affinity release that was developed for hydrogels with higher HA and MC contents.[39] Since the controlled release regime conditions were met, the cumulative release could be predicted from a master plot of cumulative release vs dimensionless time t^*, calculated using the equation $t^* = t/\frac{L^2}{D}\left(1 + \frac{C_{pep,T}}{K_D}\right)$, where K_D = 2.7 × 10^{-5} M is the dissociation constant for the peptide and SH3 domain, L = 4.0 × 10^{-3} m is the thickness of the gel, and D = 8.25 × 10^{-11} m^2/s is the estimated diffusion coefficient of the protein through water. The 7 day cumulative release values obtained with 50 and 100 times molar excess peptide to protein were predicted to be 71 and 55%, respectively, which are consistent with our experimental results of 67.9 ± 0.8 and 47.1 ± 2.9%, respectively. Thus, we demonstrated that this theoretical approach can be used to bypass experimental trial-and-error optimization to tune protein release from these new IEDDA MC hydrogels.

We evaluated the enzymatic activity of ChABC-SH3 released from MC hydrogels by measuring the rate of degradation of chondroitin sulfate A. Released ChABC-SH3 maintained approximately 25% of its initial activity after 4 days of release from hydrogels, suggesting that the extended release profile afforded by the SH3 binding peptide can extend the duration of bioactive ChABC-SH3 delivery, compared to the hydrogel without binding peptides. In contrast, previously developed ChABC delivery systems have demonstrated little activity beyond 24 h in vitro[17] while exhibiting significant activity in vivo for 14 and 28 days,[33,44] thereby suggesting that, with this new system, in vivo activity may be higher and longer lasting.

We next evaluated ChABC-SH3 release from degradable IEDDA MC hydrogels using 50 times excess binding peptide. Since the peptide was attached to the disulfide-containing MC-DTP-Tz component, which is degradable, we investigated whether the presence of a reducing agent such as glutathione would affect the affinity release strategy. We found that the release from degradable MC hydrogels was not impacted by levels of glutathione (of 2 μM) expected to be present in the brain (Figure 3D)[54] and that release profiles were similar to those for nondegradable gels. These data suggest that hydrogel degradation in vivo will likely occur slowly enough to allow ChABC-SH3 to be released over its bioactive lifetime, and the hydrogel should be resorbed after the majoritiy of ChABC-SH3 has been released. Additionally, ChABC-SH3 activity was not affected by glutathione, and approximately 25% of initial protein activity remained after 4 days, consistent with our observations from the nondegradable hydrogels (Figure 3E).

With both nondegradable and degradable peptide-containing hydrogels, cumulative ChABC-SH3 release reached a plateau before 100% protein release, and the remaining protein in the hydrogel was not detectable following cellulase degradation. This has also been observed with affinity-based release of other proteins[20] and may be a result of the equilibrium shifting to favour peptide-protein complex formation, preventing further protein release and also limiting detection. By incorporating a degradable linker between the SH3 binding peptides and MC, this equilibrium shift may be reduced in vivo as the gel begins to degrade and release any remaining bound ChABC-SH3.

3.4. Encapsulation of Neural Progenitor Cells. Primary mouse neural progenitor cells (mNPCs) isolated from adult

https://dx.doi.org/10.1021/acs.biomac.0c00357
Biomacromolecules XXXX, XXX, XXX−XXX

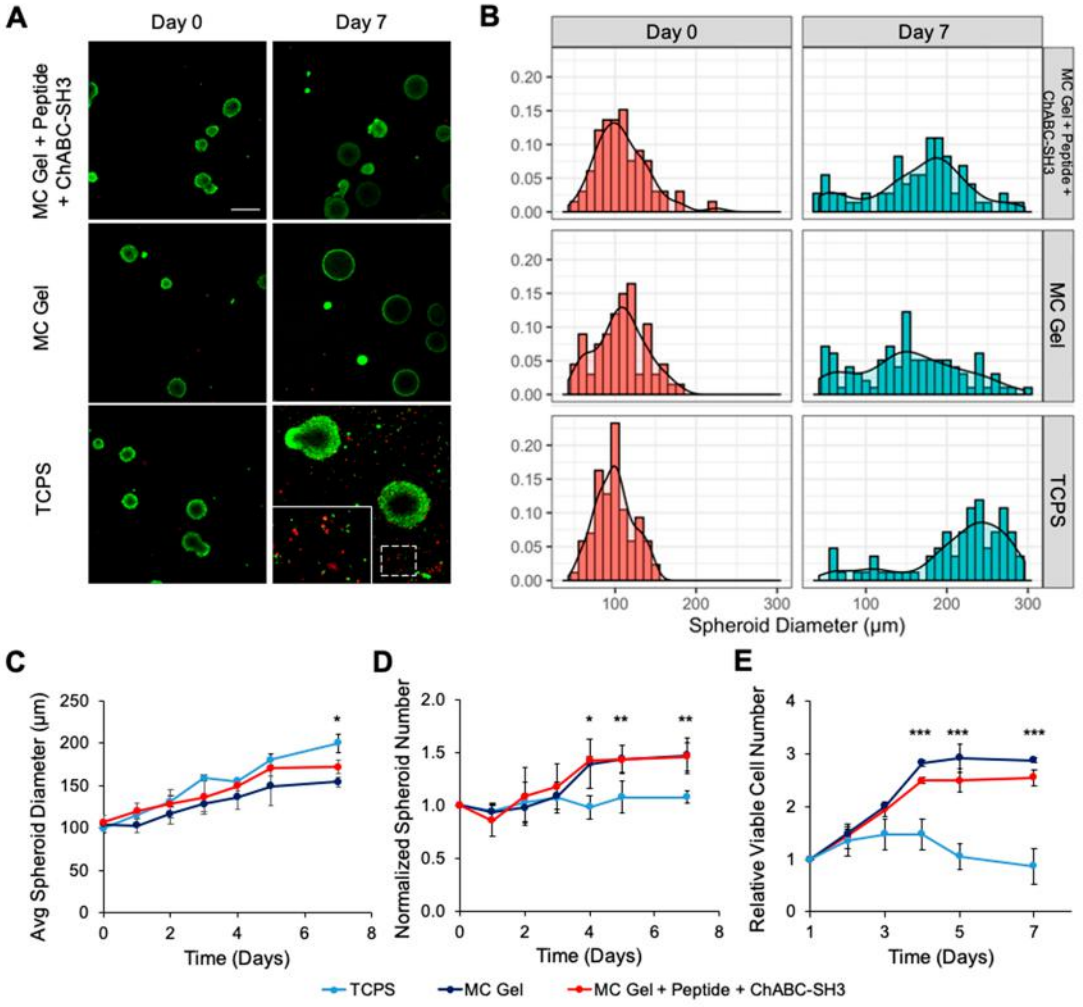


Figure 4. Cytocompatibility of degradable IEDDA MC hydrogels with primary mouse progenitor cells. (A) Representative live/dead z-stack images of mNPC neurospheres encapsulated in 1% (w/v) IEDDA MC hydrogels (MC-DTP-Tz:MC-Norb ratio of 2:1) with and without SH3 binding peptides and ChABC-SH3 vs those plated on tissue culture polystyrene (TCPS) (scale bar = 200 μm). (B) Neurosphere diameter distributions in IEDDA MC hydrogels vs those on TCPS at days 0 and 7 (n = 3, data pooled, bin size of 10 μm). (C) Average spheroid diameter. (D) Normalized spheroid number. (E) Relative change in the number of viable cells in IEDDA MC hydrogels or on TCPS over 7 days in vitro (n = 3; mean ± SD). Statistical significance was determined using two-way ANOVA with Dunnett's post-hoc test: *p < 0.05, **p < 0.01, ***p < 0.001, showing significance between MC-DTP-peptide + ChABC-SH3 and TCPS.

mouse brains were cultured as neurospheres in degradable IEDDA MC hydrogels with and without SH3 binding peptides and ChABC-SH3 for 1 week. Prior to encapsulation, single mNPCs were aggregated in AggreWells to create uniform neurospheres with an approximate diameter of 100 μm. We investigated neurosphere growth in 1% (w/v) degradable IEDDA MC hydrogels (MC-DTP-Tz:MC-Norb ratio of 2:1) and on tissue culture polystyrene (TCPS) under serum-free growth conditions. Live/dead staining immediately after cell encapsulation revealed that neurospheres were viable throughout (Figure 4A) and had a similar distribution of sizes between hydrogel groups and TCPS, with an average diameter of approximately 100 μm (Figure 4B). Very few dead cells were visible after encapsulation, suggesting that the viscosity of MC solutions before gelation was low enough to maintain neurosphere integrity during mixing. After 7 days, many neurospheres had increased in size in the hydrogels (Figure 4C), and new, smaller neurospheres had formed, creating a bimodal diameter distribution and an overall increase in the number of neurospheres (Figure 4B,D). A similar bimodal diameter distribution was observed for neurospheres encapsulated in nondegradable MC hydrogels (Figure S5). On TCPS, neurospheres grew larger than in hydrogels (Figure 4C), while fewer new spheres formed (Figure 4D), potentially due to the motile nature of neurospheres in 2D culture that enable them to fuse together.[58] More dead single cells were visible on TCPS compared to the hydrogels (Figure 4A inset), demonstrating that IEDDA MC hydrogels are cytocompatable.

We monitored the reducing potential of neurospheres over 7 days using the RealTime-Glo assay, which correlates with the total number of viable cells (Figure 4E). mNPCs proliferated for 4 days inside the IEDDA MC hydrogels and maintained metabolic activity between days 4 and 7. In contrast, the number of viable cells on TCPS decreased as spheroid size increased, likely due to limited mass transfer of oxygen or nutrients to cells in the center of the spheroids, which is consistent with the increased cell death observed in our live/dead images and by others when neurospheres exceed 250 μm in diameter.[59] The smaller neurospheres that formed in the

https://dx.doi.org/10.1021/acs.biomac.0c00357
Biomacromolecules XXXX, XXX, XXX−XXX

IEDDA MC hydrogels may have contributed to the overall increase in metabolic activity therein.

A combination strategy is especially promising in the CNS where single-target treatment approaches often have limited success due to the hostile injury microenvironment.[60] The co-delivery of neural progenitor cells and protein therapeutics, such as ChABC, can induce changes in the local microenvironment that improve cell survival and overcome the inhibitory environment, such as the chondroitin sulfate proteoglycans that make up the glial scar.[13,15,60] Collectively, our results demonstrate that degradable IEDDA MC hydrogels are suitable for cell encapsulation and hold potential for co-delivery with ChABC-SH3. Cell viability was not affected by the presence of SH3 binding peptides or ChABC-SH3, and released ChABC-SH3 collected in the cell supernatant was bioactive (Figure S6). Additionally, the presence of a degradable disulfide linker did not affect cell viability or neurosphere morphology, suggesting that this hydrogel is promising for further investigation in vivo for the co-delivery of neural stem cells and ChABC.

4. CONCLUSIONS

We demonstrate that MC modified with methylphenyltetrazine and norbornene groups undergoes rapid gelation under physiological conditions while enabling the encapsulation of bioactive proteins and cells. The addition of a disulfide-containing linker facilitates hydrogel degradation in the presence of naturally occurring free thiols. IEDDA MC hydrogels modified with affinity binding peptides enable the tunable, affinity-controlled release of bioactive chondroitinase ABC, which is predictable based on a mathematical model. Neural progenitor cells survive and proliferate in IEDDA MC hydrogels in vitro, demonstrating cytocompatibility of this hydrogel formulation. Together, these results lay the foundation for IEDDA MC hydrogels to be used for the combined delivery of ChABC and neurospheres to the CNS.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.0c00357.

^{1}H NMR of MC-norbornene (Figure S1). Physicochemical characterization of nondegradable MC hydrogels (Figure S2). Transmittance measurements of IEDDA MC hydrogels (Figure S3). Young's modulus of IEDDA MC hydrogels with immobilized SH3 binding peptides (Figure S4). Cytocompatibility of nondegradable MC hydrogels (Figure S5). Activity of ChABC-SH3 released from IEDDA MC hydrogels containing mNPC spheroids (Figure S6). Summary of compositions and physicochemical characteristics of degradable and nondegradable IEDDA MC hydrogels (Table S1). Parameters used for mathematical modeling (Table S2). Summary of results from mathematical modeling (Table S3) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Molly S. Shoichet − *Department of Chemical Engineering & Applied Chemistry and Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, ON M5S 3E5, Canada;* orcid.org/0000-0003-1830-3475; Phone: 416-978-1460; Email: molly.shoichet@utoronto.ca

Authors

Vianney Delplace − *Department of Chemical Engineering & Applied Chemistry, University of Toronto, Toronto, ON M5S 3E5, Canada*

Andrew J. Pickering − *Department of Chemical Engineering & Applied Chemistry, University of Toronto, Toronto, ON M5S 3E5, Canada;* orcid.org/0000-0002-1496-7752

Marian H. Hettiaratchi − *Department of Chemical Engineering & Applied Chemistry, University of Toronto, Toronto, ON M5S 3E5, Canada*

Spencer Zhao − *Department of Chemical Engineering & Applied Chemistry, University of Toronto, Toronto, ON M5S 3E5, Canada*

Tove Kivijärvi − *Department of Chemical Engineering & Applied Chemistry, University of Toronto, Toronto, ON M5S 3E5, Canada; Department of Fibre and Polymer Technology, KTH Royal Institute of Technology, SE-100 44 Stockholm, Sweden;* orcid.org/0000-0002-9000-0156

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.biomac.0c00357

Author Contributions

‖V.D. and A.J.P. contributed equally.

Funding

Funding for this work was provided by the Canadian Institutes of Health Research (Foundation Grant to M.S.S.), Natural Sciences and Engineering Research Council of Canada (Discovery Grant to M.S.S., Post-doctoral Fellowship to M.H.H., Undergraduate Summer Research Award to A.J.P.), the University of Toronto (Engineering Science Research Opportunities Program Fellowship to S.Z.), and Canada First Research Excellence Fund Medicine by Design (to M.S.S.).

Notes

The authors declare the following competing financial interest(s): We have submitted an invention disclosure on Chondroitinase ABC that is currently under review.

■ ACKNOWLEDGMENTS

We thank Ms. Rehnuma Islam and Professor Cindi Morshead (University of Toronto) for providing the mouse neural progenitor cells and members of the Shoichet laboratory for their thoughtful review of this manuscript.

■ REFERENCES

(1) Buwalda, S. J.; Boere, K. W. M.; Dijkstra, P. J.; Feijen, J.; Vermonden, T.; Hennink, W. E. Hydrogels in a Historical Perspective: From Simple Networks to Smart Materials. *J. Controlled Release* **2014**, *190*, 254−273.

(2) Li, J.; Mooney, D. J. Designing Hydrogels for Controlled Drug Delivery. *Nat. Rev. Mater.* **2016**, *1*, 16071.

(3) Zustiak, S. P.; Leach, J. B. Characterization of Protein Release from Hydrolytically Degradable Poly(Ethylene Glycol) Hydrogels. *Biotechnol. Bioeng.* **2011**, *108*, 197−206.

(4) Aurand, E.; Wagner, J.; Lanning, C.; Bjugstad, K. Building Biocompatible Hydrogels for Tissue Engineering of the Brain and Spinal Cord. *J. Funct. Biomater.* **2012**, *3*, 839−863.

(5) Shoichet, M. S.; Hettiaratchi, M. H. Modulated Protein Delivery to Engineer Tissue Repair. *Tissue Eng., Part A* **2019**, *25*, 925−930.

(6) Vulic, K.; Shoichet, M. S. Tunable Growth Factor Delivery from Injectable Hydrogels for Tissue Engineering. *J. Am. Chem. Soc.* **2012**, *134*, 882−885.

(7) Fu, K.; Klibanov, A. M.; Langer, R. Protein Stability in Controlled-Release Systems. *Nat. Biotechnol.* **2000**, *18*, 24−25.
(8) Leipzig, N. D.; Shoichet, M. S. The Effect of Substrate Stiffness on Adult Neural Stem Cell Behavior. *Biomaterials* **2009**, *30*, 6867−6878.
(9) Yang, C.; Tibbitt, M. W.; Basta, L.; Anseth, K. S. Mechanical Memory and Dosing Influence Stem Cell Fate. *Nat. Mater.* **2014**, *13*, 645−652.
(10) Madl, C. M.; LeSavage, B. L.; Dewi, R. E.; Dinh, C. B.; Stowers, R. S.; Khariton, M.; Lampe, K. J.; Nguyen, D.; Chaudhuri, O.; Enejder, A.; Heilshorn, S. C. Maintenance of Neural Progenitor Cell Stemness in 3D Hydrogels Requires Matrix Remodelling. *Nat. Mater.* **2017**, *16*, 1233−1242.
(11) Karimi-Abdolrezaee, S.; Eftekharpour, E.; Wang, J.; Schut, D.; Fehlings, M. G. Synergistic Effects of Transplanted Adult Neural Stem/Progenitor Cells, Chondroitinase, and Growth Factors Promote Functional Repair and Plasticity of the Chronically Injured Spinal Cord. *J. Neurosci.* **2010**, *30*, 1657−1676.
(12) Fouad, K.; Pearse, D. D.; Tetzlaff, W.; Vavrek, R. Transplantation and Repair: Combined Cell Implantation and Chondroitinase Delivery Prevents Deterioration of Bladder Function in Rats with Complete Spinal Cord Injury. *Spinal Cord* **2009**, *47*, 727−732.
(13) Führmann, T.; Anandakumaran, P. N.; Payne, S. L.; Pakulska, M. M.; Varga, B. V.; Nagy, A.; Tator, C.; Shoichet, M. S. Combined Delivery of Chondroitinase ABC and Human Induced Pluripotent Stem Cell-Derived Neuroepithelial Cells Promote Tissue Repair in an Animal Model of Spinal Cord Injury. *Biomed. Mater.* **2018**, *13*, 024103.
(14) Bradbury, E. J.; Moon, L. D. F.; Popat, R. J.; King, V. R.; Bennett, G. S.; Patel, P. N.; Fawcett, J. W.; McMahon, S. B. Chondroitinase ABC Promotes Functional Recovery after Spinal Cord Injury. *Nature* **2002**, *416*, 636−640.
(15) Nori, S.; Khazaei, M.; Ahuja, C. S.; Yokota, K.; Ahlfors, J. E.; Liu, Y.; Wang, J.; Shibata, S.; Chio, J.; Hettiaratchi, M. H.; et al. Human Oligodendrogenic Neural Progenitor Cells Delivered with Chondroitinase ABC Facilitate Functional Repair of Chronic Spinal Cord Injury. *Stem Cell Rep.* **2018**, *11*, 1433−1448.
(16) Fouad, K.; Schnell, L.; Bunge, M. B.; Schwab, M. E.; Liebscher, T.; Pearse, D. D. Combining Schwann Cell Bridges and Olfactory-Ensheathing Glia Grafts with Chondroitinase Promotes Locomotor Recovery after Complete Transection of the Spinal Cord. *J. Neurosci.* **2005**, *25*, 1169−1178.
(17) Pakulska, M. M.; Vulic, K.; Shoichet, M. S. Affinity-Based Release of Chondroitinase ABC from a Modified Methylcellulose Hydrogel. *J. Controlled Release* **2013**, *171*, 11−16.
(18) Kobayashi, K.; Huang, C.-i.; Lodge, T. P. Thermoreversible Gelation of Aqueous Methylcellulose Solutions. *Macromolecules* **1999**, *32*, 7070−7077.
(19) Tate, M. C.; Shear, D. A.; Hoffman, S. W.; Stein, D. G.; LaPlaca, M. C. Biocompatibility of Methylcellulose-Based Constructs Designed for Intracerebral Gelation Following Experimental Traumatic Brain Injury. *Biomaterials* **2001**, *22*, 1113−1123.
(20) Delplace, V.; Ortin-Martinez, A.; Tsai, E. L. S.; Amin, A. N.; Wallace, V.; Shoichet, M. S. Controlled Release Strategy Designed for Intravitreal Protein Delivery to the Retina. *J. Controlled Release* **2019**, *293*, 10−20.
(21) Bain, M. K.; Bhowmick, B.; Maity, D.; Mondal, D.; Mollick, M. M. R.; Paul, B. K.; Bhowmik, M.; Rana, D.; Chattopadhyay, D. Effect of PVA on the Gel Temperature of MC and Release Kinetics of KT from MC Based Ophthalmic Formulations. *Int. J. Biol. Macromol.* **2012**, *50*, 565−572.
(22) Bain, M. K.; Bhowmik, M.; Maity, D.; Bera, N. K.; Ghosh, S.; Chattopadhyay, D. Control of Thermo Reversible Gelation of Methylcellulose Using Polyethylene Glycol and Sodium Chloride for Sustained Delivery of Ophthalmic Drug. *J. Appl. Polym. Sci.* **2010**, *118*, 631−637.
(23) Dewan, M.; Bhowmick, B.; Sarkar, G.; Rana, D.; Bain, M. K.; Bhowmik, M.; Chattopadhyay, D. Effect of Methyl Cellulose on Gelation Behavior and Drug Release from Poloxamer Based Ophthalmic Formulations. *Int. J. Biol. Macromol.* **2015**, *72*, 706−710.
(24) Gupta, D.; Tator, C. H.; Shoichet, M. S. Fast-Gelling Injectable Blend of Hyaluronan and Methylcellulose for Intrathecal, Localized Delivery to the Injured Spinal Cord. *Biomaterials* **2006**, *27*, 2370−2379.
(25) Caicco, M. J.; Zahir, T.; Mothe, A. J.; Ballios, B. G.; Kihm, A. J.; Tator, C. H.; Shoichet, M. S. Characterization of Hyaluronan-Methylcellulose Hydrogels for Cell Delivery to the Injured Spinal Cord. *J. Biomed. Mater. Res. A* **2013**, *101*, 1472−1477.
(26) Mayol, L.; De Stefano, D.; De Falco, F.; Carnuccio, R.; Maiuri, M. C.; De Rosa, G. Effect of Hyaluronic Acid on the Thermogelation and Biocompatibility of Its Blends with Methyl Cellulose. *Carbohydr. Polym.* **2014**, *112*, 480−485.
(27) Parker, J.; Mitrousis, N.; Shoichet, M. S. Hydrogel for Simultaneous Tunable Growth Factor Delivery and Enhanced Viability of Encapsulated Cells in Vitro. *Biomacromolecules* **2016**, *17*, 476−484.
(28) Toyoda, H.; Motoki, K.; Tanikawa, M.; Shinomiya, K.; Akiyama, H.; Imanari, T. Determination of Human Urinary Hyaluronic Acid, Chondroitin Sulphate and Dermatan Sulphate as Their Unsaturated Disaccharides by High-Performance Liquid Chromatography. *J. Chromatogr. B: Biomed. Sci. Appl.* **1991**, *565*, 141−148.
(29) Stalling, S. S.; Akintoye, S. O.; Nicoll, S. B. Development of Photocrosslinked Methylcellulose Hydrogels for Soft Tissue Reconstruction. *Acta Biomater.* **2009**, *5*, 1911−1918.
(30) Gold, G. T.; Varma, D. M.; Harbottle, D.; Gupta, M. S.; Stalling, S. S.; Taub, P. J.; Nicoll, S. B. Injectable Redox-Polymerized Methylcellulose Hydrogels as Potential Soft Tissue Filler Materials. *J. Biomed. Mater. Res. A* **2014**, *102*, 4536−4544.
(31) Gold, G. T.; Varma, D. M.; Taub, P. J.; Nicoll, S. B. Development of Crosslinked Methylcellulose Hydrogels for Soft Tissue Augmentation Using an Ammonium Persulfate-Ascorbic Acid Redox System. *Carbohydr. Polym.* **2015**, *134*, 497−507.
(32) Pakulska, M. M.; Vulic, K.; Tam, R. Y.; Shoichet, M. S. Hybrid Crosslinked Methylcellulose Hydrogel: A Predictable and Tunable Platform for Local Drug Delivery. *Adv. Mater.* **2015**, *27*, 5002−5008.
(33) Pakulska, M. M.; Tator, C. H.; Shoichet, M. S. Local Delivery of Chondroitinase ABC with or without Stromal Cell-Derived Factor 1α Promotes Functional Repair in the Injured Rat Spinal Cord. *Biomaterials* **2017**, *134*, 13−21.
(34) Oliveira, B. L.; Guo, Z.; Bernardes, G. J. L. Inverse Electron Demand Diels−Alder Reactions in Chemical Biology. *Chem. Soc. Rev* **2017**, *46*, 4895−4950.
(35) Madl, C. M.; Heilshorn, S. C. Bioorthogonal Strategies for Engineering Extracellular Matrices. *Adv. Funct. Mater.* **2018**, *28*, 1706046.
(36) Alge, D. L.; Azagarsamy, M. A.; Donohue, D. F.; Anseth, K. S. Synthetically Tractable Click Hydrogels for Three-Dimensional Cell Culture Formed Using Tetrazine-Norbornene Chemistry. *Biomacromolecules* **2013**, *14*, 949−953.
(37) Desai, R. M.; Koshy, S. T.; Hilderbrand, S. A.; Mooney, D. J.; Joshi, N. S. Versatile Click Alginate Hydrogels Crosslinked via Tetrazine-Norbornene Chemistry. *Biomaterials* **2015**, *50*, 30−37.
(38) Lueckgen, A.; Garske, D. S.; Ellinghaus, A.; Desai, R. M.; Stafford, A. G.; Mooney, D. J.; Duda, G. N.; Cipitria, A. Hydrolytically-Degradable Click-Crosslinked Alginate Hydrogels. *Biomaterials* **2018**, *181*, 189−198.
(39) Vulic, K.; Pakulska, M. M.; Sonthalia, R.; Ramachandran, A.; Shoichet, M. S. Mathematical Model Accurately Predicts Protein Release from an Affinity-Based Delivery System. *J. Controlled Release* **2015**, *197*, 69−77.
(40) Vercruysse, K. P.; Marecak, D. M.; Marecek, J. F.; Prestwich, G. D. Synthesis and in Vitro Degradation of New Polyvalent Hydrazide Cross-Linked Hydrogels of Hyaluronic Acid. *Bioconjugate Chem.* **1997**, *8*, 686−694.
(41) Tam, R. Y.; Cooke, M. J.; Shoichet, M. S. A Covalently Modified Hydrogel Blend of Hyaluronan−Methyl Cellulose with

https://dx.doi.org/10.1021/acs.biomac.0c00357
Biomacromolecules XXXX, XXX, XXX−XXX

Peptides and Growth Factors Influences Neural Stem/Progenitor Cell Fate. *J. Mater. Chem.* **2012**, *22*, 19402−19411.
(42) Baker, A. E. G.; Tam, R. Y.; Shoichet, M. S. Independently Tuning the Biochemical and Mechanical Properties of 3D Hyaluronan-Based Hydrogels with Oxime and Diels-Alder Chemistry to Culture Breast Cancer Spheroids. *Biomacromolecules* **2017**, *18*, 4373−4384.
(43) Delplace, V.; Nickerson, P. E. B.; Ortin-Martinez, A.; Baker, A. E. G.; Wallace, V. A.; Shoichet, M. S. Nonswelling, Ultralow Content Inverse Electron-Demand Diels−Alder Hyaluronan Hydrogels with Tunable Gelation Time: Synthesis and In Vitro Evaluation. *Adv. Funct. Mater.* **2020**, *30*, 1903978.
(44) Hettiaratchi, M. H.; O'Meara, M. J.; Teal, C. J.; Payne, S. L.; Pickering, A. J.; Shoichet, M. S. Local Delivery of Stabilized Chondroitinase ABC Degrades Chondroitin Sulfate Proteoglycans in Stroke-Injured Rat Brains. *J. Controlled Release* **2019**, *297*, 14−25.
(45) Tropepe, V.; Craig, C. G.; Morshead, C. M.; van der Kooy, D. Transforming Growth Factor-α Null and Senescent Mice Show Decreased Neural Progenitor Cell Proliferation in the Forebrain Subependyma. *J. Neurosci.* **1997**, *17*, 7850−7859.
(46) Coles-Takabe, B. L. K.; Brain, I.; Purpura, K. A.; Karpowicz, P.; Zandstra, P. W.; Morshead, C. M.; van der Kooy, D. Don't Look: Growing Clonal Versus Nonclonal Neural Stem Cell Colonies. *Stem Cells* **2008**, *26*, 2938−2944.
(47) Saito, G.; Swanson, J. A.; Lee, K.-D. Drug Delivery Strategy Utilizing Conjugation via Reversible Disulfide Linkages: Role and Site of Cellular Reducing Activities. *Adv. Drug Delivery Rev.* **2003**, *55*, 199−215.
(48) Wu, D.-C.; Loh, X. J.; Wu, Y.-L.; Lay, C. L.; Liu, Y. 'Living' Controlled in Situ Gelling Systems: Thiol−Disulfide Exchange Method toward Tailor-Made Biodegradable Hydrogels. *J. Am. Chem. Soc.* **2010**, *132*, 15140−15143.
(49) Yang, F.; Wang, J.; Cao, L.; Chen, R.; Tang, L.; Liu, C. Injectable and Redox-Responsive Hydrogel with Adaptive Degradation Rate for Bone Regeneration. *J. Mater. Chem. B* **2014**, *2*, 295−304.
(50) Rehfeldt, F.; Brown, A. E. X.; Raab, M.; Cai, S.; Zajac, A. L.; Zemel, A.; Discher, D. E. Hyaluronic Acid Matrices Show Matrix Stiffness in 2D and 3D Dictates Cytoskeletal Order and Myosin-II Phosphorylation within Stem Cells. *Integr. Biol.* **2012**, *4*, 422−430.
(51) de Rooij, R.; Ovaert, T. C.; Wyrobek, T.; Steinmann, P.; Nay, R.; Kuhl, E.; Budday, S.; Nay, R.; de Rooij, R.; Steinmann, P.; et al. Mechanical Properties of Gray and White Matter Brain Tissue by Indentation. *J. Mech. Behav. Biomed. Mater.* **2015**, *46*, 318−330.
(52) Weickenmeier, J.; Kurt, M.; Ozkaya, E.; de Rooij, R.; Ovaert, T. C.; Ehman, R. L.; Butts Pauly, K.; Kuhl, E. Brain Stiffens Post Mortem. *J. Mech. Behav. Biomed. Mater.* **2018**, *84*, 88−98.
(53) Murphy, M. C.; Jones, D. T.; Jack, C. R., Jr.; Glaser, K. J.; Senjem, M. L.; Manduca, A.; Felmlee, J. P.; Carter, R. E.; Ehman, R. L.; Huston, J. Regional Brain Stiffness Changes across the Alzheimer's Disease Spectrum. *NeuroImage Clin.* **2016**, *10*, 283−290.
(54) Yang, C.-S.; Chou, S.-T.; Lin, N.-N.; Liu, L.; Tsai, P.-J.; Kuo, J.-S.; Lai, J.-S. Determination of Extracellular Glutathione in Rat Brain by Microdialysis and High-Performance Liquid Chromatography with Fluorescence Detection. *J. Chromatogr. B Biomed. Sci. Appl.* **1994**, *661*, 231−235.
(55) Franco, R.; Vargas, M. R. Redox Biology in Neurological Function, Dysfunction, and Aging. *Antioxid. redox signaling* **2018**, 1583−1586.
(56) Ritger, P. L.; Peppas, N. A. A Simple Equation for Description of Solute Release I. Fickian and Non-Fickian Release from Non-Swellable Devices in the Form of Slabs, Spheres, Cylinders or Discs. *J. Controlled Release* **1987**, *5*, 23−36.
(57) Leach, J. B.; Schmidt, C. E. Characterization of Protein Release from Photocrosslinkable Hyaluronic Acid-Polyethylene Glycol Hydrogel Tissue Engineering Scaffolds. *Biomaterials* **2005**, *26*, 125−135.
(58) Singec, I.; Knoth, R.; Meyer, R. P.; Maciaczyk, J.; Volk, B.; Nikkhah, G.; Frotscher, M.; Snyder, E. Y. Defining the Actual Sensitivity and Specificity of the Neurosphere Assay in Stem Cell Biology. *Nat. Methods* **2006**, *3*, 801−806.
(59) Xiong, F.; Gao, H.; Zhen, Y.; Chen, X.; Lin, W.; Shen, J.; Yan, Y.; Wang, X.; Liu, M.; Gao, Y. Optimal Time for Passaging Neurospheres Based on Primary Neural Stem Cell Cultures. *Cytotechnology* **2011**, *63*, 621−631.
(60) Führmann, T.; Anandakumaran, P. N.; Shoichet, M. S. Combinatorial Therapies After Spinal Cord Injury: How Can Biomaterials Help? *Adv. Healthcare Mater.* **2017**, *6*, 1601130.

https://dx.doi.org/10.1021/acs.biomac.0c00357
Biomacromolecules XXXX, XXX, XXX−XXX