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Diamagnetic chemical exchange saturation transfer (diaCEST) affords magnetic resonance imaging of extracellular matrix hydrogel implantation in a rat model of stroke

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ABSTRACT

Extracellular matrix (ECM) is widely used as an inductive biological scaffold to repair soft tissue after injury by promoting functional site-appropriate remodeling of the implanted material. However, there is a lack of non-invasive analysis methods to monitor the remodeling characteristics of the ECM material after implantation and its biodegradation over time. We describe the use of diamagnetic chemical exchange saturation transfer (CEST) magnetic resonance imaging to monitor the distribution of an ECM hydrogel after intracerebral implantation into a stroke cavity. In vitro imaging indicated a robust concentration-dependent detection of the ECM precursor and hydrogel at 1.8 and 3.6 ppm, which broadly corresponded to chondroitin sulfate and fibronectin. This detection was robust to changes in pH and improved at 37 °C. In vivo implantation of ECM hydrogel into the stroke cavity in a rat model corresponded macroscopically to the distribution of biomaterial as indicated by histology, but mismatches were also evident. Indeed, CEST imaging detected an endogenous "increased deposition". To account for this endogenous activity, pre-implantation images were subtracted from post-implantation images to yield a selective visualization of hydrogel distribution in the stroke cavity and its evolution over 7 days. The CEST detection of ECM returned to baseline within 3 days due to a decrease in fibronectin and chondroitin sulfate in the hydrogel. The distribution of ECM hydrogel within the stroke cavity is hence feasible in vivo, but further advances are required to warrant a selective long-term monitoring in the context of biodegradation.

1. Introduction

The extracellular matrix (ECM) constitutes 20% of brain volume [1]. Stroke related infarcts result in the acute loss of neurons and other cells, whereas the extracellular matrix is gradually cleared to create an extracellular fluid (ECF)-filled tissue cavity [2]. Although neural stem cell transplantation in the peri-infarct tissue can alleviate some behavioral deficits, it does not replace lost tissue [3]. Instead, a structural support is required for cells within the cavity to develop a *de novo* tissue [4,5]. Transplantation of neural stem cells in a hydrogel produced from extracellular matrix can efficiently repopulate the tissue cavity [6]. ECM hydrogels and sheets are extensively used for regenerative medicine in clinical settings to repair tissue defects, ranging from bladder reconstruction, muscle regeneration to breast reconstruction [7].

ECM can be formulated to reside in a liquid phase at room temperature and gel at brain temperature. These gelation properties are dependent on the collagen concentration within the preparation. Indeed, ECM hydrogels with concentrations >3 mg/mL show retention within the cavity, whereas lower concentrations show poor gelation and permeate into the peri-infarct tissue [8].

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Gelation is hence a key factor in providing a structural support within the cavity. Injection and retention of the ECM at an appropriate concentration inside a brain cavity is therefore challenging, because a minimally invasive approach is required to avoid damage to brain tissue and the skull creates a closed environment [9]. The use of injection-drainage, where the appropriate concentration of ECM material is delivered to the lesion cavity, while ECF is drained, can achieve complete coverage of the lesion cavity [8] with a significant invasion of host cells, including neural progenitors [10]. Porcine urinary bladder derived ECM was selected as it has been found to be more supportive to neurite outgrowth from neural stem cells than CNS or spinal cord-derived material [11,12]. However, non-invasive imaging, such as magnetic resonance imaging (MRI), is required to define the cavity volume, as well as the injection-drainage access points.

Injection of ECM into the fluid-filled stroke cavity changes the diffusion characteristics of this environment, which provides an indirect means to visualize changes in this environment due to the hydrogel [6,13]. Indeed, T_2- and diffusion-weighted MRI have been suggested for the monitoring of tissue development from an acellular matrix [14,15]. However, these measurements do not actually detect the material itself, nor do they afford a monitoring of the biomaterials' degradation. In some cases, the biomaterial has a very distinctive MR signature that can be used to contrast the implant versus host tissue [16,17]. However, many scaffolding materials are designed to biodegrade, as host cells invade the material and gradually replace it [10], hence becoming indistinguishable from host tissue. In these cases, MR contrast agents can be integrated into, for instance, hyaluronic acid (HA)-based hydrogels, to afford a specific visualization of their location and degradation [18,19].

Contrast agents only serve as a surrogate and do not directly visualize the injected material; similar degradation profiles are essential for these to report adequately on the presence of scaffolding material. Incorporation of contrast agents into the scaffold will also require additional safety scrutiny from regulators [20,21]. Ideally, an imaging method as an analytical tool will not require the addition of MR contrast agents and will directly detect the injected material and its degradation profile. Chemical exchange saturation transfer (CEST) is a versatile MR imaging approach [22,23] that can selectively visualize the distribution of specific proteins and metabolites in vitro [24], in vivo in animal models [25], and also in human brain [26,27]. CEST acquisition is performed with an off-resonance saturation pulse, similar to magnetization transfer (MT), where contrast can be observed in a wide frequency range (~100 kHz). However, CEST imaging is more specific in that it can only be detected around the resonance frequency of labile protons from mobile molecules [28].

As ECM molecules are known to have MR-detectable effects on water molecules that can be detected using CEST [29,30], this principle can be exploited to directly detect the scaffold material. For instance, addition of gelatin to HA hydrogels can be used to detect the HA implant using diamagnetic CEST (diaCEST) [31]. Preferably though, no additional molecules are required to afford detection. Hydrogels derived from ECM that is manufactured by decellularization of a source tissue retain most molecules and hence potentially provide a multitude of molecules that can exert CEST effects. We here describe the in vitro characterization of CEST effects induced by ECM in its liquid form and upon gelation, as well as their in vivo detection in a rat model of stroke with histological validation. MRI hence could serve as a key in vivo analytical tool to monitor the distribution of ECM hydrogel, its interaction with the host brain and potentially provide biochemical information about its biodegradation.

2. Methods

2.1. Extracellular matrix hydrogel

ECM was derived from the basement membrane and tunica propria of porcine urinary bladder (Tissue Source, Lafayette, IN). A mechanical delamination of the luminal epithelium and subjacent layers was followed by decellularization. Decellularization of the tissue was accomplished by 0.1% peracetic acid in 4% ethanol (v/v; 120 min; 300 rpm) and agitation prior to washing out of cellular components with PBS and deionized water rinses. Confirmation of decellularization was achieved using a series of measures (Hematoxylin & Eosin, 4′,6-diamidino-2-phenylindole (DAPI) staining, agarose gel electrophoresis, and measurement of DNA content). This material was lyophilized, comminuted, and solubilized with pepsin (1 mg/mL) in 0.01 N HCl prior to neutralization with 0.1 N NaOH. The final product was an injectable liquid at room temperature (21 °C) that formed a hydrogel at concentrations >3 mg/mL at brain temperature (37 °C) [8]. Concentrations of 0 (PBS only), 2, 4, 6, and 8 mg/mL were used for in vitro experiments, whereas only 8 mg/mL was used for in vivo experiments. Matristem™ (ACell Inc. Columbia, MD) was used for comparison of a commercially available UBM-ECM product.

2.2. In vitro CEST imaging

Hardware: MR images were acquired on a 9.4 T horizontal bore system interfaced to a DirectDrive 2 console (Agilent, Santa Clara, CA, USA). For phantom studies, a volume coil with an internal diameter of 38 mm was used (Agilent, Santa Clara, CA, USA). For in vivo studies, a custom made volumetric birdcage quadrature coil (Virtumed LLC) achieving a radiofrequency (RF) power input of up to 55 μT/5 s with an internal diameter of 36 mm and effective length of 25 mm was used.

CEST imaging: All in vitro imaging was carried out at room temperature (apart from the varied-temperature experiments). Two sets of imaging parameters were adopted. CEST images were acquired by 2-shot spin-echo echo planar imaging (EPI) with FOV = 50 mm × 25 mm, matrix 128 × 64, TR = 10 s, and TE = 7.4 ms. A B_1 map was first acquired to calibrate the transmit power for the CEST studies and evaluate the B_0 homogeneity. Because the spatial variation of B_1 is <15% for all of our phantoms, no correction of the B_1 inhomogeneity was applied. The CEST sequence was used to acquire Z-spectra between −8 and +8 with 43 interval offsets. The interval was chosen to be uneven since the chemical shift of most endogenous labile protons from water was less than 4 ppm. Thus the interval was 0.2 ppm from −1 to 1 ppm, 0.25 ppm from 1 ppm to 4 ppm and −1 ppm to −4 ppm, and 1 ppm from 4 to 8 ppm and −4 to −8 ppm. Additionally, images were acquired at offsets of 300 ppm for normalization. A B_0 map was obtained by using the water saturation shift referencing (WASSR) scheme [32], where a low power saturation (10 Hz) pulse was applied for 1 s, with 31 offset values ranging from −0.3 to 0.3 ppm in 0.02 ppm steps. In data sets where a significant B_0 inhomogeneity (B_0 variation >10 Hz) was detected, the RF offset of images were interpolated to a 1 Hz interval on a pixel-by-pixel basis and shifted according to the B_0 map. Optimization of the saturation power was explored using an array (65, 220, 420, 750 Hz) of saturation frequencies. For saturation power of ≤420 Hz, the saturation duration was 5 s. For the highest power of 750 Hz, the direct water saturation (DWS) became significant even at 2−3 ppm. To minimize DWS and RF heating, an off-resonance spin-lock sequence was used [33,34], and the irradiation duration was reduced to 3 s. Data were processed in MATLAB to generate magnetization transfer ratio asymmetries (MTR_{asym}), which were

calculated from Ref. [35].

$$MTR_{asym}(\Omega) = \frac{[S_{sat}(-\Omega)-S_{sat}(\Omega)]}{S_{sat}(300ppm)},$$

where $S_{sat}(\Omega)$ is the signal intensity with a saturation pulse at frequency offset of Ω. All subsequent experiments used an acquisition frequency of 220 Hz.

2.3. Temperature and pH effects on CEST signal

Chemical exchange is highly sensitive to temperature and pH. To probe the effects of temperature and pH on the CEST signal, both variables were varied and CEST images were acquired. For this, 8 mg/mL ECM hydrogel samples were created where pH was neutralized (pH 7). To adjust pH, Sodium hydroxide or Hydrochloric acid were added to the hydrogels and pH was confirmed with a pH spear (Eutech Instruments). ECM samples with an array of pH (5.5, 6.0, 6.5, 7.0) in 2 mL Eppendorfs were placed in 4% Agar holders. A temperature probe was placed into the Agar. These samples were placed into the MR scanner and temperature was controlled by circulating hot air to reach room temperature (21 °C), body temperature (37 °C) and an intermediate transition temperature (30 °C).

2.4. Liquid phase versus hydrogel ECM

In order to assess whether the gelation state of the ECM affects its detection, both liquid (i.e. pre-gel) and gelled samples were imaged. 8 mg/mL ECM was prepared, and incubated for 40 min at 37 °C to allow gelation. Meanwhile, fresh ECM was prepared (with no gelation step) and maintained on ice until imaging. Samples were imaged at 21 °C.

2.5. Preparation of individual ECM components

To ascertain which ECM components are contributing to the CEST signal, purified proteins (Table 1) and artificial cerebro-spinal fluid (aCSF, Harvard Apparatus, 597316) were imaged separately for comparison with the ECM-derived signal. Solutions were made up in PBS and samples were imaged at 21 °C.

2.6. Middle cerebral artery occlusion – a rat model of stroke

All animal procedures complied with the US Animals Welfare Act (2010) and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). As previously described, 14 male Sprague-Dawley rats (260 ± 15 g, Taconic Labs, USA) underwent transient intraluminal right middle cerebral artery (MCA) occlusion, a rat model of stroke [36]. For this, under isoflurane (4% induction, 1% maintenance in 30% O_2) anesthesia, a 5-0 silicon rubber-coated monofilament (503556PK10, Doccol, USA) was advanced to the ostium of the MCA in the circle of Willis and the MCA was occluded for 70 min prior to reperfusion. After recovery from anesthesia, animals were assessed for forelimb flexion and contralateral circling with daily post-operative care and neurological assessment until they recovered pre-operative weight [36,37].

2.7. In vivo CEST imaging

Rats were anesthetized with isoflurane (4% induction, 1% maintenance). T_2-weighted images were acquired using a Fast Spin Echo Multi Slice sequence to determine the location of lesion and to select the slices for CEST imaging (TR = 8 s, TE = 53 ms, number of segments = 16, number of averages = 4, 30 × 30 mm FOV, 128 × 128 matrix, 42 slices with 0.5 mm slice thickness, acquisition time = 9 min). Tissue volume loss was based on a hyperintense signal on T_2-weighted images that were thresholded at 1 standard deviation above the mean of a rectangular region of interest (ROI) in the contralateral hemisphere, encompassing striatum, corpus callosum and neocortex [38]. Rats with lesion volume <40 mm^3 (i.e. 40 μL) were excluded [8]. T_2-weighted images also served as anatomical reference for CEST image overlays. For a proof-of-principle of detection, 3 MCAO rats with ECM hydrogel injection (see below) underwent CEST imaging 24 h after implantation. To evaluate the time-dependence of the CEST effect, 3 groups of rats (MCAo only, n = 3; MCAo + PBS, n = 3; MCAo + ECM hydrogel, n = 4) were imaged 4 times: pre-injection (−2), 1, 3 and 7 days post-injection.

For CEST imaging, B_1 and B_0 maps were first obtained, similar to phantom studies. To acquire the Z-spectrum, a saturation pulse with a power of 220 Hz and a duration of 2 s was applied. Images were acquired immediately after the saturation with a 2-shot spin-echo EPI. 43 different RF offsets were acquired from −8 to +8 ppm, as for the phantom studies. The parameters for imaging were: TR = 6.5 s; TE = 7.6 ms; FOV = 30 × 30 mm; matrix size = 96 × 96; 5 slices with 1 mm thickness, acquisition time = 15 min. CEST images were thresholded to 3 standard deviations (s.d.) of the contralateral hemisphere to visualize only signal that is above the noise threshold and the baseline CEST signal (Supplementary Fig. 1). Total MR acquisition time was 40 min per animal.

Image Registration: The baseline MR scan of a single case was chosen as a reference and all structural MR images were rigidly (i.e. with 6° of freedom) registered to this reference using a previously described registration method [39] based on the FLIRT software [40]. For each rat at each time-point, a composite transformation was obtained by combining the transformation of each diaCEST image onto the corresponding MR image (derived from scanner positional information) with the transformation of the MR image onto the reference scan. Thus, all structural MR scans and all diaCEST images were rigidly registered into the same reference space. For accurate serial analysis, the post-treatment MR scan of each rat was further registered directly to the pre-treatment scan and the same transformation was applied to the associated diaCEST images.

Post-Processing: Mean structural and diaCEST images were computed for each group (MCAO only, PBS and ECM) in the reference space. Change in diaCEST signal in each rat over time was obtained by subtracting the pre-treatment image from the corresponding post-treatment images. To establish a suitable physiological noise level in the CEST images, a region of interest was drawn spanning the contra-lateral hemisphere in the reference MR image. This region was used to obtain the mean and standard deviation signal in each registered diaCEST image and in each diaCEST serial subtraction image. A threshold range of mean ± 3 s.d. was used to exclude all signal within the range typically seen in the

Table 1
List of individual ECM components.

Component	Concentration (mg/mL)	Company	Catalogue #
Chondroitin sulfate	3	Sigma	C9819
Collagen I	3	BD	354236
Collagen IV	1	Sigma	C7521
Fibronectin	1	Sigma	F1141
Heparin sulfate	1	Sigma	H7640
Hyaluronic acid	5	Sigma	53747
Laminin	1	Sigma	L2020
Vitronectin	0.8	Sigma	SRP3186

contra-lateral hemisphere (Supplementary Fig. 2).

2.8. Implantation of ECM hydrogel

Fourteen days post-MCAo, rats underwent the implantation procedure by placement into a stereotactic frame (Kopf, USA) under isoflurane anesthesia (1.5% in 30% O_2) prior to drilling of burr holes at the appropriate coordinates for injection and drainage using a frame-mounted drill [8]. The injection volume of biomaterial was equivalent to the lesion volume, as determined by the hyperintense volume range on MR images (40–180 μL). The liquid form of ECM was taken-up into a 250 μL Hamilton syringe with a 24 G beveled tip metal needle (Hamilton) mounted on the frame. The syringe/needle was advanced to the appropriate coordinates for biomaterial injection, whereas a 24 G cannula was placed in position to drain ECF, as previously described [8]. Injection of ECM hydrogel (8 mg/mL) was controlled using a frame mounted injection pump (World Precision Instruments, USA) at a constant speed of 10 μL/min. After the injection was complete, the needle and cannula were left in place for 5 min before being slowly removed from the brain with burr holes being filled with bone wax (Fisher) prior to suturing. LX4 (Ferndale, containing 4% Lidocaine) was topically applied as an analgesic, and Buprenex (0.05 mg/kg) was administered subcutaneously.

2.9. Immunohistochemistry

To analyze the distribution of the ECM hydrogel within the lesion cavity, rats were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (in 0.2 M PBS) 1 or 7 days post-implantation to fix brain tissue prior to its removal from the skull. Brains were post-fixed in 4% paraformaldehyde for 24 h prior to being cryopreserved in 30% sucrose with sodium azide (Sigma) at 4° C. Histological sections (50 μm thickness) were cut on a cryostat (Leica) directly onto microscopic slides. Brain sections were washed 3 × 5 min with 0.01 M PBS, followed by 1 h in blocking solution (PBS + 0.05% Triton X-100+ 10% Normal Goat Serum, NGS, Vector). Primary antibodies were then applied, consisting of rabbit anti-collagen I (1:250, Abcam, AB34710), mouse anti-fibronectin (1:150, Abcam, AB6328), mouse anti-chondroitin sulfate (1:200, Abcam, AB11570) and a chicken anti-Glial Fibrillary Acid Protein (GFAP, 1:3000, Abcam, AB4674) antibody diluted in blocking solution (0.05% Triton X-100, 10% NGS in PBS). Sections were incubated at 4 °C overnight. After rinsing of the primary antibodies (3 × 10 min PBS), appropriate secondary Alexa Fluor 488 and 555 antibodies (Life Technologies) were applied for 1 h. Secondary antibodies were washed off and sections were incubated with Hoechst (1 μg/mL, Sigma, 14533) for 10–15 min prior to 3 × 5 min washes in PBS and being coverslipped with Vectashield for fluorescence (Vector Laboratories). Visualization of antibodies was performed on a fluorescence microscope (M2 Axioimager, Zeiss) with a monochrome camera driven by StereoInvestigator image capture software (MBF Bioscience).

3. Results

3.1. In vitro CEST characteristics of extracellular matrix hydrogel

Extracellular matrix (ECM) contains a variety of molecules that can potentially affect chemical exchange saturation transfer (CEST) with water. To define an acquisition paradigm that will afford a robust detection, an array of frequencies was applied to record the chemical exchange saturation transfer (S_{Sat}/S_0) of ECM hydrogel (8 mg/mL) over a relevant range of radiofrequency (RF) offsets (Fig. 1A). Blips in the spectra are evident indicating specific

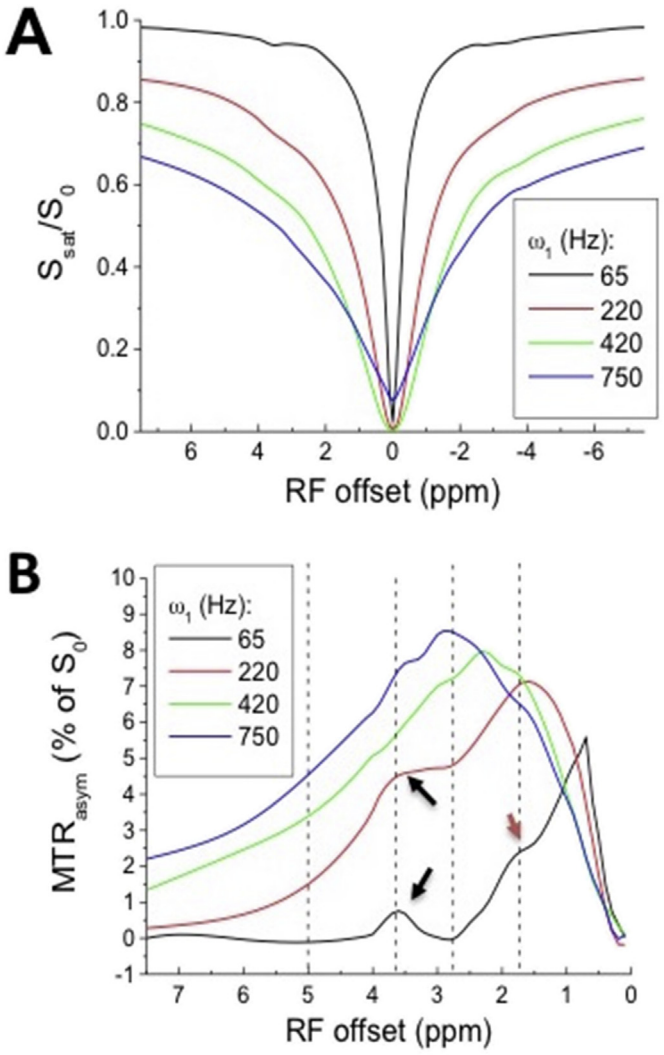

Fig. 1. Characterizing the CEST effect of ECM hydrogel. A. In vitro z spectra of 8 mg/mL ECM hydrogel at different frequencies allowed us to assay if there are any chemical exchange sites that affect the magnetization transfer from the saturation signal (S_{sat}) and could be exploited for a direct imaging approach. **B**. To more clearly visualize the exchange sites, the magnetization transfer asymmetry (MTR_{asym}) was plotted to reveal distinct exchange sites at frequencies <750 Hz. The most pronounced effect in these sites was observed at 220 Hz, which was used for all subsequent imaging.

exchange sites, especially around +3.6 ppm (65 Hz), but also a broader underlying asymmetry between the negative and positive offset at higher frequencies. Indeed, magnetization transfer asymmetry (MTR_{asym}), which was obtained by subtracting the positive from the negative offset, reveals effect sizes of up to 8.5% at 2.8 ppm with 750 Hz (Fig. 1B). Indeed, the most significant asymmetry was apparent with a frequency of 750 Hz. However, the smoothness of the curve did not clearly reveal a specific exchange site. In contrast, the lower frequencies of 220 Hz and 65 Hz reveal specific exchange sites at 3.6 ppm, 2.8 ppm, as well as at 1.8 ppm. The 65 Hz frequency provides the sharpest definition of these sites, but the effect size is markedly reduced compared to the 4.3% (3.6 ppm) and 6.5% (1.8 ppm) MTR_{asym} at 220 Hz. A 220 Hz acquisition hence provides specific exchange sites, as well as marked contrast for the ECM hydrogel. Images of the MTR_{asym} further indicate a robust visualization of the ECM hydrogel using 220 Hz compared to 65 Hz. Although 420 Hz and 750 Hz also

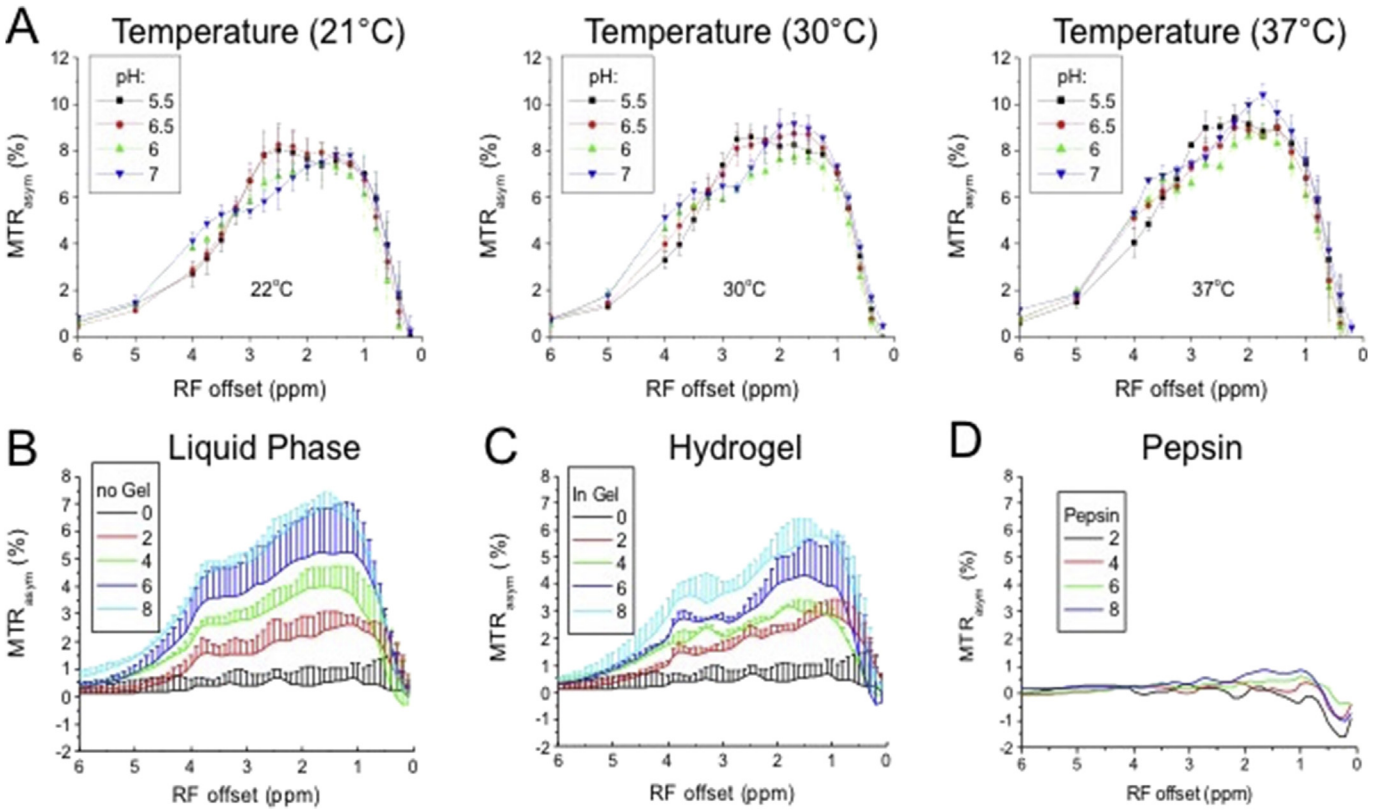

Fig. 2. Influence of experimental conditions on CEST signal. To evaluate how temperature and pH affect the detection of a specific signal from the 8 mg/mL hydrogel upon implantation into the brain, temperature (21 °C, 30 °C, 37 °C) and pH (5.5, 6.0, 6.5, 7.0) were arrayed with the acquisition of a full z spectra and displayed as MTR_{asym} plots **(A)**. Detection of ECM can be further affected by its concentration (2, 4, 6, 8 mg/mL, as indicated by differently colored lines), as well as its state being in liquid form **(B)** or cross-linked as a hydrogel **(C)**. A 0 mg/mL condition, consisting of PBS only, served as a control. Pepsin is also added to the ECM preparation in a concentration-dependent fashion (as indicated by differently colored lines for different ECM concentration, mg/mL) and could hence be a confounding factor, but no effect of pepsin on the CEST signal was found here at any concentration **(D)**.

afforded a robust detection with a more significant asymmetry at all sampled frequencies, these effects are less specific, which renders them more susceptible to unspecific MTR effects that could compromise a specific detection of ECM hydrogels in vivo. All subsequent experiments therefore used the 220 Hz frequency for acquisition.

3.2. Magnitude of ECM CEST signal is affected by temperature

As the CEST signal is affected by both temperature and pH (due to changes in the chemical exchange rate), both were arrayed to establish their effect on detection of the ECM hydrogel (8 mg/mL). Indeed, the temperature of the ECM will increase from room

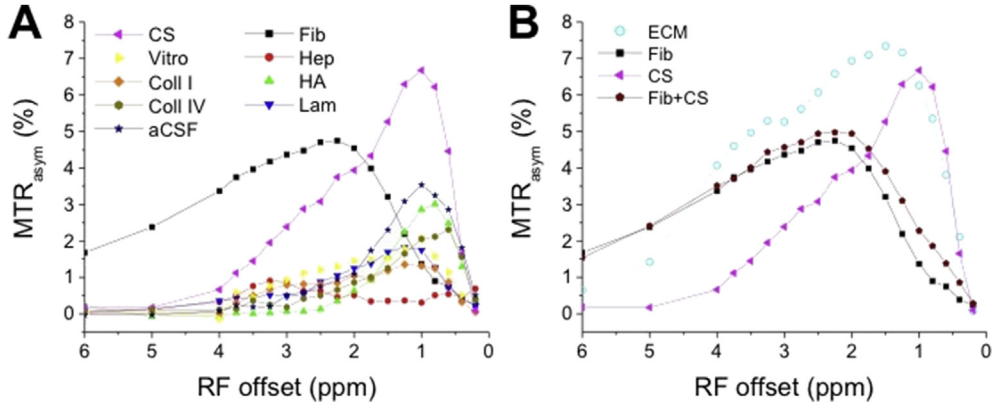

Fig. 3. Detection of individual ECM components. A. MTR_{asym} plots of individual ECM molecules found in ECM hydrogel. Specifically, Chondroitin Sulfate (CS), vitronectin (Vitro), collagen I (Coll I) and IV (Coll IV), fibronectin (Fib), Heparin Sulfate (Hep), hyaluronic acid (HA) and laminin (Lam) were evaluated as potential candidates for the signal detected in the ECM hydrogel preparation. As a potential confound in vivo, artificial cerebrospinal fluid (aCSF) was also assayed for its MTR_{asym}. **B**. As CS and Fib exhibited individual MTR_{asym} signals that resemble the ECM hydrogel when combined, both were combined into a single preparation to determine if these are the dominant source of the MTR_{asym} in the ECM hydrogel.

temperature (21 °C) to body temperature (37 °C) upon implantation and allow gelation of the material. Although pH of the ECM hydrogel is neutral upon injection, the infarcted area has a low pH and could reduce pH in the injected ECM. There was an overall effect of temperature, which increased MTR_{asym} at 1.8 ppm by ~42%, from 7.3% at 21 °C to 10.4% at 37 °C (pH 7.0) (Fig. 2A). Since the CEST signal is nearly proportional to the water T_1, this increase is likely caused by an increase of T_1, which increased from 2.8 s at room temperature to about 4.0 s at 37 °C. The same pattern was observed for all pH values, but notable differences in the z spectra were also apparent. For instance, at 21 °C, the MTR_{asym} at 2.8 ppm was much more pronounced for low pH values, but this difference gradually eroded with an increase in temperature. Furthermore at 37 °C, pH 7 was increased from the other pH values at 1.8 ppm, but only by ~16%. Temperature therefore increased the MTR_{asym} overall, which is advantageous for detection in the brain, whereas the effect of pH was negligible.

3.3. ECM concentration affects magnitude of CEST signal

As ECM is injected into the brain at 21 °C, it transitions from a liquid phase to a hydrogel state at 37 °C by spontaneous cross-

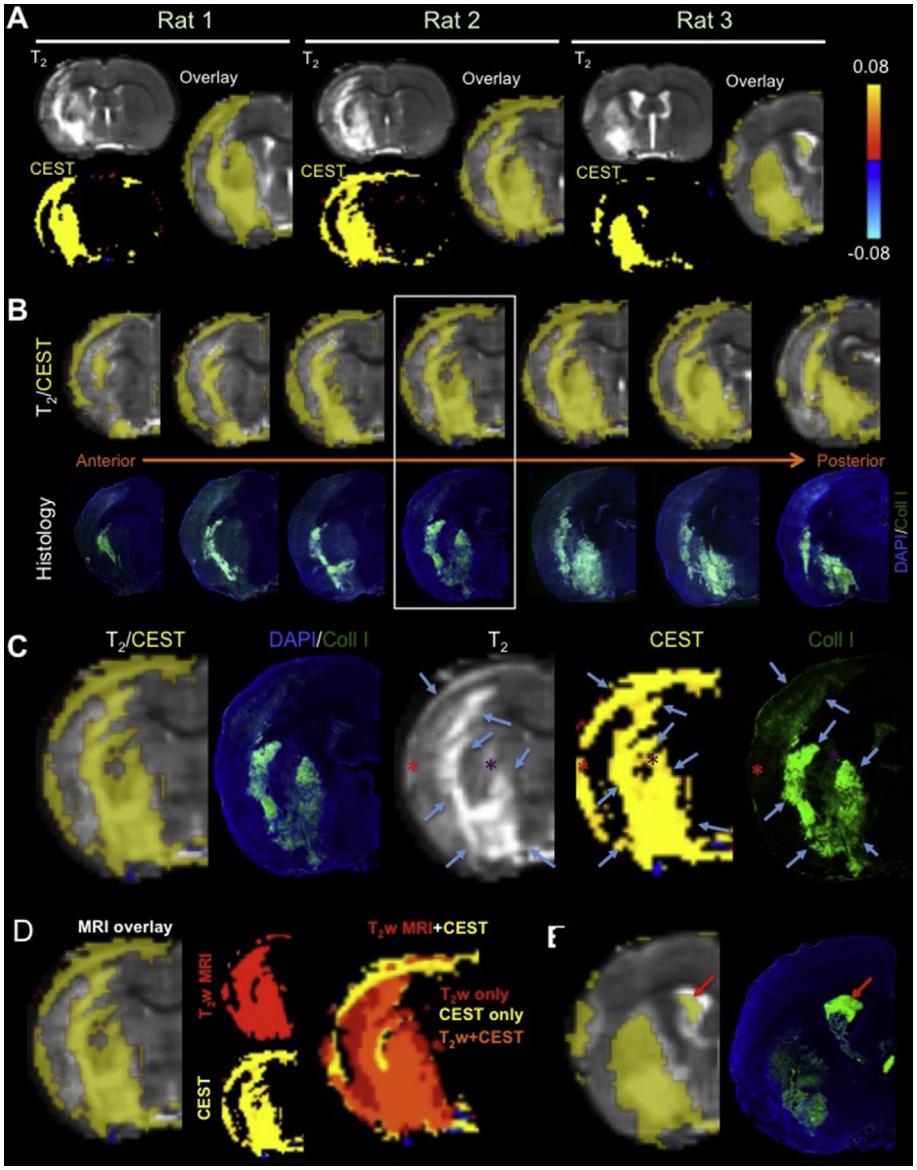

Fig. 4. Acute in vivo detection of ECM hydrogel implanted in a stroke cavity. A. CEST imaging of 8 mg/mL ECM hydrogel 24 h after injection into the stroke cavity in 3 different animals revealed a signal that corresponded to the expected coverage of the lesion cavity. **B**. Anterior-posterior slices further highlight the coverage of the entire lesion cavity and its validation by histological analysis (ECM hydrogel detected by collagen I staining). **C**. A more detailed examination of the correspondence between the CEST and histological detection of the ECM hydrogel revealed a good overlap (blue arrows), but also point out some discrepancies. In some cases, the CEST signal corresponded to the T_2-weighted lesion environment, but there was no evidence of ECM hydrogel present in this area (*). In other instances, a mismatch indicated a CEST signal in an area with damaged host tissue (*), but no significant ECM hydrogel being present. D. A quantitative comparison of T_2-weighted lesion detection (1 standard deviation threshold in red) with ECM-CEST detection (3 standard deviation threshold in yellow) overlaid. Individual voxels were quantified to determine the relative proportion of co-localization of T2-based lesion detection and ECM-CEST (orange voxels) contrasted with lesion (red) and ECM-CEST (yellow) only voxels. **E**. ECM hydrogel leaking into the lateral ventricle (red arrows) due to injection tract damage can be selectively visualized using this approach indicating its validity to assay the macroscopic distribution of ECM hydrogel injections. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

linking of proteins, such as collagen I. The cross-linking and the associated rheological changes are also dependent on the concentration of the ECM. The physical characteristics of the ECM therefore change in a concentration-dependent fashion and could affect detection using diaCEST. In the liquid phase, a robust detection of the ECM is observed at 8 mg/mL with a dose-dependent decrease in signal to 2 mg/mL (Fig. 2B). A 0 mg/mL (PBS only) condition did not produce any detectable MTR_{asym}. Formation of a hydrogel (i.e. bringing the ECM to 37 °C) also produced a dose-dependent signal (Fig. 2C). However, separation between 2 and 4 mg/mL was less clear, potentially due to a lack of gelation of 2 mg/mL. The 4 mg/mL hydrogel condition hence produces an MTR_{asym} that is akin to the 2 mg/mL liquid MTR_{asym}. It is noteworthy that these MTR_{asym} properties of ECM hydrogel are also evident in the commercially available ECM product Matristem (Supplementary Fig. 3). A potential confound of the dose-dependent ECM MTR_{asym} is pepsin, which is also added during the ECM preparation in a dose-dependent fashion. However, different doses of pepsin did not produce an MTR_{asym} that could confound the ECM detection (Fig. 2D).

3.4. Chondroitin sulfate and fibronectin are prominent components of ECM hydrogel CEST signal

As the ECM hydrogel is a collection of different molecules present in tissue, it is unclear what specific molecules contribute to the MTR_{asym}. Individual components were hence prepared to evaluate their MTR_{asym} for comparison with the ECM-induced MTR_{asym}. Some ECM molecules (collagen I, heparin sulfate) produced negligible (<1%) MTR_{asym}, whereas others (collagen IV, hyaluronic acid, laminin, vitronectin) induced minor effects of 1–2.5% (Fig. 3A). In contrast, chondroitin sulfate and fibronectin produced significant MTR_{asym} of 4–7% at 1.8 ppm. It is intriguing to observe that these two molecules produce an overlapping MTR_{asym} very similar to

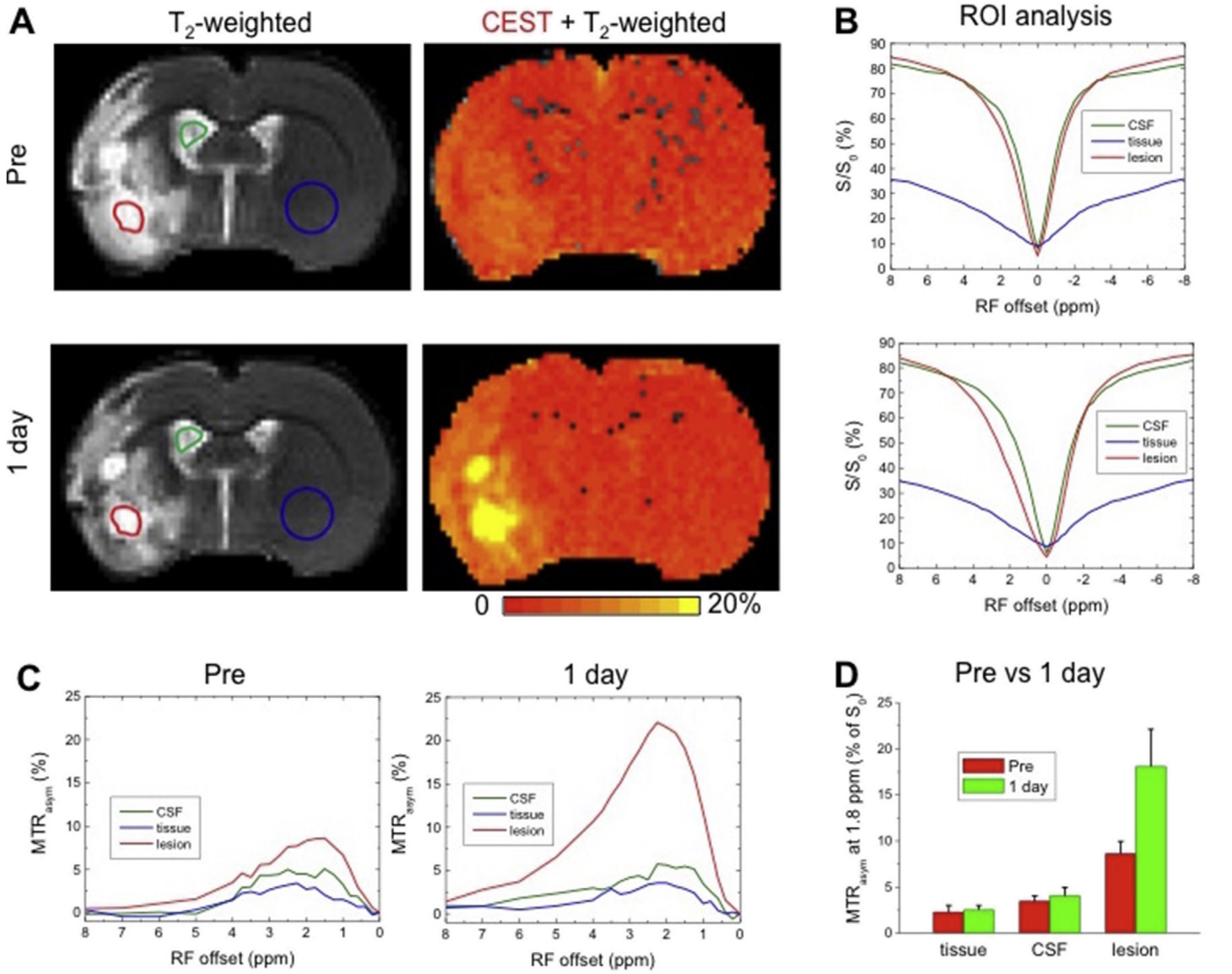

Fig. 5. Stroke microenvironment-specific CEST signals and specificity of ECM hydrogel signal. A comparison of the CEST signal within the lesion cavity, homologous contralateral tissue, as well as the lateral ventricle (**A**). Measurement of the CEST signal in the ROIs (**B**) and calculation of the magnetization transfer ratio asymmetry (MTR_{asym}) revealed a weak (<5%) endogenous CEST signal at 1.8 ppm, which is upregulated in the lesion cavity (~8%) (**C**). However, injection of ECM hydrogel within this same environment lead to an increase of the signal to >18% by 1 day post-implantation. A comparison of effect size (% of S_0) at 1.8 ppm revealed that the signal produced by the ECM at 1 day was very significantly increased ($p = 0.018$) (**D**).

ECM, with a combination of both replicating the overall shape of an ECM hydrogel-induced MTR_{asym} (Fig. 3B).

As the application of ECM hydrogel will require injection into the fluid-filled cavity caused by a stroke, another factor potentially affecting the specificity of detection is the presence of extracellular or cerebrospinal fluid (CSF). Artificial CSF produced a 3.5% MTR_{asym} at 1 ppm and 1.7% at 1.8 ppm (Fig. 3A), which is very similar to that observed from HA, but distinct from ECM. ECM hydrogel can therefore be specifically detected in contrast to potentially confounding variables (PBS, Pepsin, CSF) and produce a robust dose-dependent MTR_{asym}.

3.5. In vivo detection of ECM hydrogel distribution in a rat model of stroke

Upon ECM hydrogel implantation into the cavity 14 days post-stroke, a CEST signal covering the lesion was evident 24 h post-injection in all animals (Fig. 4A). The CEST signal highlighted the anterior-posterior distribution of ECM and its macroscopic comparison to histology further confirmed that the signal corresponded to the area filled with ECM hydrogel (Fig. 4B). However, a more detailed comparison between the CEST and histology images revealed subtle differences that indicated that some of the CEST

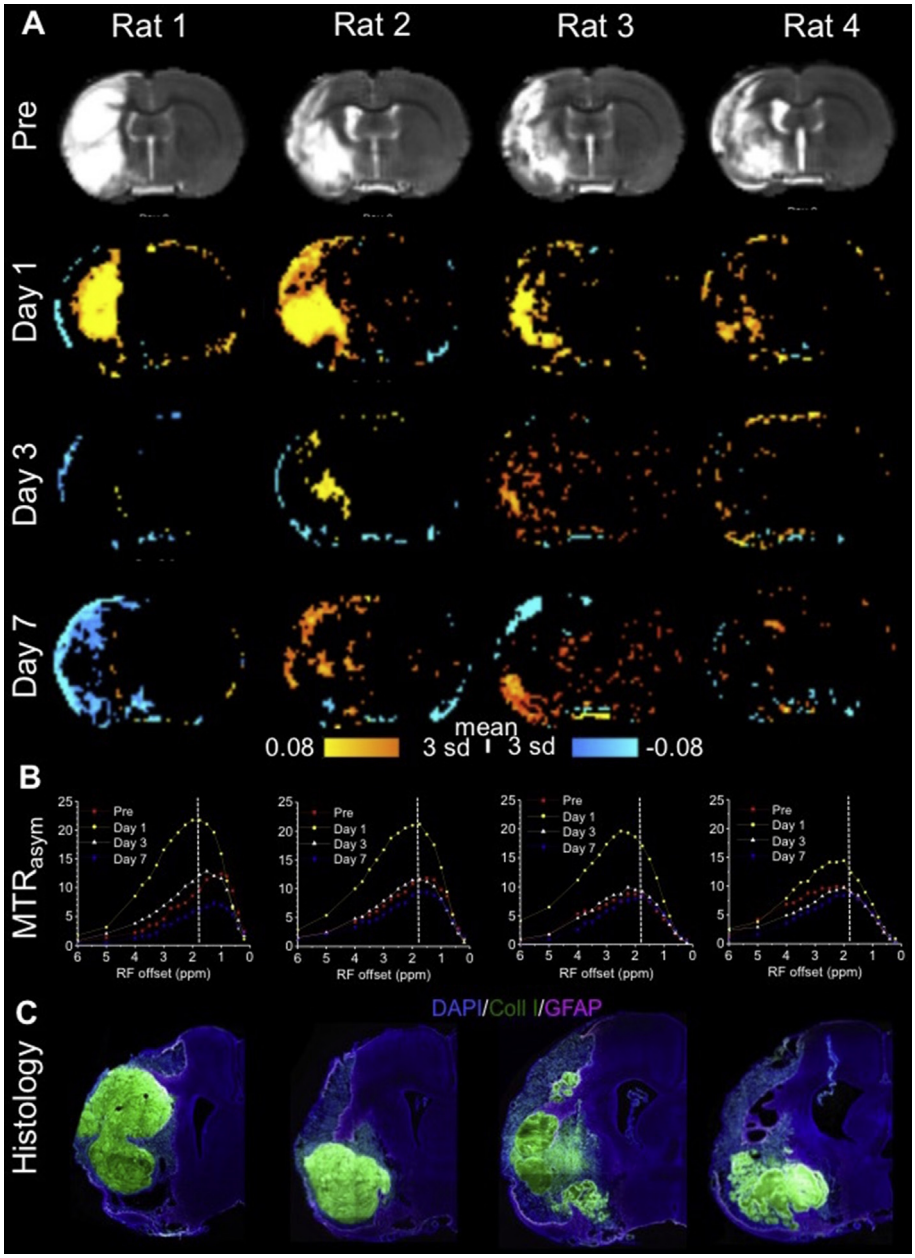

Fig. 6. Serial in vivo detection of ECM hydrogel implanted in a stroke cavity. To probe the utility of this imaging approach in monitoring the presence and potential biodegradation of the ECM hydrogel over time, serial MR imaging was performed prior to the injection of the ECM hydrogel (Pre), as well as 1, 3 and 7 days post-implantation (**A**). To account for a baseline CEST signal, the pre-implant images were subtracted from post-implant time points with thresholding of ±3 standard deviations (s.d.) being applied to filter out noise. Warm colors indicate the degree of signal increase, whereas cold colors represent the extent of signal decrease. MTR_{asym} plots show the evolution of the signal inside the lesion cavity for individual animals (**B**). Histological analyses further corroborated the detection and distribution of ECM hydrogel based on collagen I staining, as well as the delineation of the lesion cavity based on glial scarring as indicated by GFAP staining (**C**).

signal is not contained with the lesion cavity and some voxels are not specific to the injected material (Fig. 4C). Specifically, damaged peri-infarct tissue, as well as areas with a T2 hyperintense signal, also overlap with the CEST signal, but do not contain ECM hydrogel as revealed by collagen I staining, hence suggesting that some endogenous molecules might be upregulated in these areas and account for these discrepancies. A quantification of the T_2-weighted lesion (red voxels) and the ECM-based CEST signal (yellow voxels) indicated 56.37% were co-localized (orange voxels), with 24.90% only being T2w and 18.73% voxels only having a CEST signal (Fig. 4D). This quantification hence supports the qualitative observation that both signals measure different biological substrates, but also that some damaged areas (T2w signal) contain no or insufficient ECM. It is also important to note that in a couple of animals, ECM material unexpectedly leaked into the lateral ventricle (Fig. 4E) and low concentrations of ECM can also be found to permeate into damaged tissue that was below a T_2w lesion threshold, hence leading to a significant CEST signal that was beyond the T_2w-defined stroke lesion.

3.6. In vivo specificity and evolution of ECM hydrogel CEST signal

The presence of a baseline CEST signal, as well as its increase in damaged tissue, question the selectivity of the approach for ECM hydrogel. Indeed, in the contralateral undamaged hemisphere, a weak <5% CEST signal at 1.8 ppm can be found with injected ECM producing a CEST of ~20% (Fig. 5A). This "background" CEST signal is mainly from the intracellular proteins and metabolites, but the intensity is relatively low due to the short tissue T_2 (~40 ms at 9.4 T) and the large direct water saturation effect (Fig. 5B). A slightly larger signal of up to 5% is also present within the lateral ventricles, whereas the signal within the lesion cavity is significantly increased compared to undamaged brain and ventricles to 8.6% MTR_{asym} (Fig. 5C). Note the larger CEST signal in the lesion cavity does not necessarily indicate a higher protein/ECM concentration. Besides the chemical exchange mediated relaxation, the CEST signal is also affected by other relaxation effects, such as T_1, T_2 and direct water saturation. These relaxation effects are expected to be smaller in the lesion cavity due to its higher water content. Nevertheless, implantation of ECM hydrogel within this same area significantly increased the signal to >18% MTR_{asym} ($p = 0.018$) and afforded a clear distinction from baseline signals (Fig. 5D). It is hence plausible that endogenous ECM molecules produced a weak signal in the lesion cavity, but that the injection of ECM hydrogel dramatically increased this signal to a level that is very distinct in its magnitude from the endogenous MTR_{asym}. The CEST signal is hence specific to components of the ECM, but the presence of enogenous ECM in tissue produces a background signal that quantitatively is distinguishable from injected ECM hydrogel.

To selectively visualize the distribution of the ECM hydrogel, it is therefore necessary to account for this endogenous signal. This can be achieved by acquiring a pre-implantation baseline image that is subtracted from post-implantation images. Using this post-processing of the CEST images, the distribution of ECM hydrogel can be specifically and selectively visualized at different time points (Fig. 6A). Consistently injection of ECM hydrogel in 4 animals lead to an increased signal in the lesion cavity by 1 day post-implant. In two animals with a large well-defined T_2-weighted hyperintense lesion cavity, a very robust and cohesive signal increase can be observed with >20% MTR_{asym} (Fig. 6B). In one animal (rat 3), a more irregular lesion cavity also showed a clear increase in MTR_{asym} in the cavity, but patches adjacent to the main area were evident. In rat 4, the detection of the ECM hydrogel was less robust. In all animals, the signal due to the ECM hydrogel dramatically reduced within 3 days post-implantation to reach baseline levels and below baseline by day 7. These observations were also supported by MTR_{asym} plots of the lesion cavity at each time point. Nevertheless, histology at day 7 revealed a robust presence of ECM hydrogel in the lesion cavity in all animals (Fig. 6C). A quantitative comparison of the T_2-weighted lesion with ECM-CEST and ECM-histology volume (Fig. 7A) indicated no statistically significant difference (Fig. 7B). However, T_2-weighted lesion volume (145.63 mm^3) was 25% higher compared to ECM-CEST and ECM-histology, with ECM-CEST and ECM-histology revealing a very similar volume (110 mm^3 and 102.6 mm^3) at 7 days, suggesting that CEST is a reasonable surrogate of ECM hydrogel that is independent from the MR detection of the lesion cavity.

3.7. Group comparison of CEST signal with control conditions

To afford a group comparison of the CEST signal due to the ECM hydrogel injection, animals were coregistered and mean images were created for a MCAo only, a vehicle (PBS) injection, as well as an ECM injection condition. A comparison of the T_2-weighted

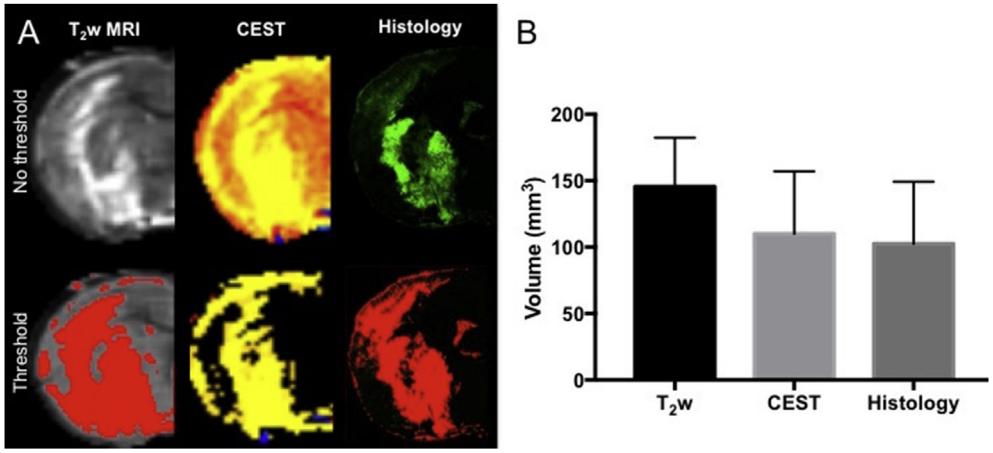

Fig. 7. Quantitative comparison of lesion volume with CEST- and histology-based ECM volumes. Acquired images were thresholded to define lesion volume (1 s.d.), as well as ECM-CEST (3 s.d.) and ECM-histology volumes (1 s.d.) (**A**). A comparison of volumes revealed no significant difference, but ECM volume (both CEST and histology) was 25% lower than T_2-weighted lesion volumes at 7 days (**B**).

hyperintensity, indicative of the stroke lesion, revealed no significant changes over 7 days (Supplementary Fig. 4). In the MCAO only condition, no change of the CEST signal in the stroke area was evident (Fig. 8). A vehicle injection revealed a slight increase in the CEST signal in damaged tissue 1 day post-injection, but upon further inspection this was only evident in 1 animal out of 4. While the averaged MTR_{asym} spectra showed little change at day 1 after the PBS injection (yellow vs. red, Fig. 7B), at day 3 and 7 the spectra became much lower. At 1.8 ppm, the MTR_{asym} is 11.1%, 11.3%, 7.3%, and 6.4% for preinjection and day 1, 3, and 7 after PBS injection, respectively. Compared to the case without injection, the significant drop of MTR_{asym} over time after the PBS injection indicated some physiological change caused by (or in response to) the injection, but the source is still unclear and requires further investigation. In contrast, in the ECM group a robust increase in the CEST signal was observed throughout the hyperintensity delineating the lesion cavity on the T_2-weighted MR image. The signal evident at 1 day post-implantation in ECM hydrogel implanted animals is hence selective to this material and reveals its in vivo distribution. However, by 3 days post-implantation the area of distribution was much reduced. By 7 days, no CEST signal above baseline could be detected. These visual representations are further supported by direct measurements of the MTR_{asym} in the lesion area (Fig. 8B). A clear peak of the CEST signal is evident at 1.8 ppm with >20% MTR_{asym} on day 1 after ECM injection. In contrast to the CEST image, at 7 days post-implantation there was still robust histological evidence of ECM hydrogel present within ECM injected animals, whereas there was no material present within MCAo only or PBS injected animals (Fig. 8C). Nevertheless, there was evidence in 1 PBS animal that there was an upregulation of collagen I in a peri-infarct area, which indicates an endogenous response to the injection of vehicle that is likely to upregulate ECM molecules to a level that is distinct from its pre-implantation state and sufficient to induce a small increase in CEST signal. However, this increase was not evident in the other animals in this group, indicating that an injection of a large volume of PBS inside damaged tissue, rather than the cavity, in this one animal might underlie these histological and imaging observations.

3.8. Time course comparison of CEST signal and ECM molecules in infarct and hydrogel

A comparison of the time course of the CEST signal with histology indicates that in MCAo only animals, the CEST signal is almost exclusively confined to the T_2-hyperintensity of the lesion, corresponding histologically to the cavity, rather than surrounding damaged tissue, as indicated by GFAP reactivity of glia limitans (Fig. 9A). Nevertheless, collagen I, chondroitin sulfate and fibronectin are all upregulated in this peri-infarct area compared to intact tissue. After ECM hydrogel injection, the cavity is filled with ECM hydrogel, as indicated by collagen I staining, corresponding to the area of increased CEST contrast. Still, some CEST signal is evident in damaged cortical regions, where no ECM hydrogel is evident. Chondroitin sulfate and fibronectin are also highly present within the ECM hydrogel hence providing a larger area of coverage, as well as a greater abundance of the molecules compared to the MCAo only condition (Fig. 9B). By 7 days, the area and magnitude of the CEST signal is reduced, but the area covered by the ECM hydrogel still corresponds to the lesion cavity, hence indicating a mismatch between the CEST signal and the ECM hydrogel due to the CEST signal returning to its baseline state. A quantitative assessment of signal intensity of fibronectin and chondroitin sulfate inside the ECM hydrogel indicate that with time ($p < 0.01$) there is a significant decrease of both within the ECM hydrogel and to a lesser degree a decrease in collagen I (Fig. 9C). This further highlights the contribution of fibronectin and chondroitin sulfate to the specific and selective imaging of ECM after injection. The loss of these molecules is likely due to constructive remodeling, which eventually leads to a biodegradation of the ECM hydrogel.

4. Discussion

The use of extracellular matrix (ECM) as an inductive scaffolding material in regenerative medicine is finding wide-ranging applications [7], yet little advancement has been achieved in monitoring the distribution and degradation of these bioscaffolds using non-invasive imaging [21]. Herein, we demonstrate that ECM hydrogel has specific diamagnetic chemical exchange saturation transfer (diaCEST) properties that can be exploited to visualize and analyze its distribution acutely after implantation into a stroke cavity in the brain. Chondroitin sulfate and fibronectin are likely to be the main source of this effect, with the decrease of these molecules in the ECM hydrogel resulting in a return to the baseline CEST signal. Chondroitin sulfate and fibronectin within ECM hydrogel therefore provide specificity to the imaging approach, but their level of abundance conveys the selectivity to visualize the injected bioscaffold against the ECM background in the brain.

4.1. Specificity of ECM hydrogel detection by CEST imaging

The ECM is a collection of molecules secreted by resident cells of all tissues and organs [41]. Upon tissue decellularization, these ECM molecules are retained and can be used for bioscaffolding purposes [7,42]. Non-invasive imaging of ECM molecules has mainly focussed on glycosaminoglycans (GAGs) due to their presence in intervertebrate discs and the clinical need to improve our ability to visualize its degradation. The assessment of GAG content can be achieved using CEST imaging [29,43]. Implantation of a biomimetic ECM to promote cartilage regeneration indicated that these materials produce MRI characteristics similar to that of the native tissue [44,45], but no gagCEST was performed to visualize the scaffold using its biochemical characteristics. Importantly four classes of GAGs can be recognized: heparin sulfate; chondroitin sulfate; keratin sulfate; and hyaluronic acid [46]. Indeed, chondroitin sulfate and hyaluronic acid are known to be present at a high concentration within the urinary bladder-derived ECM and detectable using immunohistochemistry against the lower abundance present within the native brain [8]. Our in vitro studies here showed that CS and fibronectin induced CEST effects and potential for CEST imaging. Especially at 1.8 ppm, CS showed a marked effect with a 7% MTR_{asym} effect compared to a 2.5% effect of HA. In contrast, collagen I which is highly present with ECM from urinary bladder exerted a negligible ~1% MTR_{asym}. Still, most ECM molecules tested here exhibited small effects between 0.2 and 2 ppm. Only CS and fibronectin exhibited wider chemical shifts. Indeed, chondroitin sulfate and fibronectin combined produce a MTR_{asym} generally resembling the ECM profile, suggesting that the other molecules tested here exert a minor contribution to the CEST detection of ECM hydrogel. UBM-ECM-specific CEST imaging is hence feasible based on the MTR_{asym} generated by CS and fibronectin. In contrast to ECM, the use of particular ECM molecules, such as HA, and its combination with other molecules potentially provides a mean to image those molecules more specifically in the absence of these in the native tissue [31]. However, a separation of individual molecules' signals will be challenging in a naturally mixed preparation, such as ECM, as there is a significant overlap between the MTR_{asym} of these molecules.

The interaction and complexing of molecules in a mixed environment, such as the ECM, is also likely to influence the CEST effects that the individual molecules exert. Indeed, protein composition is known to affect biodegradation and rheological properties, which

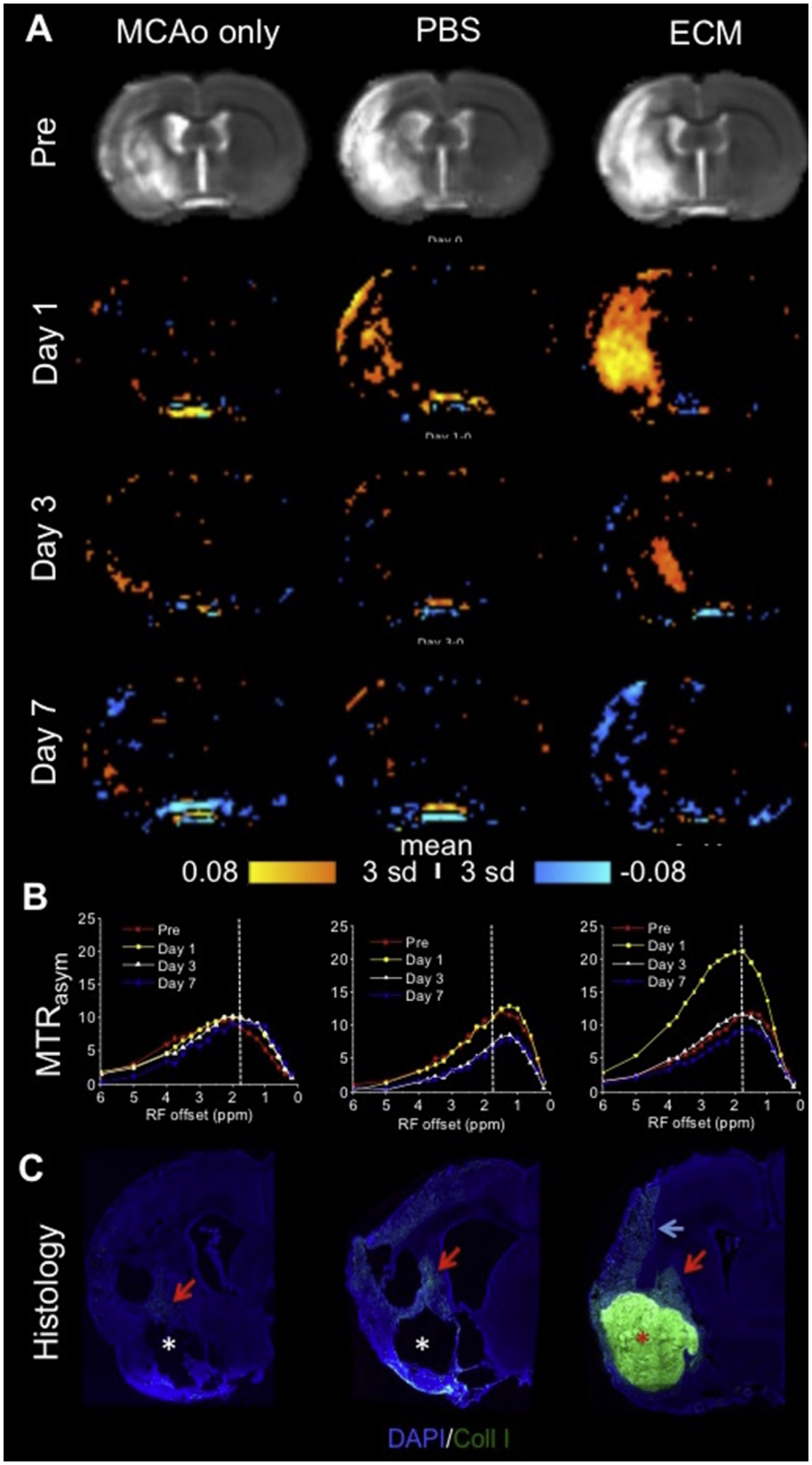

Fig. 8. Group comparison of CEST signal. A. To afford a group comparison, individual animals were co-registered and images were averaged to provide a representative mean group image for each time point. In MCAo only animals, no CEST signal was evident at any time point when accounting for the baseline signal and thresholding. For PBS (i.e. vehicle) injected animals, an acute signal change was observed. This was due to 1 animal having an elevated CEST signal on 1 day, potentially reflecting an increase in host chondroitin sulfate and fibronectin due to injection damage. However, a clear increase in CEST signal was evident in the ECM injected group at 1 day post-injection, but this signal gradually decreased by 3 days and no longer warranted detection at 7 days. Warm colors indicate the degree of signal increase, whereas cold colors represent the extent of signal decrease. **B**. Group MTR_{asym} plots for signal inside the lesion cavity further supported the changes observed on the mean images. **C**. A representative histology image further corroborated the

can affect MR parameters, such as T2 and diffusion [15,47]. It was also evident here that detection of the CEST signal was influenced by the phase of the ECM (i.e. liquid versus cross-linked into a hydrogel). An overall decrease of the CEST signal was apparent. Importantly, at lower concentration with incomplete gelation [8], little distinction was, for instance, observed between 2 and 4 mg/mL in the hydrogel preparation. Still, a robust dose-dependence was evident indicating a linear relationship between concentrations >3 mg/mL and the CEST signal. Indeed, changes in the rheological properties due to cross-linking are likely to influence T2 signal, as well as NMR properties [48]. Although gagCEST is highly correlated with the T2 signal in native tissue [49], cross-linking of a hydrogel will not only influence water content of the material leading to a decrease in T2 signal, but will also affect its NMR properties [50]. A transition here from the liquid phase to the hydrogel state resulted in an overall reduction of the MTR_{asym}. Still, an effect size of ~6% in vitro indicated potential for in situ visualization. Encouragingly, pH did not significantly affect the ECM hydrogel signal and the MTR_{asym} was actually improved at physiological normothermia (i.e. 37 °C). A specific dose-dependent CEST signal of ECM hydrogel can hence be achieved that is marginally affected by pH and temperature.

4.2. Selective in vivo monitoring of hydrogel distribution and degradation

Injection of ECM hydrogel at 8 mg/mL afforded the detection of a specific CEST signal at 1.8 ppm that covered the lesion cavity. However, noise and background ECM signal required the application of a threshold to selectively visualize the signal increase. This signal was macroscopically corroborated by immunohistochemistry for the ECM hydrogel based on collagen I staining and was consistent with previous experiments [8]. However, a more detailed investigation of the correspondence between the CEST signal and the histological distribution of ECM hydrogel revealed small patches of mismatch, suggesting that damaged host tissue also produces a CEST signal at 1.8 ppm. Indeed, it is known that a variety of ECM molecules (e.g. chondroitin sulfate; fibronectin; laminin; collagen IV; hyaluronic acid) are upregulated in the peri-infarct area [51–54], as well as after intracerebral injection [55], thus confounding the "specific" CEST signal at 1.8 ppm. To distinguish this endogenous upregulation of the CEST signal in the stroke brain from the ECM hydrogel injected signal, acquisition of a baseline pre-implantation image allowed the subtractation of this endogenous baseline signal from the post-implant CEST to reveal changes due to the injected material. This technique produced a selective visualization of the ECM hydrogel distribution at day 1, but also suggested that an additional upregulation of endogenous ECM molecules may be caused by injection damage to the peri-infarct region. These results hence indicate that ECM hydrogel can be detected selectively, but that interpretation of any CEST changes needs to include a careful consideration of alternative endogenous sources of contrast.

Considering the microenvironment inside the lesion cavity, semi-solid macromolecular magnetization transfer (MT), as well as water proton saturation (spillover), provide competing signals influencing CEST imaging within a stroke lesion [27,56]. Nevertheless, the magnitude of increase in MTR_{asym} due to the ECM injection against the stroke environment is ~12%, providing a robust detection of distribution within the lesion, but also erroneous hydrogel localization, for instance in the ventricle. This demonstrates the usefulness of this approach to map the delivery and initial distribution of the ECM hydrogel within the stroke lesion environment, akin to our previous report using immunohistochemical methods to detect ECM hydrogel [8]. Still, the degradation of the material reduced the abundance of ECM molecules and within 3 days mostly returned to baseline. Remodeling of the ECM lead to a decrease in fibronectin and chondroitin sulfate, which is likely to account for the decrease in CEST signal. However, ECM material here resulted in a fairly broad CEST effect with specific peaks at 1.8 and 3.6 ppm, which is more weighted by chondroitin sulfate and fibronectin. While the 3.6 ppm signal is likely due to the amide-water proton transfer effects [35], the exact source of the 1.8 ppm signal is not clear. The signal reaches maximum with a relatively high power of 220 Hz, suggesting a fast chemical exchange rate. Thus, it may arise from hydroxyl protons, which have a chemical shift of 1–3 ppm from water [57], or amine groups from the side chain of mobile proteins [58,59]. Interestingly, others reported similar CEST effects (also at 1.8 and 3.6 ppm) using hyaluronic acid hydrogels supplemented with gelatin [31]. In order to quantify the degradation of the biomaterial more accurately, implementation of imaging approaches geared towards measuring the macromolecular tissue volume might be needed [60], whereas monitoring the degradation of specific ECM molecules would require their tethering to contrast agents, such as gadolinium [17].

5. Conclusions

Individual constituents of ECM hydrogel produce a diamagnetic CEST effect that can be exploited to analyze its distribution and potentially biodegradation in vivo using non-invasive imaging. We demonstrated here that it affords the selective visualization of an ECM hydrogel injected into a stroke-cavity by targeting a specific frequency at which the "contrast" is tuned towards chondroitin sulfate and fibronectin. Although the imaging is robust against changes in pH and temperature, other molecules can exert similar effects, as the CEST signal is not directly detecting ECM molecules, but is sensitive towards their labile proton content. Both hydroxyl and amine signals may contribute to the signal in this frequency range. A further consideration is that endogenous ECM also exerts a minor CEST effect, which is upregulated in the area of infarction, hence requiring subtraction of a baseline CEST imaging to provide a truly selective imaging of ECM hydrogel distribution. ECM hydrogel is used clinically to promote regeneration of soft tissue, but there is a lack of non-invasive methods to monitor the remodeling of the material. CEST imaging is finding implementation in clinical practice and these methods conceivably can be adapted to provide novel ways to analyze the implantation of ECM hydrogel, but also potentially map a time course of the tissue reconstruction process. We here provide proof-of-principle that MRI can serve this analytical need, but further studies will be needed to develop additional methods to visualize individual ECM components to monitor their degradation and the gradual replacement of the hydrogel with *de novo* tissue.

lack of ECM material in the lesion cavity in MCAo only and PBS animals, but a robust coverage in ECM hydrogel injected animals (*). In a PBS injected animal, an upregulation of collagen I (Coll I) was observed in the peri-infarct area (red arrow), but this was not evident in the MCAo only animals. In ECM hydrogel injected animals, there was also evidence of an upregulation of collagen I staining in the peri-infarct area including the lesioned cortex (blue arrow). However the degree of increase was markedly higher than in PBS animals and is likely to reflect some of the ECM hydrogel having permeated into this area during the injection procedure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

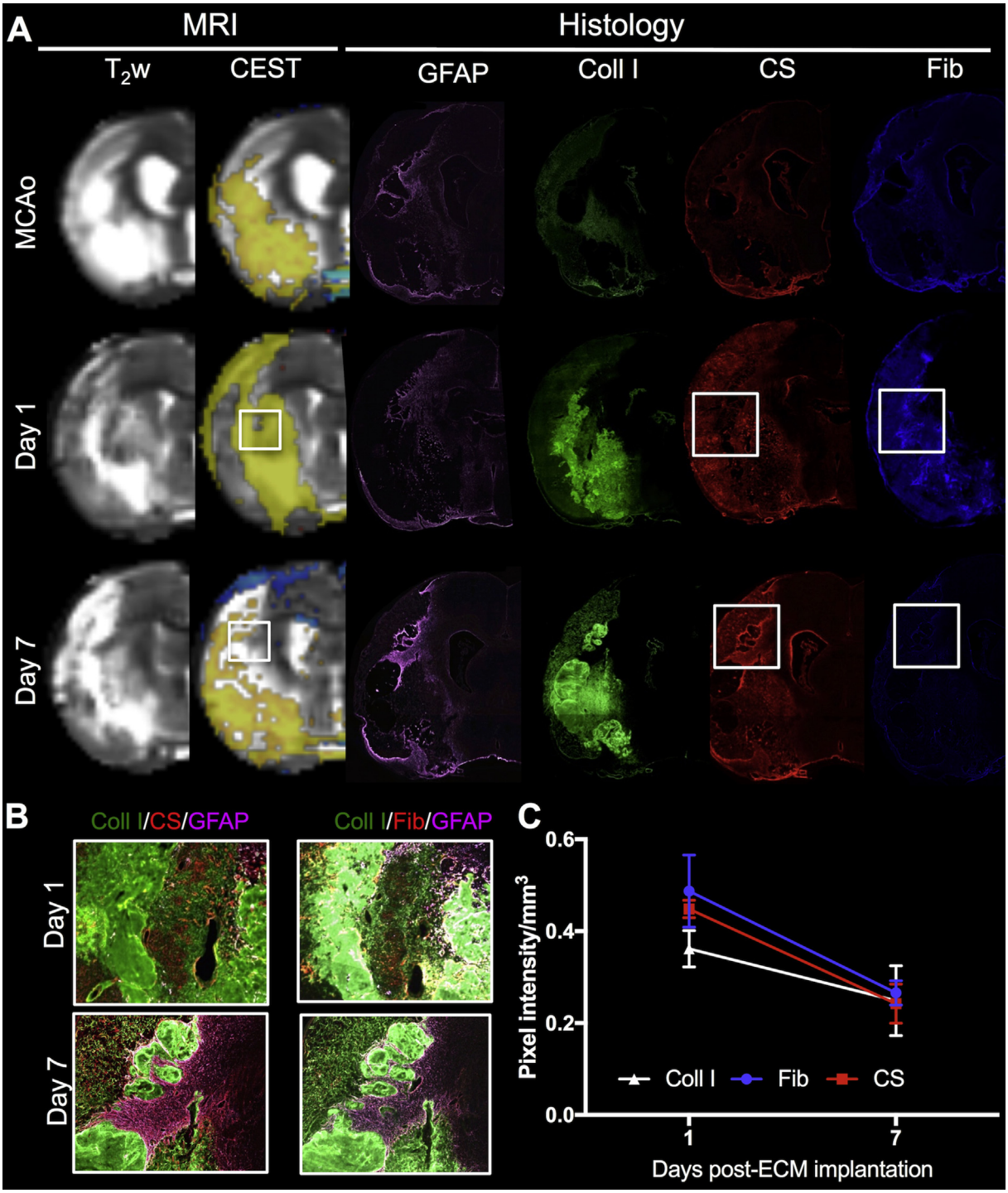

Fig. 9. Histological validation of in vivo imaging. A. A direct comparison between the lesion environment and the CEST signal indicates that a strong signal (thresholded to 3 standard deviations of the contralateral hemisphere) can be detected in the stroke area in MCAo animals. The CEST signal detected here is hence not by itself specific to the ECM hydrogel, but is based on the CEST effect of ECM molecules that are also upregulated after a stroke. Indeed, collagen I (Coll I), chondroitin sulfate (CS) and fibronectin (Fib) are found to be significantly upregulated in the lesion area compared to the contralateral hemisphere and hence provide a biological source for this increase CEST signal. Nevertheless, the injection of an ECM hydrogel further enhances this endogenous signal, specifically in the area where ECM hydrogel was injected into the lesion cavity with chondroitin sulfate and fibronectin being strongly present within the injected material at 1 day, but less so at 7 days. At 7 days post-injection, this signal is reduced to baseline. Especially fibronectin appears reduced at 7 days within the ECM hydrogel, with chondroitin sulfate showing a less marked decrease and collagen I showing little difference to the 1 day time point. **B**. Overlay images for 1 and 7 days post-injection further highlight the differences in chondroitin sulfate and fibronectin (adjacent sections) in the ECM hydrogel (collagen I), as well as at the interface with damaged host tissue. **C**. A quantitative measurement of pixel intensity inside the ECM hydrogel indicates that fibronectin and chondroitin sulfate are highly present within the ECM on day 1 post-injection, but appear to be reduced by 7 days post-injection. Collagen I is also reduced, further highlighting that biodegradation is occurring between these two time points.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2016.10.043.

Author contributions

JT set-up, acquired and developed the post-processing of diaCEST images. FJN prepared all samples, performed MCAo surgery and ECM implantation and perfused animals. WC performed co-registration, group mapping and statistical image comparisons. HG performed immunohistochemistry and acquired histology images. SFB provided the ECM hydrogel. MM conceived of the study, provided funding, oversaw the acquisition and analysis and also drafted the manuscript. All authors read and approved the manuscript.

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