A multilayered valve leaflet promotes cell-laden collagen type I production and aortic valve hemodynamics

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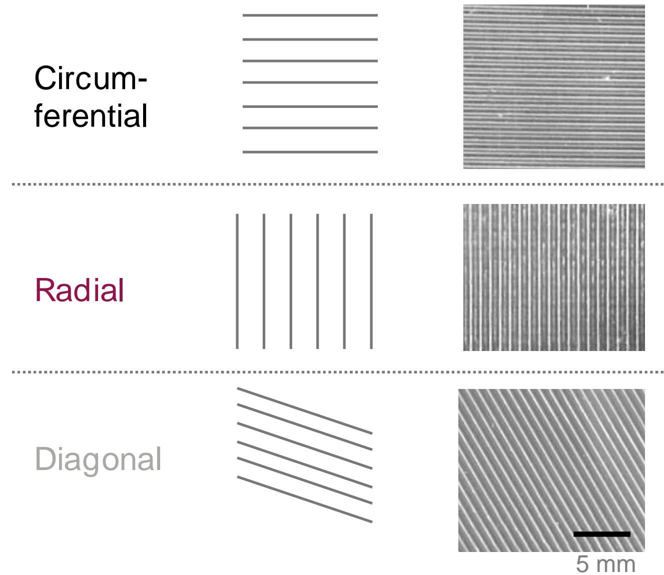
1. **Supplementary Methods:**

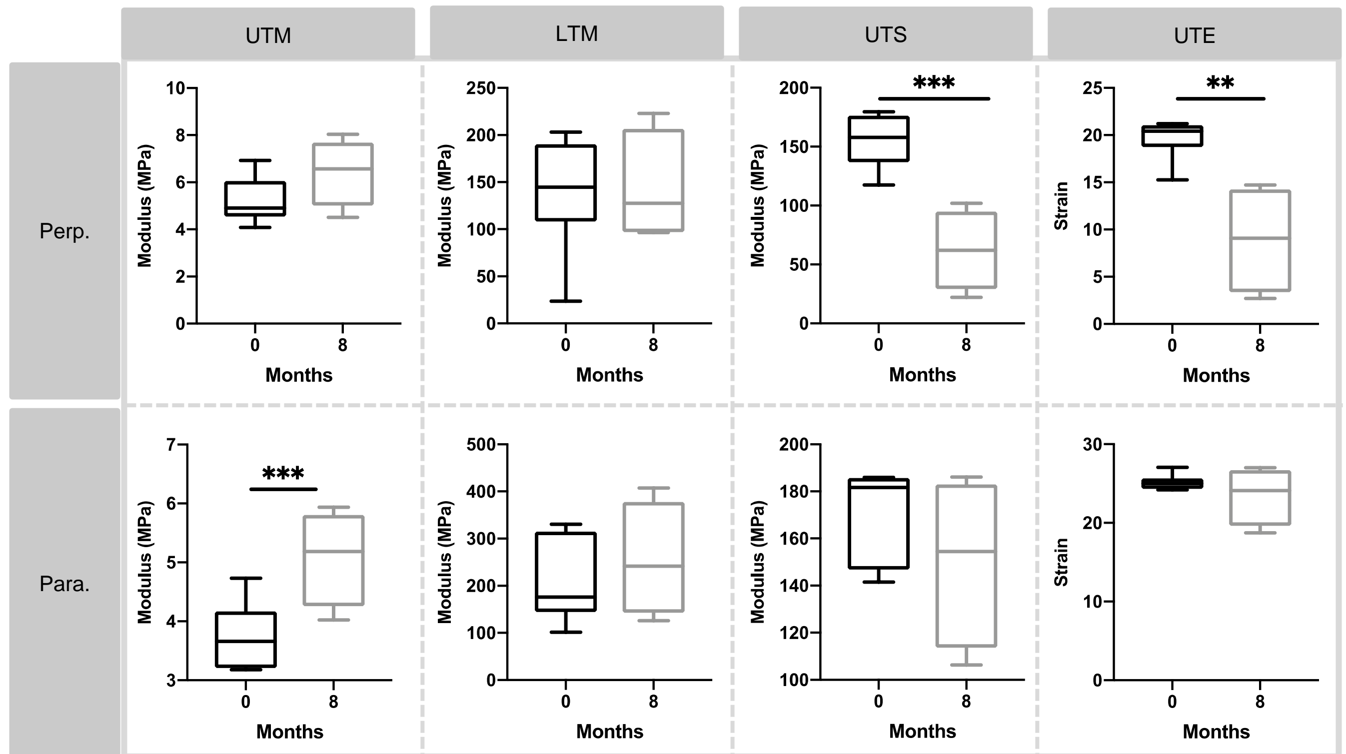
1.1. Quantification of DNA and GAG content

Multilayered scaffolds were loaded into PSS systems as described above and cultured up to D7. Controls were multilayered scaffolds at D0 and D7 static conditions. Following culture under a given condition, GelMA/PEGDA hydrogels were removed from the PCL-MAA layer of the scaffold and stored at -80°C until further processing. After freezing, hydrogels were enzymatically digested in 500 µL of a 5 mL papain enzyme solution with 25 µL of 10 mg/mL papain (Sigma-Aldrich) in PBE buffer (100 mM Na_2HPO_4, Sigma-Aldrich; 10 mM EDTA, CALIBIOCHEM; pH 6.5) with 8.35 mg of L-Cysteine (Sigma-Aldrich). The solution was added to hydrogels from each experimental group and incubated overnight at 60°C. DNA content of the digested hydrogel solution was quantified using the PicoGreen® assay according to manufacturer's instructions. Briefly, 100 µl of DNA standard or sample was combined with 100 µl of Quant-iT^{TM} reagent in the well of a 96-well plate, and the plate was incubated at room temperature for 5 minutes. Following incubation, the fluorescence was read at a 480 nm excitation wavelength and a 520 nm emission wavelength using the BioTek Synergy 2 plate reader (BioTek Instruments). Mean DNA content of each sample was then calculated from the generated DNA standard curve and the measured fluorescence value of each sample. GAG content of digested hydrogels was measured using a 9-dimethylmethylene blue chloride (DMMB, Sigma-Aldrich) assay. Briefly, 25 µl of GAG standard (Chondroitin sulfate sodium salt from shark cartilage, Sigma-Aldrich) or 25 µl of sample was combined with 5 µl of a 2.3 M NaCl (Sigma-Aldrich) solution and 200 µl of DMMB solution in the well of a 96-well plate. The absorbance was read at a wavelength of 520 nm using the BioTek Synergy 2 plate reader, and the GAG concentration was calculated from the generated GAG standard curve and measure absorbance. The GAG content was then normalized to the DNA content measured from the PicoGreen® assay by dividing a given hydrogel's measured GAG concentration by its measured DNA concentration.

2. Supplementary Results:

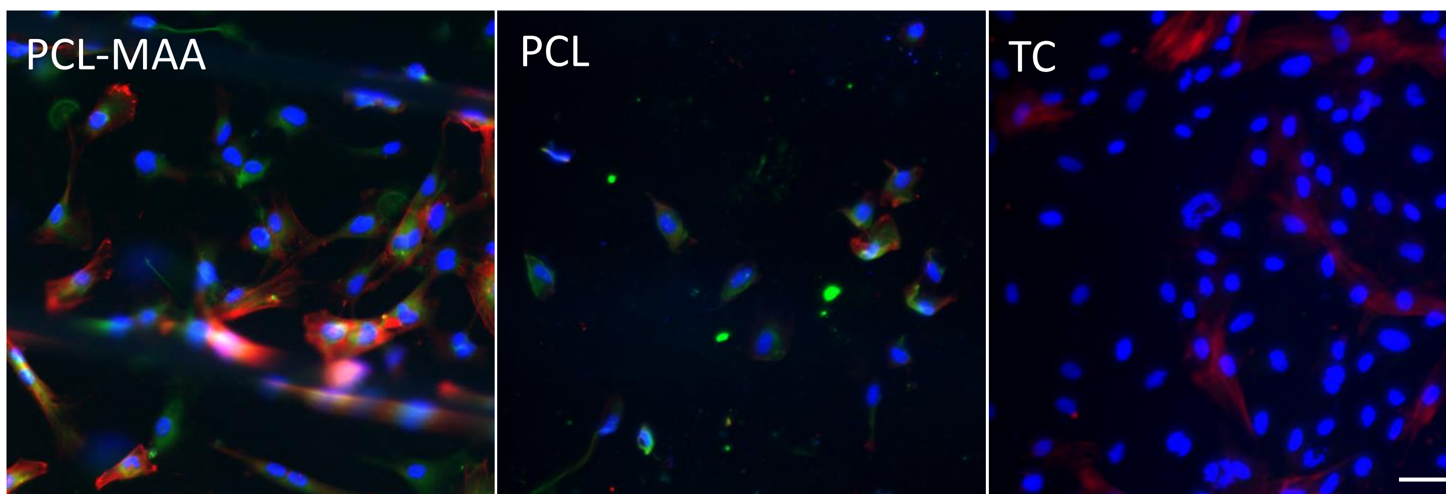


Supplementary 1. Illustration of fiber orientation. PCL was 3D printed in 3 different fiber orientation: circumferential, radial, and diagonal. Scale bar 5mm.

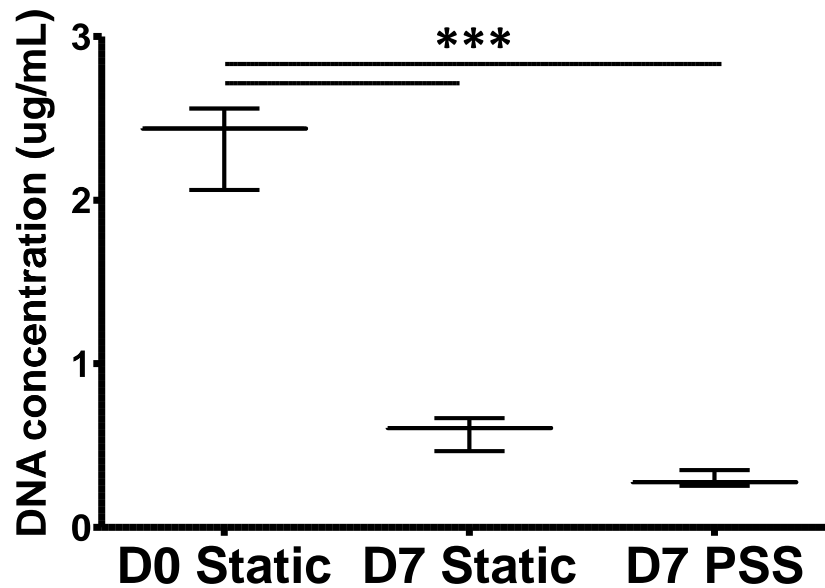


Supplementary Figure 2. Characterization of degradation of PCL scaffolds of perpendicular and parallel fiber orientation. The UTM, LTM, UTS, and UTE were characterized from uniaxial tensile testing post-degradation. Samples were maintained at 37°C in PBS for degradation studies and at 0 and 8 months the mechanical properties were characterized and compared to month 0 scaffolds. N = 3-7. Unpaired t-tests were used.
*** $p<0.001$; ** $p<0.01$.

Supplemental Figure 3. Rheological characterization of GelMA/PEGDA hydrogels over 28 days. Frequency sweeps of storage moduli and loss moduli of blank GelMA/PEGDA hydrogels and GelMA + PEGDA hydrogels with encapsulated cells over 28 days measured by rheology. Two-way ANOVA, ** p < 0.001, **** p < 0.0001.



Supplementary Figure 4. aSMA and vimentin expression of iMSCs when seeded on PCL-MAA and PCL. Immunostaining of aSM and vimentin expression of iMSCs seeded on PCL-MAA, PCL, and tissue culture (TC) after 7 days. DAPI – blue, vimentin – green, an aSMA – red. Scale bar 50 μm.



Supplementary 5. Quantification of DNA content in cell-laden multilayered scaffolds at day 0 and 7 under static and pulsatile shear stress conditions. Mean DNA content was quantified for each group using a PicoGreen® assay. N=3. One-way ANOVA with Tukey's multiple comparison test. ***$p<0.001$.

The bulk DNA content in cell-laden multilayered leaflet scaffolds under PSS was evaluated (Supplementary Figure 5). DNA content in multilayered scaffolds seeded with VICs was quantified using a PicoGreen® assay and was compared among D0, D7 static, and D7 PSS. The mean DNA content in D0 multilayered scaffolds was 2.35 ug/mL. This value was significantly greater than that of both D7 static and D7 PSS multilayered scaffolds. Further, although not statistically significant, mean DNA content in D7 static scaffolds, 0.58 ug/mL, was greater than that in D7 PSS scaffolds, 0.25 ug/mL.

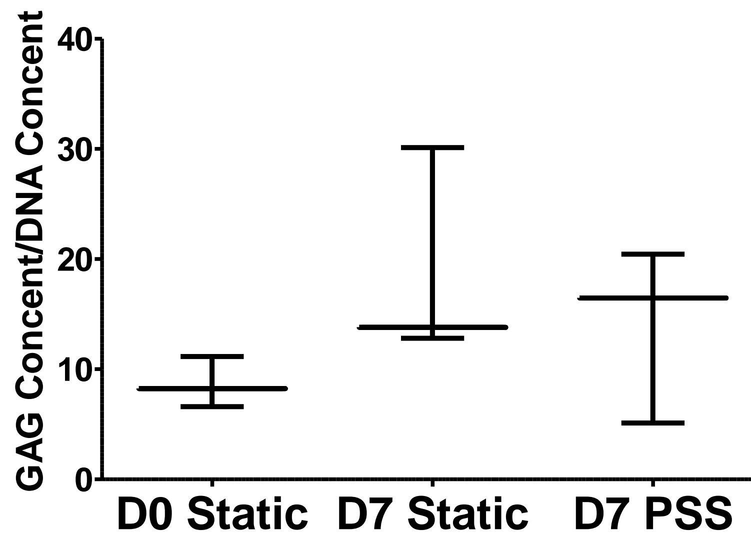


Figure 6. Quantification of GAG content in cell-laden multilayered scaffolds at day 0 and 7 under static and pulsatile shear stress conditions. Mean GAG content was quantified for each multilayered scaffold using a DMMB assay and normalized to the DNA content of the same scaffold. N=3.

GAG content in multilayered scaffolds seeded with VICs was quantified using a DMMB assay, and was compared among D0, D7 static, and D7 PSS (Supplementary figure 6). The GAG content of each scaffold was then normalized to the DNA content of the scaffold as measured by the PicoGreen® assay to account for differences in cell number among multilayered scaffolds. Following normalization, the mean GAG/DNA content ratio in D0 static, D7 static, and D7 PSS multilayered scaffolds was 8.67, 18.91, and 14.01, respectively. There were no significant differences in the GAG/DNA content ratio among each group tested.