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A hydrogel composite system for sustained epi-cortical delivery of Cyclosporin A to the brain for treatment of stroke

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ABSTRACT

Stimulation of endogenous neural stem/progenitor cells (NSPCs) with therapeutic factors holds potential for the treatment of stroke. Cyclosporin A (CsA) is a particularly promising candidate molecule because it has been shown to act as a survival factor for these cells over a period of weeks both in vitro and in vivo; however, systemically-delivered CsA compromises the entire immune system, necessitating sustained localized delivery. Herein we describe a local delivery strategy for CsA using an epi-cortical hydrogel of hyaluronan-methylcellulose (HAMC) as the drug reservoir. Three methods of incorporating the drug into the hydrogel (solubilized, particulate, and poly(lactic-*co*-glycolic) acid (PLGA) microsphere-encapsulated) resulted in tunable release, spanning a period of hours to weeks. Importantly, PLGA-encapsulated CsA released from the hydrogel had equivalent bioactivity to fresh drug as measured by the neurosphere assay. Moreover, when CsA was released from the PLGA/HAMC composite that was injected on the cortex of adult mice, CsA was detected in the NSPC niche at a constant concentration for at least 24 days post-implant. Thus this hydrogel composite system may be promising for the treatment of stroke.

1. Introduction

Stroke is a traumatic neurological event caused by occluded or ruptured cerebral blood vessels that permanently disables approximately 5 million people every year [1]. Currently, stroke is treated with either tissue plasminogen activator or an endovascular mechanical device to promote revascularization [2]; however, there are no clinical therapies capable of repairing damaged brain tissue and restoring lost function, except through rehabilitation, which has limited benefits, relying on endogenous repair and plasticity. To further enhance repair, two cell based strategies have been investigated: stem cell transplantation [3] and endogenous stem cell stimulation. The latter requires delivery of exogenous factors to stimulate the neural stem/progenitor cells (NSPCs) in the subventricular zone (SVZ) of the brain to migrate to the injury site and differentiate into mature cell phenotypes, thereby restoring the lost tissue. Therapeutic factors investigated for this purpose include epidermal growth factor [4,5], erythropoietin [4], nerve growth factor [6], colony stimulating factor [7], basic fibroblast growth factor [6], and cyclosporin A (CsA) [8,9].

CsA holds particular promise as a neuroprotective and neuroregenerative agent as it has been shown to act directly on NSPCs to enhance their survival both in vitro and in vivo. When NSPCs were cultured with CsA in vitro, the number of neurospheres increased compared with controls [9]. Interestingly, the number of neurospheres derived from adult mice treated for 14 days with CsA via subcutaneously implanted osmotic mini-pumps was significantly higher than untreated controls [9]. Stroke-injured mice treated for 18 days with daily injections of CsA possessed a significantly smaller lesion volume compared with untreated controls [8]. Moreover, stroke-injured animals treated with systemic CsA for 32 days displayed some functional recovery, as measured by the foot fault test [8]. Taken together, these results demonstrate that sustained delivery of CsA has considerable potential as a tissue-regenerative molecule for stroke treatment.

The delivery of drugs to the brain poses a unique challenge due to the blood–brain barrier (BBB), which is composed of tight junctions formed by endothelial cells lining the cerebrovasculature that limit the transport of molecules. While CsA can cross the BBB, its diffusion is attenuated [10], requiring very high doses of CsA to achieve therapeutically relevant quantities in the brain via systemic administration. Due to the potentially deleterious side effects associated with these high doses, including undesirable global immunosuppression, CsA requires localized delivery if it is to be used to treat stroke. Currently available local delivery strategies, such as intracerebroventricular or intracranial

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delivery via minipump/catheter infusion or bolus injection, are either highly invasive or incapable of delivering a sustained drug dose. Since CsA requires sustained delivery over a period of weeks to activate NSPCs [8,9] and existing systems are either highly invasive or unsustained, a novel, local delivery strategy is required.

To circumvent the BBB and sustain delivery in a less invasive manner, a drug-loaded hydrogel composite has been proposed [11–13]. Composed of polymeric particles dispersed in a physically cross-linked blend of hyaluronan (HA) and methylcellulose (MC), the HAMC composite is injected on the cortical surface and acts as a reservoir for the controlled release of therapeutics. The hydrogel is bioresorbable, injectable through a fine-gauge needle, and fast gelling at physiological temperatures [11]. Unlike minipump/catheter systems, implantation of the HAMC composite neither disrupts the brain tissue nor requires an external cannula. Soluble epidermal growth factor modified with poly(ethylene glycol) [12] and erythropoietin [13] were shown to penetrate through the ischemic cortex and reach the SVZ when delivered epi-cortically from HAMC. However, delivery of a soluble drug from HAMC is governed by Fickian diffusion and so sustained release is unlikely to be achieved from the hydrogel alone. For hydrophobic drugs, dispersion of solid drug particulates into the gel that slowly dissolve can yield sustained release, typically over 7 days [14]. In addition, encapsulation of the drug within poly(lactic-*co*-glycolic acid) (PLGA) particles prior to dispersion into the gel have been shown to increase the duration of release to a period of weeks to months [15–17].

Here we designed a HAMC hydrogel capable of releasing bioactive CsA for a period of 3–4 weeks. The in vitro release profiles of soluble, particulate, and PLGA-encapsulated CsA were compared and the PLGA-encapsulation method was found to yield the longest duration of release. Importantly, the bioactivity of the PLGA-encapsulated CsA was equivalent to free drug as measured by the neurosphere assay. HAMC containing PLGA-encapsulated CsA was injected onto the cortical surface of mice and the drug was detected at the SVZ up to 24 days post-implant. This novel biomaterial provides local, sustained, and less invasive release to the brain of a promising molecule for the treatment of stroke.

2. Materials and methods

2.1. Materials

1.4–1.8 × 10^6 g/mol sodium hyaluronate (HA) was purchased from NovaMatrix (Sandvika, Norway). 3.4 × 10^5 g/mol methylcellulose (MC) was obtained from Shin Etsu (Chiyoda-ku, Tokyo, Japan). Cyclosporin A (CsA) (>99% purity) and internal standard tacrolimus (>99% purity) were purchased from LC Laboratories (Woburn, USA). HPLC grade dichloromethane (DCM), acetonitrile, and ethanol were supplied by Caledon Labs (Georgetown, CA). Artificial cerebrospinal fluid (aCSF) was formulated as previously described [11] with distilled and deionized water (ddH_2O) prepared from a Millipore Milli-RO 10 Plus and Milli-Q UF Plus at 10 MΩ resistivity (Millipore, Bedford, USA). Acid-terminated poly(D,L-lactic-co-glycolic) acid (PLGA) 50:50 of inherent viscosity 0.16–0.24 dL/g, poly(vinyl alcohol) (PVA) (Mn 30,000–70,000), ammonium acetate (>99% purity), and all other reagents were purchased from Sigma-Aldrich (Oakville, CA) and used as received unless specified otherwise.

2.2. Hydrogel preparation

HAMC hydrogels were prepared through the physical blending of hyaluronan and methylcellulose in aCSF for a final composition of 1.4 wt.% HA and 3 wt.% MC. MC and HA were sequentially dispersed in aCSF using a dual asymmetric centrifugal mixer (Flacktek Inc., Landrum, USA) and left to dissolve overnight at 4 °C. For sterile hydrogels used in animal studies, MC and HA were dissolved in ddH_2O, sterile filtered, and lyophilized (Labconco, Kansas City, USA) under sterile conditions. The resulting sterile polymers were kept at 4 °C until use.

2.3. PLGA microsphere preparation and characterization

CsA-loaded PLGA microspheres were prepared using an oil/water emulsion solvent evaporation technique. An organic phase consisting of 0.9 mL DCM, 120 mg PLGA and 12 mg CsA was added to an 18 mL aqueous phase containing 10 mg/mL PVA. The emulsion was formed through homogenization (Kinematica, Bohemia, USA) on ice for 60 s at 4300 rpm. The emulsion was then added to 150 mL of 1 mg/mL PVA in water and stirred gently for 3 h at room temperature. The hardened microspheres were collected and washed by centrifugation, lyophilized, and stored at −20 °C until use. Microspheres used in vivo were sterilized by gamma irradiation.

Microsphere size was measured using laser diffraction (Malvern Mastersizer 2000, Worcestershire, UK) and surface morphology was examined using scanning electron microscopy (SEM). Drug loading was defined as the CsA mass per mg of particles, while encapsulation efficiency is the measured drug loading of the particles divided by the theoretical maximum drug loading. To determine CsA encapsulation efficiency, a known mass of particles was dissolved in 1 mL of acetonitrile and the resulting solution was analyzed for CsA content.

2.4. In vitro CsA release from HAMC

CsA release was quantified from three types of HAMC formulations: (1) HAMC containing solubilized CsA; (2) HAMC containing solid CsA particulates; and (3) HAMC containing CsA-loaded PLGA microspheres. For (1), an initial CsA solution in acetonitrile was prepared. For (2), an initial particulate dispersion was produced by mixing CsA powder into 0.5 wt.% MC solution. For (3), CsA-loaded PLGA microspheres were added to aCSF and dispersed via sonication for 1 min at 26 W and 20 kHz. 10 μL of the solubilized CsA solution, particulate CsA dispersion, or PLGA particle dispersion was added to the bottom of 2 mL Eppendorf tube. 90 μL of HAMC was then added to the tube and mixed into the CsA solution/dispersion using the dual asymmetric centrifugal mixer, resulting in a 100 μL flat drug-loaded HAMC disk at the bottom of the tube. The HAMC was allowed to gel for 30 min at 37 °C. At time zero, 900 μL of aCSF, a medium formulated to mimic the salt composition of the fluids surrounding the brain, was added to the tube. The aCSF was removed and replaced at various time points and analyzed for CsA content. All release studies were performed in triplicate and the cumulative release is expressed as mean ± standard deviation.

2.5. Neurosphere assay for CsA activity

All experiments were carried out in accordance with the Guide to the Care and Use of Experimental Animals developed by the Canadian Council on Animal Care and approved by the Animal Care Committee at the University of Toronto. NSPCs were isolated by dissection of the forebrain subependyma of adult male C57BL/6 mice (9–11 weeks old; Charles River, CA) as previously described [18]. Briefly, tissue was digested with enzymes (1.33 mg/mL trypsin, 0.67 mg/mL hyaluronidase, and 0.2 mg/mL kynurenic acid; all from Sigma-Aldrich) for 40 min at 37 °C. Enzyme activity was inhibited with 0.67 mg/mL trypsin inhibitor (Roche Diagnostics), and the tissue was mechanically dissociated into a single-cell suspension. For all conditions cells were plated at clonal density (10 cells/μL) [19] in 24-well polystyrene plates (VWR Scientific) with serum-free medium (SFM) supplemented with epidermal growth factor (EGF) (20 ng/mL; Sigma-Aldrich), basic fibroblast growth factor (bFGF) (10 ng/mL; Sigma-Aldrich), heparin (7.35 ng/mL; Sigma-Aldrich), and 1% penicillin/streptomycin (Invitrogen). Passage 1–4 cells were used in these studies. CsA dissolved in 1:1 ethanol growth factor (EGF/FGF2/heparin)-supplemented SFM was added to the cultures for a final concentration

of 100 ng/mL [9]. The CsA source was either stock CsA powder or CsA released from the drug delivery system. To prepare the latter source, release samples spanning a 21-day period were pooled and the combined sample was analyzed for CsA content. The solution was then lyophilized and re-dissolved in 1:1 ethanol growth factor-supplemented SFM for use in the neurosphere assay.

2.6. Drug delivery device implantation surgeries

The drug delivery system was spatially localized on the brain cortex of 9–11 week old C57BL/6 mice as previously described [12,13]. Briefly, anesthetized mice had a burr hole drilled into the skull at the coordinates 2.25 lateral to the midline and 0.6 anterior to Bregma and the exposed dura was pierced using a 26 G needle. A polycarbonate disk with a 2-mm opening was fixed over the burr hole and 3 μL of HAMC containing CsA-loaded PLGA microspheres was placed in the central opening in direct contact with the brain cortical surface. A second disk without an opening was fixed above the first disk and the skin was sutured over the disk system (Supplementary Fig. 3S). To determine the amount of CsA initially in the implant, 3 μL of the HAMC containing CsA-loaded PLGA microspheres was placed into the disk system in vitro using an identical technique as the in vivo implants. The disk system was then placed into 1.5 mL of acetonitrile and agitated overnight to extract the CsA for analysis.

2.7. Analysis of in vivo CsA penetration

Animals were sacrificed 6, 12, 18, and 24 days post-implantation and the drug delivery device containing HAMC was retrieved. The device was placed into 1.5 mL of acetonitrile and agitated overnight to extract any remaining CsA. Brains were snap frozen with $CO_{2(s)}$ cooled isopentane and stored at −80 °C. Three 1-mm coronal slices were prepared using a McIlwain tissue chopper (Mickle Laboratory Engineering Company, Surrey, UK) at the implant site and rostral and caudal to the implant site. Dorsal-ventral sections (0.5 mm) were then obtained from each coronal slice using a Leica CM3050S cryostat system operating at −18 °C. For each brain, sections at the same depth from the cortical surface were combined in 2 mL polystyrene microtubes and homogenized with 1.0-mm diameter zirconia beads in 120 μL of ethanol for analysis of CsA content. The CsA amount at each depth was divided by the corresponding tissue volume (calculated from an atlas of the mouse brain [20]) to yield the CsA concentration in the tissue.

2.8. CsA detection by LC–MS/MS

Liquid chromatography tandem mass spectrometry (LC–MS/MS) was used to quantitate CsA in release study samples and tissue homogenates. To prepare samples for analysis, 100 μL of release sample or homogenate was first mixed with 200 μL of acetonitrile containing internal standard. The resulting mixture was centrifuged at 14,000 rpm for 12 min to remove precipitated proteins. A Sciex API4000 triple quadrupole mass spectrometer (Ottawa, CA) fitted with an electrospray ionization interface was used for all analyses. The instrument was operated in electrospray positive ionization mode and was coupled to an Agilent 1100 capillary LC system (Mississauga, CA). The separation of CsA and internal standard was performed using a Spherisorb CN column (30 mm × 4.6 mm, 5 μm) (Waters, Milford, USA) with a mobile phase composed of 65% aqueous acetonitrile containing 2 mM ammonium acetate and 0.1% (v/v) formic acid operating at a flow rate of 1 mL/min and a sample injection volume of 10 μL. Both compounds eluted in less than 1 min and a total cycle time of 2.5 min was achieved. Quantitation was performed using multiple reaction monitoring of the ammonium-adduct transition masses of CsA (m/z 1220 → 1202) and internal standard tacrolimus (m/z 822 → 768). Instrument parameters were optimized for the simultaneous detection of both the drug and internal standard. Calibration curves were established using standard samples at CsA concentrations ranging from 100 ng/mL to 1 ng/mL with an internal standard concentration of 10 ng/mL. The coefficient of determination (r^2) from a 1/x-weighted least squares linear regression was found to be 0.999.

2.9. Statistics

All statistical analyses were performed using Prism 5.0 (GraphPad Software Inc.). Differences between groups were assessed by one-way ANOVA with Bonferonni correction. Significance levels were indicated by $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).

3. Results and discussion

3.1. CsA release from HAMC in vitro

To control the release of CsA from HAMC, the drug was incorporated in the hydrogel matrix in three forms (Fig. 1): solubilized, particulate, and PLGA-encapsulated. Solubilized CsA (Fig. 1A) was predicted to diffuse out of the gel rapidly [12–14], while particulate (Fig. 1B) and PLGA-encapsulated (Fig. 1C) CsA were expected to result in extended release profiles [14–17]. To test these hypotheses, HAMC containing CsA was injected onto the bottom of microcentrifuge tubes forming cylindrical disks (0.37 cm in thickness) with a planar surface. Artificial CSF was placed on top of the hydrogel and replaced at various time points for analysis of CsA content. As shown in Fig. 2A, solubilized CsA was confirmed to diffuse out of the gel relatively quickly, reaching 100% cumulative release in 2 days. Diffusion-controlled release of a drug source from a planar geometry can be estimated by the following analytical approximation [21]:

$$\frac{M_t}{M_\infty} = \frac{2}{L}\sqrt{\frac{D_A}{\pi}}\cdot t^{0.5} \quad (1)$$

where M_t/M_∞ is the fraction of drug molecules released from the hydrogel at time t, D_A is the diffusivity of the drug in the matrix, and L is the scaffold thickness. Using a fitted D_A value of $2.6\times10^{-6}\,cm^2/s$, which is characteristic of the diffusion of small molecules, the proportionality to the square root of time was maintained for the first 50–55% of release (Fig. 2B). This indicates that diffusion was the dominant release mechanism, as was found for other soluble drugs in previous work with HAMC [14,22]. Depletion of drug in the hydrogel results in a diminished concentration gradient and driving force that in turn slow the latter stages of release compared to the predictions of Eq. (1) and which is consistent with previous findings [14]. The CsA

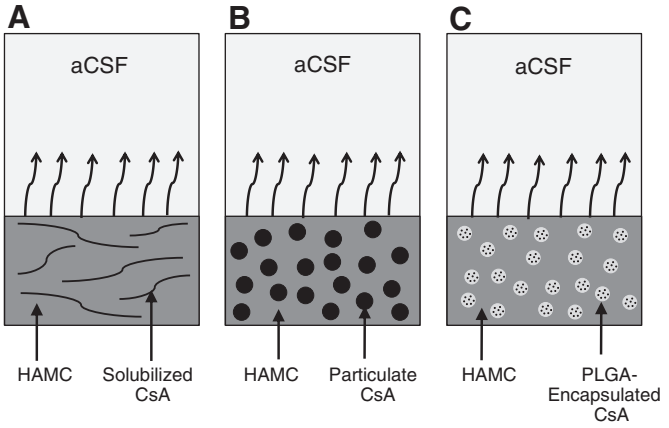

Fig. 1. Schematic of the three methods investigated for the controlled in vitro release of CsA from HAMC into aCSF: (A) solubilized CsA, (B) particulate CsA, and (C) PLGA-encapsulated CsA.

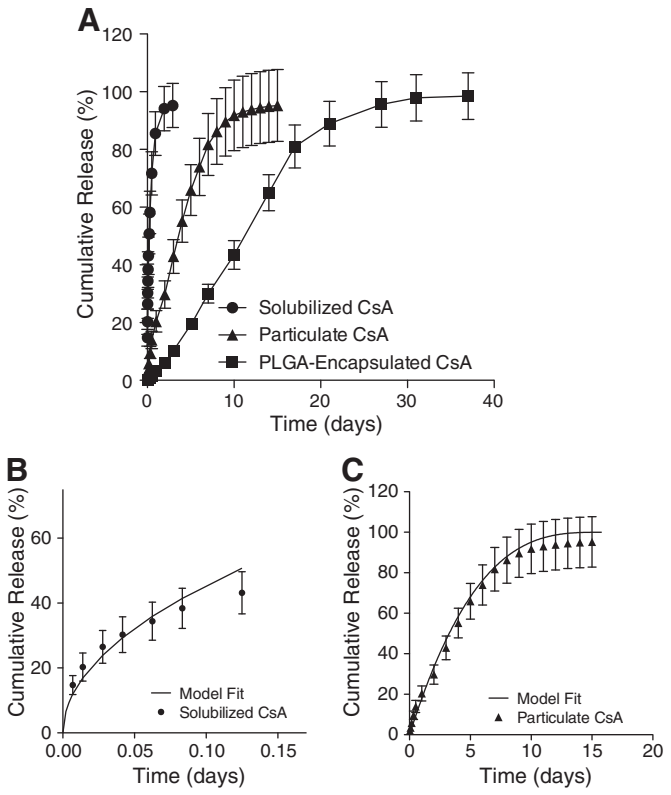

Fig. 2. In vitro cumulative release profiles of CsA from HAMC. (A) Comparison of (•) solubilized, (▲) particulate, and (■) PLGA-encapsulated CsA release. Dispersion of CsA particulates into the gel extends release to 7–8 days, while PLGA encapsulation provides sustained release for 21–28 days. (B) Release of solubilized CsA fits a diffusion-controlled release model and (C) release of particulate CsA fits a Hixson–Crowell release model. (Mean ± standard deviation, n = 3 per release study).

release profile was similar to that of nimodipine (another hydrophobic drug) despite using a different solubilizing agent (acetonitrile instead of ethanol), suggesting that release is independent of the solubilizer chosen [14].

When CsA was dispersed into HAMC in particulate form, its release was significantly slower than in the solubilized formulation. The particulate dispersion resulted in sustained release for 7–10 days (Fig. 2A), as only a fraction of the total CsA is dissolved and thus able to diffuse out of the gel at a given time [14]. Since the dispersed CsA particulates were relatively large (100 μm in diameter by laser diffraction), it was postulated that the release profile was governed by the slow dissolution of drug particulates and not diffusion of the drug out of the gel matrix. The following dimensionless number (ξ) represents the ratio of the characteristic times of these two processes [23]:

$$\xi = \frac{k_m n_p R_i^2 L^2}{D_A} \tag{2}$$

where k_m is the mass transfer coefficient of CsA dissolution in HAMC (8×10^{-5} cm/s, see Supplementary Fig. 1S), n_p is the number of particulates per unit volume in the gel (382 cm^{-3}), and R_i is the initial particulate radius (50 μm). Since the calculated ξ value of 0.042 is less than one, diffusion is indeed much faster than dissolution. Consequently, it can be assumed that the concentration of CsA in the gel matrix at any given time is negligible compared with the saturation concentration of the drug and release should follow a Hixson–Crowell profile [14,24]:

$$\frac{M_t}{M_\infty} = 1 - \left(1 - \frac{k_m C_{sat}}{\rho R_i} t\right)^3 \tag{3}$$

where C_{sat} is the saturation concentration of CsA in HAMC (45 μg/mL, see Supplementary Fig. S1) and ρ is the density of the drug particulates (~1 g/cm^3). As shown in Fig. 2C, the experimental data is in close agreement with the Hixson–Crowell prediction, further indicating that drug release from the dispersed CsA particulates is dissolution-controlled. Interestingly, this effect of particulate dissolution controlling release is enhanced by the presence of methylcellulose (MC) in HAMC, which promotes the solubilization of hydrophobic molecules [14] (shown for CsA in Supplementary Fig. 1S).

To extend release beyond the 7–10 days achieved with the particulate dispersion, CsA was encapsulated within PLGA microspheres prior to incorporation into HAMC. Drug-loaded PLGA microspheres are widely used in the field of controlled drug delivery because they are one of the few biodegradable polymers approved for therapeutic use by the FDA [25]. In these systems, sustained release results from drug diffusion through pores in the polymer matrix formed by degradation of PLGA and dissolution of entrapped drug. CsA-loaded PLGA microspheres were synthesized with a mean diameter of 25 ± 7 μm (see Supplementary Fig. 2S) and a measured drug loading of 71 μg CsA per mg microspheres. As shown in Fig. 2A, sustained release of CsA from PLGA microspheres in HAMC was achieved for 21–28 days. Interestingly, the initial burst release characteristic of PLGA particles [26,27] was nearly non-existent. This attenuation of burst release from PLGA particles when dispersed in HAMC was previously reported for encapsulated α-chymotrypsin [15], anti-NogoA [16], and neurotrophin-3 [17]. It was postulated previously [17] that two possible mechanisms may result in this behavior: (1) a reduced degradation rate of the PLGA in the particles when embedded in HAMC, resulting in an altered release profile; or (2) absorption of MC to the surface of the particles, resulting in reduced diffusion across the PLGA–hydrogel boundary and an altered release profile. It was found, via gel permeation chromatography studies, that PLGA degradation was unaffected by the presence of HAMC [17]. Consequently, it was suggested that the formation of a diffusive barrier via interaction between hydrophobic MC and PLGA at the hydrogel–particle interface is responsible for the low burst and sustained release. Interestingly, mathematical modeling of release as a sequential process whereby drug first diffuses from the bulk-eroding PLGA particles and then diffuses out of the HAMC in a Fickian manner failed to accurately predict release from the composite system [16,17], suggesting that diffusion through the PLGA particles and HAMC are not distinct processes, thereby further supporting the MC–PLGA diffusive barrier mechanism.

Through incorporation of CsA into the HAMC matrix in three distinct forms, a spectrum of release profiles was obtained spanning a period of hours to weeks. Solubilized CsA was released from the gel rapidly in a diffusion-controlled manner, particulate CsA resulted in slower dissolution-controlled release, and PLGA-encapsulated CsA extended release even further out to 3–4 weeks. As CsA must be delivered over this longer timescale to have a potential therapeutic benefit for the treatment of stroke [8,9], the PLGA encapsulation system was the only formulation examined further in terms of bioactivity and brain tissue penetration.

3.2. In vitro bioactivity of CsA released from PLGA microspheres dispersed in HAMC

The bioactivity of CsA released from PLGA microspheres dispersed in HAMC was assessed using the neurosphere assay [9,28]. In this assay, single cells isolated from the forebrain subependyma of adult mice were cultured for 7 days in vitro and NSPCs formed structures termed neurospheres during this period. These neurospheres were dissociated and re-plated in the presence or absence of CsA. When cultured in the presence of CsA, the total numbers of neurospheres that form is enhanced, representing the pro-survival effect that CsA exerts on NSPCs (Fig. 3), which is consistent with previous reports [9]. The fold-increase in the number of neurospheres is the same

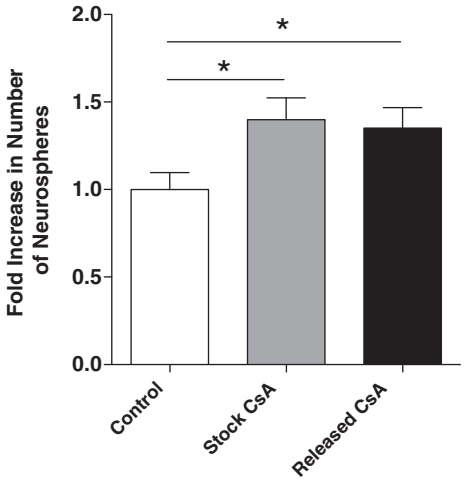

Fig. 3. CsA released over 21 days from PLGA microspheres dispersed in HAMC had equivalent bioactivity to stock CsA as measured by the neurosphere assay. Both conditions were tested at a CsA concentration of 100 ng/mL and showed significantly greater numbers of neurospheres than controls in which there was no CsA. (Mean ± standard deviation, n = 4 trials per condition, 6 wells per trial).

when cells are cultured in the presence of stock CsA and CsA released from PLGA microspheres dispersed in HAMC. This indicates that the PLGA-encapsulated CsA had equivalent bioactivity to stock CsA. Although CsA is a polypeptide, it contains no secondary or tertiary structure and so is not susceptible to the degradation often experienced by proteins during PLGA particle encapsulation [29]. Consequently, maintenance of bioactivity post-encapsulation and release was expected and achieved.

3.3. In vivo brain tissue penetration of CsA delivered from composite HAMC system

The ability of the drug delivery system to deliver a sustained dose that penetrates the brain tissue was investigated using a mouse model. HAMC containing PLGA-encapsulated CsA was injected on the cortex of adult mice using the device [12,13] depicted in Supplementary Fig. 3S. In contrast to other local delivery systems such as catheter/minipumps and bolus injection [30], this epi-cortical delivery strategy avoids the trauma and infection observed when inserting cannulas and needles directly into the brain tissue. Moreover, local delivery circumvents the blood–brain barrier and avoids the large systemic doses required to get even a small amount of CsA into the brain; however, drug penetration into brain tissue from the cortical surface can be limited by rapid elimination [30]. Consequently, it was critical to determine if CsA could diffuse out of the composite delivery vehicle and penetrate through the brain tissue to the neural stem cells in the SVZ (>1500 μm below the cortical surface at the chosen coordinates [20]).

To this end, tissue penetration profiles were quantified at 6, 12, 18, and 24 days post-implant. Six 500-μm sequential tissue sections were prepared ventral to the cortical surface and the concentration of CsA in the tissue was measured by LC–MS/MS. As shown in Fig. 4A–D, the CsA concentration was highest closest to the cortical surface and decreased with depth. Importantly, CsA was detectable out to 3000 μm at all time points tested post-implantation, which represents approximately 60–65% of the total dorsal–ventral dimension of the mouse brain [20]. While 3000-μm of tissue penetration is adequate in mouse models, we acknowledge that greater tissue depths will be required to stimulate SVZ precursor cells in human brains. Summation of the CsA content at the depth of the SVZ or below (Fig. 4E) revealed a constant drug concentration over the 24-day time period [slope of linear regression line in Fig. 4E was not statistically different from zero, determined via an F-test (p = 0.7243)]. This is critical for the therapeutic benefit of the system, as a constant concentration of CsA over a prolonged period

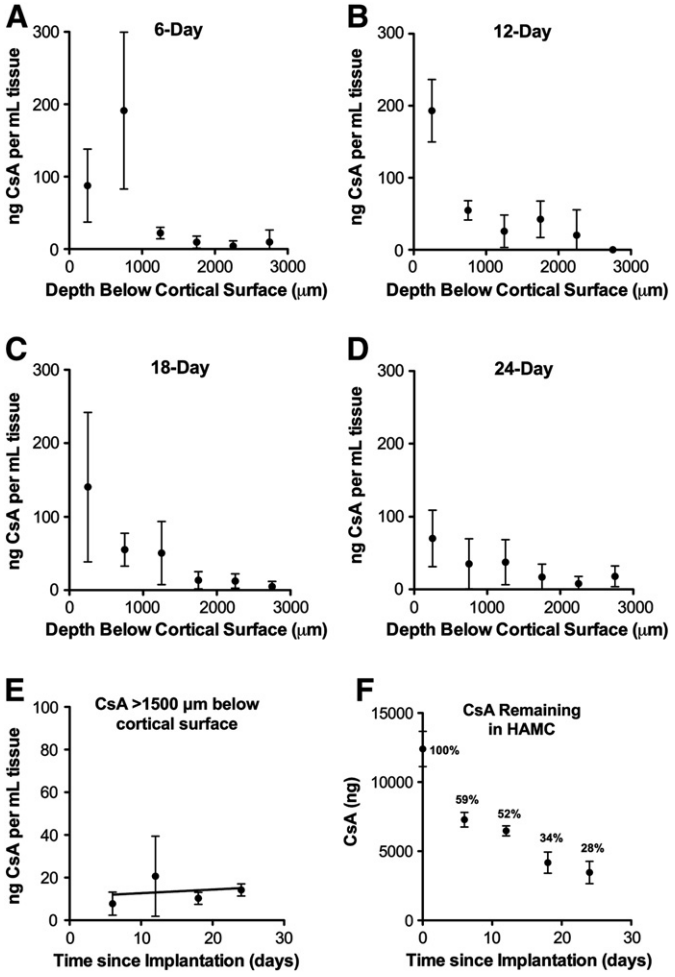

Fig. 4. Penetration profiles of CsA in uninjured mouse brain tissue at (A) 6 days, (B) 12 days, (C) 18 days and (D) 24 days post-implant. Data is plotted at midpoint of tissue section (e.g. the section spanning 500 to 1000 μm is plotted at 750 μm). (E) A constant CsA concentration was detected in the SVZ region up to 24 days post-implant. (F) CsA remaining in HAMC decreased over time. Percentages are relative to initial CsA amount in HAMC. (Mean ± standard deviation, n = 3 animals per time point).

of time is required to stimulate NSPCs [8,9]. Additionally, the HAMC implant was extracted at each time point and analyzed for CsA content. The amount of CsA remaining decreased over time (Fig. 4F), as expected from diffusion of drug out of the hydrogel and into the tissue. The total amount of CsA detected at each time point represented a very small fraction (<0.01%) of the total drug initially loaded into the implant, which is consistent with rapid clearance of small molecule and protein drugs from the brain [30].

4. Conclusions

Herein we developed a novel method for the localized and sustained epi-cortical delivery of CsA for the potential treatment of stroke. CsA release from the HAMC hydrogel system was tunable by the mechanism in which the drug was incorporated into the gel. Solubilized CsA yielded release on the order of hours, while particulate CsA extended release to days and PLGA microsphere-encapsulated CsA sustained release for a period of 3–4 weeks, a clinically relevant timescale. PLGA-encapsulated CsA released from the system was found to be bioactive and capable of penetrating to the SVZ of mice at a stable concentration over a 24-day period. Thus, this hydrogel composite system may be useful for the treatment of stroke.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jconrel.2013.01.002.

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