Supporting Information for:

A Polymer Network Architecture Provides Superior Cushioning and Lubrication of Soft Tissue Compared to a Linear Architecture

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1. Experimental Section

1.1. General methods, materials, and instrumentation.

Materials and chemicals were purchased from Sigma Aldrich (St. Louis, MO) and immediately used as received unless otherwise noted. Reactions were carried out using standard techniques. Infrared spectroscopy was performed on a Nicolet FT-IR (Thermo Scientific, Waltham, MA) in transmittance mode using non-IR absorbing potassium bromide crystal cards (International Crystal Labs, Garfield, NJ) with a resolution of 0.25 cm^{-1} and number of scans = 32. Nuclear magnetic resonance (NMR) spectra were taken on an Agilent 500 MHz VNMRS (Agilent) spectrometer with a Varian ultra-shielded magnet dissolved in D_2O, and number of scans = 256.

1.2. Lubricant synthesis

2-methacryloyloxyethyl phosphorylcholine (MPC), ethyleneglycol dimethacrylate (EGDMA, 1% mol/mol MPC) crosslinker in the case of network polymers, ammonium persulfate (0.05 w/v%), and tetramethylethylenediamine (0.1 v/v%) were mixed in deionized water and incubated at room temperature for 24 hours. Polymers were then purified via dialysis against deionized water (7,000 Da MWCO) and resuspended in deionized water to afford lubricants with varying polymer concentration (2, 5, 8, and, 12 w/v%) either with no crosslinker (linear polymers) or with EGDMA crosslinker (network polymers).

1.3. Rheological characterization

Rheometry of lubricants was performed at 25°C using an aluminum 2° cone geometry (40 mm diameter) with a gap distance of 47 μm (TA Instruments AR2000 series). Prior to testing, lubricants were presheared for 10 sec at a shear rate of 2.5 sec^{-1}. A solvent trap was used to prevent lubricant dehydration. Data acquisition intervals were logarithmic, with ten acquisition points per decade. Oscillatory stress sweeps (0.1 to 100 Pa) at 1 and 2.5 Hz were performed to obtain storage and loss moduli G' and G" in the lubricant's linear viscoelastic region. Reported moduli are averaged over the 11 data points spanning the stress decade 1-10 Pa (included in both linear and network pMPC's linear viscoelastic region, with error bars representing standard deviation. A frequency sweep (0.1 to 100 Hz) at a stress (1 Pa) determined to be within the linear viscoelastic region detected during the stress sweeps was performed to observe frequency dependence of storage and loss moduli. A continuous flow shear rate sweep (1 to 1000 s^{-1}) was performed to measure lubricant viscosity.

1.4. Molecular weight measurement

The molecular weight (M_W) of the four linear pMPC concentrations was measured by gel permeation chromatography with a phosphate buffered saline mobile phase on an Agilent 1260 II equipped with a Sepax SRT SEC-300, 5 µm, 300Å, 7.8x300 mm column and dual angle light scattering (Agilent, Santa Clara, CA). The light scattering was calibrated using a 2 mg/mL solution of bovine serum albumin (Thermo Scientific, M_W = 66,438 Da). The linear portion of each concentration of network pMPC was isolated by hydrolyzing the EGDMA crosslinks with 0.9 N sodium hydroxide for 18 hours, followed by dialysis against DI water and lyophilization. The M_W of the resulting polymer was measured in the same manner as the linear pMPC samples.

1.5. Crosslink fraction measurement

The water solubility of bisphenol A glycerolate dimethacrylate (BAGDMA) was increased by conjugation of succinic acid onto the free hydroxyls at the C2 position by a 4-dimethylaminopyridine catalyzed esterification with succinic anhydride to form succinic acid-BAGDMA (SA-BAGDMA). Network pMPC containing fluorescent crosslinker was synthesized at 2, 5, 8, and 12 w/v% of MPC with 1 mol% of SA-BAGDMA, ammonium persulfate (0.05 w/v%), and tetramethylethylenediamine (0.1 v/v%) in tris buffer (0.2 M, pH 7.4) at room temperature for 24 hours, followed by dialysis against deionized water (7,000 Da MWCO) and lyophilization. The fluorescence of the resulting network polymers was measured on a Jobin Yvon Horiba FluoroMax 3 (Horiba, Kyoto, Japan) with excitation at 294 nm and emission at 321 nm. The concentration of SA-BAGDMA within the crosslinked polymer was measured against a SA-BAGDMA standard curve and used, along with the M_W of the linear portion of the network polymer, to calculate the crosslink fraction, defined as the number of crosslinking units per MPC units in a single polymer chain.

1.6. Cytotoxicity measurements

pMPC cytotoxicity was assessed when exposed to human synoviocytes (HFLS, Cell Applications), human chondrocytes (NHAC-kn, ATCC), and mouse fibroblasts (NIH/3T3, ATCC). For cytotoxicity measurements on standard culture plates, cells were plated and allowed to adhere for 24 hours, at which point the media was aspirated and replaced with media containing pMPC resuspended at the appropriate concentration. Following 72 hours of culture, the media containing pMPC was removed and replaced with media containing MTS reagent (1:9 MTS to media). The absorbance of each well was measured on a plate reader according to the manufacturers protocol, and the viability was normalized to untreated control wells that were incubated in media lacking pMPC. For cytotoxicity measurements conducted in transwell plates, cells were plated in the bottom compartment of the well plate and allowed to adhere for 24 hours, at which point the media was aspirated and the bottom compartment was replaced with 90% of the required media. The remaining 10% of the required media was used to reconstitute pMPC at a 10X concentration and added to the top compartment of the transwell insert such that when the insert was properly placed, the pMPC was at the correct concentration. Following 72 hours of culture, the media in the lower compartment was removed, the transwell insert was discarded, and the media was replaced with media containing MTS reagent (1:9 MTS to media). The absorbance of each well was measured on a plate reader according to the manufacturers protocol, and the viability was normalized to untreated control wells that were incubated in media lacking pMPC.

1.7. Tissue specimen preparation

Osteochondral cylindrical plugs were cored from the patella and femoral groove (7 mm diameter) of skeletally mature cows using a diamond-tipped cylindrical coring bit (Starlite Industries, Bryn Mawr, PA).[1] Throughout experimentation, plugs were stored at 4°C in 400 mOsm sodium chloride containing benzamidine HCl (5 mM), Gibco Antibiotic-Antimycotic (Life Technologies, Grand Island, NY), and ethylenediaminetetraacetic acid (5 mM). Thickness measurements for strain calculation were performed via computed tomography at voxel resolution of 36 μm^3 (μCT40, Scanco Medical AG, Brüttisellen, Switzerland) using an airtight sample holder to maintain a humid environment to prevent tissue drying. The μCT data were converted to DICOM format and thicknesses were computed (Analyze, Mayo Clinic, Rochester, MN).

1.8. Mechanical characterization

Plug pairs were aligned collinearly with mated cartilages surfaces in contact, and a torsional creep regimen was applied (ElectroForce 3200, BOSE Corporation, Eden Prairie, MN). The test regimen was conducted at ambient temperature and was composed of 10080 rotations at 360°/sec (effective velocity ca. 22 mm/sec) applied under a constant compressive load of 0.78 MPa with plugs submerged in either saline or pMPC lubricant while torque, rotation, force, and displacement data were collected at 10 Hz; every 160 sec, a 10-sec lift-off was incorporated to allow reintroduction of lubricant to the tissue surfaces. In Experiment 1, three groups of plug pairs (N=3-4) were allowed to incubate with either saline or linear or network pMPC for 18 hours, then were subjected to the described torsional creep regimen while immersed in their respective lubricants. In Experiment 2, the test regimen was performed three times on a single group of plug pairs (N=3-4) on three successive days; during Test 1 (day 1) the lubricant was pMPC, during Test 2 (day 2) the lubricant was saline, and during Test 3 (day 3) the lubricant was once again pMPC. Prior to each test, the plug pairs were incubated for 18 hours at 4°C with the respective lubricant to be tested, and the tests were performed with plugs submerged in the appropriate lubricant.

1.9. *In vivo* safety study

All animal work was approved by the Institutional Animal Care and Use Committee at the Colorado State University. The middle carpal joints of skeletally mature horses were injected with 30 mg/mL network pMPC lubricant or saline as the negative control. A concentration above 5 mg/mL was used to account for dilution in the synovial fluid, which was not removed prior to treatment. Post treatment, horses were monitored for an additional 8 weeks. SF samples were serially collected from the joints and the number of white blood cells (WBCs) present was quantified. Effusion of injected carpi was also scored on a scale of 1 to 4 at 0-, 28-, and 70-days post injection. At the completion of the 8 weeks, horses were euthanized, and cartilage was harvested for histological analysis. Cartilage samples were imbedded in parafilm and sectioned prior to staining with hematoxylin and eosin (H&E).

1.10. Statistics

Significance level was set as two-tailed with P-value <0.05. Statistically significant differences (95% confidence level) were identified through ANOVA with Tukey-Kramer Multiple Comparisons using a Bonferroni correction.

2. Supplementary Results and Discussion

2.1. SEC-MALS traces

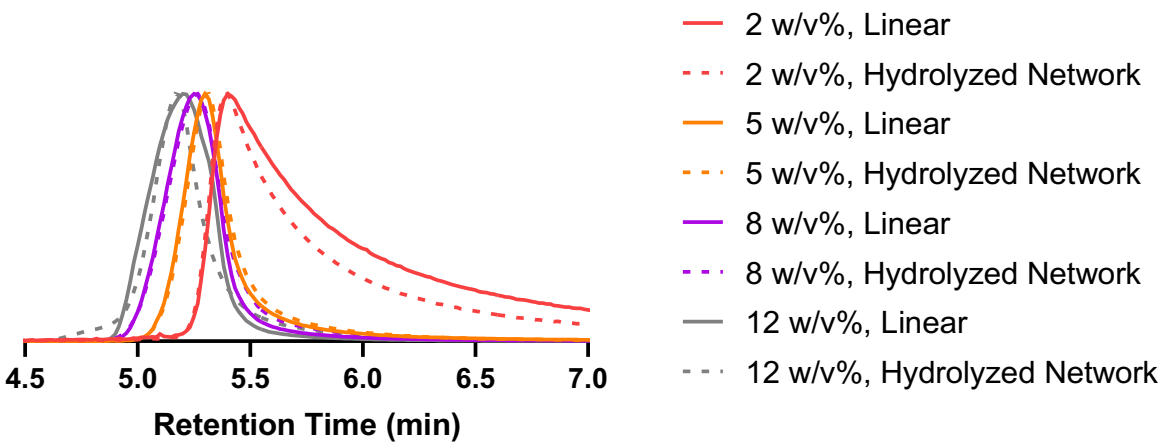

Figure S1. SEC traces for linear and hydrolyzed network pMPC solutions of varying concentrations from the 90º dual angle light scattering detector.

2.2. Confirmation of phosphorylcholine following hydrolysis of crosslinks

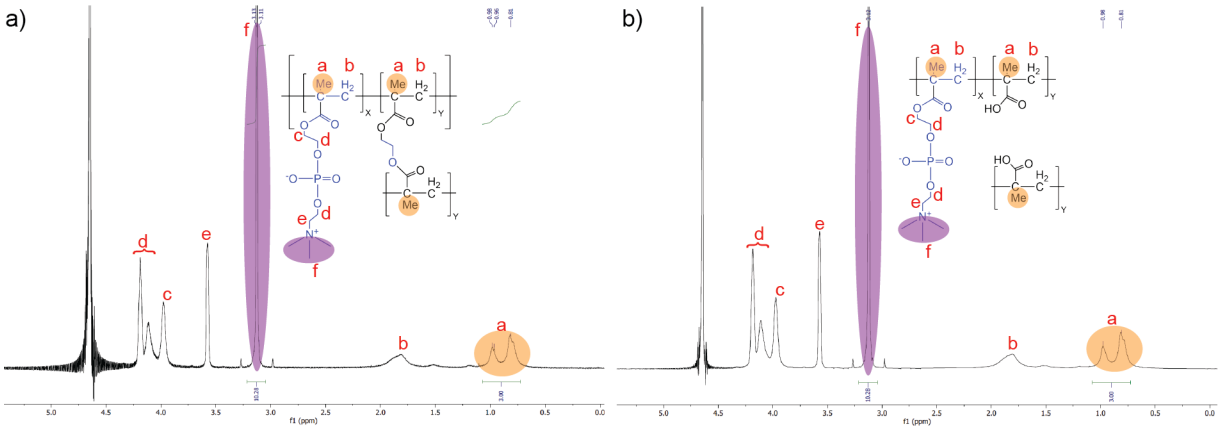

Figure S2. ^{1}H NMR spectra of 8 w/v% network pMPC were taken **a)** before and **b)** after hydrolysis of the EGDMA crosslinks and purification via dialysis to confirm the presence of the phosphorylcholine group along the hydrocarbon backbone of the polymer. The integrations were normalized to the methyl hydrogens present in the polymer backbone, which are unaffected by the addition of base, and the choline proton peak was integrated to ensure all of the hydrogens remained following hydrolysis. The integration of the choline peak remained the same, at 10.28, before and after hydrolysis, confirming that the base did not remove the phosphorylcholine side chains from the polymer.

2.3. Verification of hydrolyzed crosslinks by comparison of viscosities

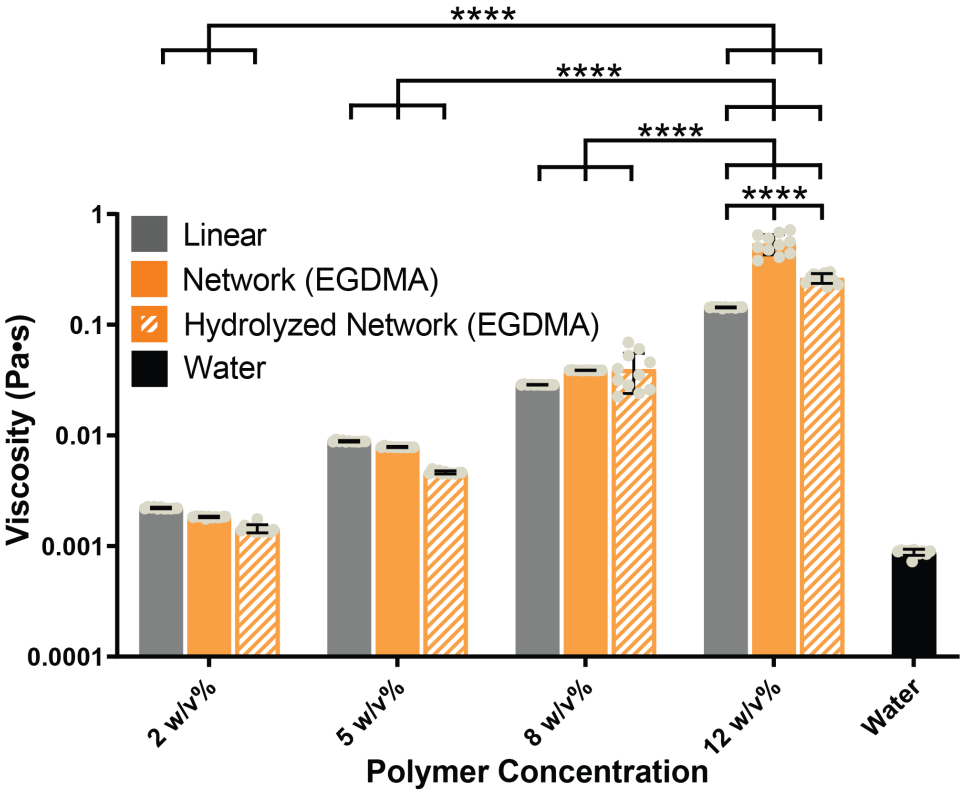

Figure S3. The viscosity of linear, network, and hydrolyzed network polymers at each of the four concentrations was averaged between shear rates of 10-100 s^{-1} to ensure that hydrolysis effectively broke the EGDMA crosslinks present in the network architectures. The average viscosity of hydrolyzed network polymers decreased below that of the network polymer at 2 w/v%, 5 w/v% and 12 w/v%. At 2 w/v% and 5 w/v%, this decrease is non-significant; however, at 12 w/v%, which is the only polymer concentration with a significant difference between the average viscosity of linear and network polymer, the decrease is significant, indicating that we are effectively breaking the EGDMA crosslinks in the network polymer. **** = $p<0.0001$. Significance bars across concentrations refer to architecture matched samples.

2.4. Storage and loss moduli complete stress sweep

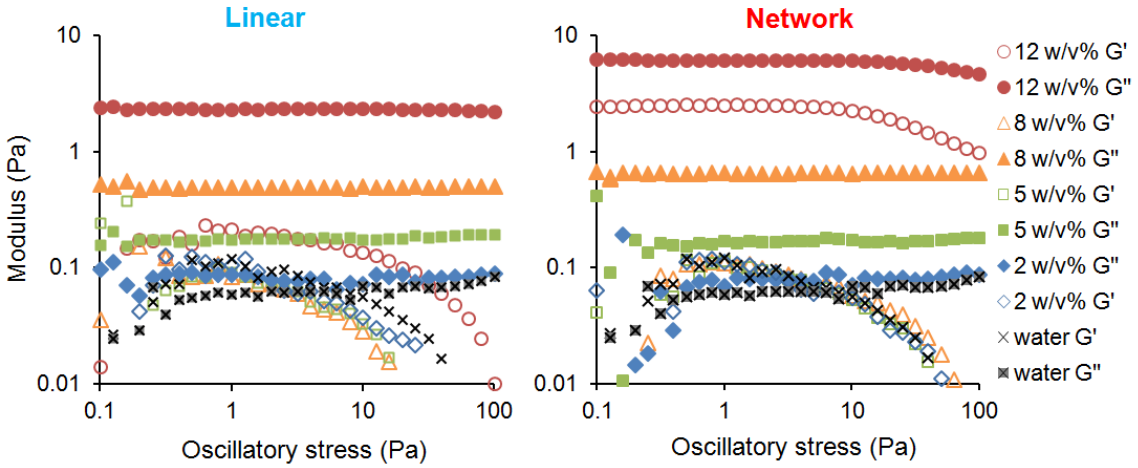

Figure S4. Storage and loss moduli over oscillatory stresses ranging 0.1 to 100 Pa, at a frequency of 2.5 Hz for linear and network pMPC solutions of varying concentration. Linear viscoelastic region spans approximately 1 to 10 Pa for both lubricant architectures and all concentrations.

2.5. Polymer concentration dependent rheology

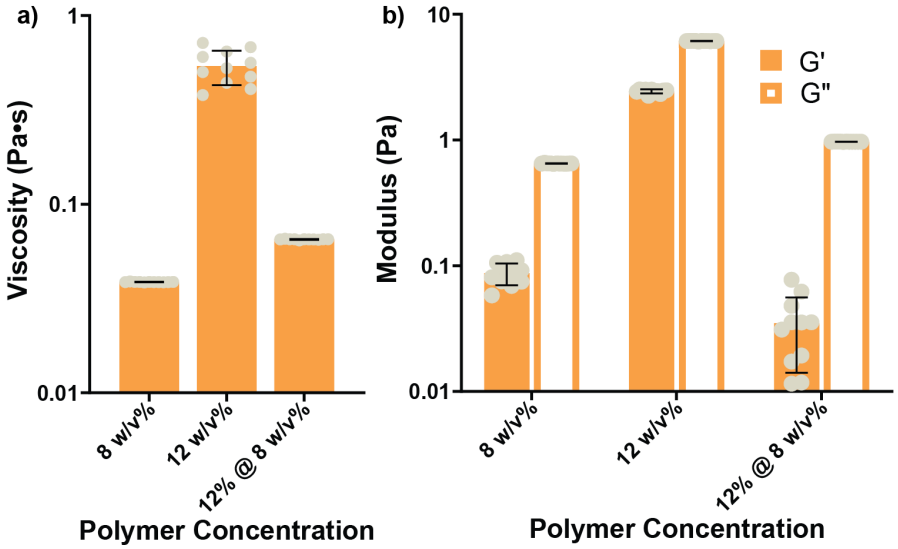

Figure S5. 12% network pMPC was resuspended at a concentration of 8 w/v% (12% @ 8 w/v%) and was rheologically characterized to identify if polymer concentration, in addition to polymer M_W and architecture, influences viscosity, storage modulus (G'), and loss modulus (G"). **a)** The viscosity of the 12% network pMPC resuspended at a concentration of 8 w/v% (0.065 Pa·s) decreased towards the level of 8% network pMPC (0.039 Pa·s), and was much lower than the viscosity of 12% network pMPC (0.54 Pa·s) **b)** Similarly, the G' and G" of 12% network pMPC resuspended at a concentration of 8 w/v% decreased towards the levels of 8% network pMPC. The G' of 12% network pMPC resuspended at 8 w/v% was 0.035 Pa, while that of 8% network pMPC was 0.087 Pa, and that of 12% network pMPC was 2.44 Pa. The G" of 12% network pMPC resuspended at 8 w/v% was 0.97 Pa, while that of 8% network pMPC was 0.65 Pa, and that of 12% network pMPC was 6.12 Pa. Therefore, polymer M_W, architecture, and concentration all contribute to the observed rheological properties of polymer suspensions.

2.6. Phase angles (δ)

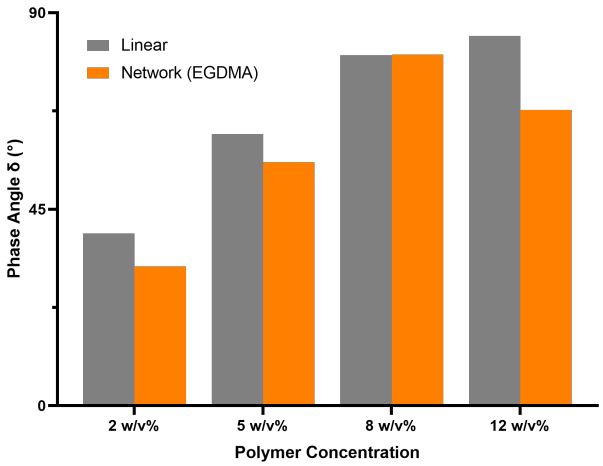

Figure S6. Phase angles at 1 Pa stress and 2.5 Hz frequency for linear and network pMPC solutions of varying concentrations.

2.7. Crossover frequencies

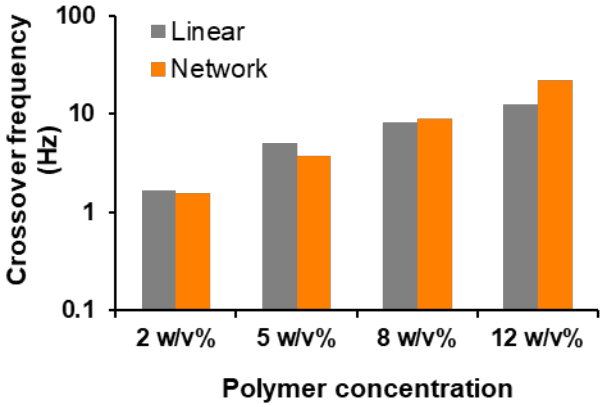

Figure S7. Crossover frequency for solutions of linear and network pMPC at each polymer concentration, obtained from a frequency sweep at 1 Pa stress.

2.8. Polymerization-negative control

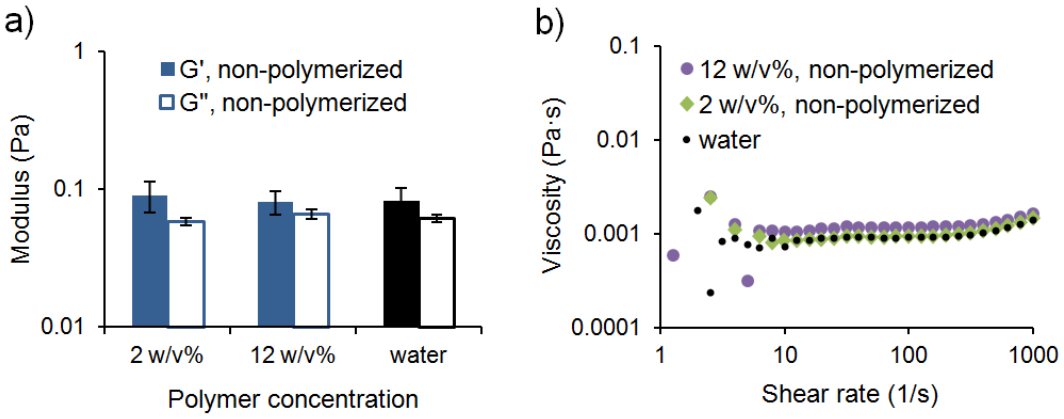

Figure S8. For solutions of MPC monomer ranging in concentration 2 to 12 w/v% and lacking the persulfate free radical initiator, storage and loss moduli **a)** and viscosities **b)** were similar to deionized water. Intermediate concentrations 5 and 8 w/v% not displayed, for clarity.

2.9. pMPC susceptibility to enzymatic degradation

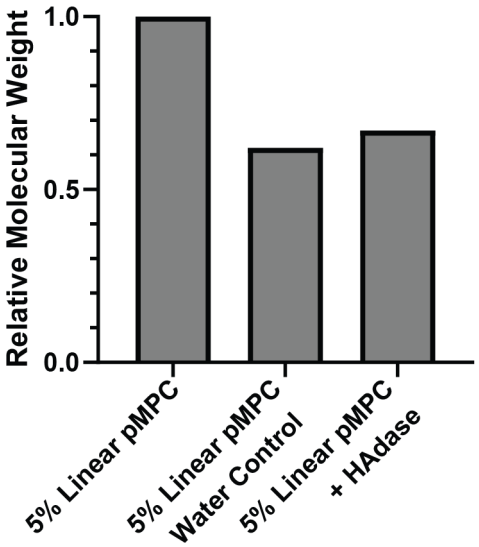

Figure S9. Given the presence of hyaluronidase (HAdase) in synovial fluid, and its increased concentration in osteoarthritic synovial fluid, pMPCs susceptibility to degradation by HAdase was assessed. 5% linear pMPC was suspended in phosphate buffered saline (PBS) containing 1 mg/mL of HAdase and incubated at 37°C for 24 hours. The 5% linear pMPC water control was incubated in PBS lacking HAdase for 24 hours at 37 °C. The MW of each polymer suspension was determined by SEC-MALS, as previously described, and compared to a freshly prepared suspension of 5% linear pMPC. The water control and polymer exposed to HAdase decreased to 62% and 67% of their original M_W, respectively. This provides evidence that pMPC is not susceptible to degradation by HAdase, given that the M_W of polymer exposed to HAdase did not decrease beyond the water control. The reduction in M_W in the water control is most likely explained by hydrolysis of the ester linkages present between the polymer backbone and the phosphorylcholine side groups.

2.10. Cytotoxicity study

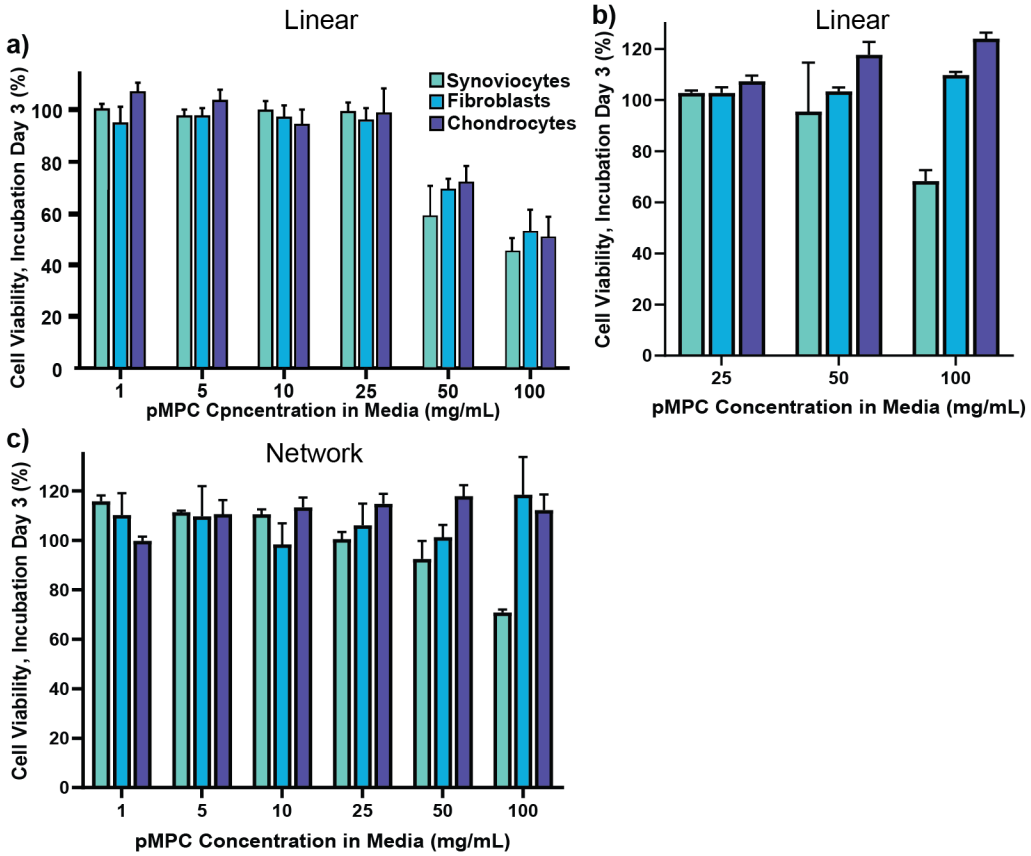

Figure S10. Cell viability of synoviocytes, fibroblasts, and chondrocytes after 72 hours of incubation in varying concentrations of pMPC (N=3). **a)** Cells were plated in standard culture plates and polymer formulations, made with 5% linear pMPC suspended in the corresponding media, were added into corresponding wells. In the standard 96-well plate, the viability of all cell types began to decrease at a concentration of 50 mg/mL ($p<0.01$). **b)** To assess whether the observed cytotoxicity with the linear polymer was due to polymer induced cytotoxicity, or due to the polymer settling on the cells and preventing cells from accessing appropriate nutrients in the media, an additional cytotoxicity experiment was conducted using a 96-well plate fitted with a transwell insert. Cells were plated in the bottom compartment of the plate while the polymer formulations, made with 5% linear pMPC in the corresponding media, were added into the transwell inserts. In this format, the fibroblasts and chondrocytes maintained viability at 50 and 100 mg/mL, while the synoviocytes had decreased viability at 100 mg/mL compared to 25 mg/mL ($p<0.05$). **c)** The transwell format was used to assess the cytotoxicity of the network polymer to the three cell types as well. Cells were plated in the bottom compartment of well plates fitted with transwell inserts. Polymer formulations, made with 5% network pMPC in the corresponding media, were added into the transwell inserts. The network polymer did not affect the viability of the fibroblasts or chondrocytes across the concentrations used, while the viability of the synoviocytes was significantly decreased at a concentration of 100 mg/mL compared to all lower concentrations ($p<0.01$). This indicates that synoviocytes are more susceptible to pMPC related cytotoxic effects than chondrocytes and fibroblasts.

2.11. pMPC sterilizability

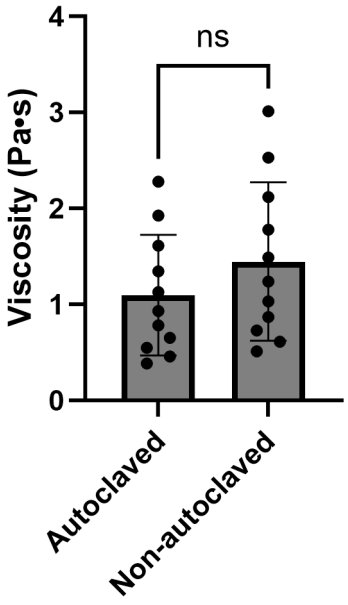

Figure S11. The viscosity of pMPC was measured before and after autoclaving to assess if this sterilization technique affects the properties of the lubricant. The average viscosity post-autoclaving is not significantly different from non-autoclaved pMPC (p=0.28), demonstrating that pMPC can be sterilized by autoclave without impacting its properties.

2.12. Linear pMPC ^{1}H NMR spectra, 500 MHz, D_2O

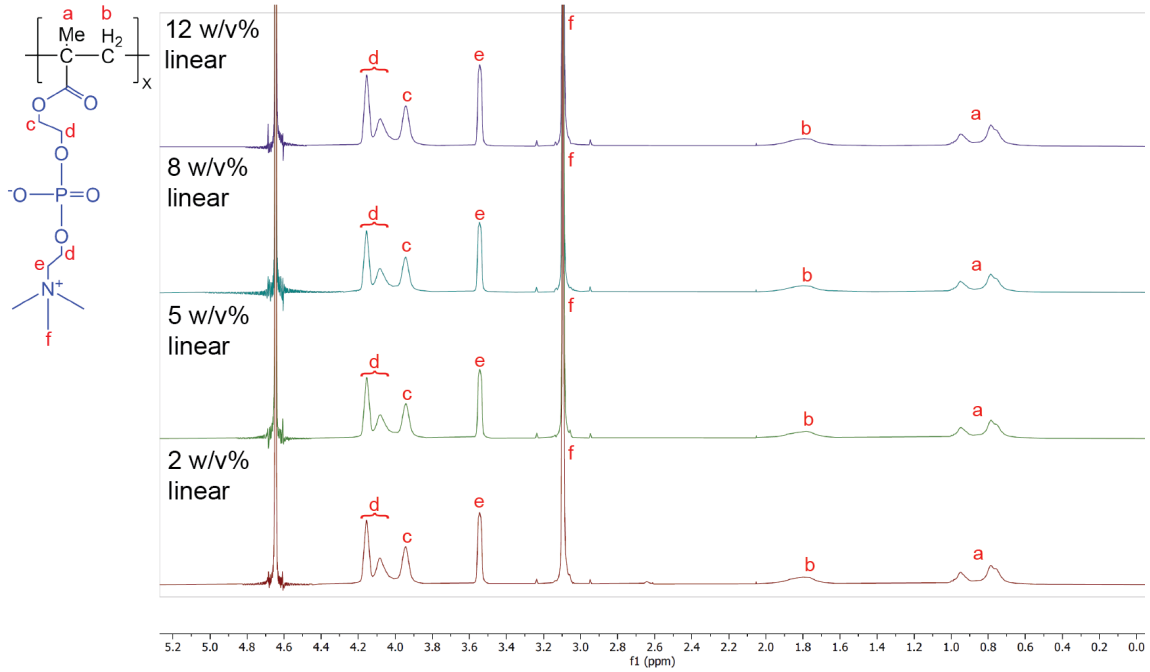

Figure S12. ^{1}H NMR of 2, 5, 8, and 12 w/v% linear pMPC.

2.13. Network (EGDMA) pMPC ^{1}H NMR spectra, 500 MHz, D_2O

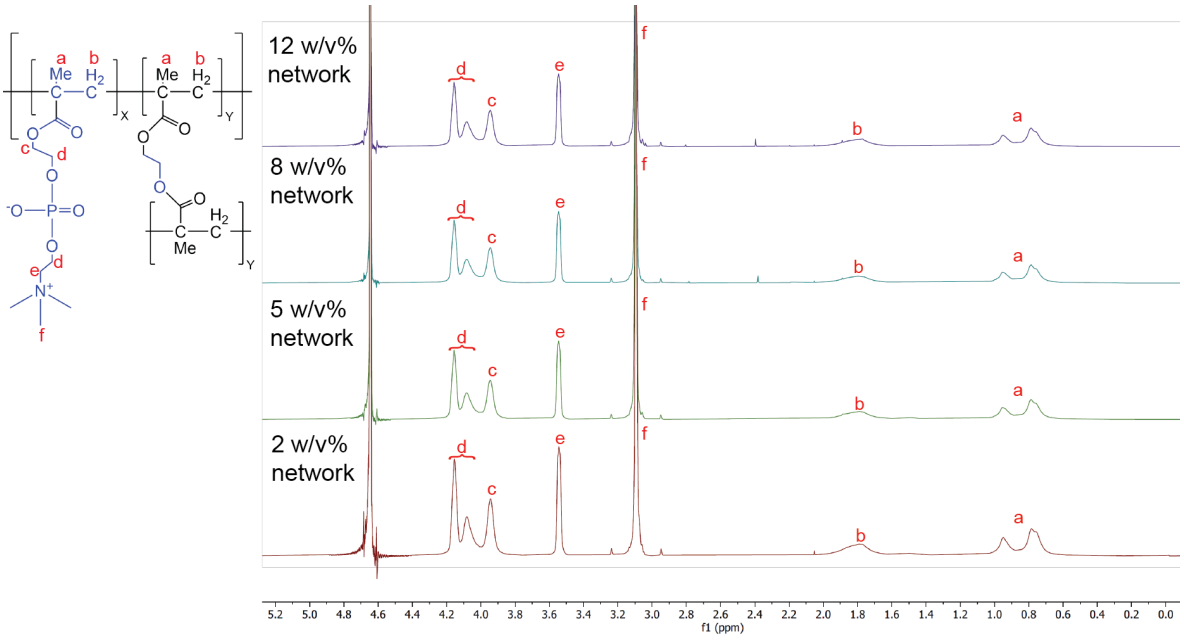

Figure S13. ^{1}H NMR of 2, 5, 8, and 12 w/v% network (EGDMA) pMPC.

Supplementary References

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