

Supplementary Information
for
Kinetics and retention of polystyrene sulfonate for proteoglycan replacement in cartilage

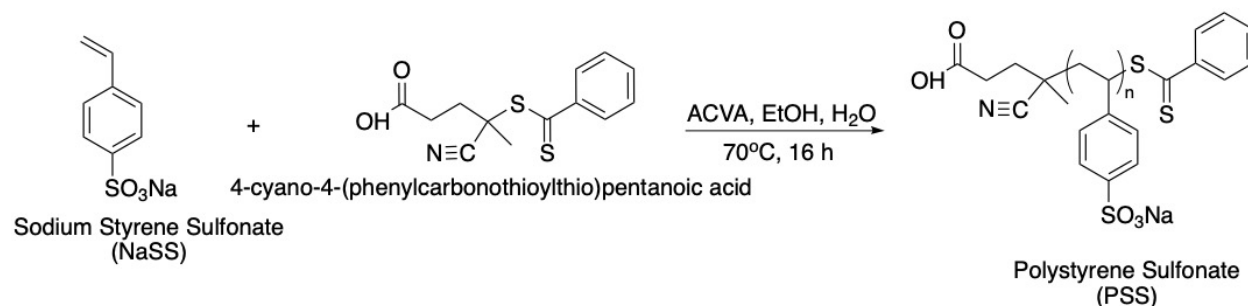
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S1. PSS Sample Preparation

S1.1. PSS Synthesis. Poly(styrene sulfonate) (PSS) preparation has been described in the Methods (**Main Text**). In short, PSS synthesis was first achieved using a reversible addition fragmentation chain-transfer (RAFT) polymerization reaction (**Scheme S1**).



Scheme S1. Synthesis of poly(styrene sulfonate) (PSS) from sodium 4-styrenesulfonate (NaSS) as prepared by RAFT polymerization), resulting in polymer samples with varying molecular weights.

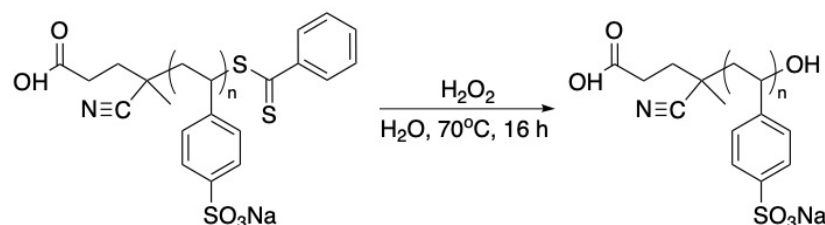
Synthesis reaction conditions and a summary of resulting PSS (with the RAFT ends still attached) are provided in **Table S1**.

Table S1. Summary of PSS polymers obtained by RAFT polymerization

Polymer ^a	[RAFT]:[M]:[I]	Time (h)	M_n (kDa) ^b	M_w (kDa) ^b	Dispersity, D^b
PSS-13kDa	1 : 43.11 : 0.2	16	9.9	12.8	1.30
PSS-30kDa	1 : 107.78 : 0.2	16	24.5	30.1	1.22
PSS-223kDa	1 : 1021.03 : 0.2	24	177.0	223.0	1.26

^a Example for PSS-13kDa of the naming convention used in this article: 13 kDa refers to 13 kg mol⁻¹, the weight averaged molecular weight of the PSSNa polymer. ^b Obtained by GPC.

S1.2. PSS RAFT Chain End Removal. In order to remove the RAFT end (the dithiobenzoate end group) for biocompatibility¹, a cleavage reaction using H₂O₂ was performed on the PSS from **Table S1**, as described in the Methods (**Main Text**) and below (**Scheme S2**).



Scheme S2. Cleavage of the dithiobenzoate end group of the synthesized PSS.

A summary of the resulting PSS polymers, after cleavage of the end group, is provided in **Table S2**. As a result of this treatment with H₂O₂, the polymer number-average molar mass (M_n) and the weight-average molar mass (M_w) were found to decrease, while the dispersity (\mathcal{D}) was found to increase. The final PSS-11kDa, PSS-20kDa and PSS-65kDa polymers were subsequently used throughout for tissue experimentation.

Table S2. Summary of final PSS polymers after RAFT end group cleavage

Final PSS Sample ^a (after end group removal)	PSS Sample Used ^a (with end group)	M_n (kDa) ^b (final)	M_w (kDa) ^b (final)	Dispersity, \mathcal{D} ^b (final)
PSS-11kDa	PSS-13kDa	7.5	10.8	1.45
PSS-20kDa	PSS-30kDa	13.0	20.4	1.57
PSS-65kDa	PSS-223kDa	26.7	64.6	2.42

^a Example for PSS-11kDa of the naming convention used in this article: 11 kDa refers to 11 kg mol⁻¹, the weight averaged molecular weight of the PSS polymer. ^b Obtained by GPC.

A visual color change of the samples from pink-orange to yellow-white was observed as depicted in **Figure S1**, showing the progression of the reaction and successful removal of the RAFT chain end. Samples with the RAFT chain end still attached have an intrinsic pink-orange color, given by the RAFT end group.¹



Figure S1. Color changes were observed after removal of the RAFT chain end group. The left vial of each pair is the original PSS synthesized with the end group still attached (PSS-13kDa, PSS-30kDa and PSS-223kDa). And the right vial of each pair is the corresponding final PSS sample, obtained after the cleavage reaction, with the end group removed (PSS-11kDa, PSS-20kDa and PSS-65kDa).

This color change, indicative of the successful removal of the RAFT chain end, can be also seen in the corresponding UV-Vis absorption spectra collected on the PSS samples before and after the cleavage reaction (**Figure S2**). Samples were dissolved in DI water (100 mg/mL) and the spectra were recorded on a spectrophotometer (UV-3600 UV-Vis-NIR Spectrophotometer, Shimadzu). After the initial RAFT polymerization reaction, there was an absorbance peak around $\lambda \sim 500$ nm, indicating presence of the RAFT dithiobenzoate end group in the original PSS polymers.¹ After the cleavage reaction using H_2O_2 , this absorbance peak around $\lambda \sim 500$ nm disappears, indicating successful removal of the RAFT chain end group.

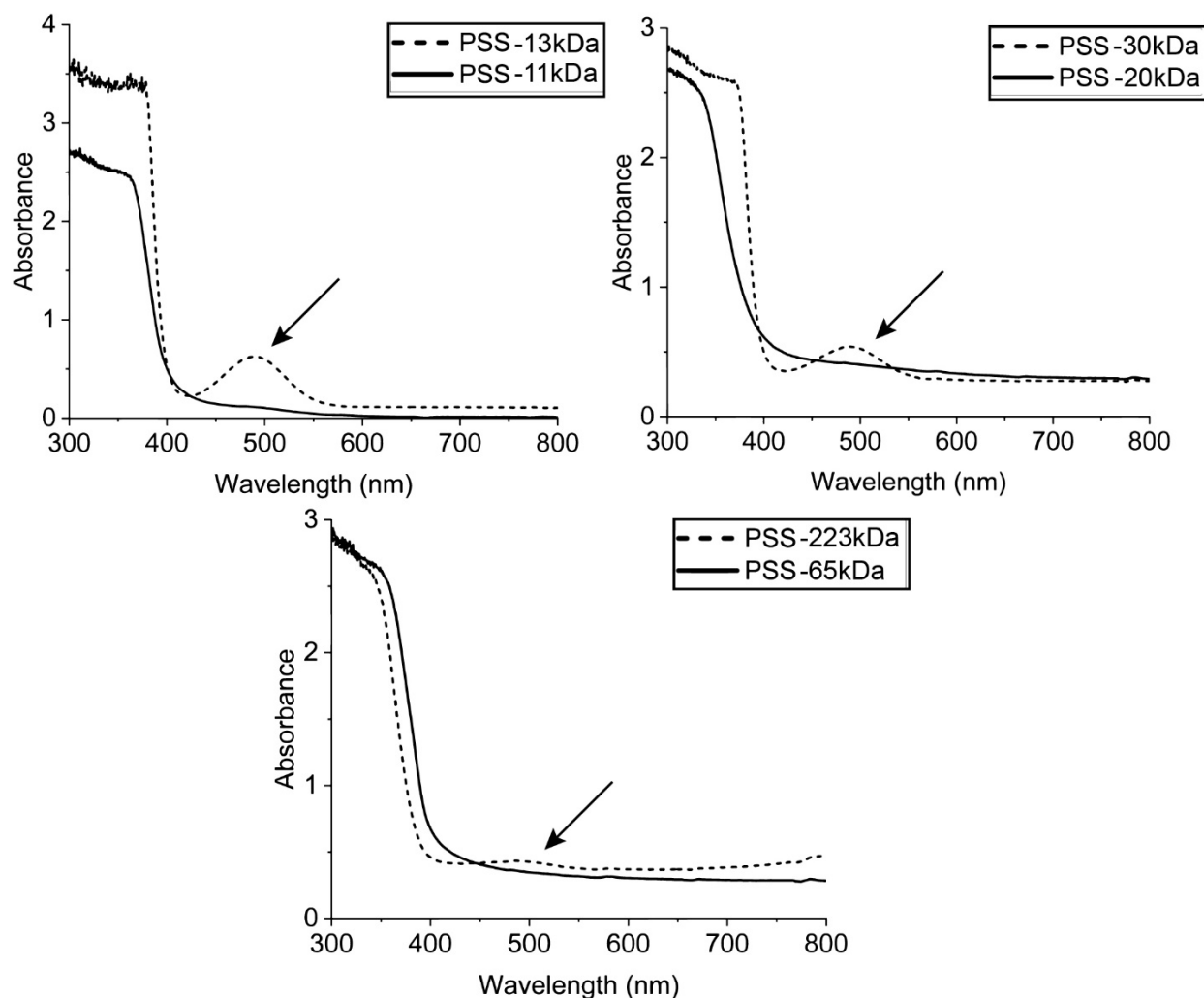


Figure S2. UV-Vis spectra indicating the removal of the RAFT end group after cleavage with H_2O_2 . The presence of the RAFT end group is indicated visually by an intrinsic orange-pink color of the sample and correspondingly by the presence of the absorbance peak around $\lambda \sim 500$ nm in the visible absorbance spectra.

S2. Determination of Polymer Molecular Weight and Dispersity.

Gel permeation chromatography (GPC) was used to determine the number-average molar mass (M_n), the weight-average molar mass (M_w), and the dispersity (D) of the PSS (before and after

removal of the RAFT chain ends) and of CS-A (Chondroitin Sulfate A sodium salt, 90+% purity, ThermoFisher Scientific) reference standard. GPC traces have been provided in **Figure S3**.

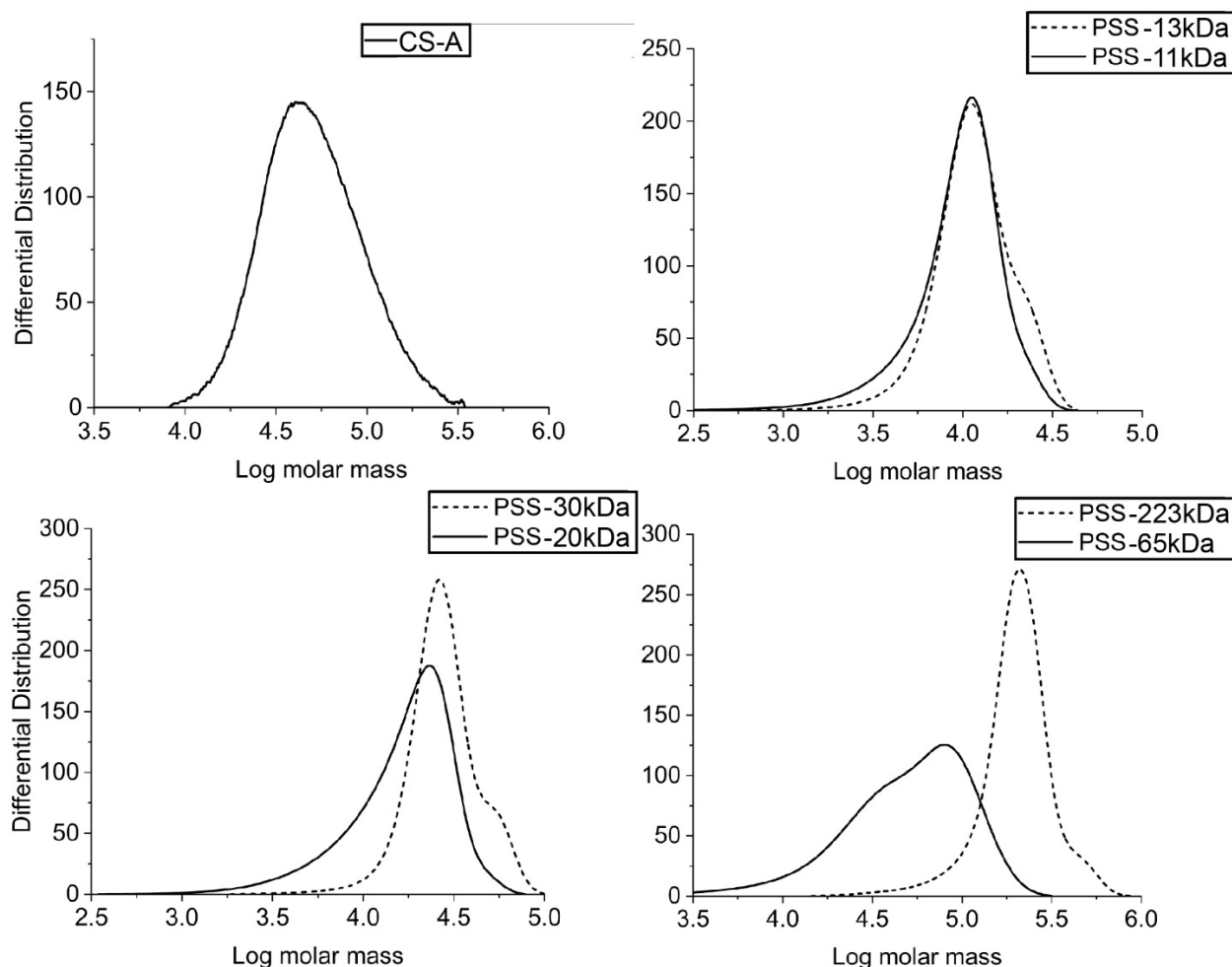


Figure S3. GPC traces of polymers. Dashed lines indicate PSS before cleavage of the RAFT chain end. Solid lines indicate PSS after cleavage of the RAFT chain end and also the reference CS-A (chondroitin sulfate A). All samples indicated by solid lines were subsequently used in tissue experimentation.

S3. PSS UV-Vis Spectroscopy Calibration Curves.

PSS concentrations in cartilage explant supernatant was quantified using UV-Vis spectroscopy. **Figure S4A** presents the absorbance spectra of 1X PBS versus 0.05 mg/mL PSS-65kDa dissolved in PBS. UV transparent 96-well plates were used (Santa Cruz Biotechnology) to reduce background absorbance at $\lambda = 225$ nm. The insert in Figure S4A demonstrates the overlap in absorbance at $\lambda = 225$ nm for PSS and a common protease inhibitor cocktail (5mM benzamidine hydrochloride (BHCl) + 5 mM ethylenediamine tetraacetic acid (EDTA)) used in cartilage explant studies². This overlap would bias PSS quantification for supernatant samples. As a result, we decided to lower the temperature to 4 °C for the diffusion periods, instead of using external protease inhibitors to reduce the impact of trypsin activity that may still be present within the tissue after digestion that could affect transport kinetics. **Figure S4B** provides sample calibration curves

of all three PSS (PSS-11kDa, PSS-20kDa and PSS-65kDa) used in the main text, taken at $\lambda = 225$ nm and $\lambda = 230$ nm. Calibration curves indicate strong linearity (all r^2 values > 0.99).

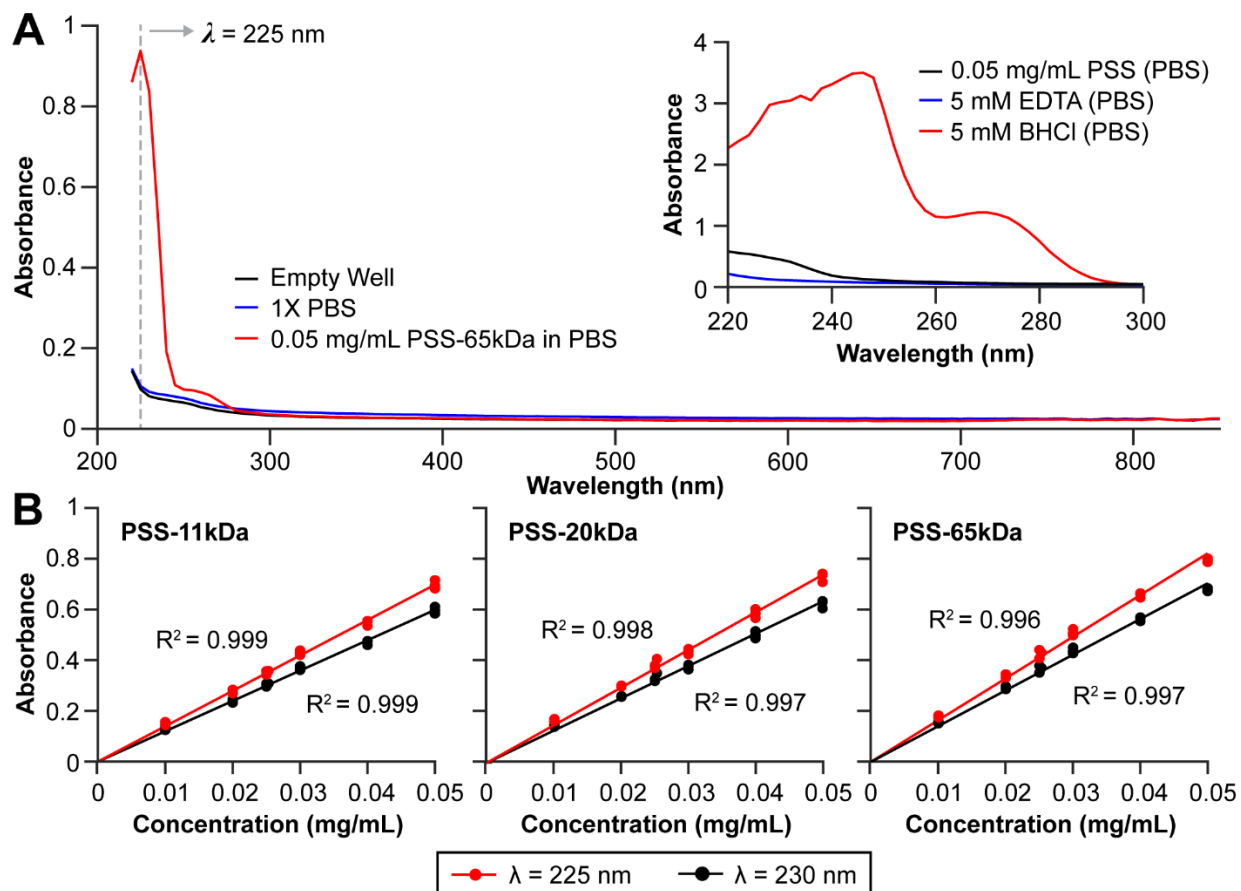


Figure S4. (A) UV-Vis absorbance spectra curves from taken from $\lambda = 220$ nm and $\lambda = 850$ nm. Each absorbance spectrum line is the average of $n = 3$ wells. Insert presents absorbance overlap at $\lambda = 225$ nm for PSS and common protease inhibitor cocktail (5 mM BHCl + 5 mM EDTA) using in cartilage studies. Each absorbance spectrum line is the average of $n = 3$ wells. (B) Example UV-Vis calibration curves of PSS-11kDa, PSS-20kDa and PSS-65kDa, taken at $\lambda = 225$ nm and $\lambda = 230$ nm. $n = 3$ per each concentration and at each wavelength. Calibration curves were background subtracted against the average of $n = 3$ wells of 1X PBS (buffer solution).

S4. Tissue Processing and Staining Protocols for Histology.

Histology was performed by the DCMR Histology Core, University of Delaware, Newark, DE. Upon fixation, cartilage explants were processed on an automated tissue processor (ASP6025S, Leica Biosystems) with the protocol presented in **Table S3**.

Table S3. Tissue Processing Protocol for Automated Tissue Processor

Reagent	Duration (h : min)	Temp	Pressure/Vacuum	Delay
Formalin	00:02	37	Pressure + Vacuum	PreTest
Processing Water	00:05	-	-	-
Ethanol (70%)	00:25	-	-	-

Ethanol (80%)	00:25	-	-	-
Ethanol (95%)	00:25	-	-	-
Ethanol (100%)	00:35	45	-	-
Ethanol (100%)	00:40	45	-	-
Ethanol (100%)	01:10	45	-	-
Xylene	00:25	-	-	-
Xylene	00:40	45	-	-
Xylene	00:45	45	-	-
Paraffin	00:45	65	-	-
Paraffin	00:45	65	Vacuum	-
Paraffin	01:00	65	Vacuum	-

It can be difficult to get cartilage to adhere to slides throughout the staining process since the cellular makeup of the tissue fails to provide enough negatively charged ions to bind to positively charged slides. As a result, following tissue processing and sectioning at 4 μm thickness, tissue slices were then adhered to slides using the following protocol: 1) Tissue sections were picked up from the water bath, 2) Slides were dried upright on a rack for 6-24 hours at room temperature or until no visible water remained, 3) Slides were placed on a hot plate or in an oven (at 55 °C) overnight, and 4) Slides were removed from the hot plate or oven and cooled back to room temperature (for 1 h) before continuing to the staining process.

Slides were then stained with Safranin O/Fast Green (Safranin O Cartilage Staining Kit, American MasterTech). This was done by the following staining protocol: 1) Deparaffinize slides in xylene for 5 minutes (x2 changes), 2) Rinse slides in 100% alcohol for 1 minute each (x3 changes), 3) Rinse slides in running tap water, 4) Immerse slides in Wiegert's hematoxylin for 5 minutes, 5) Rinse slides in running water for 1 minute, 6) Immerse slides in 0.2% Fast Green for 5 minutes, 7) Without rinsing, immerse slides in 1% acetic acid for 15 seconds with slight agitation, 8) Immerse slides in 1% Safranin O for 5 minutes, 9) Dehydrate in absolute alcohol for 1 minute each (x3 changes), 10) Clear in xylene for 1 minute each (x3 changes), and 11) Coverslip slides using permanent medium.

S5. Safranin O – Polyanion Absorbance Spectra.

UV-Vis absorbance spectra were captured after addition of CS-A and PSS standard solutions to a diluted Safranin O staining solution. Safranin O stain dilutions and respective absorbance spectra are provided in **Figure S5**. Dilutions were prepared by diluting the original Safranin stock solution with DI water. The 1:200 Safranin O solution was chosen for the experiment since the absorbance spectra lies below an absorbance of 1 throughout the wavelength range from $\lambda = 400 \text{ nm}$ to $\lambda = 600 \text{ nm}$.

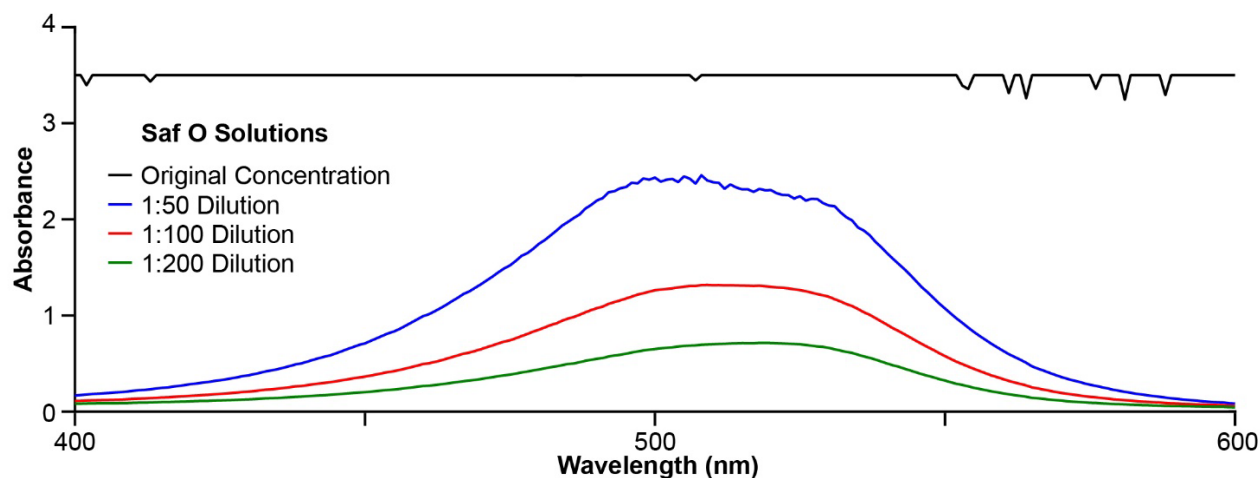


Figure S5. Absorbance spectra of Safranin O staining solution dilutions, as determined through UV-Vis Spectroscopy. Each absorbance spectrum line is the average of $n = 3$ wells.

S6. CS-A Diffusion in Cartilage.

To confirm that CS-A diffusion at 50 mg/mL does not result in positive Safranin O staining, we ran the CS-A diffusion experiment from the main text (**Figure S6A**) again on a separate dissection day. 6 mm full-thickness cartilage explants were harvested aseptically, using a cylindrical biopsy punch, from freshly sacrificed bovine (*Bos taurus* or cattle) metacarpal phalangeal joints (Sudlersville Meat Locker, MD). Explants were either assigned to control groups or treated with CS-A. All explants were then fixed and stained with Safranin O after the experiment was complete. Resulting staining pictomicrographs are provided in **Figure S6B**.

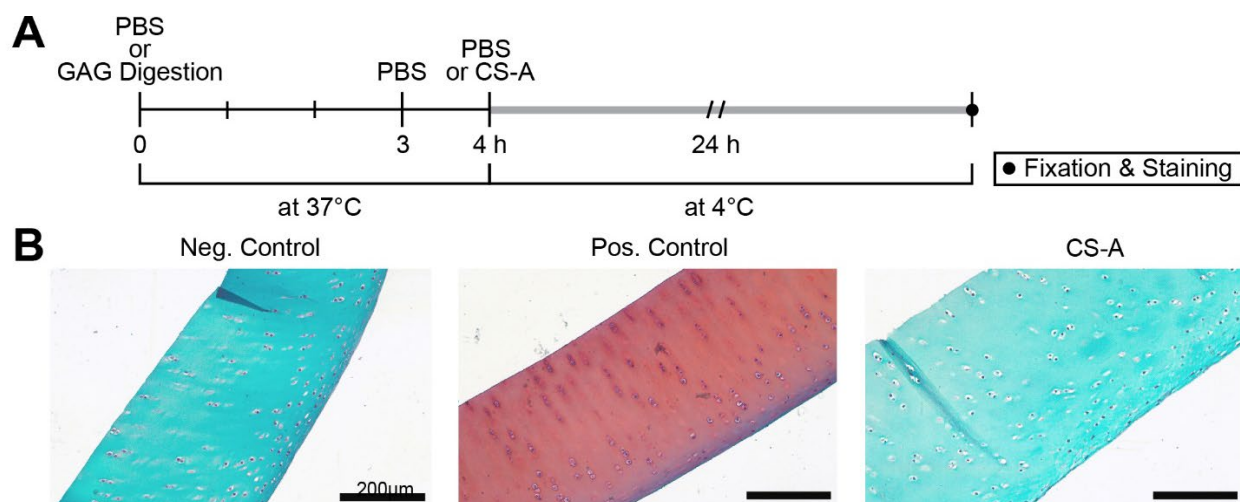


Figure S6. Repeat experiment of CS-A diffusion at 4 °C. **(A)** Timeline of diffusion experiment. Treated group was treated with 50 mg/mL CS-A solution following GAG (glycosaminoglycan) digestion. Controls and treated samples were fixed and stained after 24 h diffusion. **(B)** Pictomicrographs of Safranin O staining. All images oriented the same way with respect to cartilage zones.

S7. PSS Diffusion Coefficient Analysis.

Safranin O pictomicrographs (Main text, **Figure 7**) were batch analyzed in MATLAB (Mathworks). These images were for $t = 5$ min diffusion across all three PSS used (PSS-11kDa, PSS-20kDa and PSS-65kDa). Stain intensities were first converted to corresponding gray scale and images were oriented parallel to the superficial size of the tissue. An example for the image processing has been provided in **Figure S7A** for PSS-20kDa. Pixel intensities were then averaged across each row, parallel to the superficial side of the tissue, to calculate for the average pixel intensity. The average pixel intensity was then plotted against tissue depth from the superficial side of the tissue (**Figure S7B**). The resulting plot was fitted with the solution of Fick's second law for a semi-infinite medium to calculate for the respective diffusion coefficients as described in the main text.

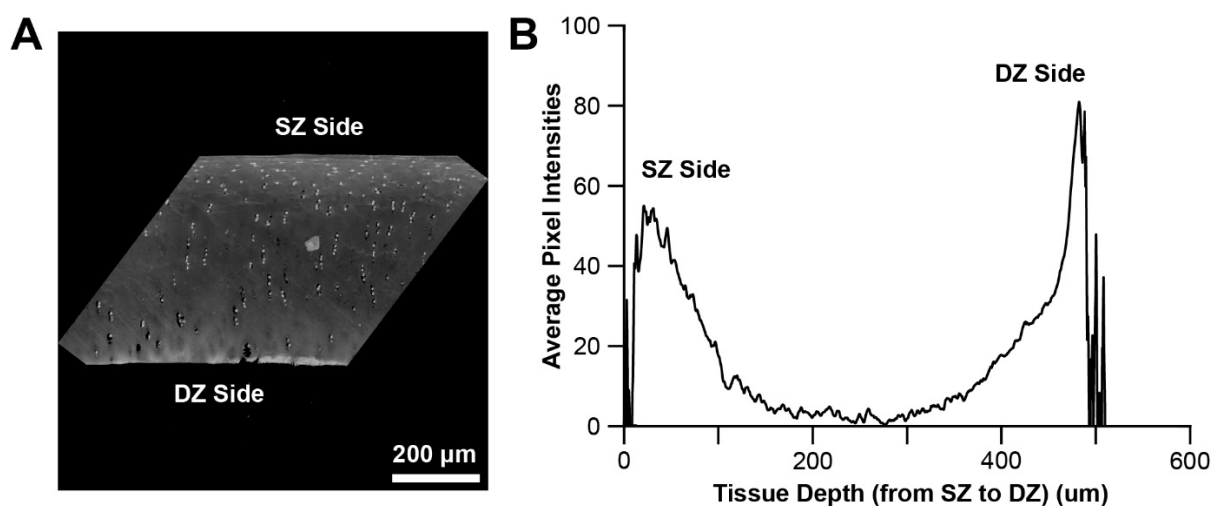


Figure S7. Example of diffusion analysis to calculate PSS diffusion coefficients. SZ = superficial zone, DZ = deep zone. **(A)** Image conversion and image rotation of an example Safranin O pictomicrograph (PSS-20kDa, 5 min diffusion time). **(B)** Average Safranin O pixel intensities parallel to the cartilage surface over tissue depth. Diffusion profiles were fitted with Fick's second law to calculate diffusion coefficients from both sides of the tissue.

S8. PSS-65kDa Desorption Under Loading.

PSS-65kDa desorption from cartilage explants under unconfined compression loading was also determined. While it is traditionally common to perform compression testing on cartilage osteochondral plugs (with the subchondral bone attached), we found that there was significant background absorbance at $\lambda = 225$ nm after trypsin digestion, which would overlap with the PSS quantification (**N. Control – red data point, Figure S8**). As a result, we used biopsies (full thickness cartilage explants without harvesting the underlying subchondral bone) for the loading experiments since the negative biopsy controls (**N. Control – black & blue data points, Figure S8**) had minimal absorbance at $\lambda = 225$ nm compared to the 1X PBS solution and the UV transparent 96-well plates that were used (Santa Cruz Biotechnology).

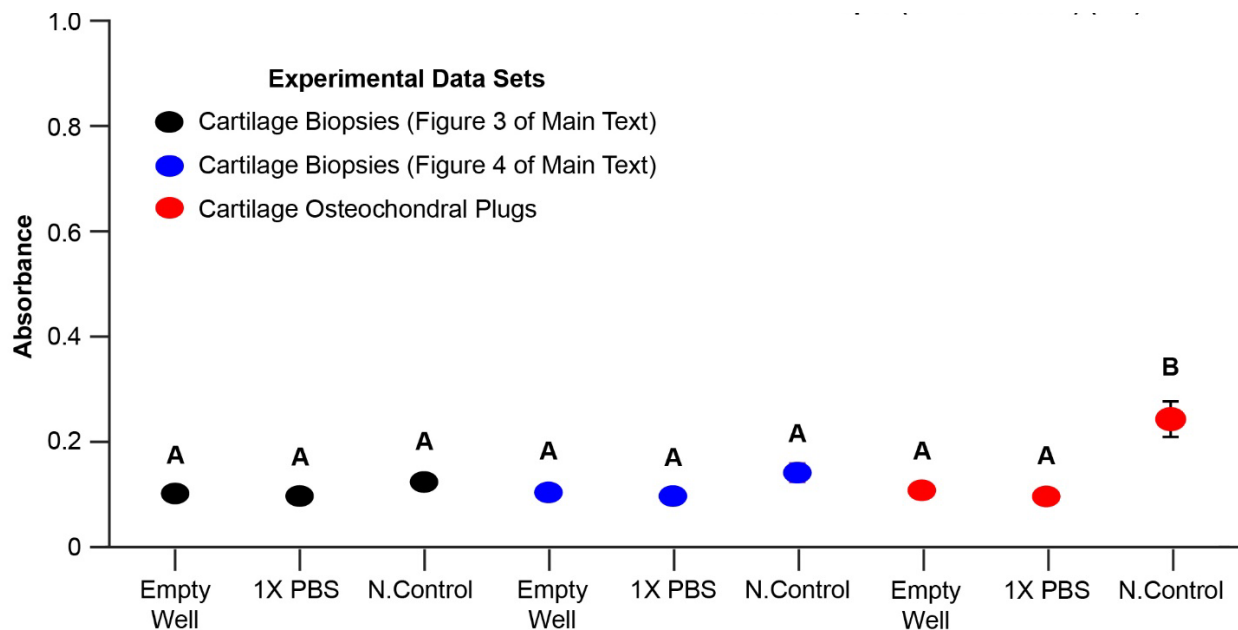


Figure S8. Comparison of UV-Vis background absorbance at $\lambda = 225$ nm for negative controls. Graph depicts negative controls from experiments run with cartilage biopsies (full thickness tissue with no bone attached) versus osteochondral plugs (full thickness tissue with bone attached). All samples were 6 mm in diameter and cylindrical in shape. Error bars indicate standard error of the mean where $n = 3$ explants. Statistical significance determined with a Tukey's *post-hoc* multiple comparisons test (groups connected by the same letter are not significant at p -value of 0.05).

Loading on the 6 mm cartilage biopsies were performed by loading 5, 15, 30 and 60 unconfined compression cycles, in sequence, with a target applied force of approximately 3 N (**Figure S9**). This translates to approximately 106.2 kPa of applied pressure. The experimental timeline has been provided in **Figure 5** of the main text. Loading was performed to determine how much PSS is forced out of the tissue upon compression compared to desorption under static conditions at 25 °C.

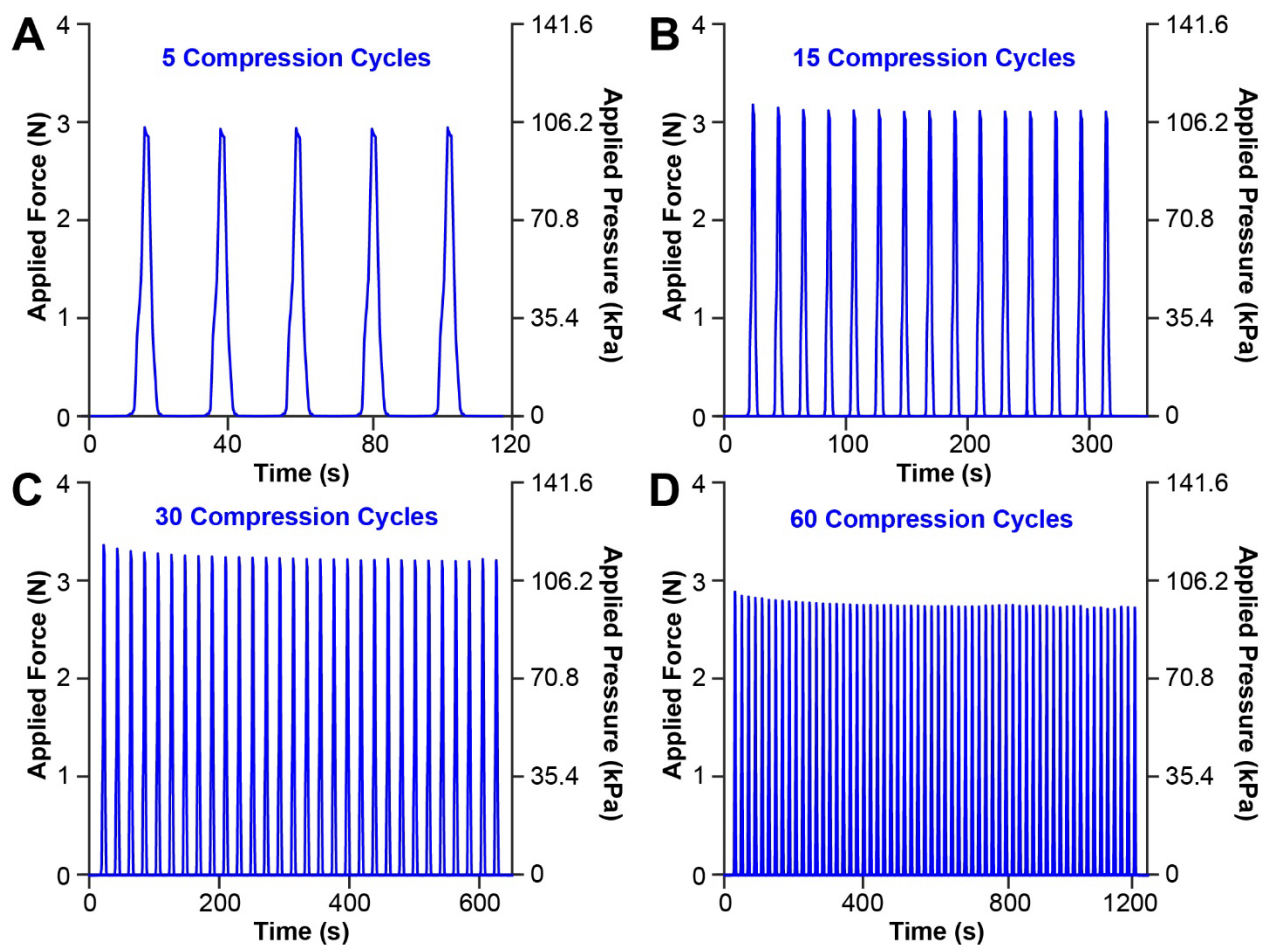


Figure S9. Example loading cycles for PSS desorption from cartilage biopsies under unconfined compression testing with a target applied force of approximately 3 N. Loading velocity = 0.2 mm/s. **(A)** 5 compression cycle period. **(B)** 15 compression cycle period. **(C)** 30 compression cycle period. **(D)** 60 compression cycle period.

S5. SAXS Data Analysis.

Solution-based SAXS data for CS-A and PSS (PSS-11kDa, PSS-20kDa and PSS-65kDa) were fitted with a Guinier analysis as seen in **Figure S10**. However, due to the large deviations from linearity, a full Guinier analysis could not be performed to determine R_g , the radius of gyration. The downturn in the PSS curves indicates strong repulsive interactions occurring in the solution, which would lead to underestimation of R_g with a Guinier fit in the low- q region.^{3,4} In contrast, the upturn in the CS-A curve indicates attractive interactions between chains and nonspecific aggregation, which would lead to overestimation of R_g with a Guinier fit in the low- q region.^{3,4}

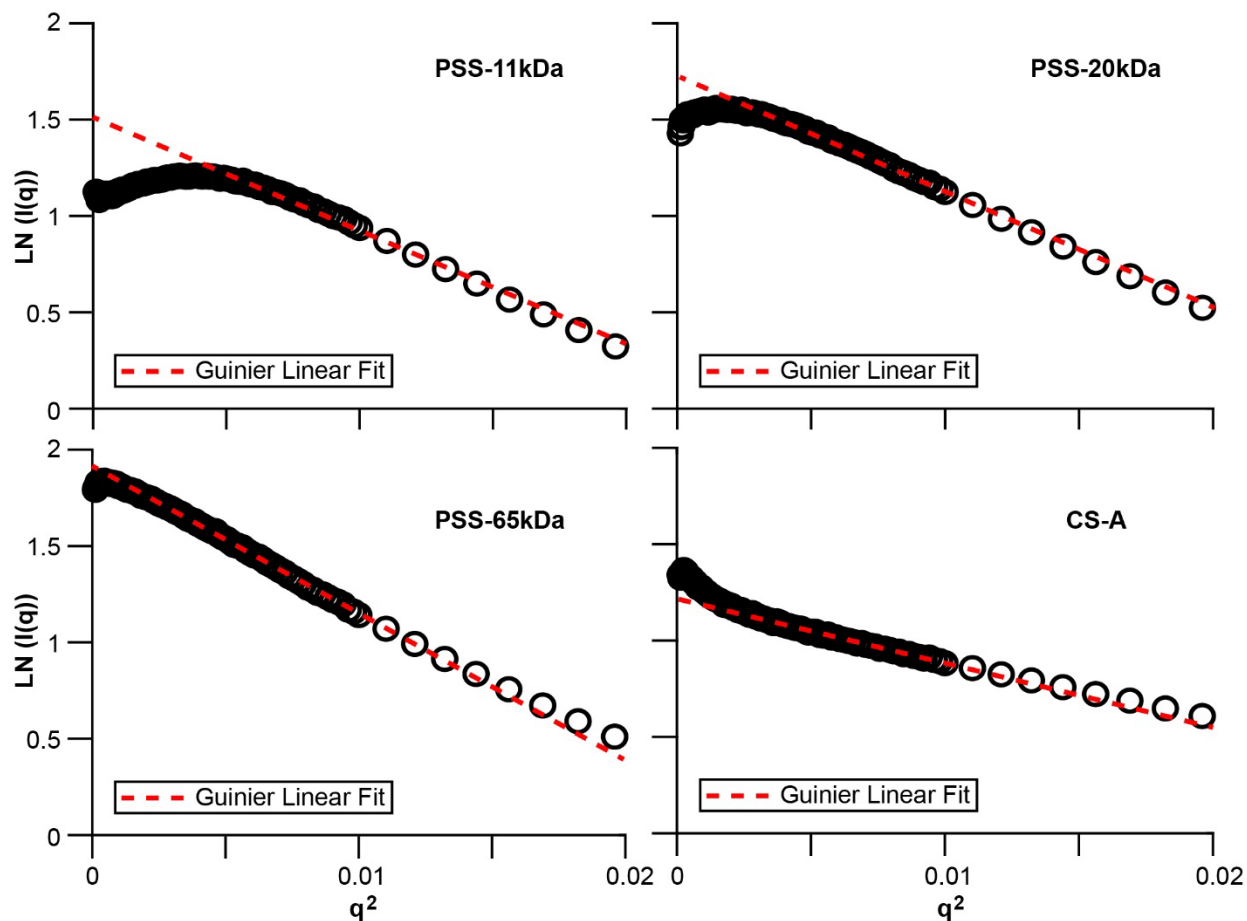


Figure S10. Guinier analysis (linearization and regression fits) for SAXS data of CS-A versus PSS (PSS-11kDa, PSS-20kDa and PSS-65kDa).

The SAXS data were then fitted with a linear regression in order to determine the Porod exponent (D) as described in the main text. **Figure S11** presents the resulting linearization and regression fits ($R^2 > 0.99$) demonstrating the conformational differences between CS-A and PSS (PSS-11kDa, PSS-20kDa and PSS-65kDa).

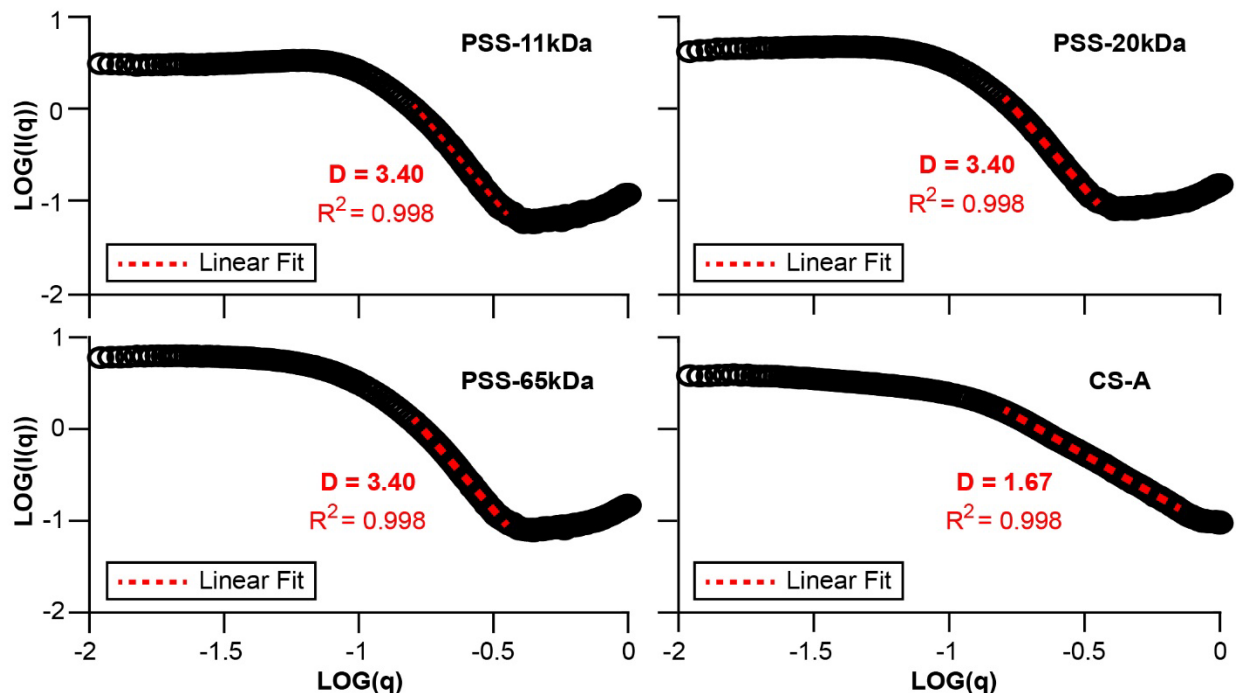


Figure S11. Analysis of SAXS curves through linearization and regression fits to determine the Porod exponent (D) for CS-A versus PSS (PSS-11kDa, PSS-20kDa and PSS-65kDa).

Figure S12 presents Kratky and Porod-Debye plots of the CS-A and PSS (PSS-11kDa, PSS-20kDa and PSS-65kDa) SAXS curves.

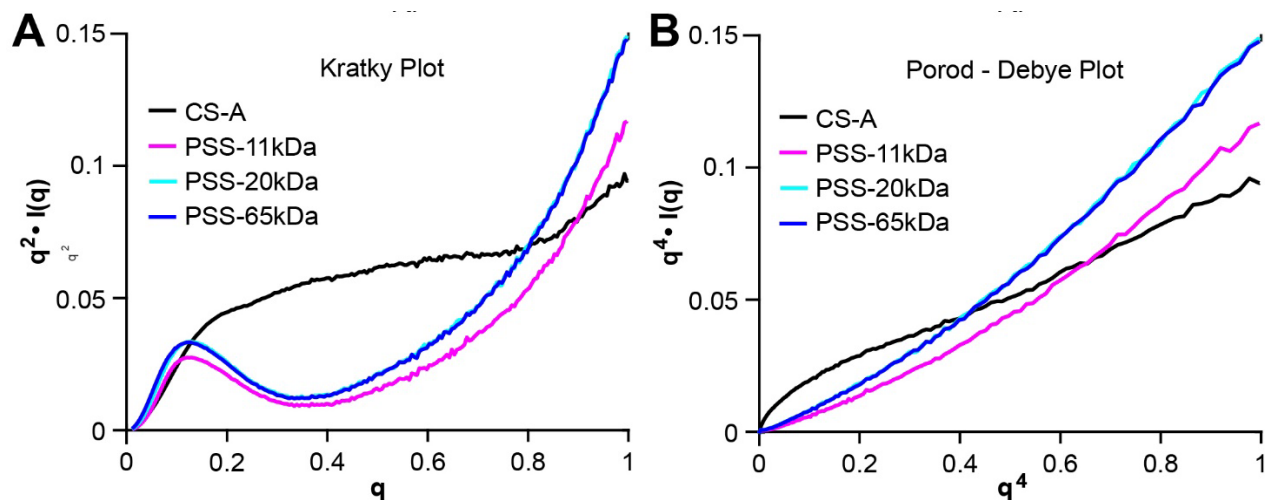


Figure S12. (A) Kratky plot for SAXS data of CS-A versus PSS (PSS-11kDa, PSS-20kDa and PSS-65kDa). (B) Porod-Debye plot for SAXS data of CS-A versus PSS (PSS-11kDa, PSS-20kDa and PSS-65kDa).

S6. Safranin O – Polyanion Absorption Spectra.

We studied the effect of increasingly adding CS-A, PSS-11kDa, PSS-20kDa and PSS-65kDa to a Safranin O dye solution (**Figure 7, Main Text**). We found that the original dye solution (0 mg/mL

of polyanion added) had an orthochromatic absorbance peak that occurs at $\lambda = 519$ nm. **Table S2** provides the resulting absorbances of the absorption peak in the orthochromatic region (that occurs at $\lambda = 519$ nm) upon adding increasing amounts of the polyanions.

Table S2. Average absorption maximums in the orthochromatic region ($\lambda = 519$ nm)

Dye Alone	0.706 ± 0.036			
Concentration	CS-A	PSS-11kDa ^a	PSS-20kDa ^a	PSS-65kDa ^a
0.001 mg/mL	0.652 ± 0.005	0.684 ± 0.011 (n.s.)	0.689 ± 0.009 (n.s.)	0.665 ± 0.015 (n.s.)
0.01 mg/mL	0.569 ± 0.002	0.607 ± 0.008 (n.s.)	0.585 ± 0.012 (n.s.)	0.571 ± 0.015 (n.s.)

$n = 3$ wells. Error bars indicate standard error of the mean. ^a Statistical significance compared against CS-A as determined with a Dunnett's pairwise comparisons test (ns = not significant).

When the polyanion concentration exceeds 0.01 mg/mL, for both CS-A and the three PSS polymers, there are appearances of metachromatic absorbance peaks that occur due to color changes of the dye – polyanionic solution. **Table S3** provides the wavelengths (in nm) at which these metachromatic absorption peaks occur at upon adding increasing amounts of the polyanions.

Table S3. Average wavelengths (λ) of absorption maximums in the metachromatic region

Concentration	CS-A	PSS-11kDa	PSS-20kDa	PSS-65kDa
0.1 mg/mL	473.3 nm ± 0.3	498.0 nm ± 0.0 (****)	498.0 nm ± 0.6 (****)	500.7 nm ± 0.9 (****)
1 mg/mL	490.0 nm ± 2.5	506.7 nm ± 0.3 (****)	507.7 nm ± 0.3 (****)	508.7 nm ± 0.3 (****)
10 mg/mL	507.7 nm ± 0.7	534.0 nm ± 0.0 (****)	534.0 nm ± 0.0 (****)	534.0 nm ± 0.0 (****)

$n = 3$ wells. Error bars indicate standard error of the mean. ^a Statistical significance compared against CS-A as determined with a Dunnett's pairwise comparisons test (**** $p < 0.0001$).

S7. References.

- (1) Jesson, C. P.; Pearce, C. M.; Simon, H.; Werner, A.; Cunningham, V. J.; Lovett, J. R.; Smallridge, M. J.; Warren, N. J.; Armes, S. P. H 2 O 2 Enables Convenient Removal of RAFT End-Groups from Block Copolymer Nano-Objects Prepared via Polymerization-Induced Self-Assembly in Water. *Macromolecules* **2017**, *50* (1), 182–191. <https://doi.org/10.1021/acs.macromol.6b01963>.
- (2) Kupratis, M. E.; Rahman, A.; Burris, D. L.; Corbin, E. A.; Price, C. Enzymatic Digestion Does Not Compromise Sliding-Mediated Cartilage Lubrication. *Acta Biomater.* **2024**, *178*, 196–207. <https://doi.org/10.1016/j.actbio.2024.02.040>.
- (3) Lenton, S.; Fagerberg, E.; Tully, M.; Skepö, M. From Dilute to Concentrated Solutions of Intrinsically Disordered Proteins: Interpretation and Analysis of Collected Data; 2023; pp 299–330. <https://doi.org/10.1016/bs.mie.2022.09.021>.
- (4) Kikhney, A. G.; Svergun, D. I. A Practical Guide to Small Angle X-Ray Scattering (SAXS) of Flexible and Intrinsically Disordered Proteins. *FEBS Lett.* **2015**, *589* (19), 2570–2577. <https://doi.org/10.1016/j.febslet.2015.08.027>.