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Electronic Supplementary Information

Injectable Amine Functionalized Graphene and Chondroitin Sulfate Hydrogel with Potential for Cartilage Regeneration

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Supplementary experimental section

Name	***Description***	***Catalog Number***	***CAS Number***	***Lot Number***	***Supplier***	***Production Location***
p-Toluenesulfonic acid monohydrate	ReagentPlus®, 98.5%	T35920-100G	6192-52-5	MKBH8370V	Sigma Aldrich	Japan
Concentrated sulfuric acid	Certified ACS Plus	A300-212 (2.5 L)	7664-93-9	174051	Fisher Chemical	United States of America
Graphite	Flake, natural, 325 mesh, 99.8% (metal basis)	43209 (250G)	7782-42-5	F01X009	Alfa Aesar	United States of America
Potassium permanganate	ACS, 99.0% min	14307 (500G)	7722-64-7	T18D006	Alfa Aesar	United States of America
Hydrogen peroxide	Certified ACS, 30%	H325-500ML	For H_2O_2 7722-84-1, For H_2O 7732-18-5	131932	Fisher Scientific	Saudi Arabia
SnakeSkin® dialysis tubing	3.5K MWCO, 35 mm dry I.D., 35 feet	Q9214926	N/A	N/A	Thermo Scientific	N/A
Triethyl orthoacetate	98%	L06004 (2500ML)	78-39-7	5001A13M	Alfa Aesar	China
Sodium hydroxide	Certified ACS	S318-500G	1310-73-2	164106	Fisher Chemical	United States of America
Ethyl alcohol	200 proof, absolute, anhydrous, ACS/USP grade	111000200 (1Gallon)	64-17-5	K17C31234	Pharmco-AAPER	N/A
p-Dioxane	For HPLC, Spectrophotometry, and GC	DX2091-1	123-91-1	55222	OmniSolv®	China
Molecular sieves	Davidson®, Type 4A, Grade 514, 8-12 mesh, beads, effective pore size 4Å	M514-500 (500GM)	N/A	052746	Fisher Chemical	N/A
N, *N*-Dimethylformamide	Spectranalyzed®	D131-1L	68-12-2	162304	Fisher Chemical	Saudi Arabia
Thionyl chloride	ReagentPlus® ≥99%,	230464-100ML	7719-09-7	SHBD9658V	Sigma Aldrich	Germany
Chondroitin sulfate A	Sodium salt from bovine trachea, lyophilized powder	#C9819-25g	39455-18-0	SLBJ4544V	Sigma Aldrich	China
Phosphate-buffered saline	PBS, 10×, PH7.4	J62036 1L	N/A	P18B901	Alfa Aesar	United States of America
1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide	N/A	024810 (100G)	25952-53-8	024810J25L	Oakwood Chemical	China
N-Hydroxysuccinimide	98+%	A10312 (25G)	6066-82-6	10195147	Alfa Aesar	Great Britain
Chondroitinase ABC	Lyophilized powder from Proteus vulgaris	C2905	9024-13-9	126M4082V	Sigma Aldrich	Israel
Picric acid	Saturated solution, 1.3%	P6744-1GA	89-89-1	SHBB2249	Sigma Aldrich	United States of America

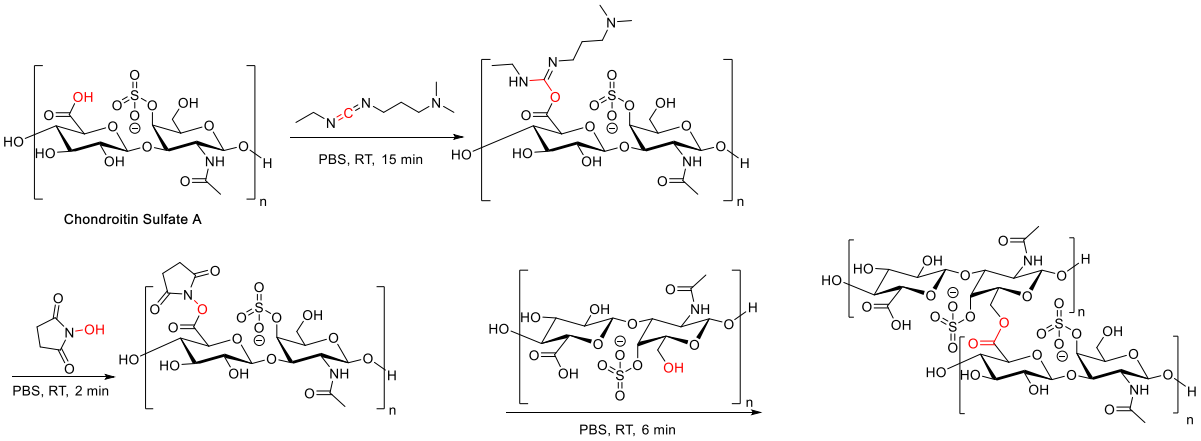
Graphene oxide (GO) synthesis. In a 4 L flask on ice behind a blast shield, graphite flakes (5 g) were added to concentrated sulfuric acid (125 mL) under stirring. Over 30 min, potassium permanganate (10 g) was slowly added. The mixture stirred for 1 h, was slowly warmed to room temperature, and then continued stirring for 1 h. The mixture was slowly heated to 35 °C and stirred for 90 min. After turning the heat off, deionized (DI) water (225 mL) was added slowly, followed by ice-cold DI water (700 mL) that was slowly added over 30 min. Hydrogen peroxide (10 mL) was added over 5 min, followed by additional ice-cold DI water (200 mL). The quenched reaction stirred at room temperature overnight.

The product was collected via centrifugation (3290×*g* / 4200 rpm, 10 min). The supernatant was discarded, and pellets were collected into 3500 Da molecular weight cutoff dialysis tubing and dialyzed in DI water for a week, changing the DI water daily. The remaining solids were collected, placed in a -80 °C freezer overnight, and lyophilized for three days, yielding a dry powder of GO.

Claisen graphene synthesis. A 1 L round bottom flask outfitted with a condenser column and stir bar was flame dried, placed under nitrogen, and charged with GO (1.98 g) and triethyl orthoacetate (402 mL). The reaction was sonicated (240 W, 42 kHz ultrasonic cleaner, Kendal) for 10 min to suspend the GO, charged with *p*-toluenesulfonic acid (34 g), and stirred and refluxed at 140 °C for 2 days under nitrogen, yielding Claisen graphene 1 (CG1).

Still under stirring, the reaction was cooled to room temperature. Sodium hydroxide (3.2 g) that was dissolved in ethanol (110 mL) was added slowly, and the reaction proceeded for 3 h under atmosphere. Then, the reaction was centrifuged (3290×*g* / 4200 rpm, 10 min) to collect the product. The supernatant was discarded, and the pellet was washed three times with acetone and two times with DI water. The final product was dried under vacuum, yielding Claisen graphene 2 (CG2).

Acid Chloride Graphene Synthesis. A flame-dried, 1 L round bottom flask was charged with dioxane (240 mL) that was dried with 4 Å molecular sieves, Claisen graphene 2 (2 g), and anhydrous dimethylformamide (2 mL) under nitrogen. The reaction mixture was sonicated for 10 min to disperse the Claisen graphene 2. Next, thionyl chloride (9.6 mL) was added slowly, and the mixture was allowed to react overnight at room temperature under nitrogen. The reaction mixture was centrifuged (3290×*g* / 4200 rpm, 10 min), the supernatant discarded, and the pellet was dried, yielding acid chloride graphene. To prevent degradation of acid chloride graphene, it was immediately used for the synthesis of ethylenediamine graphene (EDAG).



Scheme S1 Synthesis of CS–CS gels. EDC/NHS activate the carboxylic acid groups of the CS backbone. Then, the less sterically hindered hydroxyl group of CS acts as a nucleophile, forming covalently crosslinked, CS–CS gels.

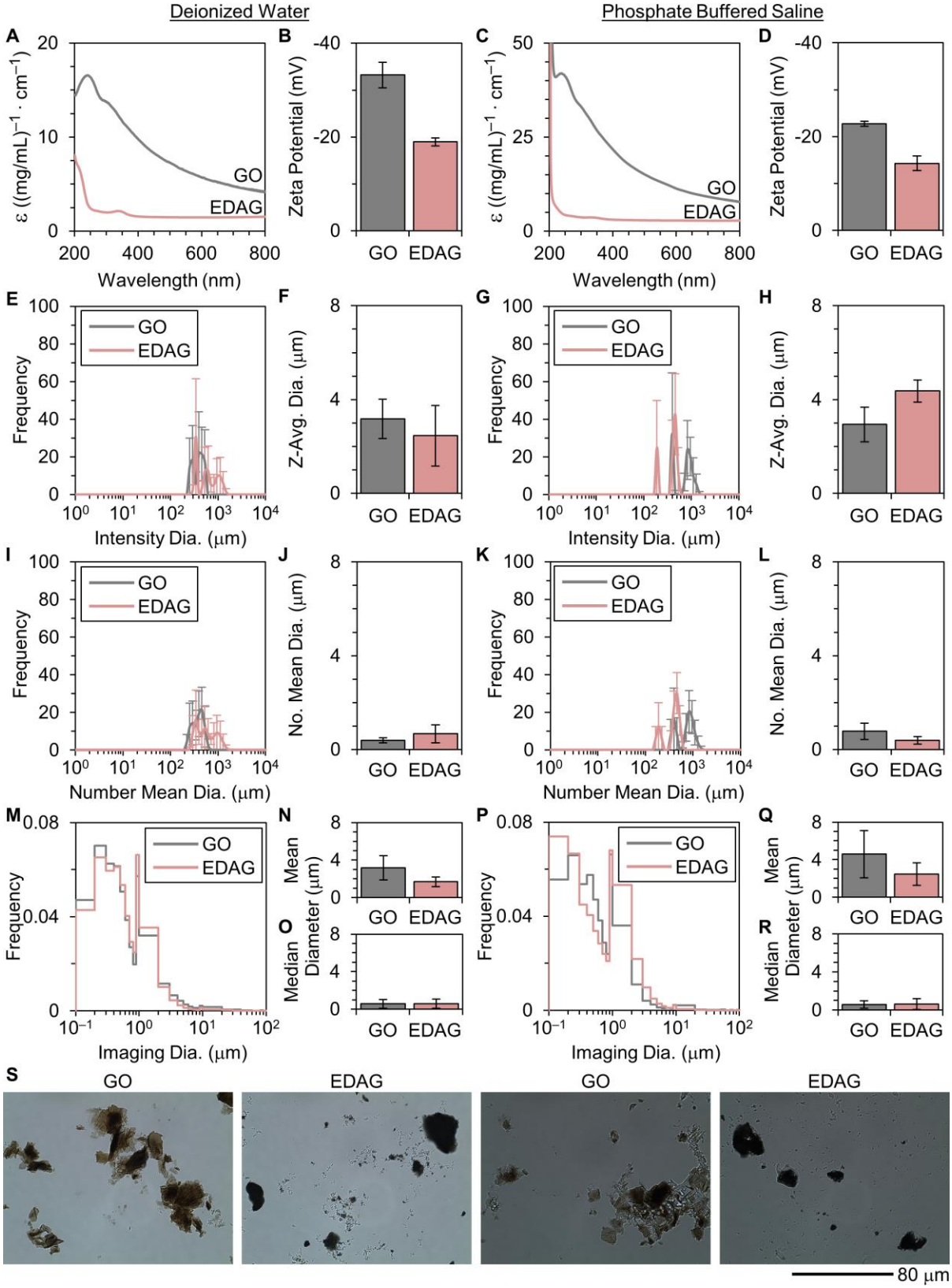


Fig. S1 Particle characterization of dispersions of GO and EDAG in DI water and PBS. (A,C) Absorbance spectroscopy and (B,D) average zeta potential. (E–F) Dynamic light scattering-determined data. (E,G) Intensity-based distributions of particle size. (F,H) Z-average (cumulants) mean diameters. (I,K) Number-based distributions of particle size. (J,L) Number-based mean diameters. (M–S) Optical imaging-determined data. (M,P) Distributions of particle sizes and (N,Q) mean and (O,R) median diameters determined from optical imaging. (S) Representative color images of GO and EDAG particles.

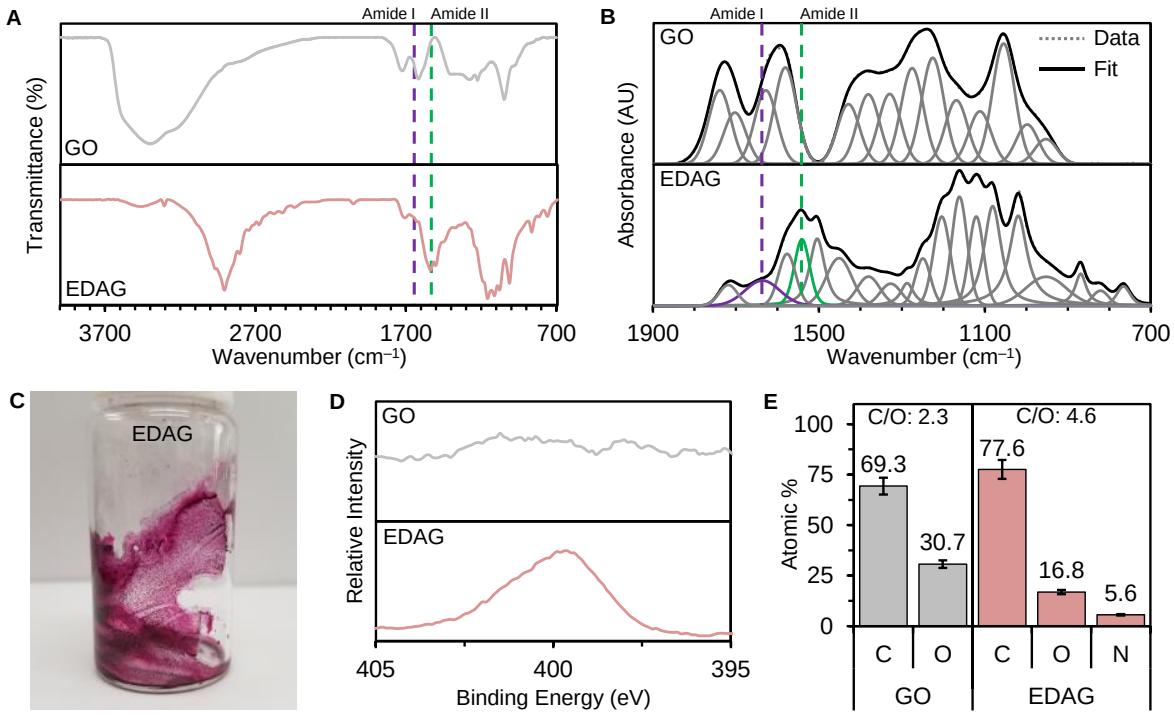


Fig. S2 Compositional characterization of EDAG compared to GO. (A) FTIR spectra. (B) Peak fitting of the FTIR spectra over 700–1900 cm^{-1}. The peaks at 1637 and 1541 cm^{-1} that correspond to Amide I and Amide II are colored purple and green, respectively, and confirm amide functionalization for EDAG. (C) Optical image of EDAG subjected to the ninhydrin amine test. (D) XPS high resolution nitrogen (N1s) spectra. (E) Elemental composition as determined from XPS. No nitrogen was detected for GO.

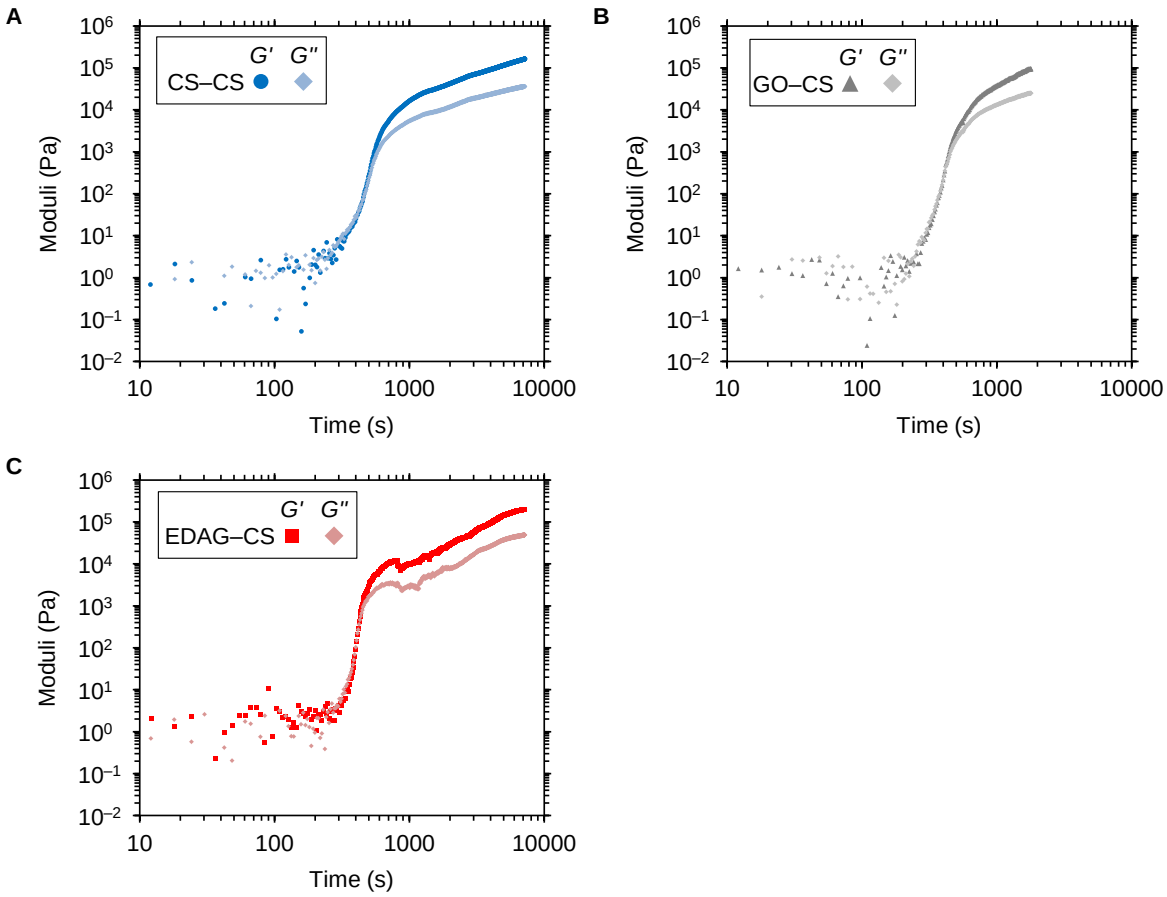


Fig. S3 Representative shear storage (*G′*) and loss (*G″*) moduli for reactions of (A) chondroitin sulfate (CS) only, (B) graphene oxide (GO) and CS, and (C) ethylenediamine graphene (EDAG) and CS that were prepared and immediately place on the rheometer for analysis. Gelation time is defined as when *G′* increases above (i.e., crosses over) *G″*.

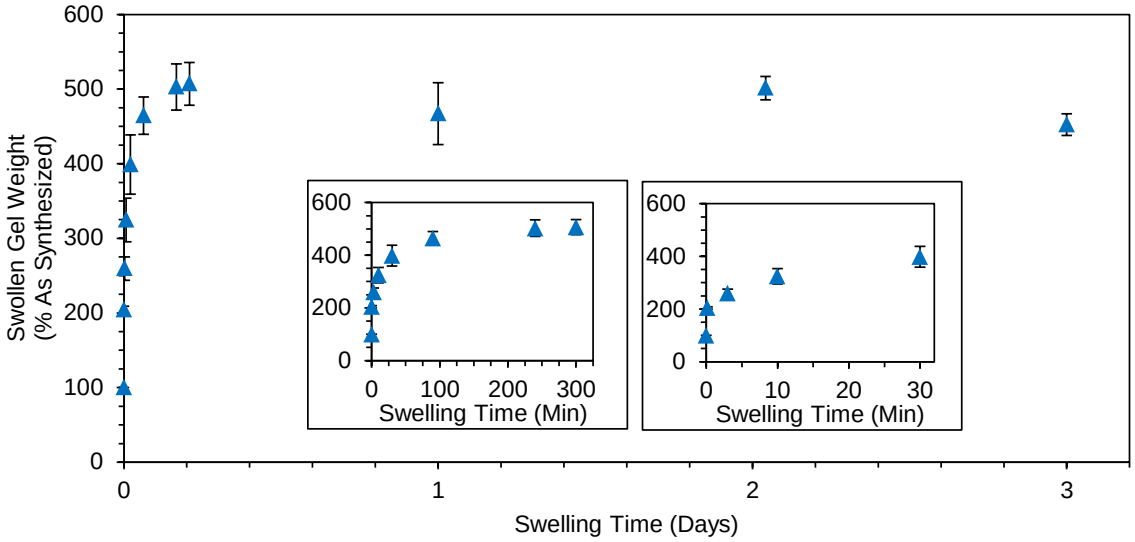


Fig. S4 Time-dependent swelling of CS–CS gels prepared in molds for 90 min then placed into pre-weighed scintillation vials. The t = 0 time point is for the as-produced gel that was not exposed to a semi-infinite bath of DI water. Subsequent time points indicate the time for which the gels were exposed to a semi-infinite bath of DI water (10 s to 3 days). Two inset plots are included for clarity. Equilibrium is reached after 4 h.

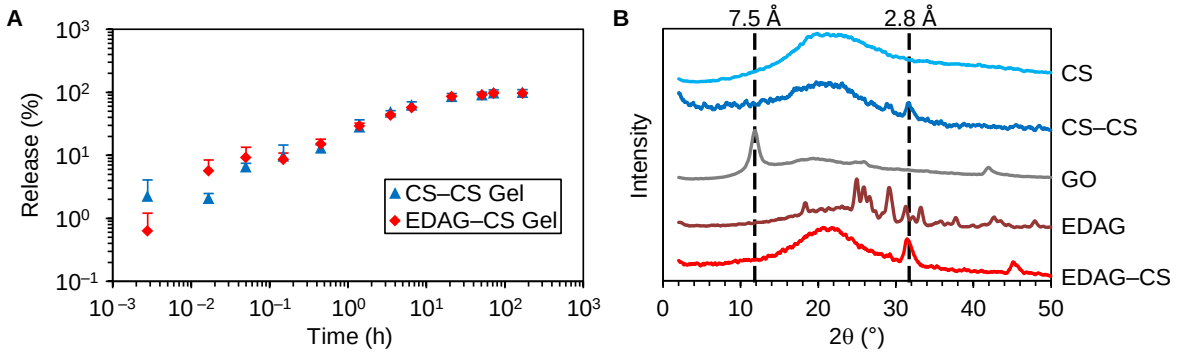


Fig. S5 Structural characterization. (A) Chondroitin sulfate (CS) only (CS–CS) and ethylenediamine graphene (EDAG)–CS gels that were reacted in solution containing a small molecule chromophore and then placed in a semi-infinite bath of deionized water nearly completely released the small molecule chromophore over time, suggesting an interconnected porous structure. Error bars are sample standard deviation. (B) Powder X-ray diffraction indicates lack of crystallinity for gels that were synthesized and lyophilized to dryness for analysis. The line at 11.8° corresponds to d_{hkl} = 7.5 Å of GO. The line at 31.73° (2.8 Å) is centered at the small peak observed for the gels but not for the starting materials.

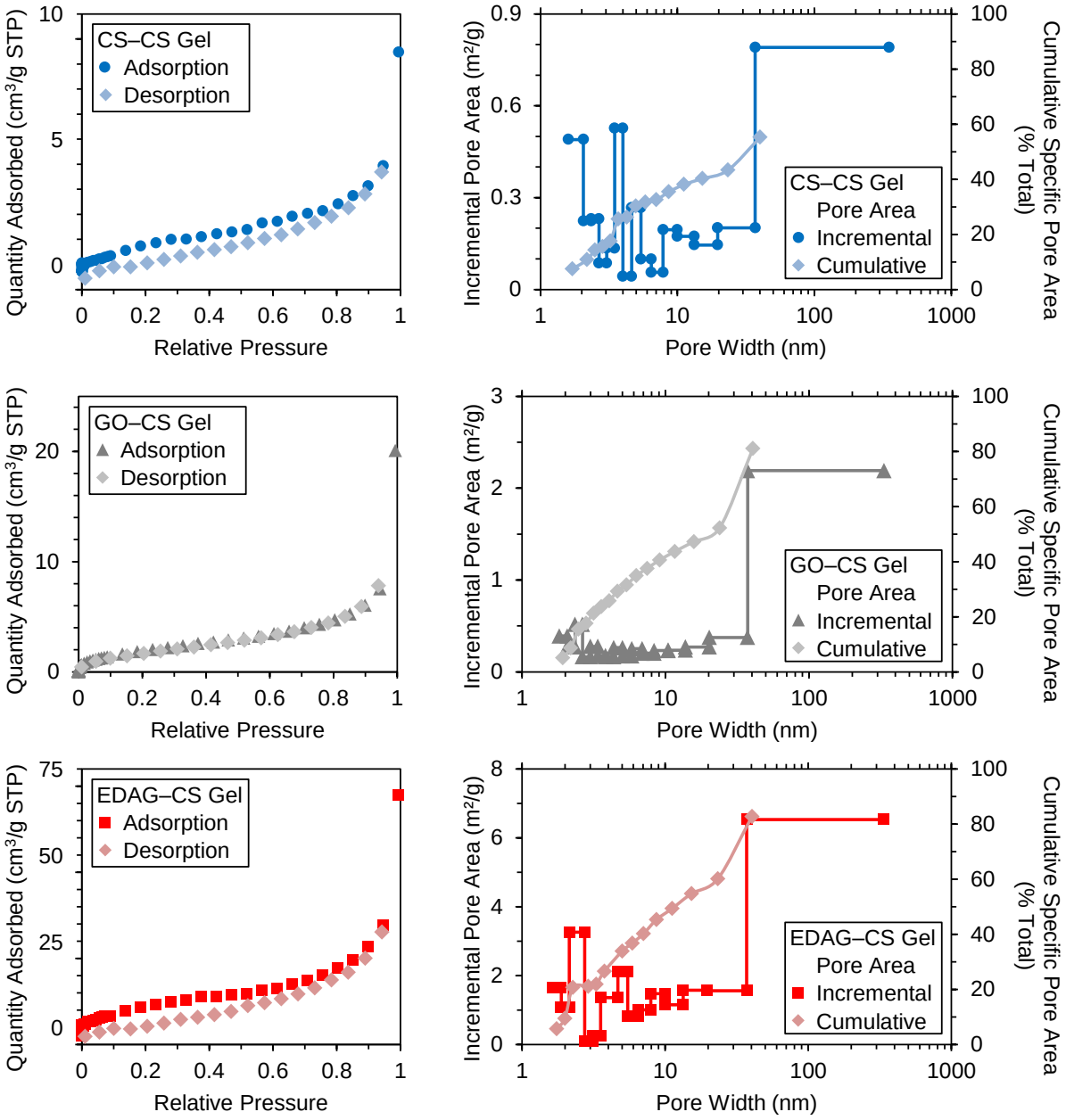


Fig. S6 Porosimetry adsorption/desorption isotherms and pore size distributions.

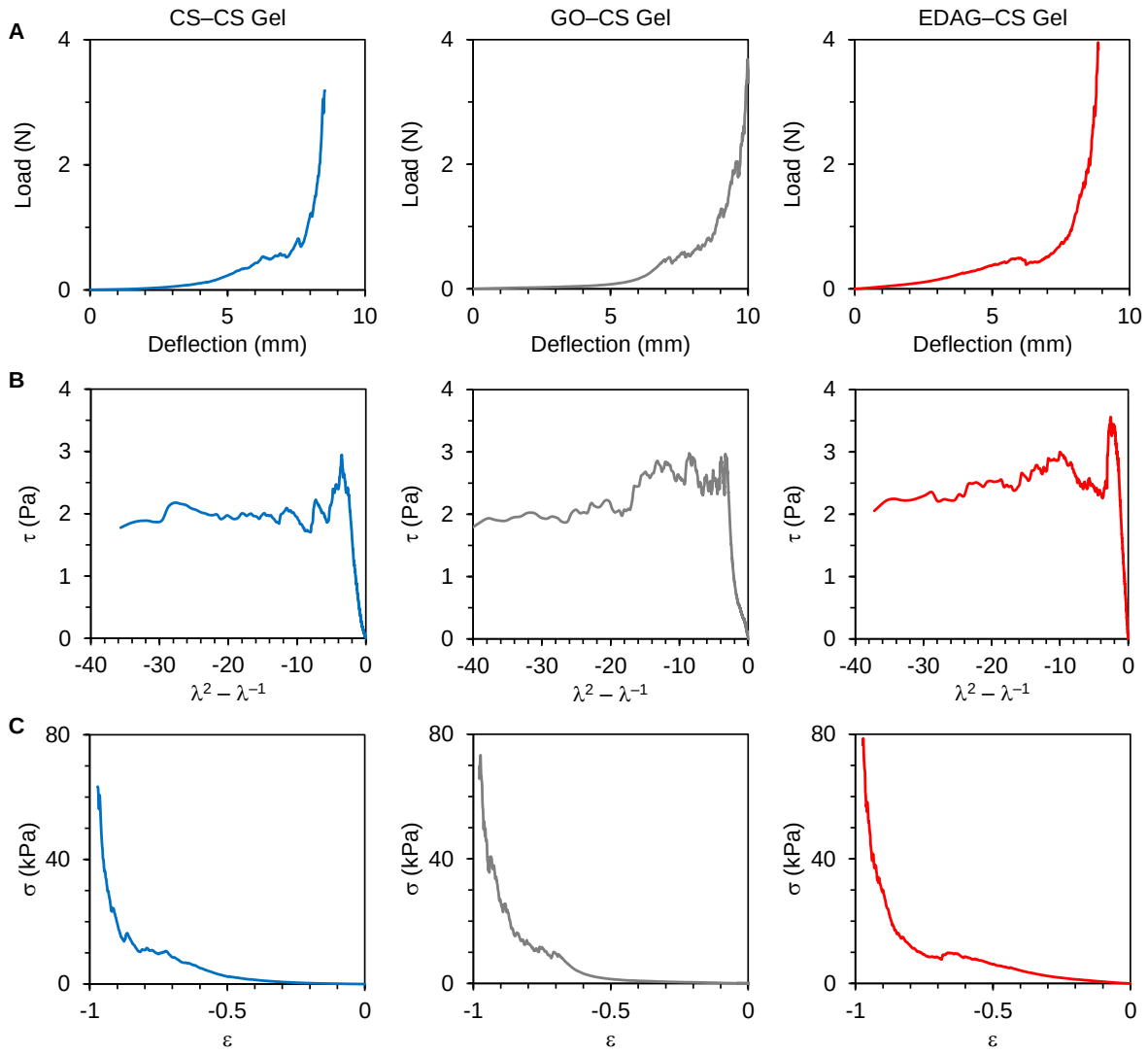


Fig. S7 Representative axial compression data. (A) As-acquired load (force) deflection (distance) data. (B) True stress (τ) versus adjusted stretch (λ). (C) Engineering stress (σ) versus engineering strain (ε). For comparison, the three plots for each type of gel are from the same sample.

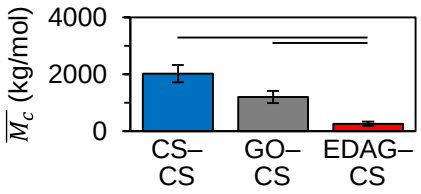


Fig. S8 Average molecular weight between crosslinks ($\overline{M_c}$) as determined from axial compression measurements and rubber elasticity theory. n = 9, 10, and 7 for CS–CS, GO–CS, and EDAG–CS gels, respectively. ANOVA F-value 12.8 and p-value 1.8×10^{-4}. Sidak post hoc test two-tailed p-value for CS–CS to EDAG–CS gels is 1.2×10^{-4} and for GO–CS to EDAG–CS gels is 0.033. Error bars are SEM and lines indicate a p-value <0.05.

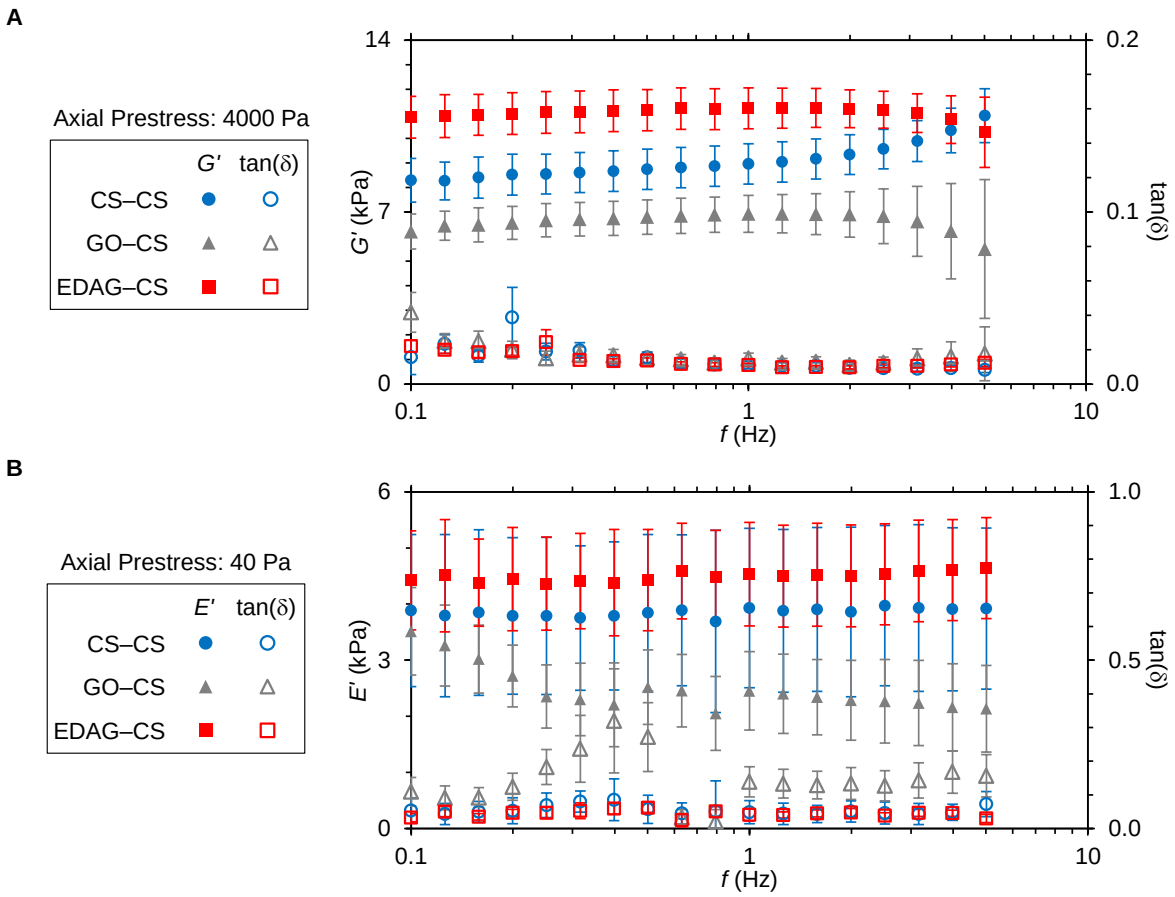


Fig. S9 Assessment of the mechanical properties of gels using dynamic techniques. (A) Shear storage modulus (G') and tanδ measured with an axial prestress of 4000 Pa. The total shear response of EDAG–CS is stiffer than that of CS–CS. n = 15, 3, and 9 for CS–CS, GO–CS, and EDAG–CS gels, respectively. ANOVA F-value 371.4 and p-value 4.0×10^{-31}. Error bars are SEM. (B) Axial compressive dynamic mechanical analysis storage (E') and loss (E'') moduli measured with an axial prestress of 40 Pa show that EDAG–CS gels are stiffer than CS–CS gels. n = 3. ANOVA F-value 329.8 and p-value 6.7×10^{-30}. Error bars are SEM.

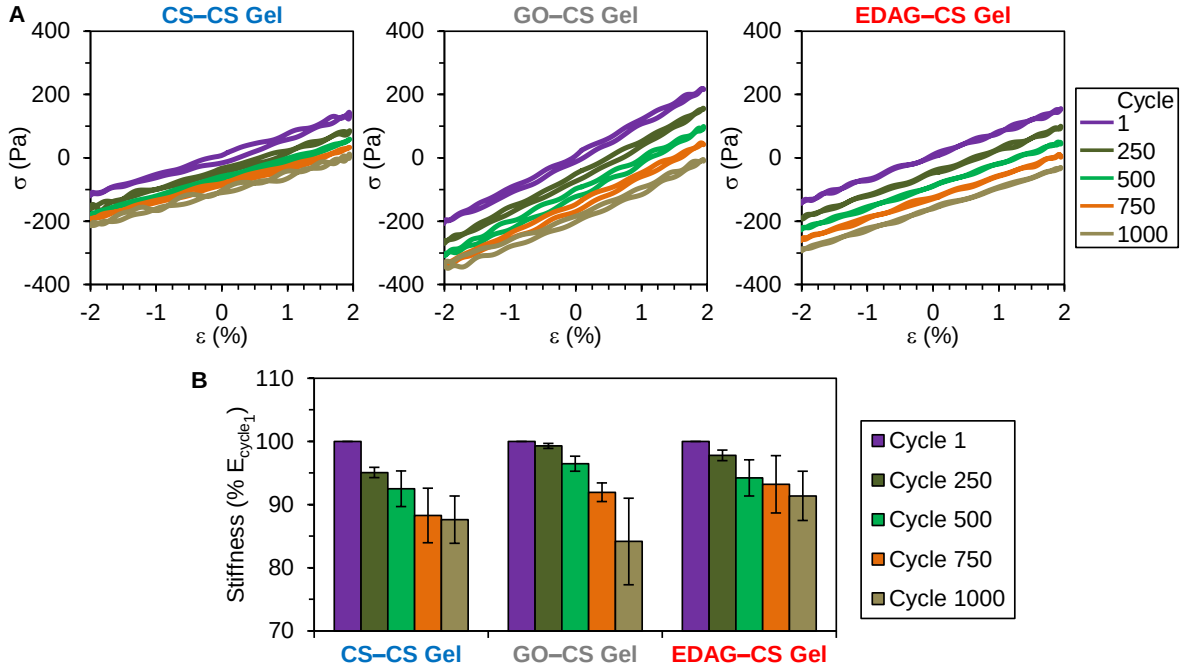


Fig. S10 Fatigue testing. (A) Stress/strain curves for the indicated cycle number. (B) Gel stiffness with increasing cycle number as a percent of the initial stiffness. Note that the preforce on the GO–CS gel was 42% larger than the CS–CS and EDAG–CS gels and, thus, resulted in a stiffer response.

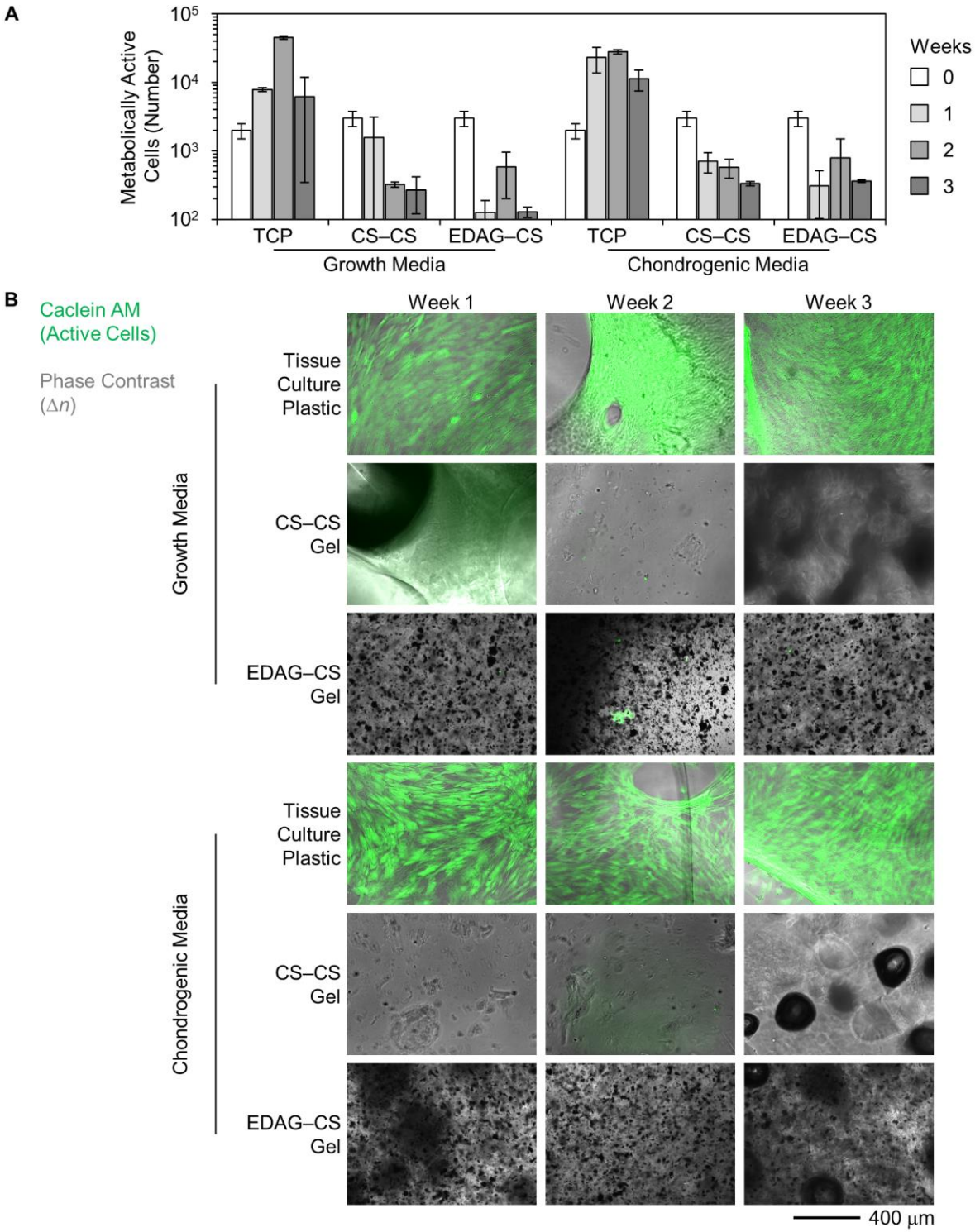


Fig. S11 Cellular vitality analysis of human mesenchymal stem cells (hMSCs) encapsulated within gels. (A) Number of viable cells over time for different experimental conditions assessed using Calcein AM that fluoresces green when internalized into cells and converted into a fluorescent form by intracellular esterases. (B) Representative images of Calcein AM fluorescence overlaid on bright field images of hMSCs.

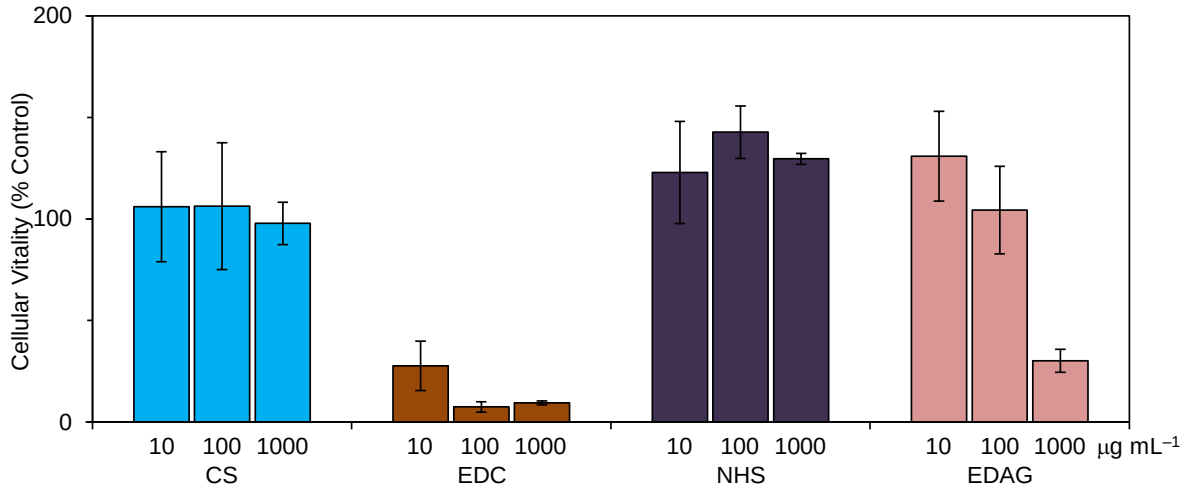


Fig. S12 Cytocompatibility of starting materials assessed using Calcein AM that fluoresces green upon internalization into cells and conversion into a fluorescent form by intracellular esterases. n = 3, and error bars are sample standard deviation.

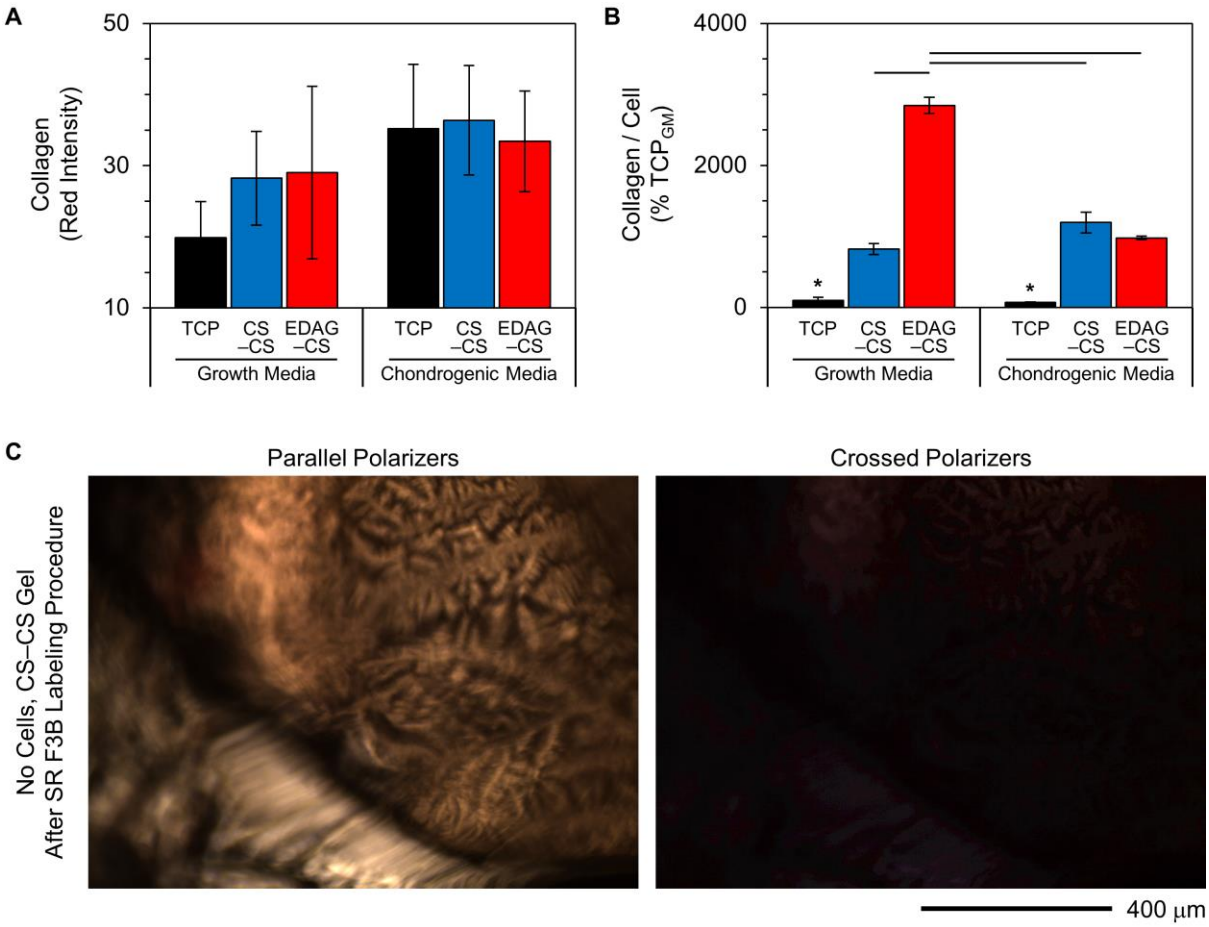


Fig. S13 Quantification of total collagen from Sirius red F3B labeling. (A) Quantification of the intensity of sirius red F3B that labels collagen red from hMSCs. Average values from CS–CS gels without cells that were subjected to the same labeling procedure were small and subtracted from the reported average values for the cell-containing samples. Note that "TCP" refers to monolayer cell culture on tissue culture plastic. n = 4, ANOVA F-value 0.55 and p-value 0.74, and error bars are Sidak post hoc test two-tailed p-value for TCP growth media to TCP chondrogenic media is 0.045, and error bars are SEM. (B) Collagen intensity was divided by cell number and reported as a percent of the value of the TCP sample maintained in growth media. n = 4, ANOVA F-value 146.2 and p-value 6.4×10^{-14}, and error bars are SEM. Sidak post hoc test two tailed p-values for TCP growth media to CS–CS growth media, EDAG–CS growth media, CS–CS chondrogenic media, and EDAG–CS chondrogenic media are 1.4×10^{-4}, 1.2×10^{-13}, 4.6×10^{-7}, and 1.1×10^{-5}, respectively; for TCP chondrogenic media to CS–CS growth media, EDAG–CS growth media, CS–CS chondrogenic media, and EDAG–CS chondrogenic media are 8.1×10^{-5}, 9.7×10^{-14}, 3.0×10^{-7}, and 6.7×10^{-6}, respectively; and for EDAG–CS growth media to CS–CS growth media, CS–CS chondrogenic media, and EDAG–CS chondrogenic media are 2.3×10^{-11}, 6.9×10^{-10}, and 8.8×10^{-11}, respectively. Note that lines indicates a p-value <0.05 and * indicates p-values <0.05 compared to all gel samples. (C) Representative color images of chondroitin sulfate only (CS–CS) gels that had no cells or media and were subjected to the collagen labeling procedure with sirius red F3B.

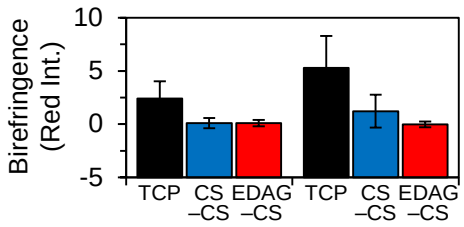


Fig. S14 Quantification of red intensity of birefringence of sirius red F3B labeled human mesenchymal stem cells imaged with crossed polarizers. n = 3, ANOVA F-value 1.3 and p-value 0.34, and error bars are SEM.

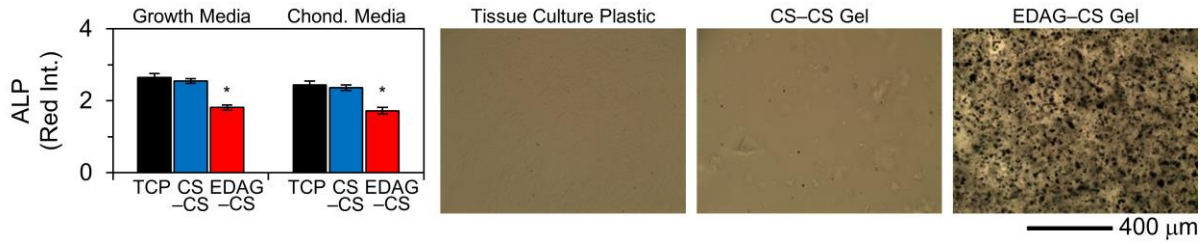


Fig. S15 Human mesenchymal stem cells (hMSCs) were encapsulated in chondroitin sulfate (CS–CS) or ethylenediamine graphene (EDAG)–CS gels or grown on tissue culture plastic (TCP) as a control, and after 3 days in growth media, were cultured for an additional 2 weeks in either growth media or chondrogenic (Chond.) differentiation media. To determine if the hMSCs were undergoing endochondral ossification towards a bone phenotype, they were stained for alkaline phosphatase (ALP), a marker of osteoblasts. Here, ALP would stain red, and the lack of red intensity suggests that hMSCs were not undergoing endochondral ossification. The ALP intensity was slightly but significantly reduced for hMSCs encapsulated and cultured in EDAG–CS gels. $n = 3$. ANOVA F-value 20.2 and p-value 1.8×10^{-5}. Sidak post hoc test two-tailed p-values for EDAG–CS growth media to TCP growth media, CS–CS growth media, TCP chondrogenic media, and CS–CS chondrogenic media are 3.0×10^{-4}, 9.5×10^{-4}, 4.2×10^{-3}, and 1.1×10^{-2}, respectively; and for EDAG–CS chondrogenic media to TCP growth media, CS–CS growth media, TCP chondrogenic media, and CS–CS chondrogenic media are 1.1×10^{-4}, 3.2×10^{-4}, 1.3×10^{-3}, and 3.2×10^{-3}, respectively. Note that * indicates a p-value <0.05 compared to TCP and CS–CS gels for both growth and chondrogenic medias.