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Tunable diblock copolypeptide hydrogel depots for local delivery of hydrophobic molecules in healthy and injured central nervous system

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Abstract

Many hydrophobic small molecules are available to regulate gene expression and other cellular functions. Locally restricted application of such molecules in the central nervous system (CNS) would be desirable in many experimental and therapeutic settings, but is limited by a lack of innocuous vehicles able to load and easily deliver hydrophobic cargo. Here, we tested the potential for diblock copolypeptide hydrogels (DCH) to serve as such vehicles. *In vitro* tests on loading and release were conducted with cholesterol and the anti-cancer agent, temozolomide (TMZ). Loading of hydrophobic cargo modified DCH physical properties such as stiffness and viscosity, but these could readily be tuned to desired ranges by modifying DCH concentration, amino acid composition or chain lengths. Different DCH formulations exhibited different loading capacities and different rates of release. For example, comparison of different DCH with increasing alanine contents showed corresponding increases in both cargo loading capacity and time for cargo release. *In vivo* tests were conducted with tamoxifen, a small synthetic hydrophobic molecule widely used to regulate transgene expression. Tamoxifen released from DCH depots injected into healthy or injured CNS efficiently activated reporter gene expression in a locally restricted manner in transgenic mice. These findings demonstrate the facile and predictable tunability of DCH to achieve a wide range of loading capacities and release profiles of hydrophobic cargos while retaining CNS compatible physical properties. In addition, the findings show that DCH depots injected into the CNS can efficiently deliver small hydrophobic molecules that regulate gene expression in local cells.

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Keywords

Biomaterials; hydrogels; steroids; anti-cancer drugs; brain; spinal cord; reactive astrocytes; neural stem cells

1. Introduction

There are many hydrophobic small molecules whose delivery into the central nervous system (CNS) is of interest either for experimental investigations or for potential therapeutic applications. Such hydrophobic molecules include many naturally occurring and synthetic compounds, ranging from steroid hormones to anticancer drugs. These compounds have the potential to influence or regulate an enormous number of cellular functions in the healthy and injured CNS, including gene expression [1–3], epigenetic activities [4], signal transduction [5] and other cellular mechanisms [6], with a wide range of potential applications including anticancer treatment, neuroprotection, repair and regeneration or behavioral modification.

At present, most hydrophobic molecules are delivered for CNS applications by systemic injection, either dissolved in aprotic solvents such as dimethyl sulfoxide (DMSO) and injected intraperitoneally or intramuscularly for acute delivery, or dissolved in corn oil depots and injected subcutaneously for sustained delivery. In many cases, global systemic delivery is associated with a high potential for side effects because such compounds often influence fundamental mechanisms among many cell types, both within and outside of the CNS. Unwanted side effects can be particularly problematic in the CNS, where there are many topographically distinct CNS neural systems that subserve different functions but share similar operational mechanisms at the cellular and molecular level. For these reasons, locally restricted CNS delivery is desirable or essential in many experimental and therapeutic settings. Although acute local delivery can in some cases be achieved using DMSO, there is a lack of innocuous vehicles that are able to load hydrophobic cargo and that can be easily injected directly into CNS tissue without causing damage and can efficiently deliver hydrophobic molecules for prolonged local delivery.

Hydrogels that can be injected as local depots represent a promising means of achieving sustained locally restricted delivery of different types of molecules in the CNS [7]. Hydrogels that are suitable for CNS applications will need to exhibit physical properties and functional features such as rigidity, porosity and surface chemistry that are compatible with CNS tissue [8]. Because these types of properties are incompletely characterized for CNS tissue in the context of injury or disease, there is a need for versatile materials where such features can be easily tuned to meet the requirements of specific applications and modified in accordance with practical experience.

Amphiphilic diblock copolypeptide hydrogels (DCH) are synthetic materials with many features that make them attractive for CNS applications that are likely to require progressive adjustment and fine-tuning of material properties [9]. We have used a combination of chemical synthesis and structural characterization to establish a detailed understanding of DCH structure-property relationships that allows a high level of control over gel stiffness, gel porosity, gel functionality and media stability, and many of these properties can be adjusted independently of each other [10–12]. Based on information from these prior studies, DCH physical properties can be varied readily and predictably by altering copolymer chain length, architecture or composition [9] and therefore have the potential for continual refinement and incremental optimization in response to experimental or clinical experience. DCH are physically associated gels that can be deformed and thinned by applied

stress and injected through small-bore cannulae, after which they rapidly re-assemble into rigid gel networks [11], providing DCH with the capacity for minimally invasive delivery *in vivo*. We have shown that DCH formulations with storage modulus (G′) values somewhat lower than that of brain tissue are easily injected, and after injection self-assemble into well formed deposits of rigid gel networks that integrate well with host CNS tissue *in vivo* [8]. We also found that these DCH are fully biodegradable over a time frame of about eight weeks after injection into the forebrain and exhibited no detectable toxicity or adverse inflammatory reaction [8]. These observations suggested that deposits of DCH may be useful as depots for local delivery of potentially therapeutic molecules over a sustained subacute timeframe.

We have recently reported a combination of *in vitro* and *in vivo* evidence that DCH can serve as depots for sustained local release of hydrophilic effector molecules for investigative and potential therapeutic applications in the CNS [8, 13]. *In vitro*, we found that release time of either a small, water soluble probe, Rhodamine B, or a representative protein, lysozyme, dissolved in DCH was dependent on DCH charge, storage modulus (G′) and viscosity (G″), and ionic strength of the media. *In vivo* we found that DCH depots loaded with the protein growth factor, nerve growth factor (NGF) and injected into the forebrain, provided significantly prolonged delivery of NGF bioactivity (quantified as induction of hypertrophy of local forebrain cholinergic neurons) over a four week period in comparison to NGF injected in phosphate buffered saline (PBS). These findings showed that DCH depots injected into CNS can provide sustained delivery within the blood-brain barrier of a hydrophilic, bioactive protein growth factor that exerts a predicted, quantifiable effect on local cells over a prolonged subacute time [13].

Here, we present *in vitro* and *in vivo* proof of principle tests on the ability of DCH to dissolve and release hydrophobic molecules. DCH differ from most hydrogels in that they are inherently amphiphilic and contain hydrophobic segments essential for their gelation properties. Most hydrogels are typically considered poor carriers for hydrophobic molecules due to their high water content. To achieve loading of hydrophobic compounds, non-amphiphilic hydrogels require modification with hydrophobic patches or by covalent attachment of hydrophobic molecules, which may adversely affect hydrogel properties and may require additional or complex fabrication steps [14]. Because DCH are inherently amphiphilic, we hypothesized that the hydrophobic segments of these polypeptides, which are critical for hydrogel formation, may also be useful for the entrapment and release of hydrophobic compounds. We designed and prepared a series of DCH in which hydrophobic segments were varied both in terms of amino acid type as well as chain length, and compared the ability of these different DCH formulations to load different representative hydrophobic molecules, including various steroids and anti-cancer agents, and release these molecules *in vitro*. For *in vivo* evaluations, we used tamoxifen, a small synthetic hydrophobic molecule widely employed to regulate the expression of transgene constructs, and tested the ability DCH depots injected into healthy or injured CNS to deliver tamoxifen and activate reporter gene expression in nearby CNS cells in transgenic mice.

2. Methods

2.1. Preparation of DCH

2.1.1. Materials and instrumentation—Tetrahydrofuran (THF), hexanes, and methylene chloride were dried by purging with nitrogen and passed through activated alumina columns prior to use. $Co(PMe_3)_4$ and amino acid N-carboxyanhydride (NCA) monomers were prepared according to literature procedures [11]. All other chemicals were purchased from commercial suppliers and used without further purification unless otherwise noted. Fourier Transform Infrared (FTIR) measurements were taken on a Perkin Elmer RX1

FTIR spectrophotometer calibrated using polystyrene film. ^{1}H NMR spectra were acquired on a Bruker ARX 400 spectrometer. Tandem gel permeation chromatography/light scattering (GPC/LS) was performed at 60 °C using an SSI Accuflow Series III pump equipped with Wyatt DAWN EOS light scattering and Optilab rEX refractive index detectors. Separations were achieved using 10^5, 10^4, and 10^3 Å Phenomenex Phenogel 5 μm columns at 60 °C with 0.1 M LiBr in DMF as eluent and sample concentrations of 5 mg/mL. Pyrogen free deionized water (DI) was obtained from a Millipore Milli-Q Biocel A10 purification unit.

2.1.2. General polypeptide synthesis—DCH samples were designed with average lengths of around 200 residues, containing poly-L-leucine (L), poly-L-alanine (A), poly-L-leucine-*stat*-poly-L-alanine (L/A) or poly-L-phenylalanine (F) as the hydrophobic domain, and poly-L-lysine (K) as the hydrophilic domain exposed to tissue (Fig. 1). DCH of $K_{180}L_{20}$ and other compositions were prepared on the basis of previous studies showing that these formulations at injectable concentrations self-assemble into well-tolerated deposits after injection into mouse forebrain [8].

DCH were synthesized by sequential polymerization of desired NCA monomers using the transition metal initiator $Co(PMe_3)_4$ according to published procedures [13, 15]. All polymerization reactions were performed in a dinitrogen filled glovebox. Isolated yields of the deprotected copolymers ranged between 85% and 90%. Relative copolypeptide compositions (Table 1) were determined using ^{1}H NMR and were found to be within 5% of predicted values [8]. Chain lengths of initial, protected K_m segments were determined using GPC/LS and were found to be within 1% of predicted values. ^{1}H NMR of deprotected polymers in deuterated TFA (*d*-TFA) indicated a 97%–98% removal of benzyloxycarbonyl protecting groups from lysine residues.

2.1.2. Representative synthesis of a DCH sample: $K_{180}A_{30}$—In a dinitrogen filled glovebox, a solution of $Co(PMe_3)_4$ (22 mg, 0.059 mmol) in THF (20 mg/ml) was rapidly added, *via* syringe, to a solution of N_ε-carbobenzyloxy-L-lysine NCA (1.00 g, 3.3 mmol) in THF (50 mg/ml). The reaction was stirred at 20 °C and polymerization progress was monitored by FTIR. Polymerization reactions were generally complete within 45 minutes. Immediately upon polymerization completion, an aliquot was removed for GPC/LS analysis to determine the length of the polylysine segment. A solution of L-alanine NCA (57 mg, 0.54 mmol) in THF (50 mg/ml) was then added and the polymerization was monitored by FTIR. Polymerization was generally complete within 45 minutes. After complete consumption of L-alanine NCA, the reaction was removed from the glovebox and solvent removed under vacuum prior to deprotection. The protecting groups of the N_ε-carbobenzyloxy-L-lysine residues were removed by addition of 10 ml of 33 wt% HBr in acetic acid to a solution of the copolymer in TFA (50 mg/ml) at 0 °C for 1 hour. Diethyl ether (30 ml) was then added to precipitate the copolymer. The product was isolated by centrifugation and was washed with ether twice more before resuspending in water. The deprotected copolymer was dissolved in nonpyrogenic DI water and then placed in a dialysis bag (MWCO = 2000 Da) and dialyzed exhaustively against NaCl for two days and then nonpyrogenic DI water for two days. Lyophilization of the resulting solution gave 510 mg poly(L-lysine)$_{180}$-*block*-poly(L-alanine)$_{30}$ ($K_{180}A_{30}$) (91% yield) as a fluffy white solid. ^{1}H NMR analysis of this material was used to determine the copolymer composition, which was found to be $K_{180}A_{29}$ (Table 1).

2.1.3. Preparation of fluorescence-tagged DCH (AMCA-X-DCH)—For a few test cases, DCH were conjugated with a fluorescent dye to track their location *in vivo*. Fluorescent tagging of lysine ε-amine groups was performed using AMCA-X [6–((7-Amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid] (AnaSpec, Cat# 81207) as a blue

fluorescent tag, which was attached using NHS-EDC coupling chemistry. In this procedure, $K_{180}L_{20}$ or $K_{180}A_{30}$ powder (3.2 μmol) was dissolved in PBS buffer (pH = 6.5, 30 ml, 0.1 M). To the polypeptide solution, 2 equivalents of NHS (N-hydroxysuccinimide) in PBS buffer (pH = 6.5), 10 equivalents of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) in PBS buffer (pH = 6.5), and 1 equivalent of AMCA-X in DMSO per copolypeptide chain (corresponding to 2.8% of the available lysine amines) were added, and the mixture was stirred for 16 hours. For purification, the sample was covered in Al foil to protect from light, and then dialyzed (MWCO = 2 kDa) for 5 days with pyrogen free water, with dialyzate changes every 12 hours. The tagged polymer (AMCA-X-$K_{180}L_{20}$ or AMCA-X-$K_{180}A_{30}$) was isolated by lyophilization. For injection *in vivo*, AMCA-X-$K_{180}L_{20}$ or AMCA-X-$K_{180}A_{30}$ was mixed with unlabeled DCH sample at a ratio of 1:10.

2.2. Rheology measurements

DCH were prepared by rehydrating freeze-dried samples in phosphate buffered saline (PBS) pH = 7.4. Rheological measurements (dynamic) were performed on a strain controlled Reometrics fluids spectrometer RFS II in a cone-plate geometry with diameter of 25 mm and cone angle of 0.02 rad similar to previously described [12]. Strain was set at 10% and the sweep frequency was 1 rad/s. For each sample, small-deformation linearity was checked before performing oscillatory measurements.

2.3. Loading of hydrophobic molecules in DCH

Cholesterol (Sigma, molecular weight (m.w.) 386.65) was used as representative small hydrophobic molecule, while ambroxol (Sigma, m.w. 378.56), estradiol (Sigma, m.w. 272.39), taxol (Sigma, m.w. 853.91), temozolomide (TMZ) (Sigma, m.w. 194.15), camptothecin (Sigma, m.w. 348.35) and tamoxifen (Sigma, m.w. 371.51) were used as representative biologically active hydrophobic molecules to study the maximum loading in DCH. Cholesterol and TMZ loaded DCH were also prepared for rheology measurements and in vitro release studies.

Cholesterol and TMZ stock solutions were prepared by dissolving them separately in ethanol (3.5 mg/ml). DCH ($K_{180}A_{20}$, $K_{180}A_{30}$, $K_{180}A_{40}$, $K_{180}(L/A)_{20}$, $K_{180}L_{20}$, $K_{180}L_{30}$, or $K_{180}F_{20}$) was dissolved at 15 mg/ml in a 90% ethanol and 10% water mixture to yield a clear, fluid solution. As an example, DCH and cholesterol solutions were mixed together at a desired ratio and then transferred to a flat-bottomed vial having a 1 cm diameter. After evaporating the ethanol and water under vacuum to dryness, a homogeneous transparent film formed on the bottom of the vial. Millipore deionized (DI) water or PBS buffer was added to rehydrate the film, which yielded a transparent DCH hydrogel loaded with the desired amount of cholesterol.

2.4. Release of hydrophobic molecules from DCH in vitro

0.5 ml of DCH ($K_{180}A_{20}$, $K_{180}A_{30}$, $K_{180}A_{40}$, $K_{180}(L/A)_{20}$, $K_{180}L_{20}$, $K_{180}L_{30}$, or $K_{180}F_{20}$ prepared at different concentrations in PBS buffer) containing test molecules (cholesterol or TMZ, at different concentrations) were injected into dialysis cassettes (Thermo Scientific) with membrane molecular weight cut-offs of 20 kDa. The dialysate was PBS for cholesterol and TMZ, which contained 10 mg/ml BSA to help capture the released hydrophobic molecules. To study release kinetics, a dialysis cassette containing DCH with loaded test molecule was placed into dialysate (total volume 200 ml) in an amber bottle at 37°C. Aliquots of dialysate (1 ml each) were sampled for analysis after 0, 1, 2, 4, 8, 12, 24 and 48 hours. After 2 days, the time interval between sampling was adjusted according to the release kinetics of each sample. The concentrations of test molecules in dialyzate samples were measured using a calibrated gas chromatography (GC) method for cholesterol and a high performance liquid chromatography (HPLC) method for TMZ, respectively.

The cholesterol was extracted from an aliquot of dialysate with toluene, and its concentration was quantified using a Shimdazu 6890N gas chromatographic system and a DB-17 capillary column (30 m × 0.250 mm × 0.15 mm, Agilent Technologies Inc., CA, USA). The DB-17 column has mid-polarity and is suitable for analysis of free steroids. One microliter (1.0 μl) of analyte in toluene was injected into GC system with split/splitless injector and flame ionization detector. The inlet temperature was 250 °C and the split ratio was 10:1. The carrier gas was helium at 2.5 ml/min constant flow. The oven was programmed to start at 250 °C, hold for 5 min, followed by increasing temperature at a rate of 5 °C/min up to 260 °C, and then holding again for 8 min. Total time for gas chromatographic determination was 15 min. The detector was set at 300 °C with 200 ml/min air flow, 80 ml/min hydrogen flow, and 40 ml/min helium makeup flow.

For TMZ, stability in DCH was studied first to be able to calibrate the in vitro release data. 3% DCH loaded with 2% TMZ were stored at ambient temperature for different periods (0, 1, 2, 4, 8, 12, 24 or 48 h). The TMZ was then extracted from the DCH with ethyl acetate. The organic layer was transferred into a 15 ml conical polypropylene test tube, the solvent was evaporated to dryness under a stream of nitrogen at 45°C, and the residue was dissolved in 200 μl mobile phase. The samples were transferred to autosampler vial inserts and 5 μl of each was injected onto the reverse phase C18 column for HPLC analysis using an Agilent 1100/1200 HPLC system equipped with G1361A preparative pumps, a G1314A autosampler, a G1314A VWD, and a G1364B automated fraction collector. The mobile phase consisted of MeOH:0.5% HOAc in water (20:80, v/v) and was delivered at 1.1 ml/min. For study of release from DCH over time, TMZ was extracted from an aliquot of dialysate with ethyl acetate, and transferred into a 15 ml conical test tube. The solvent was evaporated to dryness and the residue was dissolved in 200 μl mobile phase for HPLC analysis described as above.

2.5. In vivo delivery to CNS of hydrophobic cargo in DCH

2.5.1. Preparation of Tamoxifen in DCH for in vivo injections—Tamoxifen (m.w. 371.51; Sigma-Aldrich, USA) was dissolved in ethanol at 5 μg/μl. $K_{180}A_{30}$ was dissolved at 15 mg/ml in a 90% ethanol and 10% water mixture to yield a clear solution. $K_{180}A_{30}$ and tamoxifen samples were mixed together at a desired ratio (to give final concentrations of 3% $K_{180}A_{30}$ and 0.2% tamoxifen) and then transferred to a flat bottom vial having a 1cm diameter. After evaporating the solvent under vacuum to dryness, a homogeneous transparent film formed on the bottom of the vial. Dried DCH films were reconstituted to yield concentrations of 3% in $K_{180}A_{30}$ on wt/vol basis using PBS. The final DCH preparation consisted of 3% $K_{180}A_{30}$ loaded with 0.2% Tamoxifen, which exhibited in a G' of 47 Pa. As a control we used unloaded 3.5% $K_{180}A_{30}$, which had a comparable G′ of 51 Pa.

2.5.2. Animals—*In vivo* experiments were conducted using transgenic mice obtained from JAX Laboratories (Bar Habor, Maine). GLAST-CreERT mice (JAX strain Tg(Slc1a3-cre/ERT)1Nat/J, stock # 012586) were crossed with Ai38 reporter mice (JAX strain B6;129S-*Gt(ROSA)26Sortm38(CAG-GCaMP3)Hze*/J; stock # 014538). Offspring expressing both transgenes were referred to as GLAST-CreERT-Ai38 reporter mice and were used for experiments. Mice were housed in a 12 hour light/dark cycle in an SPF facility with controlled temperature and humidity and allowed free access to food and water, and all surgical procedures and experiments were conducted according to protocols approved by the Chancellor's Animal Research Committee of the Office for Protection of Research Subjects at UCLA.

2.5.3. Surgical Procedures—All surgical procedures were performed under sterile conditions with isoflurane in oxygen-enriched air as the general anesthesia and using an operating microscope (Zeiss, Oberkochen, Germany) and rodent stereotaxic apparatus (David Kopf, Tujunga, CA). For injections into the caudate putamen nucleus, the skull was exposed and a burr hole was drilled with a high speed dental drill. Sterile solutions of 2μl of DCH were injected stereotaxically into the center of the caudate putamen nucleus using the target coordinates of 0.5 mm anterior to Bregma, 2.0 mm lateral to Bregma and a depth of 3.0 mm below the cortical surface. For injections into the spinal cord, mice were given crush spinal cord injuries (SCI) as described in detail elsewhere [13, 16, 17]. Two days after SCI, DCH were injected into the center of the clearly visible SCI lesion immediately lateral to the central dorsal vein and to a depth of 0.6 mm below the spinal cord surface. All injections were made at a speed of 0.2μl per minute using glass micropipettes (pulled and ground to 25 to 50 μm tips) connected via specialized connectors and high pressure tubing (Kopf and Hamilton) to a 10μl syringe (Hamilton) under the control of a microinfusion pump (Harvard Instruments). All animals were given analgesic prior to wound closure and every 12 hr for at least 48 hr post-surgery.

2.6. Histological procedures

At survival times of 21 days after forebrain or spinal cord injections, mice received terminal anesthesia by barbiturate overdose and were perfused transcardially with PBS followed by 10% formalin in PBS and prepared for histological processing as described in detail elsewhere [13, 16, 17]. Briefly, brains or spinal cords were removed, post-fixed overnight and cryoprotected in buffered 30% for at least 2 days. Thirty or 40 μm coronal frozen sections were prepared using a cryostat microtome (Leica, Nussloch, Germany) and processed for fluorescence immunohistochemistry. Primary antibodies were: rabbit anti-GFAP (1:1500; Dako); goat anti-GFP (1:1000, Novus). Anti-rat and anti-rabbit secondary developing antibodies were conjugated to Alexa 488 (Molecular Probes) or to Cy3 (Vector Labs and Chemicon), and 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; 2ng/ml; Molecular Probes) was used as a general nuclear stain. Sections were coverslipped using ProLong Gold anti-fade reagent (Invitrogen, Grand Island, NY). Stained sections were examined and photographed using fluorescence microscopy and scanning confocal laser microscopy (Zeiss, Oberkochen, Germany).

3. Results

3.1. In vitro studies

3.1.1. Loading and release of cholesterol—In an initial study to test if small hydrophobic molecules can be dissolved and loaded into DCH, we examined the ability of 3% $K_{180}L_{20}$ DCH to dissolve cholesterol. This DCH formulation was previously found to be useful as an injectable hydrogel capable of good tissue integration, as well as depot formation for release of hydrophilic protein cargos [13]. Here, cholesterol was chosen due to its low water solubility, similarity in size to many hydrophobic therapeutics, its low cost, stability, and ability to be readily assayed. Because cholesterol, like many therapeutic small molecules, is sparingly soluble in water, its direct dissolution into preformed DCH, which in this case is 97 % water, was found to be slow and inefficient. To assist dissolution of cholesterol in DCH, we developed a protocol to first prepare separate homogeneous solutions of cholesterol and $K_{180}L_{20}$ in 90:10 ethanol:water, followed by mixing in desired ratios to give homogeneous mixtures, and then solvent evaporation to yield a solvent-free, clear amorphous film. Rehydration of this film with water, or aqueous buffer, afforded a transparent DCH formulation containing dissolved cholesterol. The amounts of cholesterol were varied to determine the maximum loading limit, the highest cholesterol concentration that yielded a transparent and homogeneous DCH sample, which was found to be 2% (w/v)

cholesterol in 3% $K_{180}L_{20}$ in water. The amount of cholesterol that could be loaded in DCH was substantially greater than its water solubility of ~1×10^{-5}%. Above the loading limit, precipitates of cholesterol were observed to form in the DCH samples upon aqueous rehydration. At or below that maximum loading limit, the cholesterol loaded DCH were found to be stable, with no changes in homogeneity, transparency, or mechanical properties when stored at 4 °C or 25 °C for weeks.

When cholesterol is dissolved in DCH, it likely segregates into the assembled hydrophobic domains of the block copolypeptides. Since these same assemblies give rise to DCH physical properties, namely hydrogel formation, intercalation of cholesterol was expected to have some effect on the gel storage modulus, G′, of DCH formulations. Before measuring rheological properties of DCH loaded with cholesterol, we examined if our new processing method, where DCH were treated with ethanol/water, had any effects on DCH physical properties by itself. DCH consisting of 3% and 4% $K_{180}L_{20}$ in Millipore water were prepared using two different methods: direct dissolution of $K_{180}L_{20}$ in water, or dissolution of $K_{180}L_{20}$ in ethanol:water (90:10), followed by solvent evaporation and then film rehydration with Millipore water (Fig. 2A). Rheology measurements on these two sets of samples revealed that all samples formed hydrogels, and that the more highly concentrated samples gave stiffer gels (higher G′), as expected from earlier work. The only differences were that the ethanol:water processing method gave DCH with slightly lower G′, and slightly higher loss moduli, G″, as compared to samples prepared by direct dissolution in water (Fig. 2A). Although the ethanol:water processing method does affect DCH properties, the effect was found to be small and predictable, and could be countered by slight adjustments in DCH concentration if desired (*vide infra*).

To study the effects of cholesterol on DCH rheological properties, measurements were made on 3% $K_{180}L_{20}$ that had been loaded with different amounts of cholesterol up to the loading limit of 2% in water (Fig. 2B). It was found that dissolution of a small amount of cholesterol (0.1%) in this DCH resulted in an increase in G′, in effect stiffening the hydrogel, while increasing amounts of cholesterol resulted in a progressive decrease in G′ to about half of the original value (Fig. 2B). Although high concentrations of cholesterol did weaken the DCH significantly, small increases in DCH concentration could readily counteract this change as needed since G′ of cholesterol loaded $K_{180}L_{20}$ were found to increase significantly with copolypeptide concentration (Fig. 2C), similar to cholesterol-free DCH.

In addition to concentration, physical properties of DCH can also be tuned by variation of copolypeptide composition. In particular, the lengths and amino acid make-up of the hydrophobic segments of DCH are known to strongly influence their G′ values [9]. Since the use of amino acid components with different hydrophobicities, and of different lengths, may also be means to adjust the cholesterol loading capacity of DCH, we prepared a series of DCH compositions (Table 1) and measured their maximum cholesterol loading capacity as described above (Fig. 2D). All DCH carriers were prepared at similar concentrations, which allowed the influences of amino acid composition and hydrophobic segment length to be studied. It was observed that longer hydrophobic segments (e.g. $K_{180}L_{30}$ vs. $K_{180}L_{20}$) allowed dissolution of greater amounts of cholesterol, which makes intuitive sense since there is more hydrophobic volume in the hydrogel. Also observed was that cholesterol loading capacity increased with decreasing amino acid hydrophobicity (F < L < L/A < A), with alanine based DCH able to dissolve the greatest amounts of cholesterol. This trend may seem counterintuitive, yet it is important to note that in order for cholesterol to dissolve into the gel, the packing of hydrophobic α-helical segments in the DCH must be partially disrupted. The phenylalanine segments form rigid helices that interact strongly with each other in water, while the α-helical alanine segments are more flexible and less strongly associated. Thus, hydrophobic molecules may load more easily into the alanine based gels

since these have more deformable and more easily disrupted hydrophobic regions. Overall, the alanine based DCH were found to have the best capacity for loading cholesterol. While $K_{180}A_{20}$ forms a less rigid hydrogel (lower G′) compared to $K_{180}L_{20}$ samples used earlier, straightforward preparation of samples with longer hydrophobic segments (e.g. $K_{180}A_{40}$) can correct for this to give materials with comparable physical properties and higher cholesterol loading capacity (Fig. 2E).

Once dissolved in DCH, cholesterol was found to be released over time when the hydrogels were placed in constant volume dialysis cassettes and dialyzed against PBS buffer containing BSA at 37 °C [8]. BSA was added to the dialyzate to capture released cholesterol, which would otherwise precipitate in the aqueous media. Cholesterol release from different concentration $K_{180}L_{20}$ DCH samples was monitored over time, and in all cases *ca*. 70–90% of the loaded cholesterol was released into the dialysate over a period of days, with the remainder still encapsulated in the DCH (Fig. 3A). Increasing the DCH carrier concentration resulted in slower release of cholesterol, which can be advantageous for avoiding a “burst” type release of molecules. The cholesterol release profile was found to also be controlled by the nature and size of the DCH hydrophobic segments. Least hydrophobic, alanine based $K_{180}A_{30}$ released cholesterol significantly faster than leucine based $K_{180}L_{20}$ or phenylalanine based $K_{180}F_{15}$, which released cholesterol the slowest of the three (Fig. 3B). In this experiment, the lengths of the hydrophobic domains were varied to give samples with similar stiffness (G′), which is known to also affect diffusion of molecules through the hydrogels [13]. Hydrophobic segment length also affected cholesterol release rate. A study of cholesterol release from 3% $K_{180}A_{20}$, $K_{180}A_{30}$ and $K_{180}A_{40}$ formulations showed that increasing hydrophobic alanine segment length resulted in slower cholesterol release (Fig. 3C). Overall, these data show that the many adjustable parameters available within DCH can be used to predictably tune both loading capacity and release profiles of cholesterol in these hydrogels.

3.1.2. Loading and release of other hydrophobic molecules—After identifying DCH parameters and formulations that are suitable for loading and release of cholesterol, we further tested the ability of DCH to load other hydrophobic molecules by dissolving increasing amounts of different poorly water soluble bioactive molecules in either 3% $K_{180}L_{20}$ or 3% $K_{180}A_{30}$ DCH formulations using the ethanol/water method (Table 2). All molecules studied were found to have much greater solubility in DCH compared to PBS buffer, or even PBS buffer mixed with organic solvent (Table 2). It is worth noting that although ethanol is used in our loading method, it is removed during the process so that the final DCH and test molecule samples contain no organic solvents. Similar to the cholesterol data, all of the molecules were found to have greatest solubility in the alanine based DCH, indicating these gels are useful for dissolving a wide range of hydrophobic compounds. For most of the molecules studied, their solubility was approximately ten times greater in DCH compared to PBS buffer, which allows a substantial enrichment in local concentration within the hydrogel depot.

To quantify the release of a representative bioactive hydrophobic molecule from DCH, we studied the release of temozolomide (TMZ). TMZ is an orally delivered alkylating agent used to treat a variety of cancers including glioblastoma multiforme. TMZ is sparingly soluble in water and is also unstable and decomposes in aqueous environments, especially at elevated pH [18]. We first determined that TMZ dissolved in DCH in PBS buffer pH = 7.4 and remains stable at 20 °C for nearly 2 weeks. Similar to cholesterol, TMZ (2%) dissolved in $K_{180}L_{20}$ was found to also release into dialyzate *in vitro* over a number of days without decomposition (Fig. 4A). As before, release rates were slowed in a predictable manner by increasing the DCH concentrations. To further demonstrate the possibilities of DCH tunability for controlling release of hydrophobic molecules, we compared the release

profiles of TMZ and cholesterol, both dissolved at 2% in 3% $K_{180}L_{20}$ DCH (Fig. 4B). It was observed that the more polar TMZ was released faster than cholesterol from DCH. However, adjustment of the TMZ formulation by increasing the DCH concentration from 3% to 4% $K_{180}L_{20}$, was able to correct for the molecular differences between TMZ and cholesterol so that both had near identical release profiles (Fig. 4C). These results show that a range of different hydrophobic, bioactive molecules can be loaded into DCH formulations, and their release from the hydrogel depots can be predictably tuned by adjustment of DCH compositions and concentrations.

3.2. In vivo studies

3.2.1. In vivo experimental design—Our next objective was to determine as a proof of principle, whether DCH depots injected into the CNS could be used to deliver a bioactive hydrophobic molecule to exert an expected effect *in vivo*. To do so, we took advantage of the availability of a widely employed transgenic mouse model in which the hydrophobic small molecule, tamoxifen, is used to activate transcription from gene constructs that include a modified version of the promoter region of the estrogen receptor (ER) gene [1]. Tamoxifen interacts with estrogen receptors and is used as an antitumor agent in for certain kinds of estrogen sensitive cancers [19]. Tamoxifen is a synthetic molecule not expressed by mammals, making it a useful tool for transgene regulation. Tamoxifen sensitive estrogen receptor gene constructs (ERT) are efficient tools to regulate gene expression, which occurs only when tamoxifen is present [20]. For our experiments, we used transgenic mice in which (i) expression of the enzyme Cre-recombinase is under regulation of the GLAST promoter controlled by ERT, and (ii) a GFP-related reporter molecule is under regulation of a loxP-flanked STOP signal. These mice are referred to here as GLAST-CreERT-Ai38 mice, and the GFP-related reporter protein is GCaMP3, which is used to detect changes in intracellular calcium and can be detected by using immunohistochemistry for GFP [21]. When GLAST-CreERT-Ai38 mice are given tamoxifen, expression of Cre-recombinase is activated, which in turn cleaves the STOP signal and activates expression of the GFP-related reporter in those cells that would normally express GLAST. Cells that express GLAST in the adult CNS are of the astroglial cell lineage, including astrocytes and adult neural stem cells in the healthy CNS, as well as reactive and scar forming astrocytes after CNS injury [22, 23]. Using this reporter system, we tested the ability of DCH depots injected into the CNS to deliver hydrophobic tamoxifen to exert the predictable effect of activating reporter gene expression (a) in astroglia and adult neural stem cells in the healthy mouse forebrain (Fig. 5) and (b) in reactive and scar forming astroglia after spinal cord injury (Fig. 6). For DCH samples, we used 3% $K_{180}A_{30}$ loaded with 0.2% tamoxifen, which exhibited in a G′ of 47 Pa. As a control we used unloaded 3.5% $K_{180}A_{30}$, which had a comparable G′ of 51 Pa.

3.2.2. Tamoxifen release from DCH depots in healthy forebrain—Reporter mice expressing GLAST-CreERT-Ai38 were injected into the caudate putamen with 2 μl of DCH that either was, or was not, loaded with tamoxifen (Fig. 5A). Mice were perfused for histological evaluation 21 days after DCH injection. DCH deposits with or without tamoxifen exhibited biocompatibility in a manner similar to that described previously [8, 13] and DCH injected mice exhibited no detectable adverse effects at any time during the duration of the experiments. In mice injected with unloaded DCH, there was no detectable expression or staining for GFP-reporter protein. In mice injected with DCH loaded with tamoxifen, essentially all of the astroglia cells in the immediate vicinity of the DCH depots expressed immunoreactive GFP reporter protein (Fig. 5C1) and could be identified as astroglial cells on the basis of double staining with glial fibrillary acid protein (GFAP) (Fig. 5C1–C3), the canonical marker of astroglia [23].

Along the medial border of the caudate putamen is a brain region referred to as the subventricular zone, which contains a population of adult neural stem cells that remain active and retain multipotent neural potential throughout life in mammals [24]. These adult neural stem cells belong to the family of astroglial cells and express astroglial markers such as GFAP and GLAST [24, 25]. Because of the proximity of the subventricular zone to the DCH depots releasing tamoxifen, we tested the ability of tamoxifen to activate reporter gene expression in these adult neural stem cells, which in the uninjured brain give rise to proliferating progenitor cells and neuroblasts that migrate through the rostral migratory stream (RMS) (Fig. 5B) and give rise to a substantial number of neurons and a small number of glia in the olfactory bulb (OB) [24, 25]. In GLAST-CreERT-Ai38 reporter mice that received tamoxifen loaded DCH depots into the caudate putamen near the subventricular zone (Fig. 5B), we consistently found many GFP-reporter expressing cells in the subventricular zone and along the rostral migratory stream (not shown), as well as scattered, but regularly present, newly born neurons and a few newly generated astroglia that expressed the GFP-reporter protein in the olfactory bulb (Fig. 5D,E1–E3). Reporter gene expression was not observed in any of these cell types in reporter mice with injections of unloaded DCH (not shown).

3.2.3. Tamoxifen release from DCH depots in spinal cord lesions—After focal injury in the brain or spinal cord, astrocytes form scars around areas of damaged tissue and exhibit a graded reactivity that tapers with distance from the lesion [16, 17, 23]. These scar-forming and reactive astroglia selectively express GFAP, GLAST and other astrocyte markers [22]. We tested whether tamoxifen delivered via DCH could modify gene expression in reactive and scar forming astroglia adjacent to focal CNS injuries. To do so, we injected DCH depots into the center of the lesion core two days after spinal cord injury in GLAST-CreERT-Ai38 reporter mice (Fig. 6A). We found that tamoxifen released from DCH depots injected into the lesion core strongly activated reporter gene expression in most if not all scar forming astroglia that immediately surrounded the lesion on all sides (Fig. 6B1–B3). In addition, we found that reporter gene expression was also activated for up to several mm away from the lesion in the majority of the more moderately reactive astroglia that surround such lesions (Fig. 6C1–C3,D1–D3). As expected [17], these more moderately reactive astroglia located at some distance away from the lesion retained their basic morphology and preserved their individual cellular domains (Fig. 6D1–D3).

4. Discussion

In this study, we show that DCH can efficiently load and provide sustained release of various hydrophobic compounds. *In vitro*, we found that different DCH formulations exhibited different loading capacities and different rates of release, and that DCH have many adjustable parameters that can be used to predictably tune both loading capacity and release profiles of hydrophobic cargo. *In vivo*, we found that DCH depots injected into healthy or injured CNS can deliver bioactive hydrophobic compounds that have predictable effects on gene expression of local cells. Our findings show that DCH can function as vehicles for local CNS delivery of hydrophobic compounds for investigative and potential therapeutic applications.

4.1. Facile tunability of DCH

CH are highly versatile hydrogels whose amino acid compositions can be altered in various ways while retaining their basic gel properties [9–12]. This versatility affords a number of advantages. For example, DCH component amino acids and chain lengths, as well as DCH concentration, can all be altered to achieve desired properties, including loading capacities and release profiles for different types of cargos. In the first part of this study we determined

the loading capacity of DCH with representative hydrophobic compounds such as the steroid, cholesterol, and the anti-cancer drugs, TMZ and taxol. Starting with the DCH formulation, 3% $K_{180}L_{20}$, which we have characterized extensively in previous studies [8, 13], we found that 3% $K_{180}L_{20}$ could dissolve hydrophobic compounds, and that loading capacity could be increased simply by increasing the concentration of $K_{180}L_{20}$. We then tested the effects of altering DCH component amino acids on DCH ability to load hydrophobic cargo. When hydrophobic compounds are dissolved in DCH, they likely segregate into the assembled hydrophobic domains of the block copolypeptides. We hypothesized that altering the amino acid composition and chain length of the hydrophobic domains would alter the capacity of DCH to load hydrophobic compounds. We found this to be case, such that alanine chains were more efficient than leucine chains, and that hydrophobic loading capacity increased in proportion with increasing alanine segment chain length.

We also examined the effects of hydrophobic cargos on DCH physical properties. Addition of different types of molecules to hydrogels, including potential cargo, will alter their mechanical properties [26]. Because mechanical properties such as stiffness (G′) and viscosity (G″) influence the interaction of biomaterials with host tissues [27, 28], it is important to match hydrogel properties to those of the host tissue into which they are injected as *in vivo* delivery vehicles. Another advantage of DCH is that their mechanical properties, such as G′ and G″, can finely and incrementally be adjusted by altering the ratio of hydrophilic to hydrophobic residues, changing component amino acids or varying the sample concentration [9–12]. In this manner, DCH can readily be tuned to retain optimal physical properties that may have been altered by addition of cargo molecules. We have previously found that DCH prepared to have G′ within the range of 50–100 Pa, which is just below that of healthy brain tissue at ~200 Pa, exhibited good depot formation and good compatibility with CNS tissue, with no detectable toxicity and full degradation of depots within several months after injection [8, 13]. In this study we found, as expected, that DCH G′ and G″ were altered by admixture with hydrophobic compounds but that G′ and G″ could easily be tuned to achieve a wide variety of ranges by altering amino acid chain length and sample concentration. Thus, our *in vitro* studies showed that although mechanical properties of DCH are altered by loading with hydrophobic cargos, these properties of DCH mixtures can readily be tuned to achieve previously determined optimal ranges.

The ability to control and adjust rates of cargo release from delivery vehicles is desirable. We therefore quantified the release rates of different hydrophobic cargo *in vitro*, and determined the effects of altering DCH properties on release rates. We found that cargo release rates could be altered in various ways by altering DCH concentration or composition. For example, either increasing DCH concentration, or increasing DCH hydrophobic chain length, both resulted in significantly decreased release rates. Taken together, our *in vitro* findings demonstrate the facile and predictable tunability of DCH to achieve a wide range of loading capacities and release profiles of hydrophobic cargos as might be required by different applications, while retaining CNS compatible physical properties.

4.2. Local CNS delivery of hydrophobic molecules via DCH

In the second part of this study, we tested the ability of DCH depots injected into the healthy or injured CNS to deliver hydrophobic molecules that exert predictable biological effects on local CNS cells. To do so, we used a well-characterized transgenic mouse model in which the small hydrophobic molecule, tamoxifen, regulates the expression of transgenes [1]. In the specific model that we used, tamoxifen interacts with a transgene construct to activate the expression of a GFP-related reporter protein in a specifically targeted subset of CNS

cells, astrocytes, which are essential regulators of homeostasis in healthy tissue and critical responders to CNS injury and disease [23]. We found that in non-transgenic animals, as well as in transgenic animals given DCH depots that did not contain tamoxifen, there was no reporter gene expression. In contrast, transgenic mice given DCH depots containing tamoxifen exhibited easily detected reporter gene expression in local astrocytes and in nearby astrocyte-related progenitor cells, in both healthy and injured tissue, as predicted by the transgenic targeting. These findings show that DCH depots injected in the healthy or injured CNS are able to deliver hydrophobic molecules that exert predictable effects on the gene expression of nearby CNS cells.

4.3. DCH depots in CNS for investigative and therapeutic applications

Many hydrophobic small molecules, including steroid hormones and drug candidates, are of potential experimental or therapeutic interest because of their abilities to regulate or influence a wide range of activities in CNS cells, including gene expression [1, 2], epigenetic activities [4], signal transduction [5] and other cellular mechanisms [6]. Investigation of such compounds in the CNS is often limited by lack of context appropriate delivery options. Because the compounds exhibit effects on cells throughout the body or CNS, local delivery is required to achieve effective concentrations while restricting activity to specific target regions and limiting side effects in other areas. There is a need for delivery mechanisms that can reliably and safely achieve local, site-specific, prolonged and controlled delivery into the CNS of different kinds of hydrophobic molecules for both experimental and therapeutic purposes. Hydrogels that can be injected as local depots represent a promising means of achieving local delivery of bioactive compounds in the CNS [7]. We have previously shown that the synthetic hydrogels, DCH, can safely be injected into the CNS [8], and can serve as depots that provide sustained delivery within the blood-brain barrier of hydrophilic bioactive protein growth factors that exert predicted, quantifiable, specific effects on local nerve cells over a prolonged subacute time [13]. In the present study we extend these findings by showing that DCH depots injected in the healthy or injured CNS are able to deliver hydrophobic molecules that exert predictable effects on the gene expression of CNS cells in a locally restricted manner. It is noteworthy that DCH may be particularly useful for local molecular delivery in experimental studies of CNS injury models in which there are focal lesions of severely damaged tissue, such as after local contusive trauma or stroke. Our present findings indicate that injection of DCH depots into such lesions cause little or no additional damage and can be used to deliver molecules that regulate local cells involved in the injury and repair responses, such as scar forming astrocytes. In this regard, the fine tunability of DCH may be an advantage over many other materials. After injury, damaged CNS tissue in and around such lesions will be edematous and may be vulnerable to further damage by infusion of materials that are too stiff or rigid. It will be important to fine-tune any injected material to the low G′ of the damaged and surrounding CNS tissue. This type of tuning is easily done with DCH. DCH depots placed into lesions after CNS trauma or stroke could be used to deliver sustained release of local molecular gradients over prolonged subacute periods, which might beneficially influence tissue repair or the plasticity and rehabilitation of the surrounding neural tissue. There are now realistic targets for influencing numerous specific mechanisms in the repair and rehabilitation processes that take place after CNS injury by delivering specific molecules to exert predictable effects, including protein growth factors or small hydrophobic regulators of gene expression. Our findings suggest that DCH can serve as safe and efficient depots that can provide prolonged release of bioactive molecules at effective concentrations, including both hydrophilic and hydrophobic compounds, and can deliver these molecules in a locally restricted manner without causing unwanted side effects on cells in other CNS regions or other tissues. Used in this manner, DCH-depots represent powerful tools for experimental

investigation of cellular and molecular mechanisms and to test compounds for therapeutic potential in locally restricted CNS sites.

5. Conclusions

In this study we tested whether DCH could serve as vehicles for delivery of hydrophobic compounds to locally restricted sites in the CNS. Our *in vitro* results show that different kinds of hydrophobic compounds can be loaded into DCH and released over prolonged and controllable time frames, and that both the loading capacity and release profiles can be predictably, easily and finely tuned by adjustment of DCH composition and concentration. *In vivo*, we show that DCH depots can deliver hydrophobic molecules that alter gene expression of CNS cells in a locally restricted and predicted manner after injection into the forebrain or after spinal cord injury. We have previously shown that DCH injected into the CNS are non-toxic and fully biodegradable. Our findings show that DCH depots can serve as efficient, versatile and readily tunable vehicles that can provide locally restricted, sustained and controlled delivery of bioactive hydrophobic molecules for investigative and therapeutic applications in the healthy and injured CNS.

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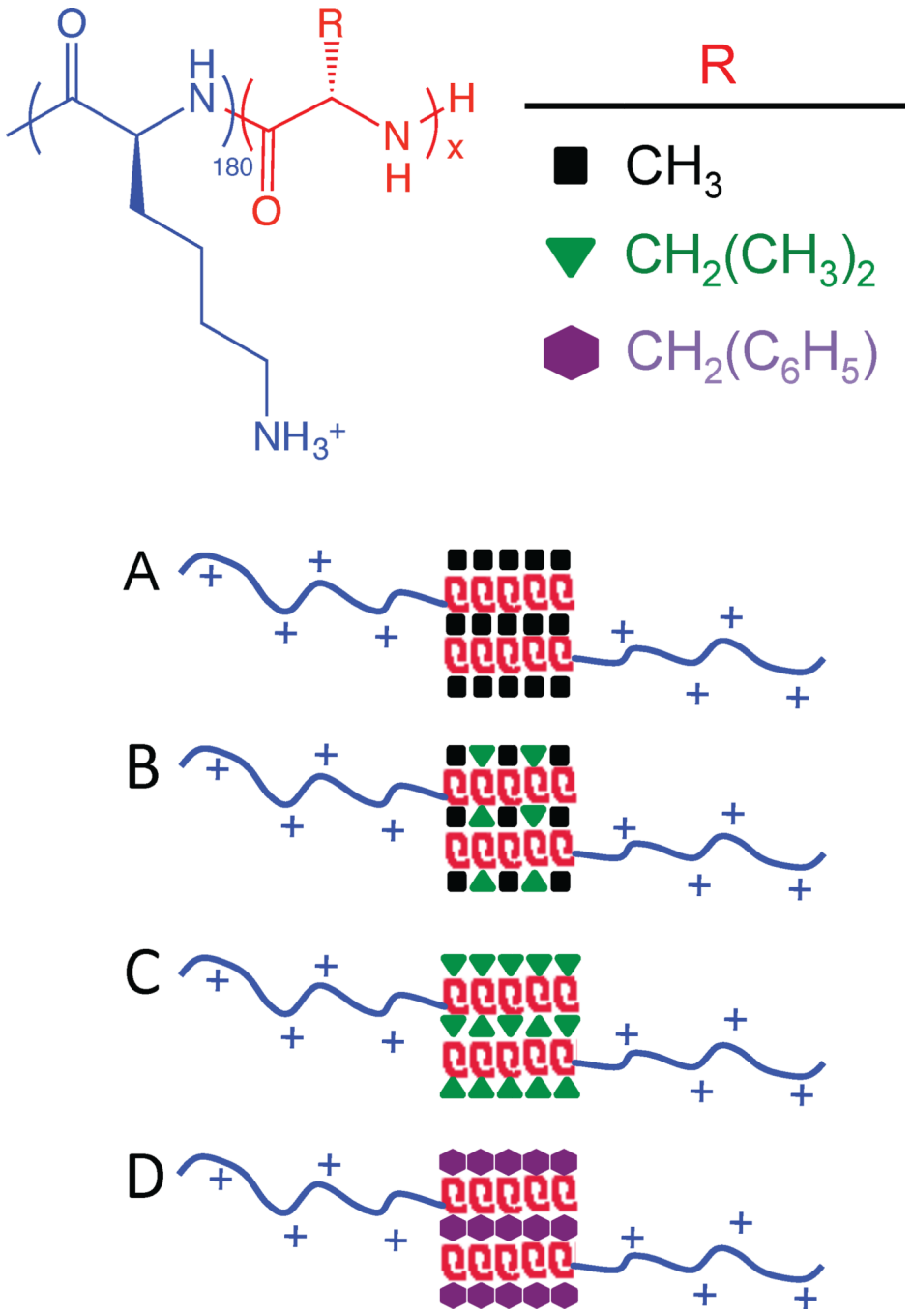
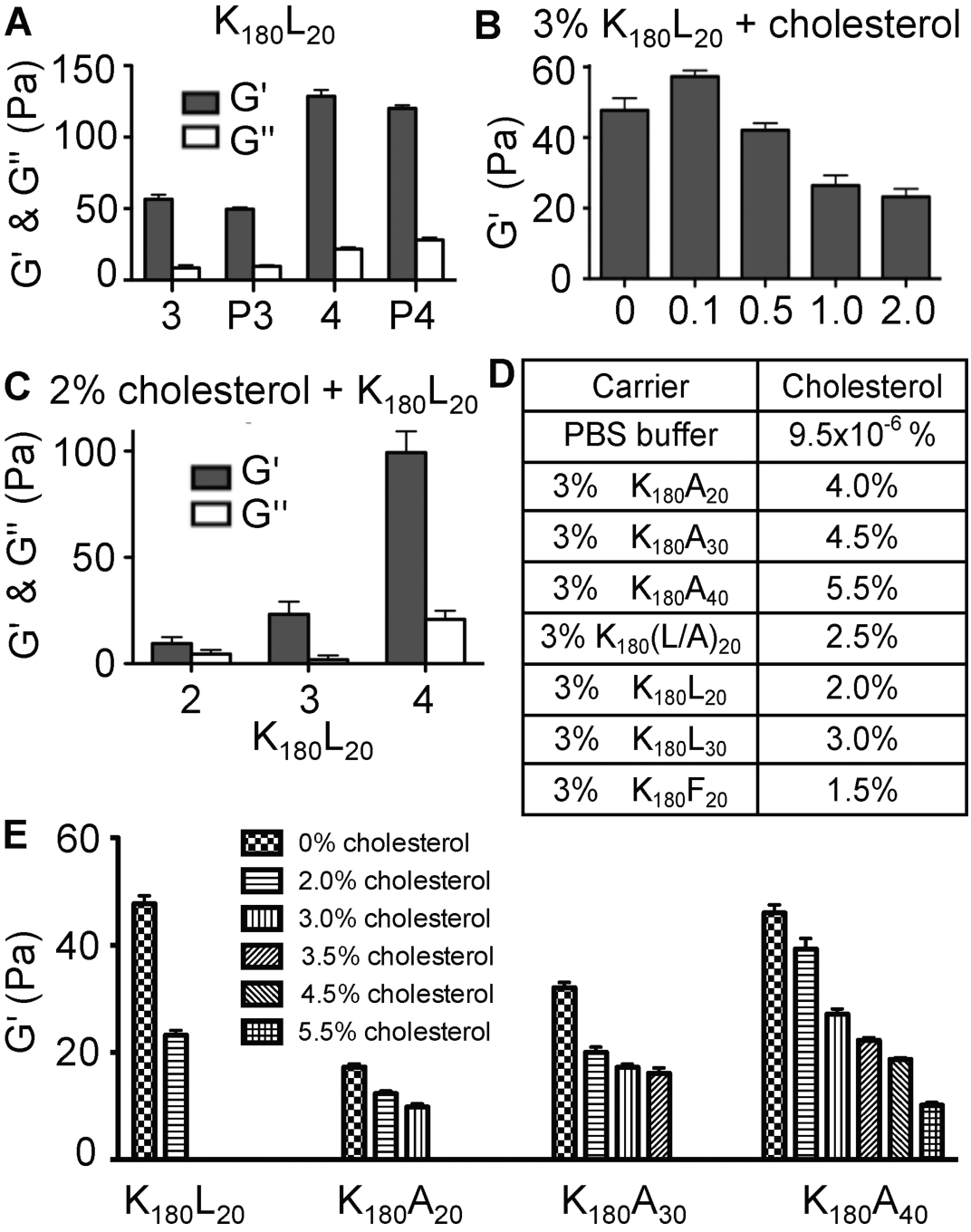


Figure 1. Schematic representations of DCH compositions and structures

Amphiphilic DCH are composed of variable-length chains of hydrophilic (blue) and hydrophobic (red) amino acids. In this study, compositions of the hydrophobic domains were varied by incorporating different amino acids (different R groups) that are shown in the schematic as black squares representing L-alanine, green triangles representing L-leucine, and purple hexagons representing L-phenylalanine. (A) Representation of K_mA_n DCH. (B) Representation of $K_m(L/A)_n$ DCH. (C) Representation of K_mL_n DCH. (D) Representation of K_mF_n DCH.



Carrier	Cholesterol
PBS buffer	$9.5x10^{-6}$ %
3% $K_{180}A_{20}$	4.0%
3% $K_{180}A_{30}$	4.5%
3% $K_{180}A_{40}$	5.5%
3% $K_{180}(L/A)_{20}$	2.5%
3% $K_{180}L_{20}$	2.0%
3% $K_{180}L_{30}$	3.0%
3% $K_{180}F_{20}$	1.5%

Figure 2. Loading of hydrophobic cargo molecules in DCH and tuning of physical properties

(A) Graph showing the effects on stiffness (storage modulus, G′) and viscosity (loss modulus G″) for different concentrations of $K_{180}L_{20}$ by formulation either via direct dissolution in water or use of ethanol/water processing. On the x-axis, 3 and 4 represent 3% and 4% DCH concentrations for direct dissolution, and P3 and P4 represent 3% and 4% DCH concentrations for ethanol/water processing. (B) Graph showing the effects on stiffness of a 3% $K_{180}L_{20}$ sample that has been loaded with different concentrations of cholesterol. On the x-axis, numbers are concentrations of cholesterol. (C) Graph showing the effects on stiffness and viscosity for different concentrations of $K_{180}L_{20}$ loaded with 2% cholesterol. On the x-axis, numbers are concentrations of $K_{180}L_{20}$. (D) Data showing

maximum concentrations of cholesterol that can be homogeneously loaded into different DCH formulations. (E) Graph showing the effects on stiffness (G′) of 3% DCH samples that have been loaded with different concentrations of cholesterol. Pa, Pascal units.

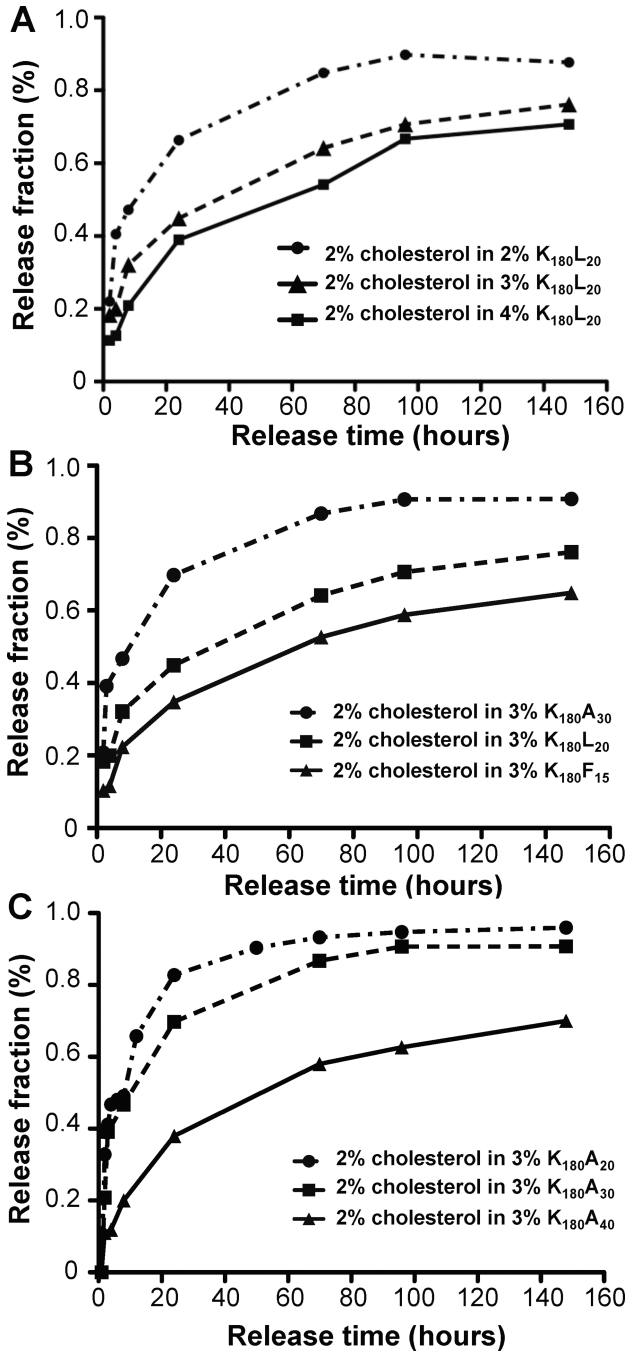


Figure 3. Cholesterol release kinetics from DCH *in vitro*

Graphs showing amount of cholesterol released (release fraction) over time from different DCH formulations at 37 °C in PBS buffer containing 10 mg/ml BSA. (A) Cholesterol release from different concentrations of $K_{180}L_{20}$ loaded with 2% cholesterol. (B) Cholesterol release from different DCH formulations containing different hydrophobic amino acids loaded with 2% cholesterol. Hydrophobic segment lengths were chosen to give samples with similar G′ values. (C) Cholesterol release from 2% $K_{180}A_n$ formulations of different alanine segment lengths loaded with 2% cholesterol.

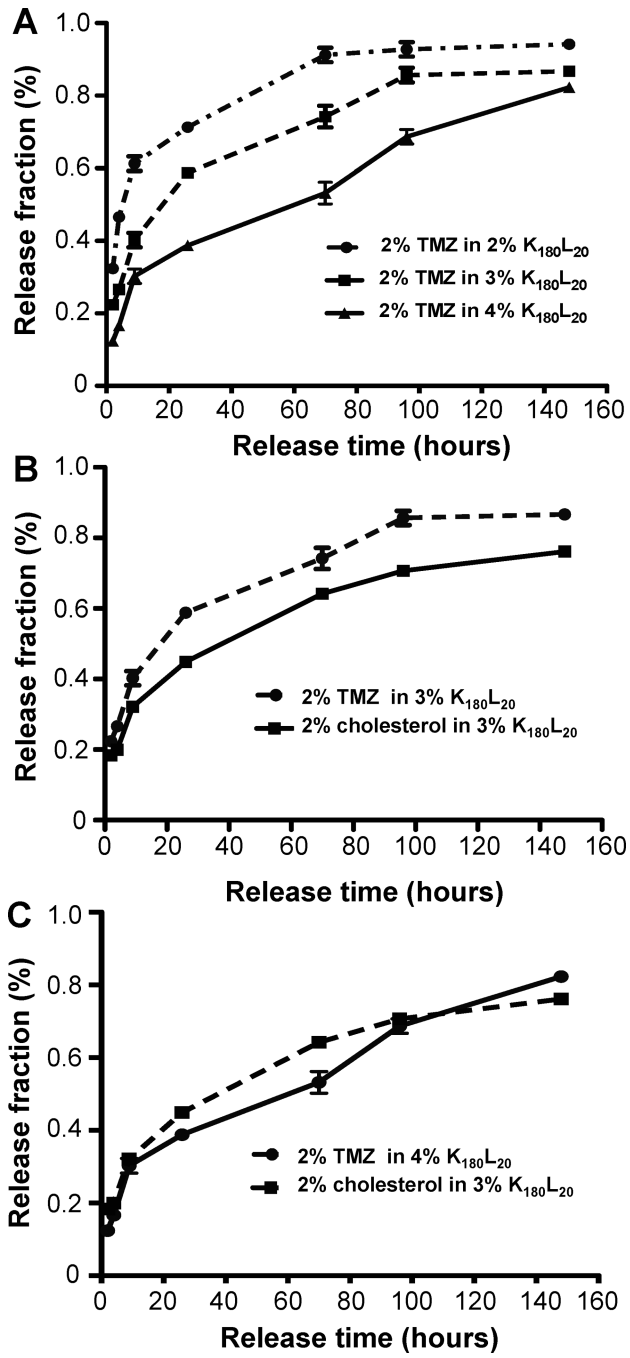


Figure 4. Effects of DCH concentration on release kinetics of cholesterol and TMZ from DCH *in vitro*

Graphs showing amount of temozolomide (TMZ) or cholesterol released (release fraction) over time from different $K_{180}L_{20}$ DCH formulations at 37 °C in PBS buffer containing 10 mg/ml BSA. (A) TMZ release from different concentrations of $K_{180}L_{20}$ loaded with 2% TMZ. (B) Comparison of 2% TMZ and 2% cholesterol release from 3% $K_{180}L_{20}$ formulations. (C) Comparison of 2% TMZ and 2% cholesterol release from different concentration $K_{180}L_{20}$ formulations, which illustrates ability to tune release rates to give similar profiles for different cargos.

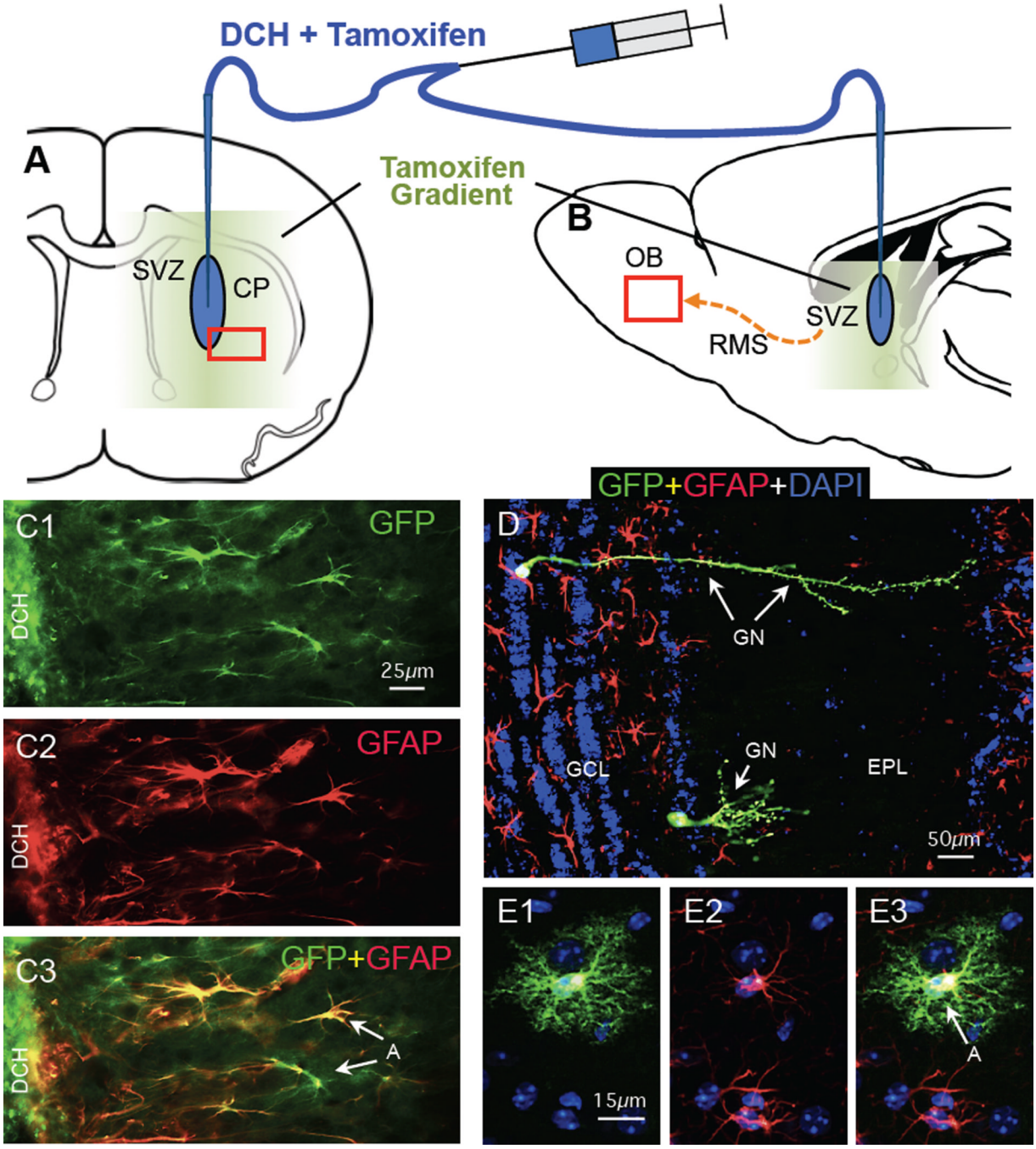


Figure 5. Tamoxifen released from DCH depots activates transgenic reporter gene expression in local forebrain astrocytes in caudate putamen (CP) and in adult neural stem cells (NSC) in nearby subventricular zone (SVZ)

(A) Schematic drawing of a coronal (frontal) section of the mouse forebrain showing the location of tamoxifen-releasing DCH depots injected into the CP. Red box shows the location of images shown in C. (B) Schematic drawing of a sagittal section of the mouse forebrain showing the location of tamoxifen-releasing DCH depots injected into the CP. Red dashed line demarks the approximate pathway of the rostral migratory stream (RMS) followed by progenitors and neuroblasts that migrate from the SVZ to the olfactory bulb (OB). Red box shows the approximate location in the OB of images shown in D and E. In

both A and B, note the proximity of the DCH depots to the SVZ. (C-E) Double labeling immunohistochemistry for the reporter protein GFP (green) and the astrocyte marker GFAP (red) and the nuclear marker DAPI in D,E. In C, note that many GFAP-positive astrocytes (A) near the DCH depot also express the GFP reporter protein and therefore appear yellow (arrows). In D, note that two granule neurons (GN) in the granule cell layer (GCL) of the OB are labeled with reporter GFP, but not GFAP. These neurons derive from NSC in the SVZ, whose progeny neuroblasts have migrated to the OB via the RMS. In E, note that occasional GFAP-positive astrocytes are also labeled with reporter GFP and also derive from NSC in the SVZ. EPL, external plexiform layer

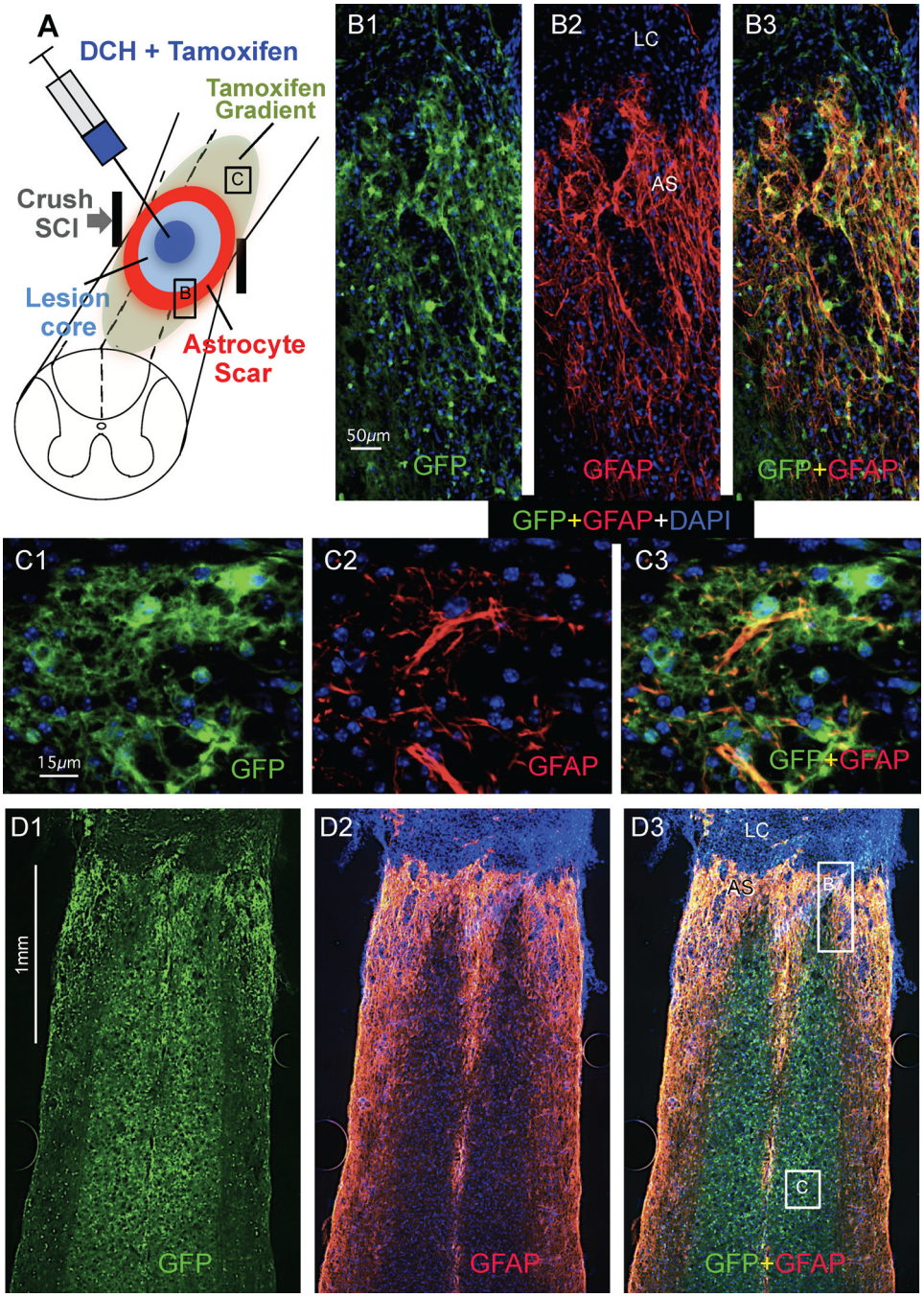


Figure 6. Tamoxifen released from DCH depots activates transgenic reporter gene expression in scar-forming astrocytes and in nearby hypertrophic reactive astrocytes adjacent to spinal cord injury (SCI)

(A) Schematic drawing of mouse spinal cord showing the location of tamoxifen-releasing DCH depots injected after crush SCI. Two boxes show the approximate location of images shown in B,C. (B–D) Double labeling immunohistochemistry for the reporter protein GFP (green) and the astrocyte marker GFAP (red), together with the nuclear marker DAPI in C,D. In B, note that most, if not all, of the elongated GFAP-positive scar-forming astrocytes in the astrocyte scar (AS) that lines the SCI lesion core (LC) express the GFP reporter protein and therefore appear yellow in the overlay (B3). In C, note that GFAP-positive

hypertrophic reactive astrocytes some distance distal to the SCI lesion also express the GFP reporter protein. (D) In this survey image, note that GFP-expression has been activated in GFAP-positive astrocytes up to several mm away from the DCH depot placed into the center of the SCI lesion. Two boxes show the approximate location of images shown in B,C.

Table 1

Synthesis and properties of DCH formulations

Predicted composition	M_n[(a)]	M_w/M_n[(b)]	K_m length[(a)]	Found[(c)] compostion	Yield[(d)] (%)	G′ of 3% polymer in PBS (Pa)
$K_{180}A_{20}$	47,400	1.05	K_{181}	$K_{181}A_{20}$	92	23
$K_{180}A_{30}$	47,320	1.06	K_{180}	$K_{180}A_{29}$	95	31
$K_{180}A_{40}$	47,320	1.06	K_{180}	$K_{180}A_{40}$	91	45
$K_{180}(L/A)_{20}$	47,400	1.05	K_{181}	$K_{181}(L/A)_{19}$	90	40
$K_{180}L_{20}$	47,300	1.09	K_{180}	$K_{180}L_{21}$	90	53
$K_{180}L_{30}$	47,300	1.09	K_{180}	$K_{180}L_{31}$	91	565
$K_{180}F_{15}$	47,400	1.05	K_{181}	$K_{181}F_{16}$	95	63
$K_{180}F_{20}$	47,400	1.05	K_{181}	$K_{181}F_{20}$	92	88

[a] M_n for initial protected K_m segment determined using GPC/LS.

[b] M_w/M_n = Polydispersity index.

[c] Determined using ^{1}H NMR analysis of deprotected samples.

[d] Total isolated yield of deprotected, purified block copolypeptide.

Table 2

Loading of hydrophobic molecules into DCH formulations

cargo / carrier	Ambroxol	Estradiol	Taxol	Temozolomide	Camptothecin	Tamoxifen
PBS buffer	0.4%	0.02%[a]	0.06%[b]	0.3%	0.1%	0.02%
3% $K_{180}L_{20}$	1%	0.5%	0.2%	2%	0.3%	0.1%
3% $K_{180}A_{30}$	2%	1%	0.5%	3%	1%	0.2%

[a] DMSO:PBS=1:4

[b] Ethanol:PBS=1:1