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Low Molecular Weight Supramolecular Hydrogels for Sustained and Localized *In Vivo* Drug Delivery

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Hydrogels were formed by dissolving cationic gelator Fmoc-Phe-DAP (**1**), Fmoc-3F-Phe-DAP (**2**), or Fmoc-F^5-Phe-DAP (**3**) to produce a final concentration of 33.7 mM gelator in 1 mL of water. The gelation protocol proceeded by the following steps. The gelator was first suspended in 800 μL of deionized water. The suspension was sonicated until a uniformly fine suspension of the gelator was formed. This solution was then heated to 80 ºC until the solid was completely dissolved. After solubilization, 200 μL of 570 mM NaCl solution was added to give a final concentration of 114 mM of NaCl, and final gel volume of 1 mL. Immediately following salt addition, the vial was briefly mixed by vortex and the hydrogel formed within a few seconds.

Hydrogelation Conditions with Diclofenac

Diclofenac-containing hydrogels were prepared by dissolving diclofenac sodium salt (Sigma Aldrich) (5 mg) in 800 μL water. Gelators **1**, **2**, or **3** were then added to this solution and dissolved by sonication followed by heating (80 ºC) until the gelator was fully solubilized. To this solution of gelator and diclofenac was added 200 μL of 570 mM NaCl solution, which was briefly mixed by vortex resulting in formation of hydrogels within a few seconds. The final gel volume was 1 mL with a gelator concentration of 33.7 mM, a diclofenac concentration of 5 mg mL^{-1} (15.7 mM), and a NaCl concentration of 114 mM.

Transmission Electron Microscopy

10 μL of sample was pipetted onto a 200 mesh carbon coated copper grid and allowed to stand for 1 minute. Residual solvent was wicked off via capillary action with filter paper. Grids were then stained with 10 μL of uranyl acetate for 1 minute, which was removed by capillary action. Grids were then allowed to air dry for 5 minutes. Images were obtained on a Hitachi 7650 transmission electron microscope with an accelerating voltage of 80 kV at magnifications between 50k and 200k.

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Oscillatory Rheology

Rheological measurements were conducted using a TA Instruments AR-G2 rheometer. A 20 mm parallel plate geometry was used for experiments. Gels were formed in a 1 mL Eppendorf tube and then a razor blade was used to cut off the bottom of the Eppendorf tube to produce a 0.5 mL cylindrical gel. This gel was then transferred to the Peltier plate. The experiments were performed using a 500 μm gap size operating in oscillatory mode. Strain sweep experiments were performed to determine the linear viscoelastic region at 25 ºC for 0.1 – 100% strain at a frequency of 6.283 rad/s. Frequency sweep experiments were performed at 25 ºC from 0.1 – 100 rad/s with 1% strain, which falls within the linear viscoelastic region for this gel.

Shear thinning experiments were performed by cyclic application of low strain (0.1%) followed by high strain (40-60%) to record the viscoelastic properties of the gels as they were broken and upon reformation. Low strain was applied for 300 seconds followed by high strain for 200 seconds and this experiment was repeated twice on each gel at an oscillatory frequency of 6.283 rad/s while G′ and G″ (Pa) were recorded. Each application of low and high strain was run immediately after one another with the exception of 5 seconds for instrument reset.

In Vitro Drug Release Studies

Hydrogels containing diclofenac were prepared as described above to form a 1 mL hydrogel with 33.7 mM gelator, 5 mg/mL diclofenac, and 114 mM NaCl. Phosphate buffered saline (pH 7, 4 mL) was slowly pipetted over the top of the gel, and this two-phase gel/solution mixture was sealed in a vial and incubated at 37 ºC. Aliquots of the buffer solution (100 μL) were removed at 20 min, 40 min, 60 min, 2 h, 3 h, 4 h, 6 h, 24 h, 48 h, and 72 h from the time the buffer was initially layered on top of the gel. After removing each aliquot, the buffer solution was immediately replaced by an equal volume. The concentration of diclofenac in each aliquot was determined by injection onto an analytical HPLC instrument (Shimadzu 2010A) equipped with a Phenomenex Gemini 5 micron C18 column (250 × 4.6 mm) and correlation of the integrated peak area of diclofenac (see **Figure S8** for an example) to a standard concentration curve. A gradient of water and acetonitrile containing 0.1% TFA was used as the mobile phase eluent at a flow rate of 1 mL/min and UV detection was monitored at 215 nm (see Table S4.1 for conditions). The correlative concentration curve (**Figure S9**) was constructed by injection of serial dilutions of a solution of known diclofenac concentration onto the HPLC under the defined

mobile and stationary phase conditions. This enabled interpolation of the amount of diclofenac released into the 4 mL solution at each particular time point by conversion of concentration of diclofenac to μmol of diclofenac and the diffusion constant was determined using **Equation 1**. This is a non-steady state diffusion model equation, where M_t/M_∞ (unitless) is the ratio of molecules of diclofenac released to the total molecules of diclofenac in the system, t is the time (min), λ is gel thickness (height, m), and D is the diffusion constant (m^2 min^{-1}).[1–3]

Equation 1. $$\frac{M_t}{M_\infty} = 4\sqrt{\frac{Dt}{\pi\lambda^2}}$$

For each timepoint, the concentration of diclofenac in the aliquot was used to calculate the total amount of diclofenac present in the 4 mL phosphate buffer layer in μmol. For the first timepoint, this value was used directly as M_t, but for subsequent timepoints the amount calculated for M_t was adjusted to include the amount of diclofenac removed in prior aliquots so that M_t reflected the total amount of diclofenac released from the gel from the start time to time t. The data were collected in triplicate and were plotted initially as M_t/M_∞ against time (min) with the error reported as the standard deviation about the mean (**Figure 6A**). A second plot was constructed by plotting M_t/M_∞ against $t^{1/2}$ ($min^{1/2}$) from the initial linear section of the first plot (comprising approximately the first 240 minutes of the release study) (**Figure 6B**). **Equation 1** can be rearranged to yield a linear relationship between M_t/M_∞ and $t^{1/2}$ ($min^{1/2}$) as follows:

$$\frac{M_t}{M_\infty} = 4\sqrt{\frac{D}{\pi\lambda^2}} \times \sqrt{t}$$

Thus, the diffusion coefficient, D (m^2 min^{-1}), was determined by measuring the slope of M_t/M_∞ against $t^{1/2}$ ($min^{1/2}$) in this second plot and setting this value equal to the coefficient of $t^{1/2}$ ($min^{1/2}$) above in order to solve for the value of D (m^2 min^{-1}).

In Vivo Drug Delivery

Adult male and female C57BL/6 mice aged 2-5 months (Jackson Laboratory) were used for the study. The animals were housed in a room with a 12 h day/night cycle. All experiments with live animals were performed in accordance with institutional guidelines at the University of Rochester. All procedures were approved by the University Committee on Animal Resources (UCAR-2014-038).

Acute inflammatory pain was induced by an intra-articular administration of complete

Freund's adjuvant (CFA, 10 µl, Sigma-Aldrich, St. Louis, MO) into a hind limb ankle joint using a syringe with 33 G needle.[4] Two days after the pain induction, diclofenac solution (0.1 mg/ml in physiological saline, 10 µl, Sigma-Aldrich, St. Louis, MO), diclofenac in Fmoc-F_5-Phe-DAP (**3**) hydrogel (5 mg/ml, 10 µl), or vehicle (dH_2O) in Fmoc-F_5-Phe-DAP (**3**) hydrogel (10 µl) was administered with 33 G needle to the ipsilateral hind limb ankle joint under anesthesia (1% isoflurane). A naïve animal group did not receive CFA administration, and Fmoc-F_5-Phe-DAP (**3**) hydrogel (10 µl) was administered into a hind limb ankle joint using a syringe with 33 G needle.

Mechanical sensitivity was measured using the Semmes-Weinstein von Frey Aesthesiometer (Stoelting Co, Wood Dale, IL) touch test, as previously described.[4] Briefly, animals were individually housed in an acrylic chamber with a metal grid at the bottom (IITC Life Science, Woodland Hills, CA), and a thin filament of 0.04 g force was gently applied to the hind paws of the animals. The rapid retraction or tapping of the foot was counted as a positive response, and the data are presented as percent of the positive responses out of the total trials. The evaluator did not participate in the experimental design and the animal assignment, thus had no prior knowledge of the animal groups. All behavioral measurements were done at the same time of the day during the 12 h light cycle. Ankle swelling was evaluated by measuring circumferences of the ankles at the joint from both left and right hind limbs, and the data are presented as percent of contralateral circumferences.

One-way ANOVA with Tukey-Kramer multiple comparison procedure was used to compare differences. A paired t test was used to compare the pain level before and immediately after the treatment. The significance level was set at 0.05 for all comparisons.

1. Sutton, S. *et al.* Controlled Release From Modified Amino Acid Hydrogels Governed By Molecular Size Or Network Dynamics. *Langmuir* **25**, 10285–10291 (2009).
2. Nagai, Y., Unsworth, L. D., Koutsopoulos, S. & Zhang, S. Slow Release Of Molecules In Self-Assembling Peptide Nanofiber Scaffold. *J. Control. Release* **115**, 18–25 (2006).
3. Panda, J. J., Mishra, A., Basu, A. & Chauhan, V. S. Stimuli Responsive Self-Assembled Hydrogel Of A Low Molecular Weight Free Dipeptide With Potential For Tunable Drug Delivery. *Biomacromolecules* **9**, 2244–2250 (2008).
4. Fujita, T., Feng, C. & Takano, T. Presence Of Caffeine Reversibly Interferes With Efficacy Of Acupuncture-Induced Analgesia. *Sci. Rep.* **7**, 3397 (2017).

FIGURES AND TABLES

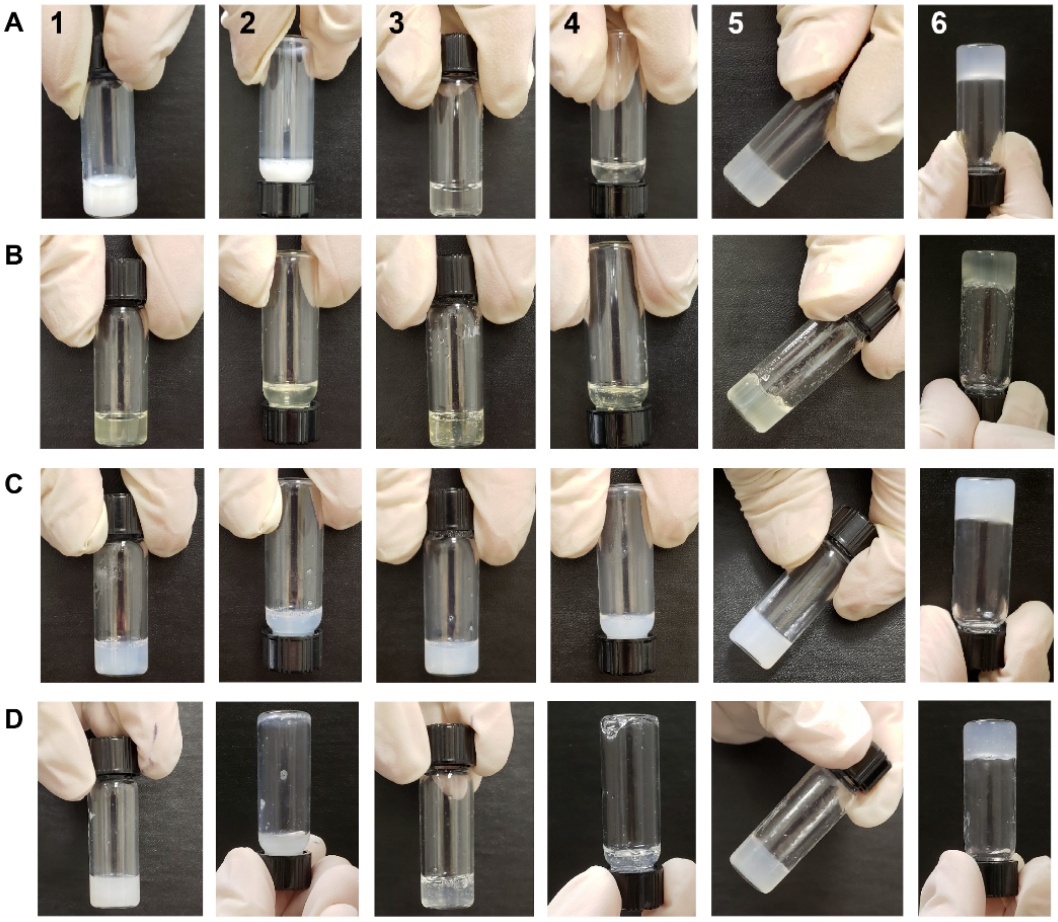

Figure S1. Formation of 33.7 mM hydrogels. **A)** Fmoc-Phe-DAP hydrogel, **B)** Fmoc-3F-Phe-DAP hydrogel, **C)** Fmoc-F^5-Phe-DAP hydrogel, **D)** 1:1 Fmoc-Phe-DAP : Fmoc-F^5-Phe-DAP hydrogel. Panels 1 and 2 depict the solutions that do not pass vial inversion after sonicating the gelator in 800 µL of water. Panels 3 and 4 depict the clarification of solutions after heating to 80 ºC. Panels 5 and 6 shows hydrogel formation immediately after addition of 200 µL of 570 mM NaCl.

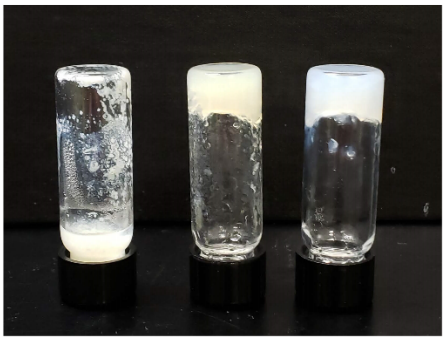

Figure S2. Picture of hydrogels incubated at 37 ºC for two weeks. From left to right: Fmoc-Phe-DAP (**1**), Fmoc-3F-Phe-DAP (**2**), and Fmoc-F^5-Phe-DAP (**3**) (33.7 mM hydrogels). Hydrogels of **1** were less mechanically stable over two weeks, while hydrogels of **2** and **3** retained mechanical stability over two weeks.

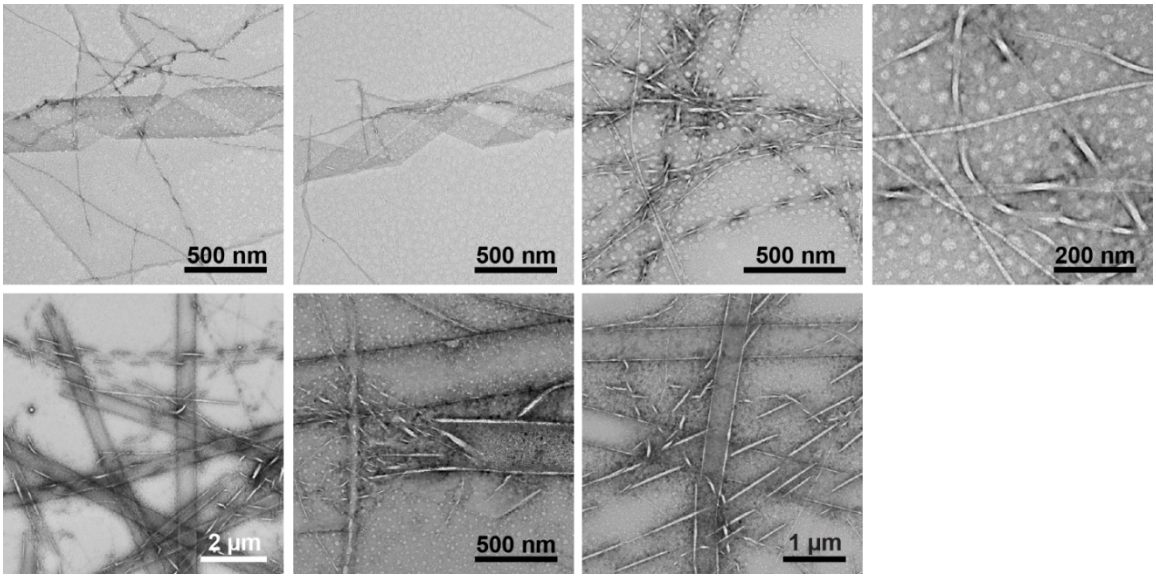

Figure S3. Representative TEM images of Fmoc-Phe-DAP (**1**) hydrogels (33.7 mM) after 24 hours (top row) and after 72 hours (bottom row) of incubation at 37 ºC.

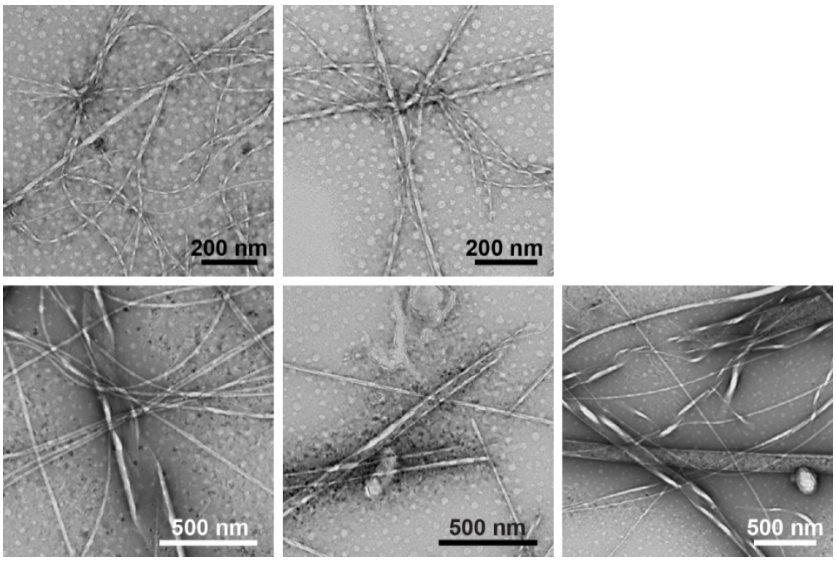

Figure S4. TEM micrographs of Fmoc-3F-Phe-DAP hydrogels. Representative TEM images of Fmoc-3F-Phe-DAP (**2**) hydrogels (33.7 mM) after 24 hours (top row) and after 72 hours (bottom row) of incubation at 37 ºC.

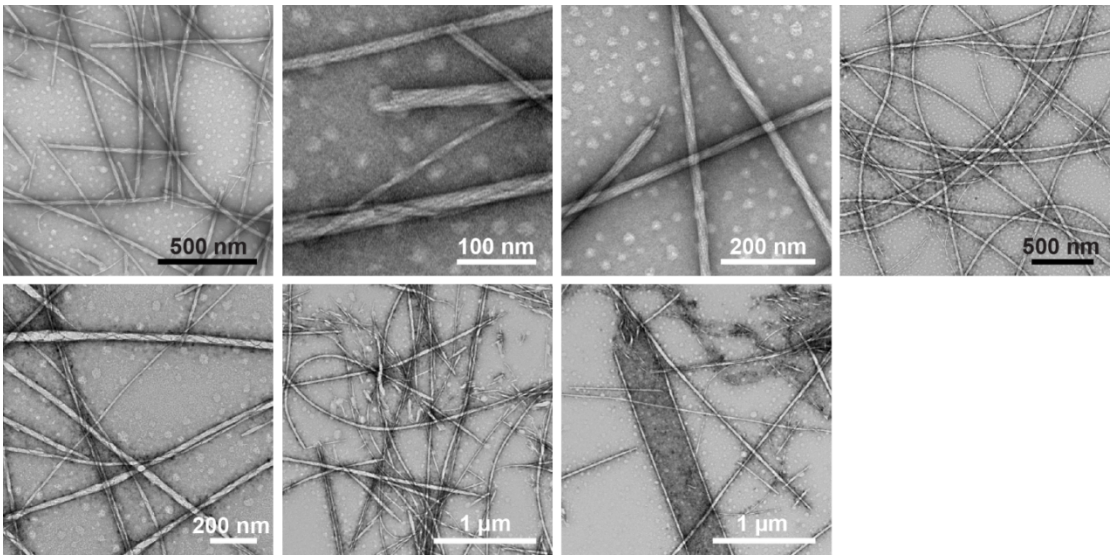

Figure S5. TEM micrographs of Fmoc-F^5-Phe-DAP hydrogels. Representative TEM images of Fmoc-F^5-Phe-DAP hydrogels (33.7 mM) after 24 hours (top row) and after 72 hours (bottom row) of incubation at 37 ºC.

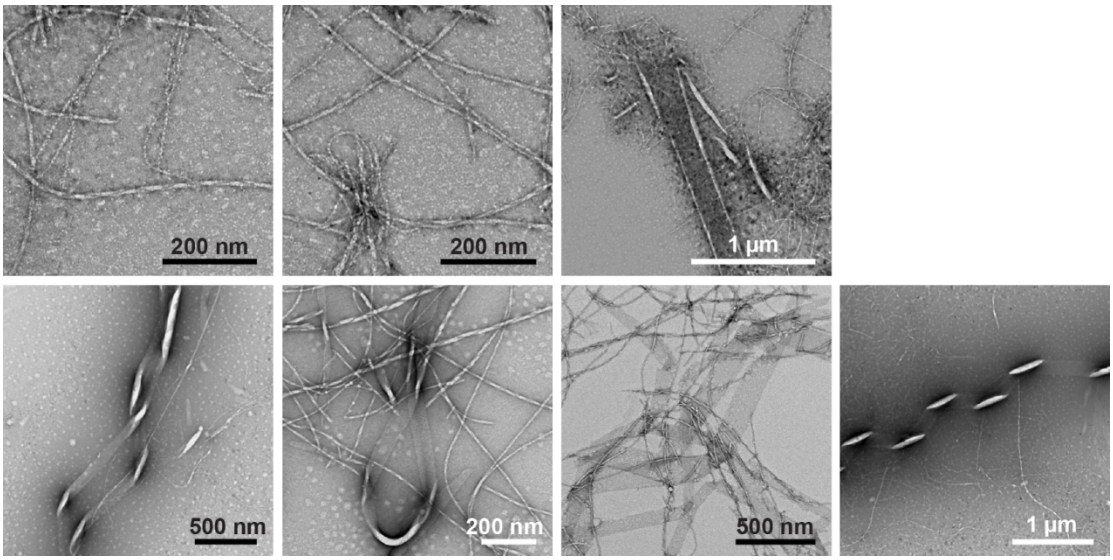

Figure S6. TEM micrographs of 1:1 Fmoc-Phe-DAP: Fmoc-F^5-Phe-DAP hydrogels. Representative TEM images of a 1:1 mixture of Fmoc-Phe-DAP (**1**) : Fmoc-F^5-Phe-DAP (**3**) hydrogels (33.7 mM total gelator) after 24 hours (top row) and after 72 hours (bottom row) of incubation at 37 ºC.

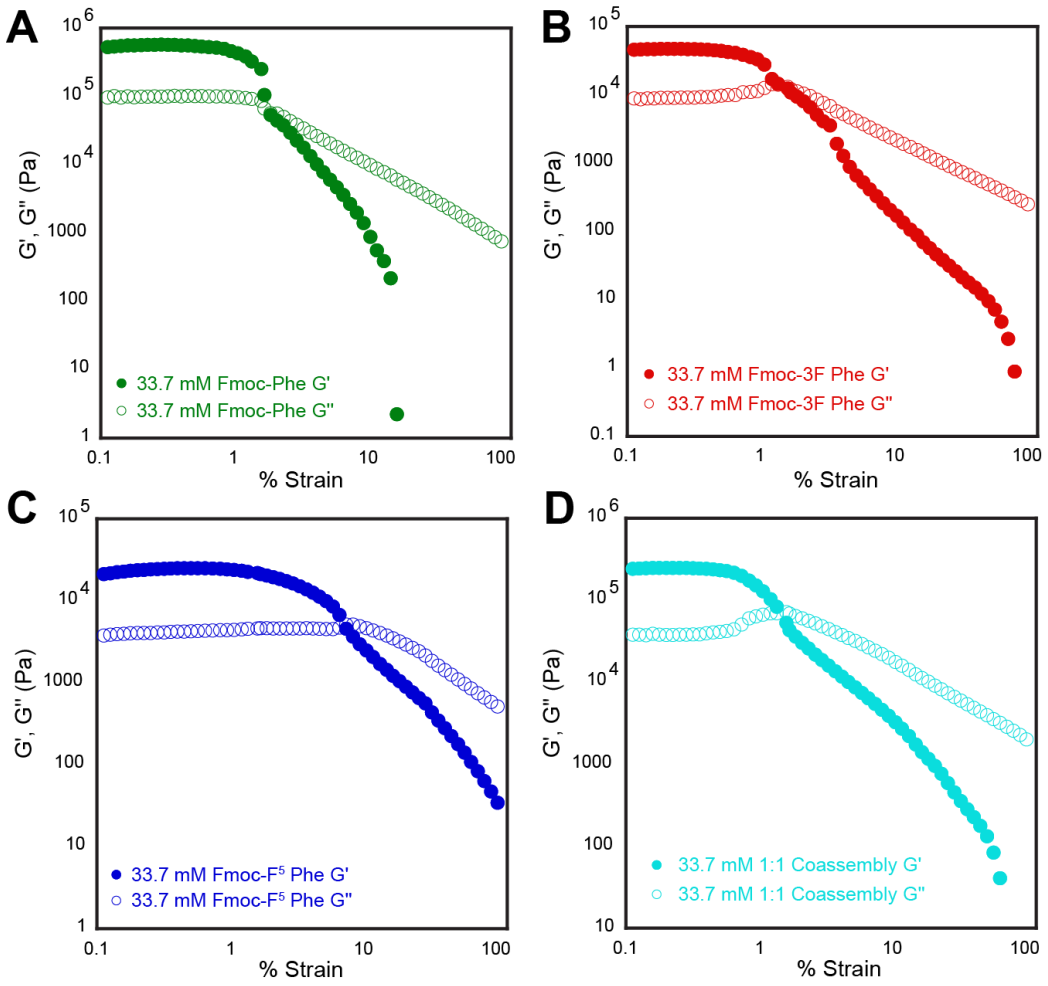

Figure S7. Rheological strain sweep measurements for 33.7 mM hydrogels: **(A)** Fmoc-Phe-DAP (**1**) hydrogel (green), **(B)** Fmoc-3F-Phe-DAP (**2**) hydrogel (red), **(C)** Fmoc-F^5-Phe-DAP (**3**) hydrogel (blue), and **(D)** 1:1 Fmoc-Phe-DAP (**1**) : Fmoc-F^5-Phe-DAP (**3**) hydrogel (cyan).

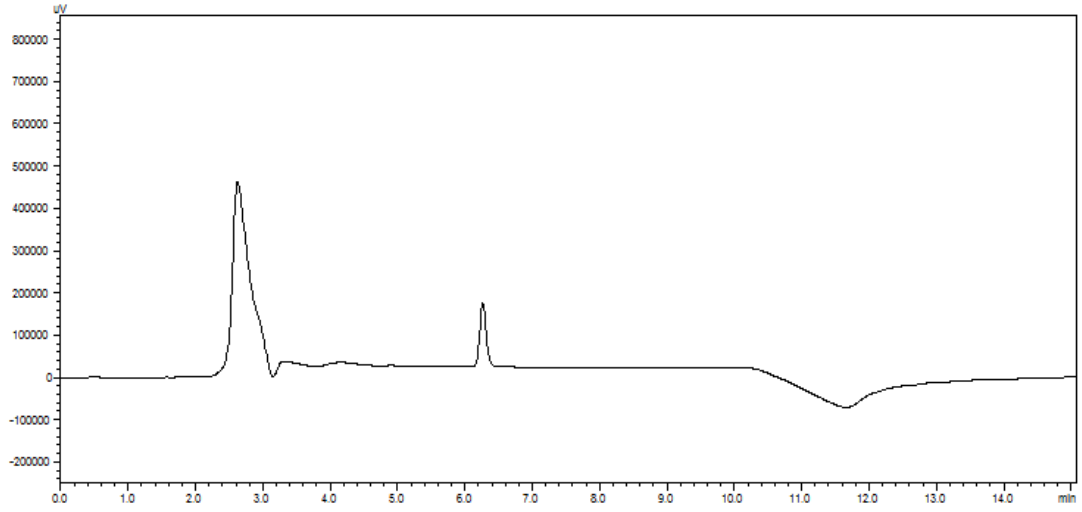

Figure S8. Analytical HPLC trace (215 nm) of diclofenac (mobile phase conditions shown in Table S1 below).

Table S1. Analytical HPLC conditions for determination of diclofenac concentration

Compound	**R_t (min)**	**Gradient** (solution **A**: water/0.5% TFA; solution **B**: acetonitrile/0.5% TFA)
Diclofenac	6.3	Isocratic 62% **B**, 1 min; 62-70% **B**, over 6 min; 70-95% **B**, over 1 min; 95% **B**, 5 min; 95-62% **B**, over 1 min; Isocratic 62% **B**, 1 min

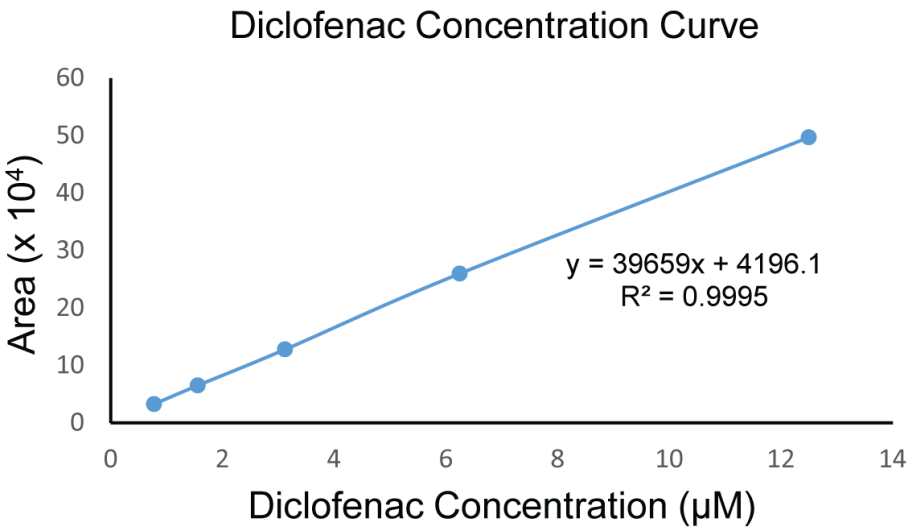

Figure S9. Diclofenac concentration curve.

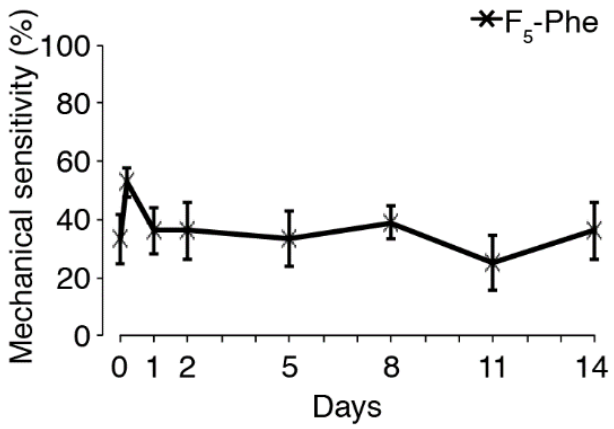

Figure S10. Mechanical sensitivity was measured over time for a control group of 6 mice treated with Fmoc-F^5-Phe-DAP (**3**) hydrogel (33.7 mM, 10 uL) that were not subjected to the CFA-induced pain model.

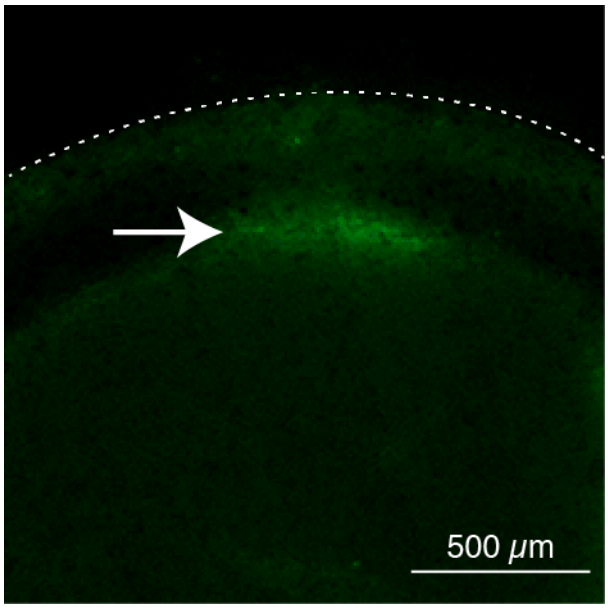

Figure S11. Fluorescence image of a fluorescein-containing Fmoc-F5-Phe-DAP (**3**) hydrogel 10 days after injection into a mouse hind limb. The image confirms that the hydrogel (white arrow) maintains its integrity in the hind limb of the mouse over the course of the experiment. Dashed line indicates the surface epidermis of the limb.