SUPPLEMENTARY INFORMATION

Tuning and predicting mesh size and protein release from step growth hydrogels

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Supplemental Materials and Methods

Evaluation of swelling ratio and calculation of mesh size of protein-loaded hydrogels

Swelling measurements were performed on hydrogels formed in cylindrical molds according to the protocol described in Section 2.2. Hydrogels were formed at a final composition of 10% w/w for PEG-4Nb (5k molecular weight), 40 mM PEG-2SH (i.e. equimolar Nb and SH), and 0.05% w/w LAP. Proteins were added to hydrogel precursor solutions at one of three concentrations: (*i*) no protein, (*ii*) BSA at 0.06 mg/mL, or (*iii*) the protein cocktail (i.e. aprotinin at 0.35 mg/mL, myoglobin at 0.60 mg/mL, BSA at 0.06 mg/mL, lactoferrin at 0.07 mg/mL, and thyroglobulin at 0.09 mg/mL). Swelling measurements were conducted as described in Section 2.3, and mesh sizes were calculated by equilibrium swelling theory as described in Section 2.6.

Generation of BSA ribbon structure

In order to generate a ribbon structure of BSA, the PDB file of the structure of BSA was obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank[1,2] deposited by Majorek et al.[3] The PDB file was imported into Maestro software (version 10.5.014, Schrödinger, LLC, New York, NY, 2016). The options for "Show Ribbons for All Residues" and "Undisplay Atoms" were selected.

Evaluation of PDGF bioactivity

In order to evaluate the bioactivity of PDGF after exposure to free radicals, mimicking the gelation protocol, 23.3 ng/µl PDGF-BB was first mixed with 20% w/w, 10k molecular weight PEG-4Nb, 80 mM L-cysteine (Sigma-Aldrich), and 0.05% w/w LAP photoinitiator in PBS. These concentrations represent concentrations used to form 20% w/w, 10k MW hydrogels (which are equal to concentrations used to form 10% w/w, 5k MW hydrogels), except with the monofunctional cysteine used instead of the difunctional

crosslinker, to avoid gelation after polymerization. After mixing, the solutions were irradiated at 365 nm light and 10 W/cm^2 for 1 minute, again mimicking the gelation protocol.

Human mesenchymal stem cells (hMSCs; Lonza) were seeded on tissue culture plates at 2000 cells/cm^2, mimicking a procedure for assessing PDGF bioactivity from Gharibi and Hughes.[4] hMSCs were cultured for 14 days in low-glucose DMEM (Gibco) with 10% FBS (Gibco), 1% penicillin/streptomycin (Gibco), and 0.2% Fungizone (Gibco), with the addition of either *i*) no exogenous PDGF-BB, *ii*) 10 ng/mL pristine PDGF-BB, or *iii*) 10 ng/mL PDGF-BB exposed to PEG and radicals as described above. Medium was changed every 2 days.

After 14 days of culture, cells were stained with DAPI, collagen I, and tenascin-C following a previously published protocol.[5] Briefly, samples were fixed overnight in 4% paraformaldehyde (Alfa Aesar). Samples were washed with PBS, permeabilized for 10 minutes in PBS + 0.1% Triton-X (Fisher Scientific), washed, blocked in PBS + 50 mg/mL bovine serum albumin (Sigma-Aldrich) for 2 hours, washed again, and incubated overnight at 4°C with rabbit polyclonal to collagen I (Abcam) and mouse monoclonal to tenascin-C (Abcam), each diluted 1:200 in PBS + 15 mg/mL BSA. The next day, samples were washed with PBS + 0.05% Tween-20 (Sigma-Aldrich), incubated for 1 hour in Alexa Fluor 647 goat anti-rabbit (Life Technologies) and Alexa Fluor 488 goat anti-mouse (Life Technologies), each diluted 1:500 in PBS + 15 mg/mL BSA, and stored overnight at 4°C in PBS. Lastly, cells were stained with 700 nM DAPI (4',6-diamidino-2-phenylindole; Life Technologies) in PBS, washed, and imaged immediately on an Axio Observer Z1 microscope (Zeiss, Germany). The DAPI images were analyzed using the Cell Counter module on ImageJ (National Institutes of Health, Bethesda, MD).

Supplemental Figures and Tables

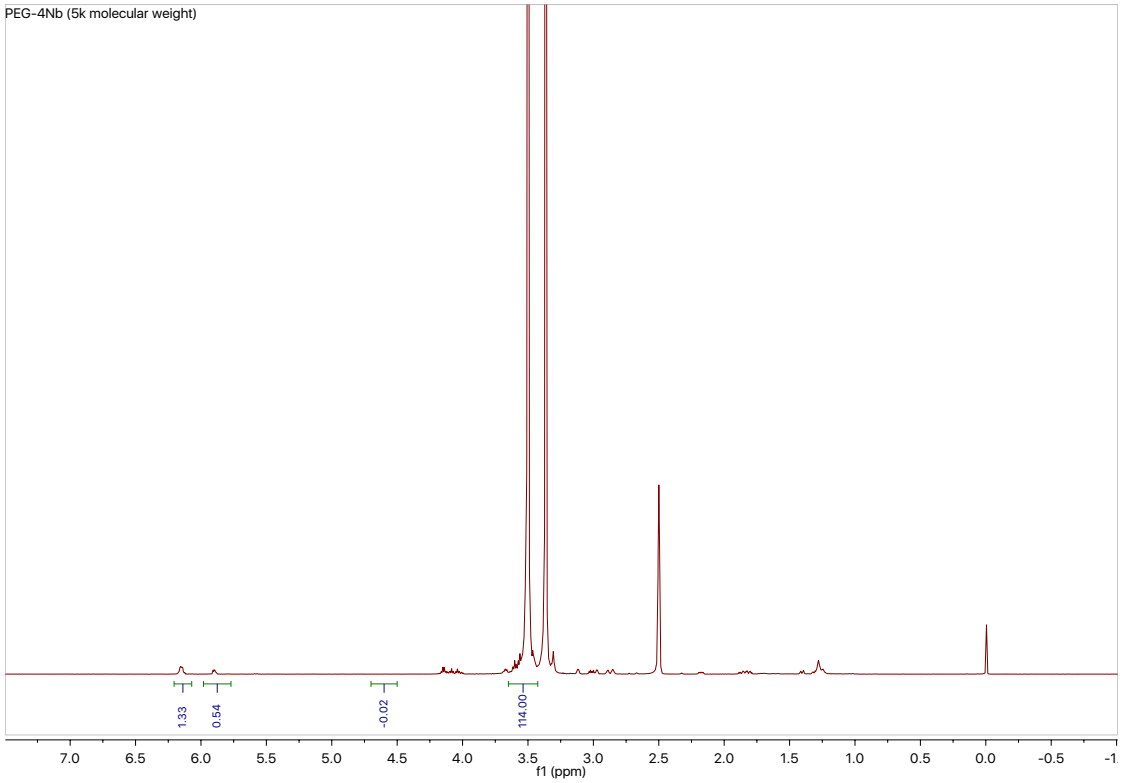


Figure S1. ^{1}H-NMR of PEG-4Nb (5k molecular weight). Successful reaction was confirmed by ^{1}H-NMR in DMSO-d6: 400 mHz δ 6.20 to 5.86 (m, 2H; norbornene double-bond peaks), δ 3.65 to 3.40 (m, 114H; PEG backbone peak) and disappearance of the -OH peak at δ 4.60 to 4.50 (t, 1H). Modification was 94% by integration of norbornene peaks.

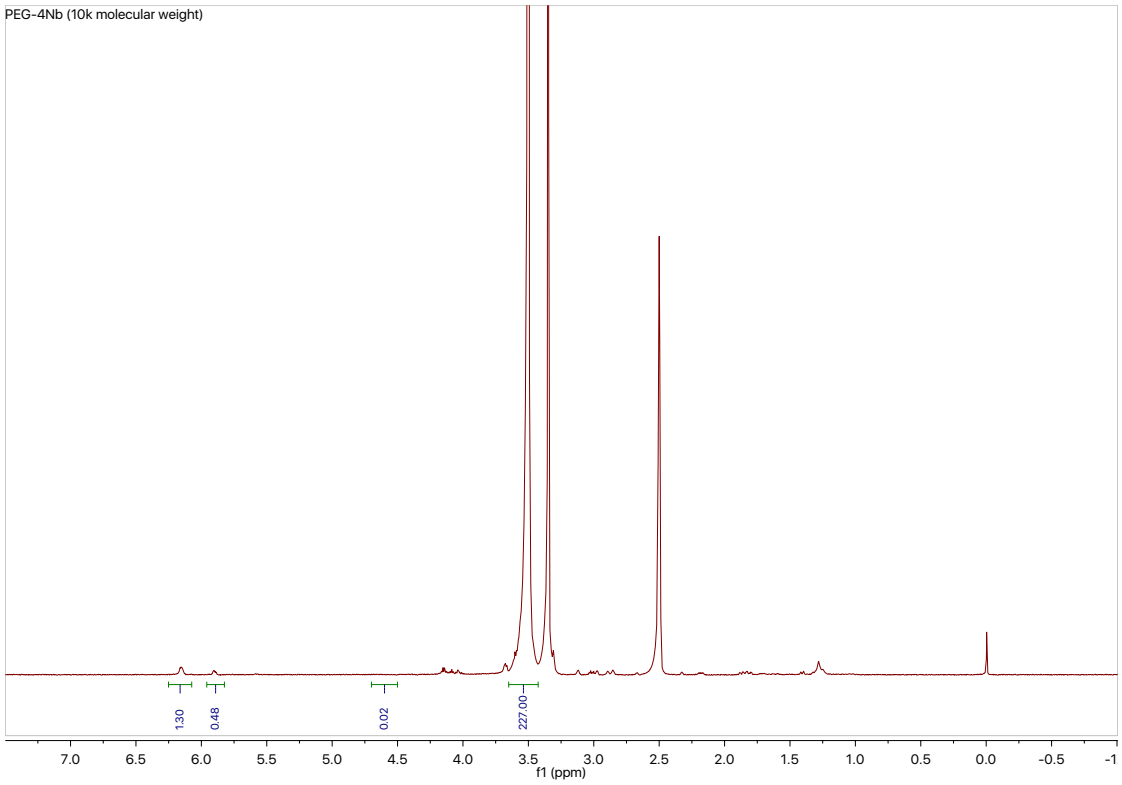


Figure S2. ^{1}H-NMR of PEG-4Nb (10k molecular weight). Successful reaction was confirmed by ^{1}H-NMR in DMSO-d6: 400 mHz δ 6.20 to 5.86 (m, 2H; norbornene double-bond peaks), δ 3.65 to 3.40 (m, 227H; PEG backbone peak) and disappearance of the -OH peak at δ 4.60 to 4.50 (t, 1H). Modification was 89% by integration of norbornene peaks.

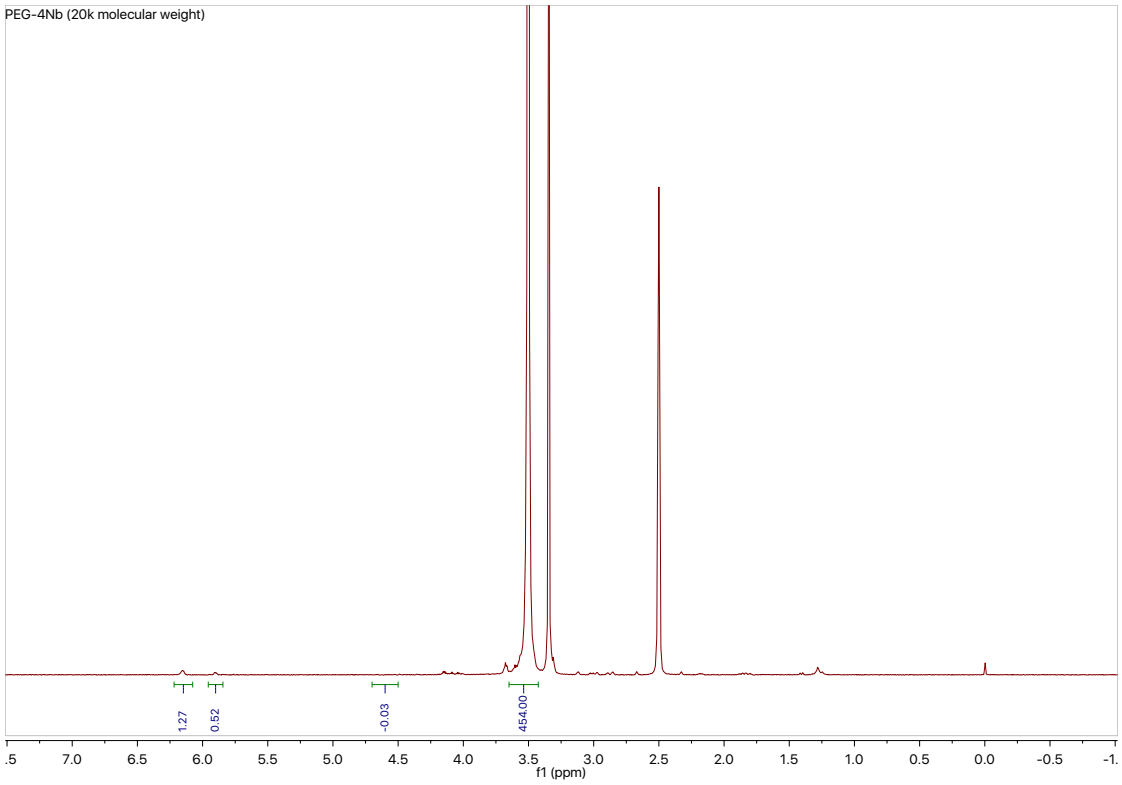


Figure S3. ^{1}H-NMR of PEG-4Nb (20k molecular weight). Successful reaction is confirmed by ^{1}H-NMR in DMSO-d6: 400 mHz δ 6.20 to 5.86 (m, 2H; norbornene double-bond peaks), δ 3.65 to 3.40 (m, 454H; PEG backbone peak) and disappearance of the -OH peak at δ 4.60 to 4.50 (t, 1H). Modification was 90% by integration of norbornene peaks.

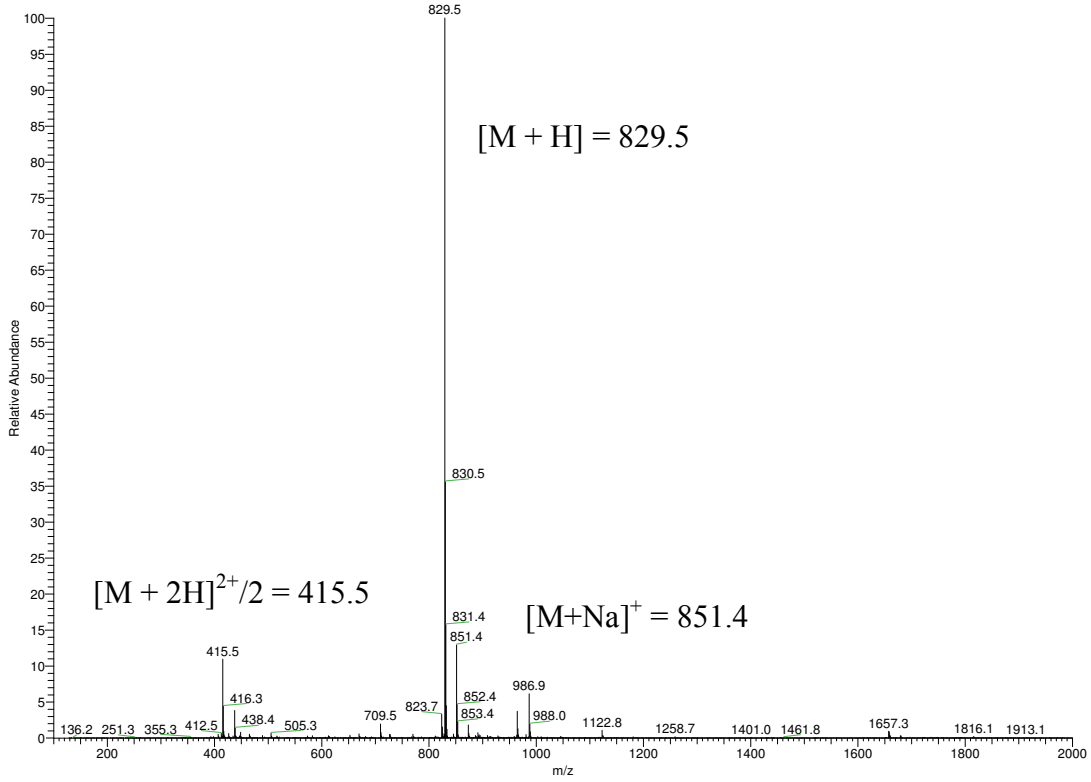


Figure S4. ESI-MS of synthesized NondegXlink. Expected MW: 828.92 g/mol. Observed MW: $[M+H]^+$ = 829.5 g/mol; $[M + 2H]^{2+}/2$ = 415.5; $[M+Na]^+$ = 851.4

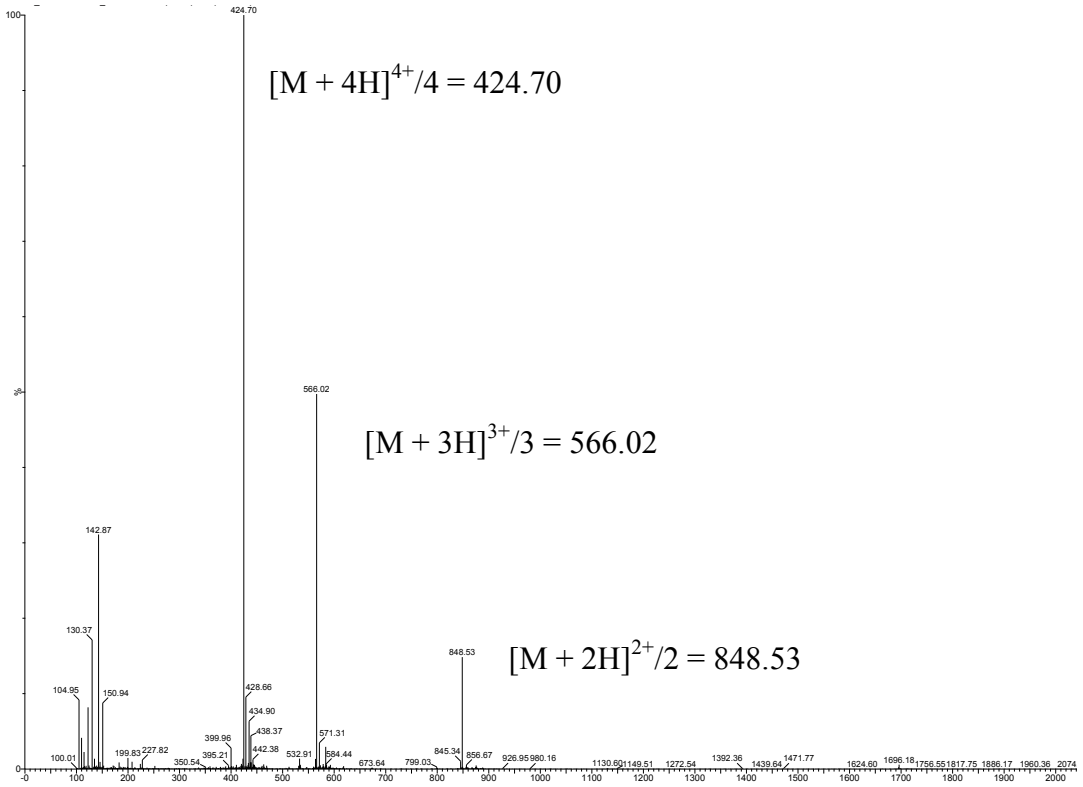
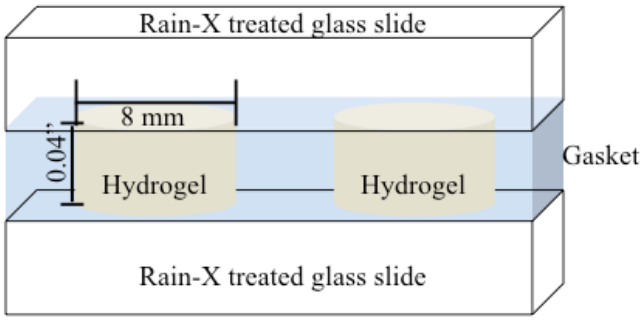


Figure S5. ESI-MS of synthesized DegXlink. Expected MW: 1695.98 g/mol. Observed MW: $[M + 2H]^{2+}/2 = 848.53$; $[M + 3H]^{3+}/3 = 566.02$; $[M + 4H]^{4+}/4 = 424.70$;



Note: diagram not to scale.

Figure S6. A schematic of the molds used to form hydrogels for swelling and protein release measurements in this study.

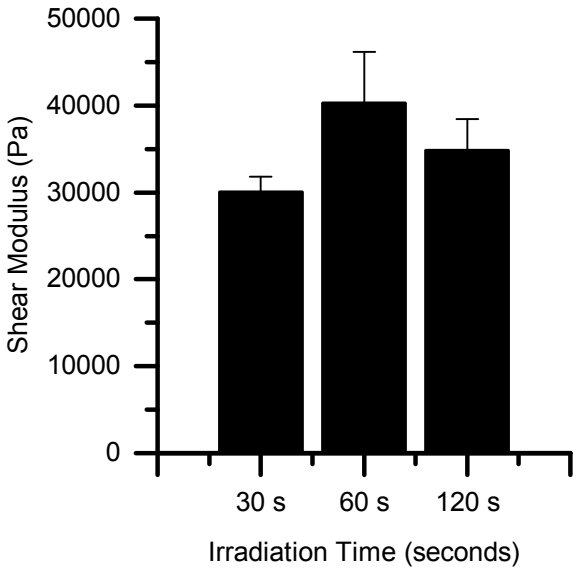


Figure S7. The effects of irradiation time on the modulus of 20% w/w, 10 kDa PEG-4NB hydrogels. Irradiating for 30 seconds or longer led to a roughly constant modulus, indicating that gel formation is essentially complete by 30 seconds. To ensure complete gel formation, 60 seconds of irradiation was selected for the studies presented in this manuscript.

Table S1. Extinction coefficients used to calculate the concentrations of protein stock solutions.

Protein	Extinction Coefficient $(g/100\ mL)^{-1}\ cm^{-1}$	Reference
Aprotinin	8.4	Manufacturer
Myoglobin	8.2	Manufacturer
BSA	6.6	Manufacturer
Lactoferrin	14.6	Manufacturer
Thyroglobulin	10.0	Ref. 6

Table S2. Mesh sizes (presented as mean ± standard error) for the hydrogel compositions displayed in Figure 2.

Composition	Calculated Mesh Size (nm)	
	Rubber Elasticity Theory	Equilibrium Swelling Theory
10 kDa PEG-4Nb; 4 wt%	12.3 ± 1.2	11.2 ± 0.6
10 kDa PEG-4Nb; 10 wt%	9.8 ± 0.9	8.3 ± 0.8
10 kDa PEG-4Nb; 20 wt%	7.6 ± 0.5	6.3 ± 0.2
5 kDa PEG-4Nb; 10 wt%	6.6 ± 0.2	5.9 ± 0.1
20 kDa PEG-4Nb; 10 wt%	15.2 ± 0.7	13.9 ± 0.5

Table S3. Mesh sizes (presented as mean ± standard error) for the hydrogel compositions displayed in Figure 3.

Identity of Crosslinker-2SH	Calculated Mesh Size	
	Rubber Elasticity Theory	Equilibrium Swelling Theory
PEG-2SH	7.6 ± 0.5	6.3 ± 0.2
NondegXlink	6.9 ± 0.3	5.2 ± 0.3
DegXlink	7.7 ± 0.4	6.3 ± 0.2

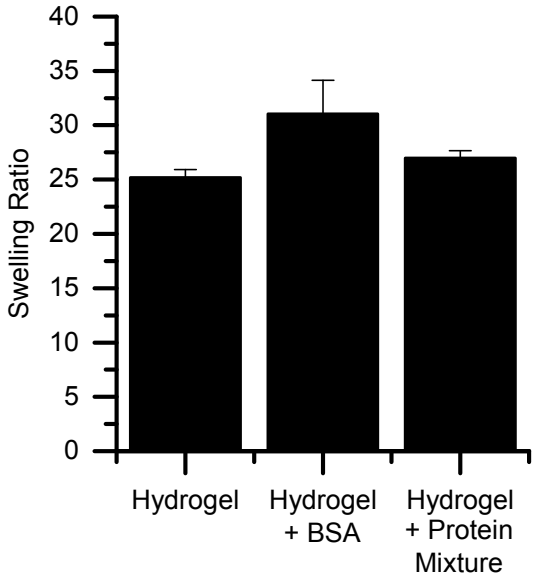


Figure S8. Evaluation of effects of protein encapsulation on the swelling of the resulting hydrogels. No significant differences were observed between the swelling ratio of hydrogels (here, 10% w/w, 5 kDa PEG-NB composition) *i)* without protein (Hydrogel), *ii)* loaded with BSA at the concentrations tested in this manuscript (Hydrogel + BSA), or *iii)* loaded with the full protein cocktail at the concentrations tested in this manuscript (Hydrogel + Protein Mixture), as determined by One-Way ANOVA ($p = 0.14$).

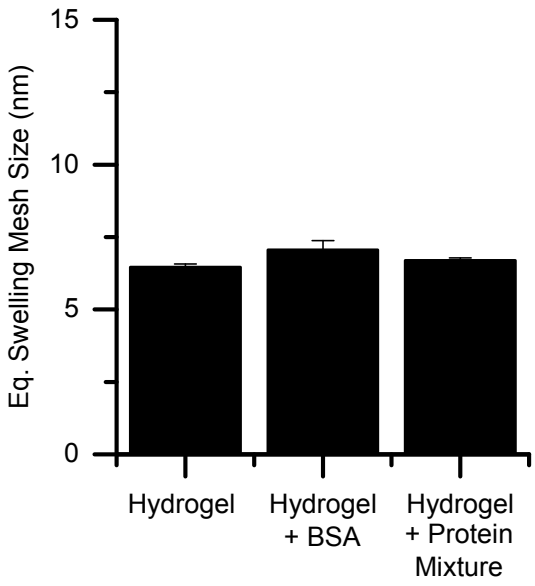


Figure S9. Evaluation of effects of protein encapsulation on the mesh size of the resulting hydrogels. No significant differences were observed between the mesh sizes of of hydrogels (here, 10% w/w, 5 kDa PEG-NB composition) *i)* without protein (Hydrogel), *ii)* loaded with BSA at the concentrations tested in this manuscript (Hydrogel + BSA), or *iii)* loaded with the full protein cocktail at the concentrations tested in this manuscript (Hydrogel + Protein Mixture), as determined by One-Way ANOVA ($p = 0.15$).

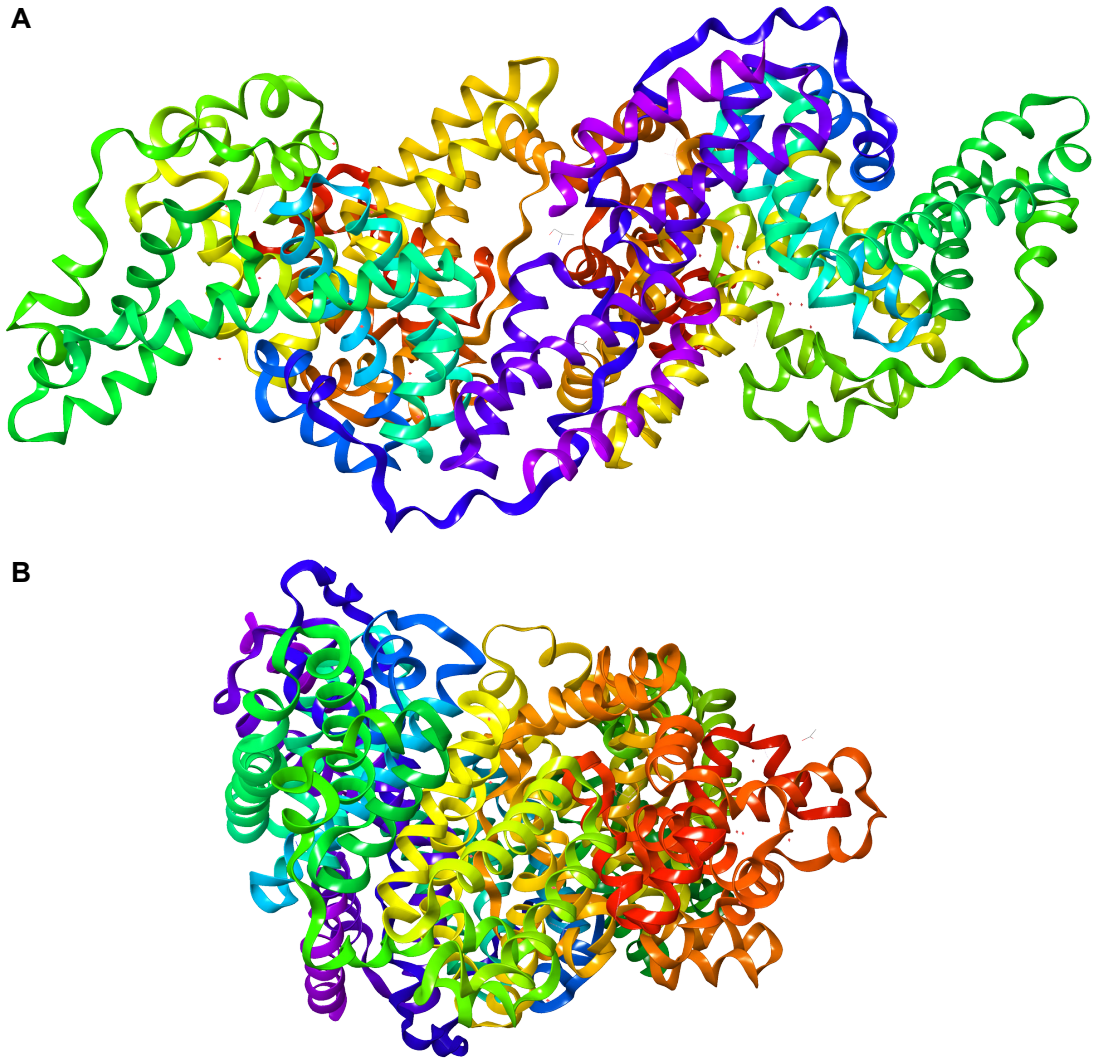


Figure S10. Model of BSA, generated based on a PDB file from Majorek et al.[3] (A) The ribbon structure of BSA is oriented to show its longest dimension. (B) The same ribbon structure as above is rotated 90º to display the shorter dimensions of BSA. We speculate that the fact that the shortest dimension of BSA is ~ 4 nm[7,8] partly explains why it is not fully retained in networks with mesh sizes on the order of ~ 6-7 nm (Figure 4.5E), despite having a hydrodynamic diameter that is greater than 7 nm.[9] The model was generated using Maestro (Schrödinger Release 2016-1: Maestro, Schrödinger, LLC, New York, NY, 2017).

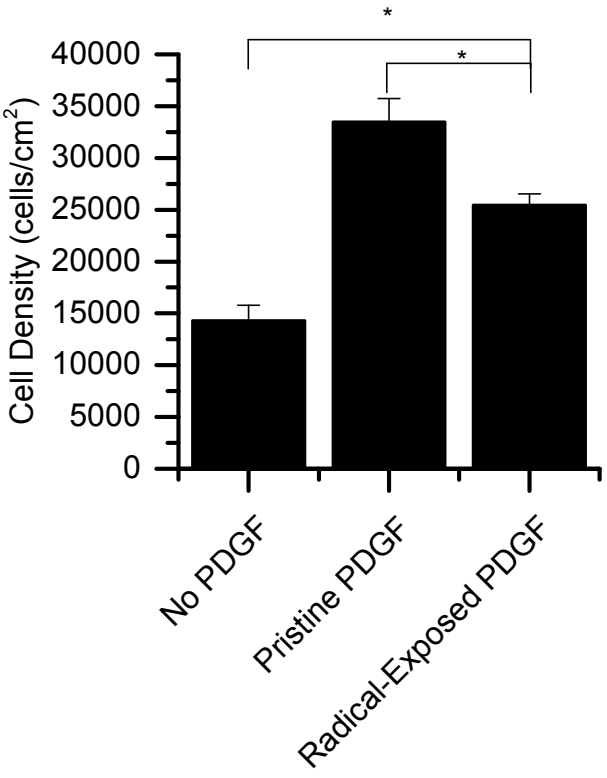


Figure S11. **The effect of polymerization conditions (radical exposure) on the bioactivity of PDGF**. hMSCs were cultured in the presence of no PDGF, unmodified (pristine) PDGF, or PDGF exposed to polymerization conditions (radical-exposed PDGF) for 14 days, and the cell density was determined to evaluate the bioactivity of PDGF, a known mitogen. The radical-exposed PDGF had significantly greater bioactivity than the no PDGF condition, demonstrating that, while bioactivity was slightly impacted by polymerization conditions, the radical-exposed PDGF was still able to exert bioactive effects on hMSCs. * $p < 0.05$ by t-test.

Supplementary Information References

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