ACCSCIENCE PUBLISHING

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RESEARCH ARTICLE

Mixed ultrashort peptide bioinks for improved 3D bioprinting of self-healing trachea-like constructs

Supplementary file

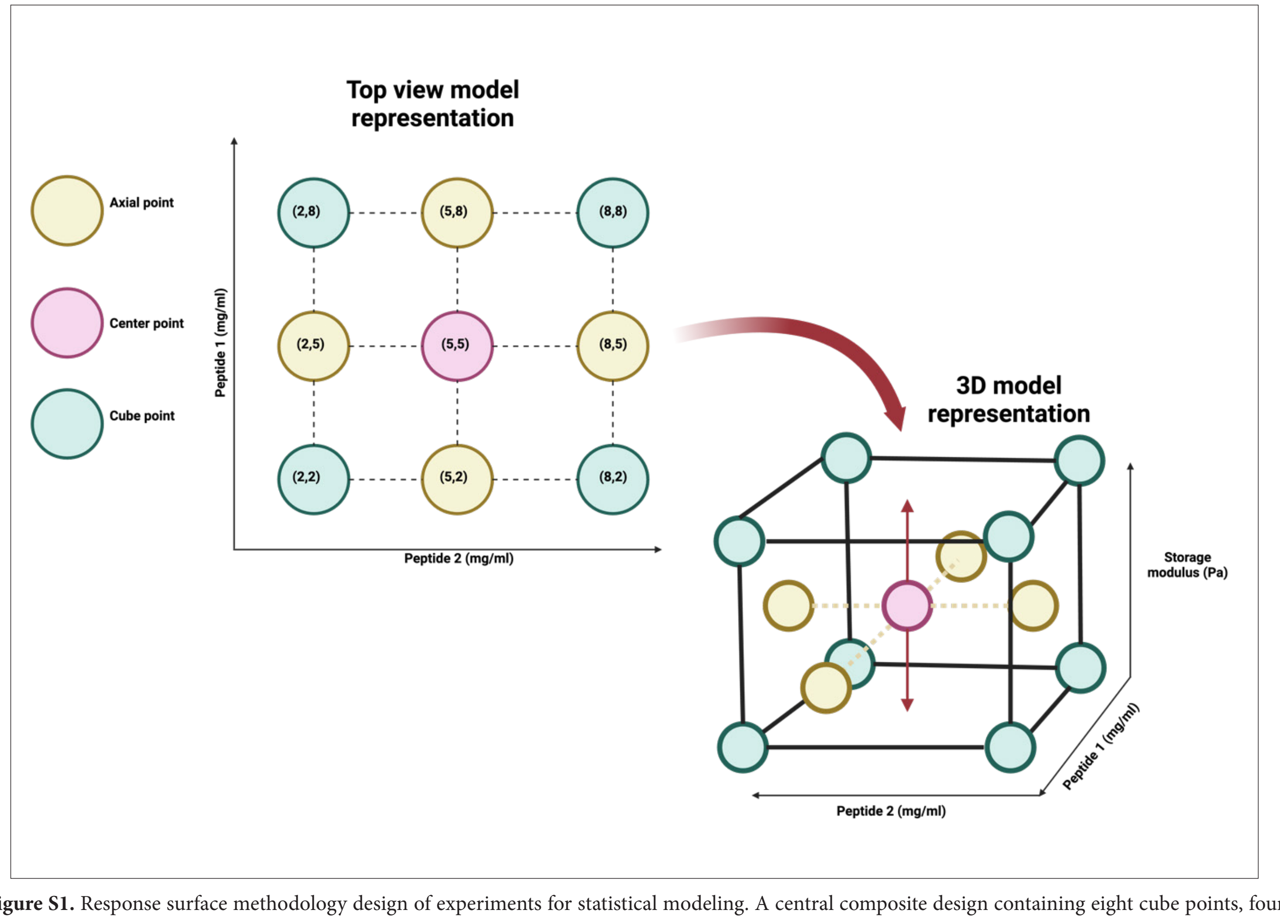


Figure S1. Response surface methodology design of experiments for statistical modeling. A central composite design containing eight cube points, four axial points, and one center point was selected to build the model.

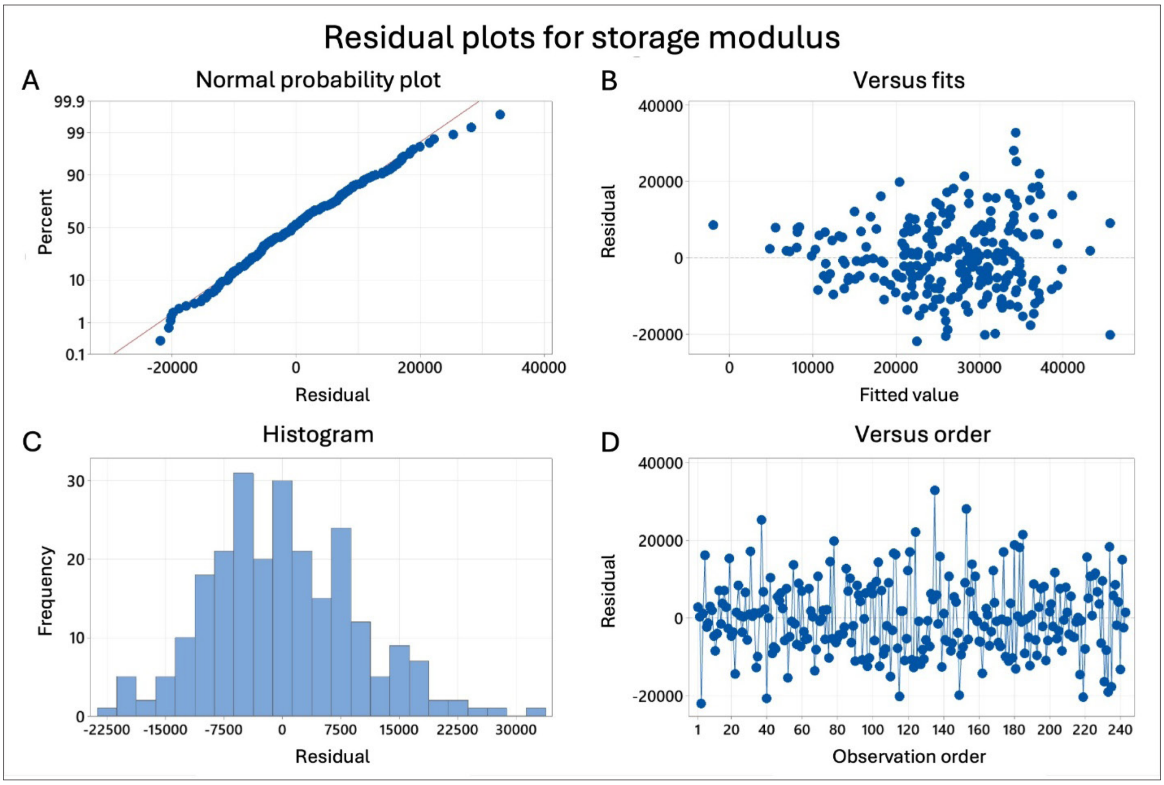


Figure S2. Adjustment of the empirical data to a full quadratic equation model with residual plots. The data distribution was evaluated through their residuals and plotted in the form of (A) a normal probability plot, (B) residuals versus fits, (C) histogram, and (D) residuals versus observation.

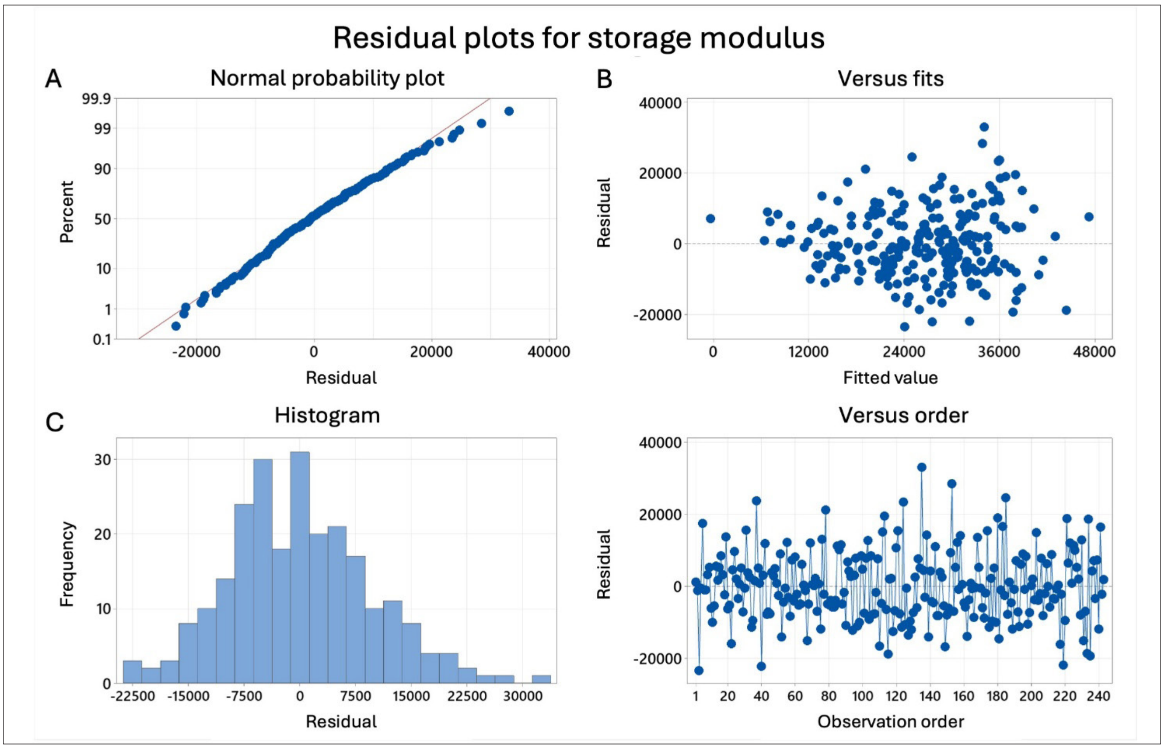


Figure S3. Adjustment of the empirical data to a linear equation model with term interaction. The data distribution was evaluated through their residuals and plotted in the form of (A) a normal probability plot, (B) residuals versus fits, (C) histogram, and (D) residuals versus observation.

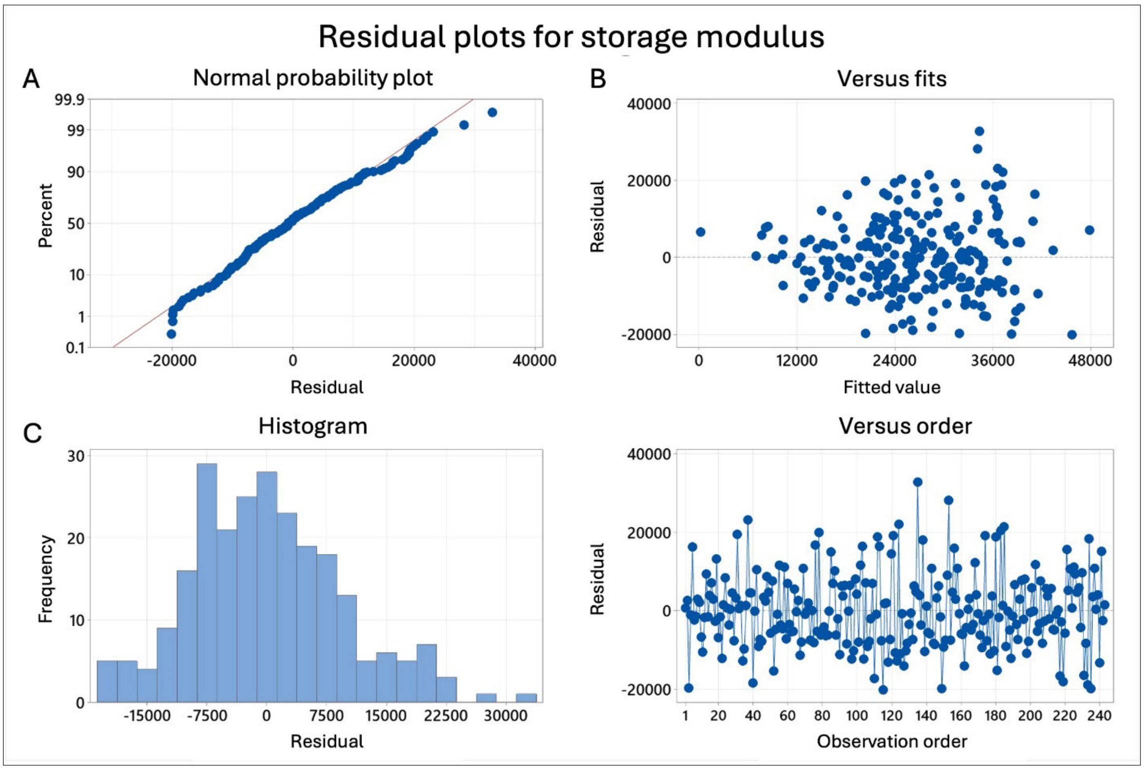


Figure S4. Adjustment of the empirical data to a linear equation model with squared terms. The data distribution was evaluated through their residuals and plotted in the form of (A) a normal probability plot, (B)residuals versus fits, (C) histogram, and (D) residuals versus observation.

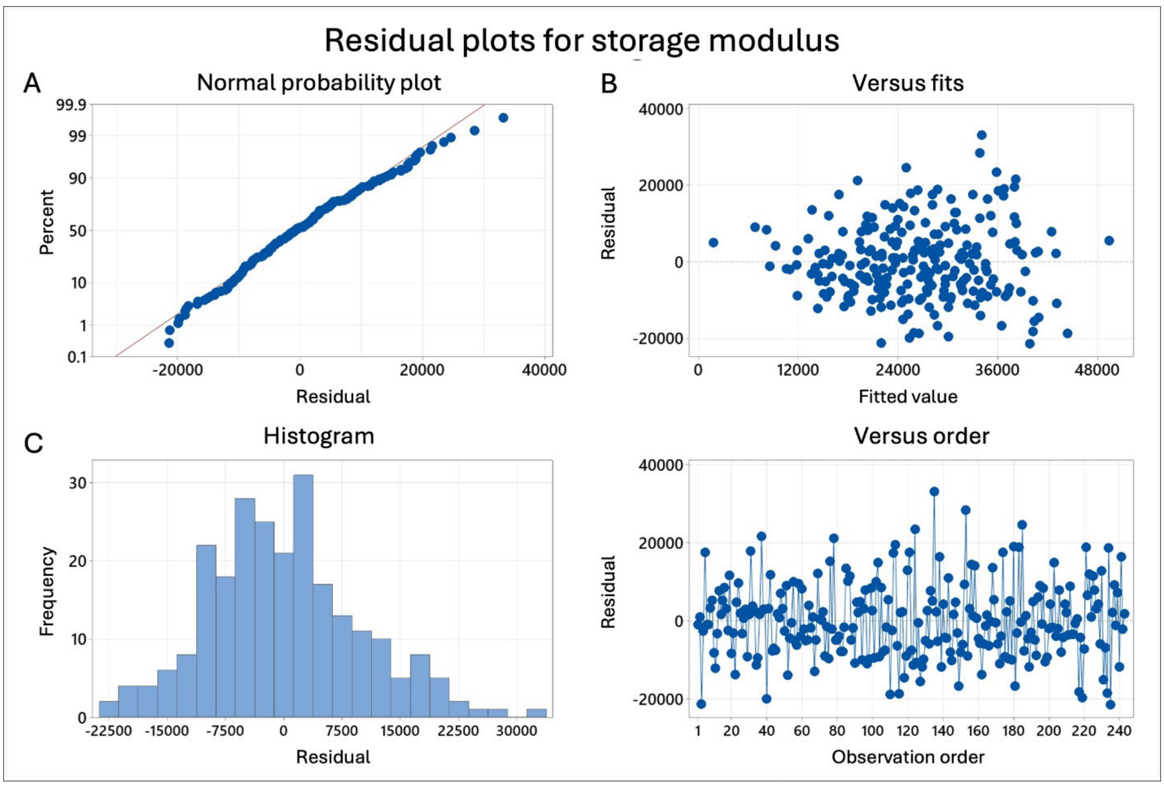


Figure S5. Adjustment of the empirical data to a linear equation model. The data distribution was evaluated through their residuals and plotted in the form of (A) a normal probability plot, (B) residuals versus fits, (C) histogram, and (D) residuals versus observation.

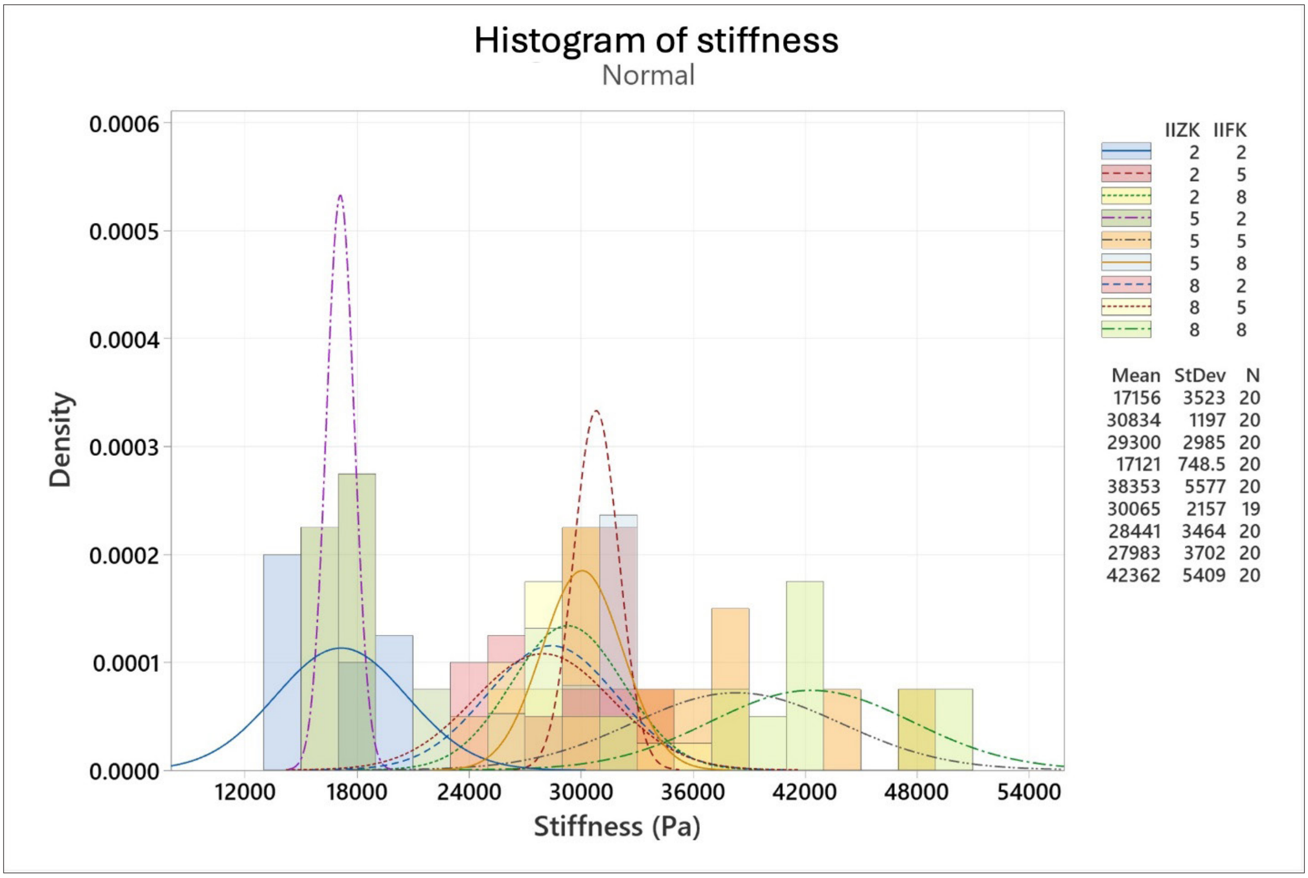


Figure S6. Normal distribution plot for rheology storage modulus data.

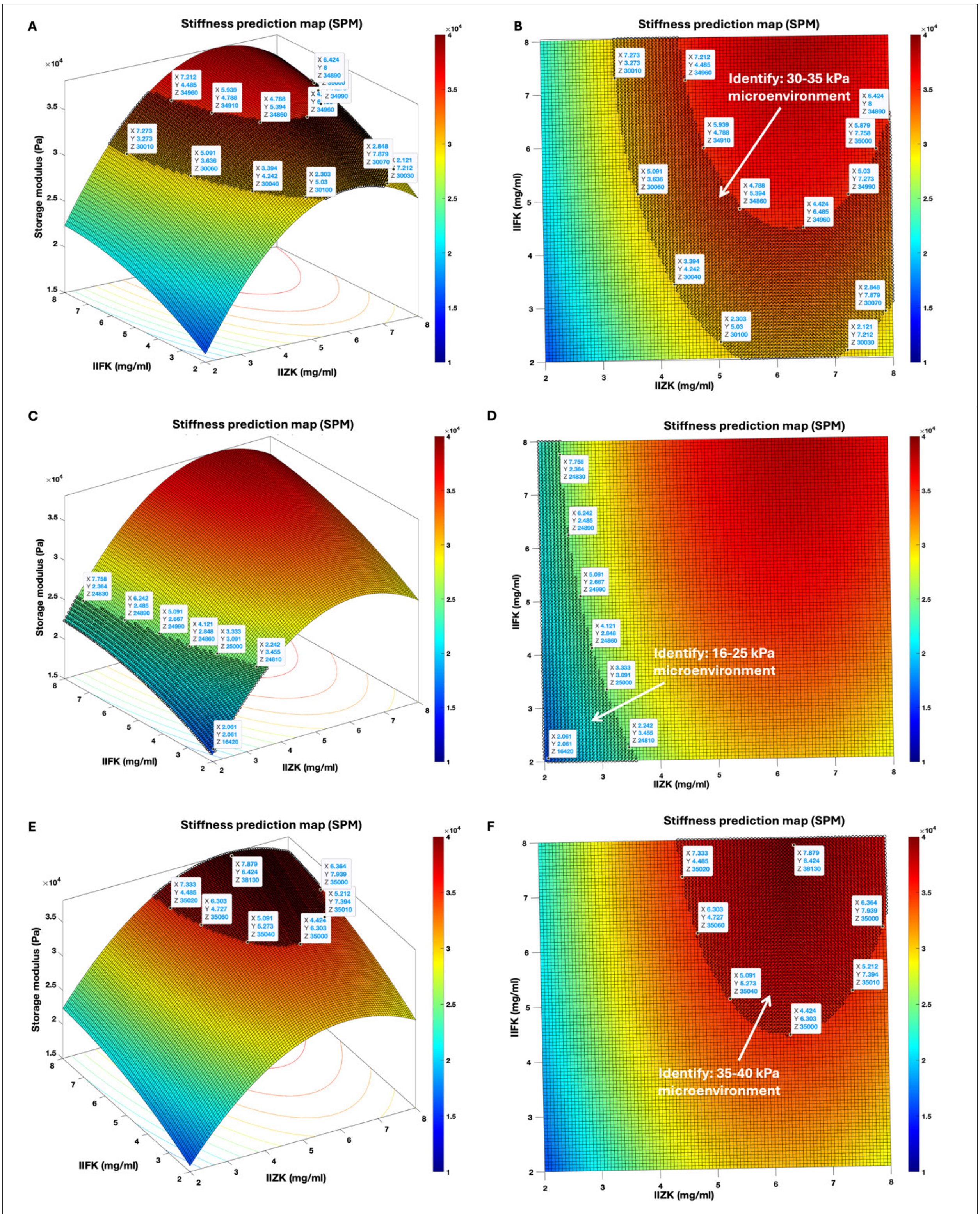


Figure S7. Stiffness peptide map to identify specific stiffnesses for the scaffold design. The map was used to formulate distinct stiffnesses through mixtures of peptide solutions between peptides IIFK and IIZK, where (A & B) stiffness of 30–35 kPa corresponds to a cartilage-like microenvironment, (C & D) stiffness of 16–25 kPa corresponds to a softer than cartilage microenvironment, and (E & F) stiffness of 35–40 kPa corresponds to a stiffer than cartilage microenvironment.

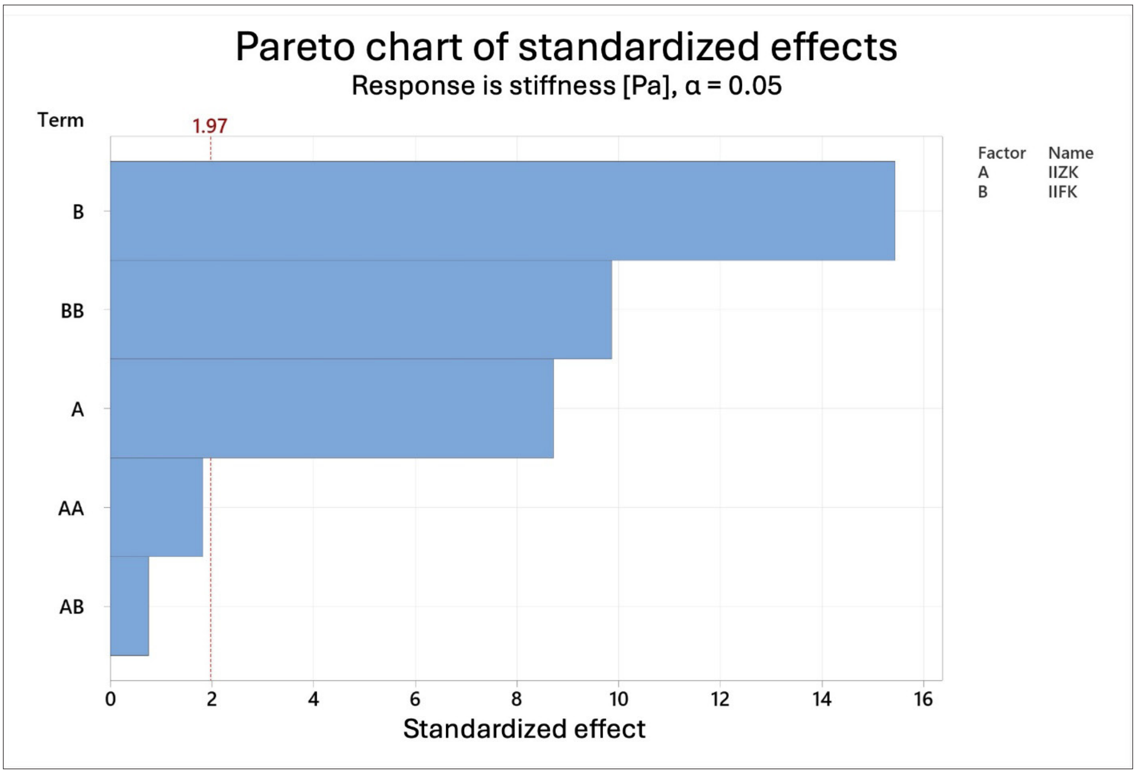


Figure S8. Pareto chart for the standardized effects of the single-peptide and mixed peptide bioinks composed of IIZK and IIFK for response surface methodology modeling. The Pareto chart identifies if the contribution of a peptide (e.g., IIZK or IIFK) is significant to predict the resulting stiffness from the mixed peptide bioink.

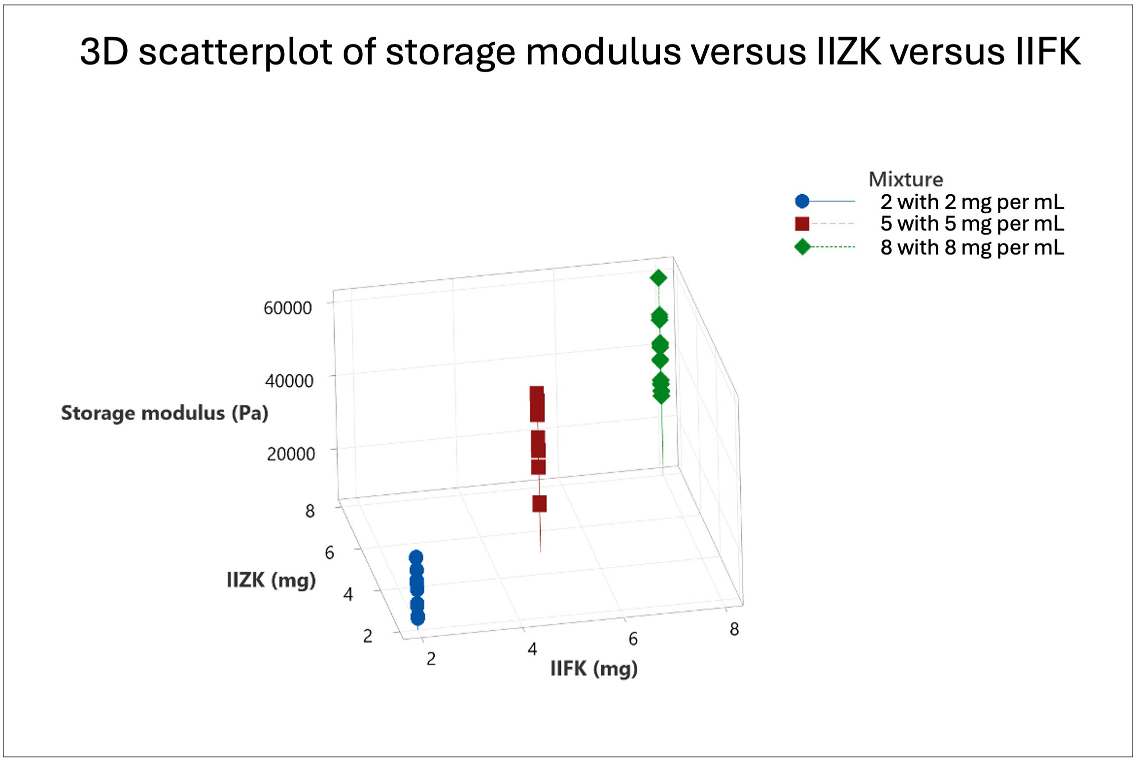


Figure S9. The effects of mixed peptide concentrations on scaffold stiffness.

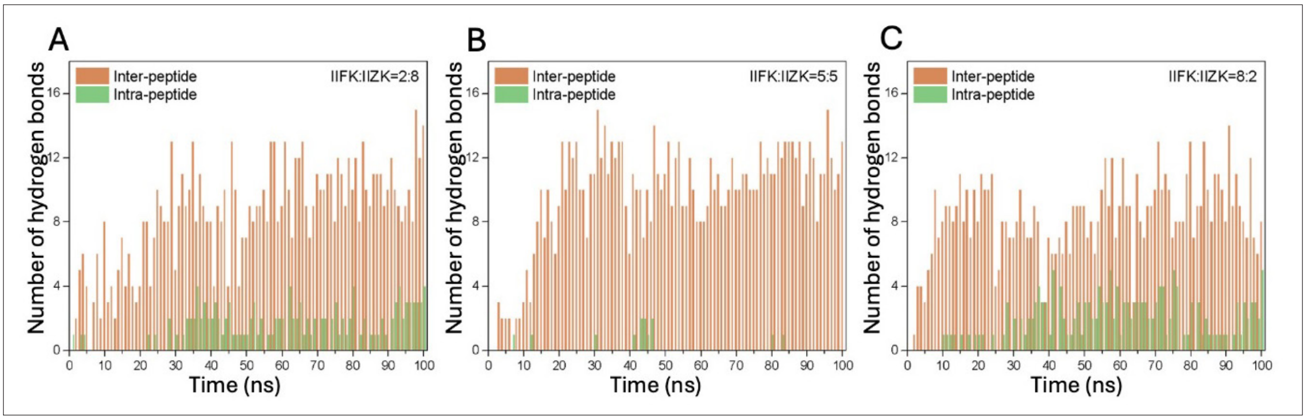


Figure S10. Hydrogen bond analysis during the self-assembly simulations using higher amounts of peptide molecules (IIZK and IIFK): (A) 2:8 IIFK:IIZK, (B) 5:5 IIFK:IIZK, and (C) 8:2 IIFK:IIZK.

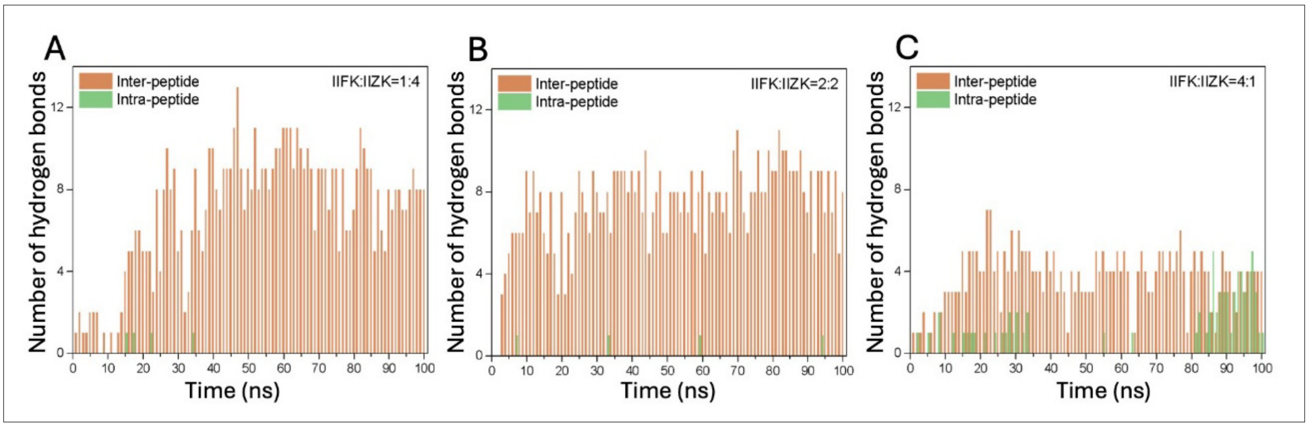


Figure S11. Hydrogen bond analysis during the self-assembly simulations at lower amounts of peptide molecules (IIZK and IIFK): (A) 1:4 IIFK:IIZK, (B) 2:2 IIFK:IIZK, and (C) 4:1 IIFK:IIZK.

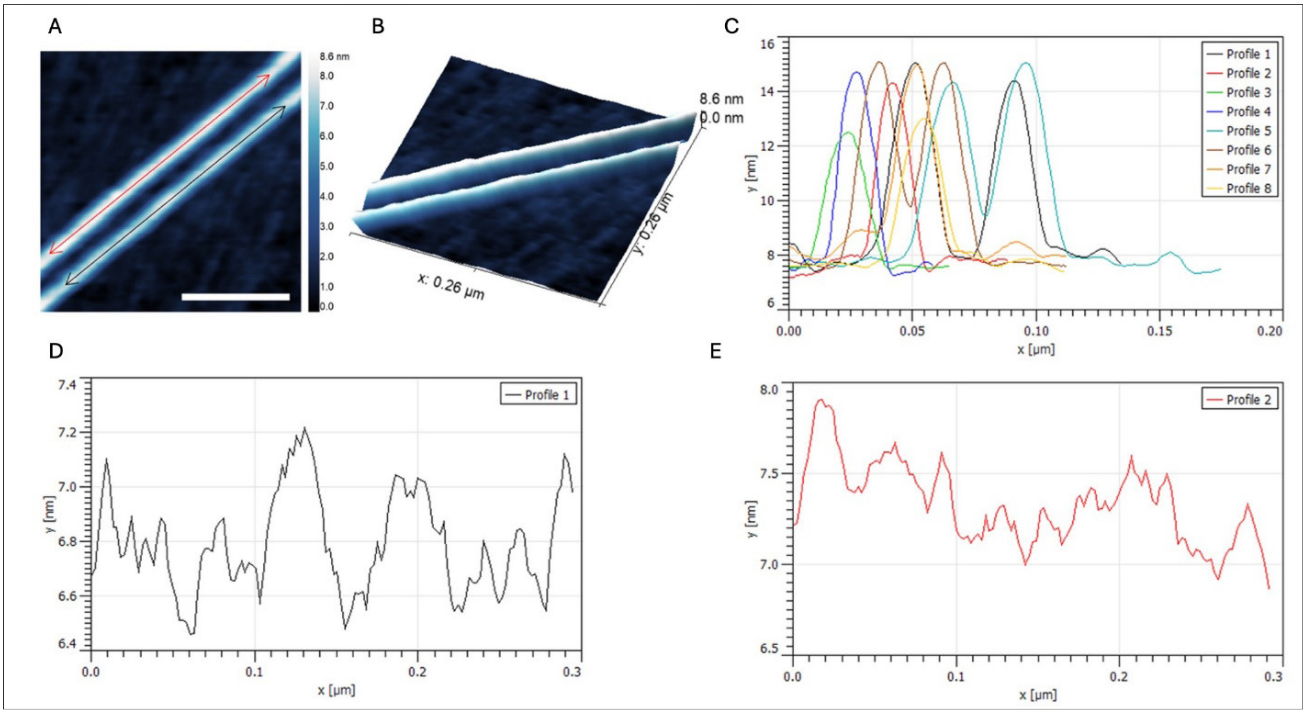


Figure S12. Atomic force microscopy (AFM) topography and profiles analysis of 3:1 IIZK:IIFK bioinks. (A) Magnification of AFM height image from **Figure 3H** (left, red area) with two longitudinal profiles indicated by a line (scale bar: 100 nm). (B) Image in **Panel A** rendered in 3D to appreciate the absence of helical pitch. (C) Exemplification of orthogonal profiles extracted from the AFM topography height images. (D) Longitudinal fiber Profile 1, as indicated by the black line in **Panel A**. (E) Longitudinal fiber Profile 2, as indicated in **Panel A**.

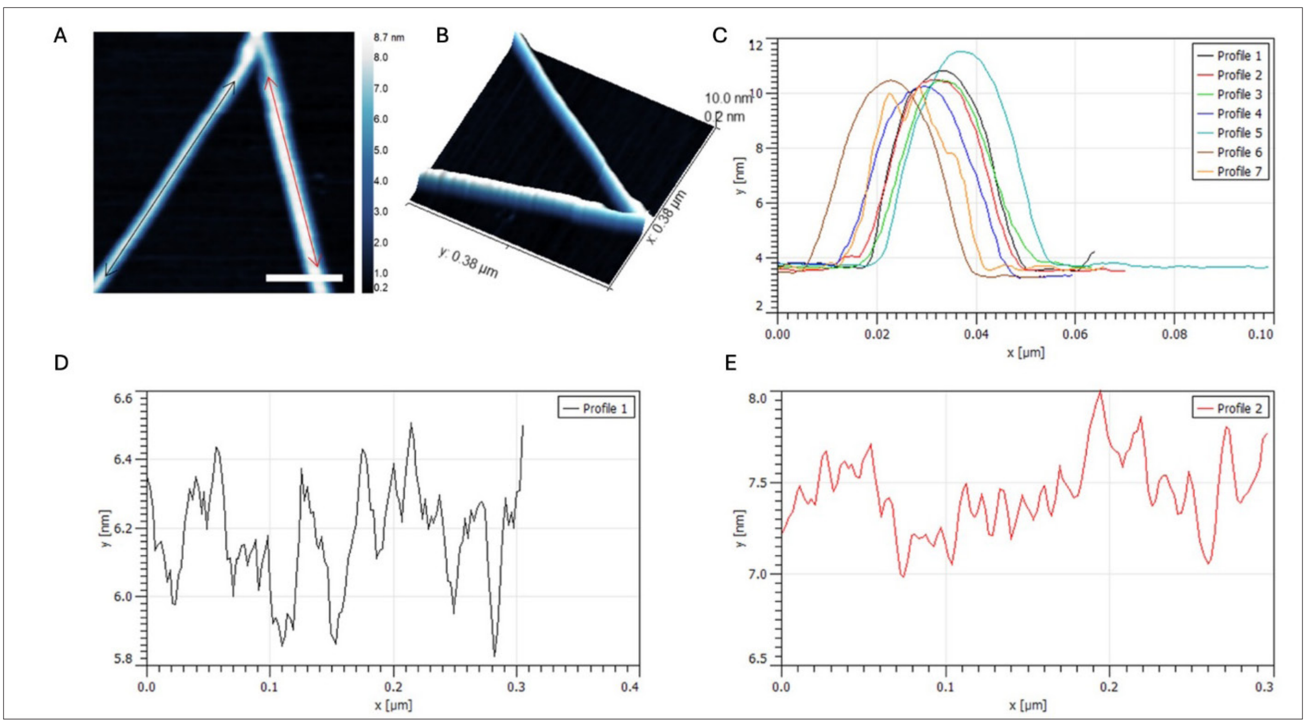


Figure S13. Atomic force microscopy (AFM) topography and profiles analysis of 1:1 IIZK:IIFK bioinks. (A) Magnification of AFM height image from **Figure 3H** (center, red area) with two longitudinal profiles indicated by a line (scale bar: 100 nm). (B) Image in **Panel A** rendered in 3D to appreciate the absence of helical pitch. (C) Exemplification of orthogonal profiles extracted from the AFM topography height images. (D) Longitudinal fiber Profile 1, as indicated by the black line in **Panel A**. (E) Longitudinal fiber Profile 2, as indicated in **Panel A**.

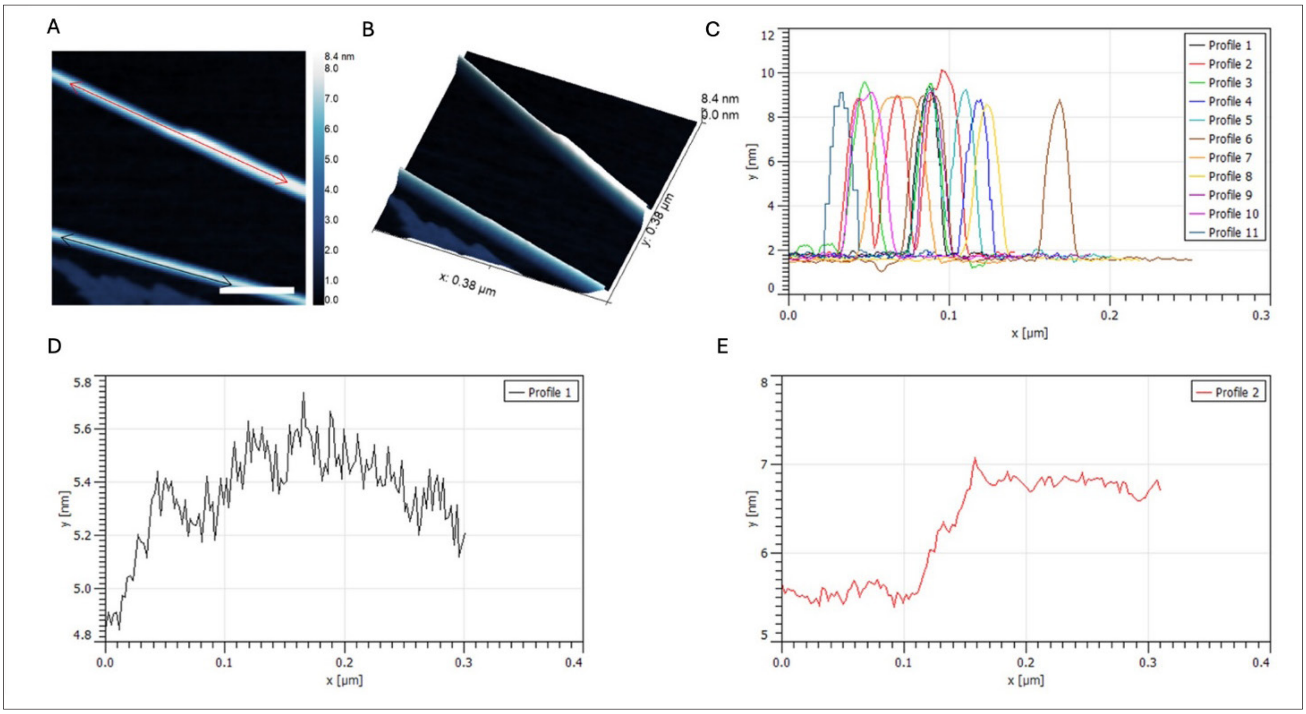


Figure S14. Atomic force microscopy (AFM) topography and profiles analysis of 3:1 IIFK:IIZK bioinks. (A) Magnification of AFM height image from **Figure 3H** (right, red area) with two longitudinal profiles indicated by a line (scale bar: 100 nm). (B) Image in **Panel A** rendered in 3D to appreciate the absence of helical pitch. (C) Exemplification of orthogonal profiles extracted from the AFM topography height images. (D) Longitudinal fiber Profile 1, as indicated by the black line in **Panel A**. (E) Longitudinal fiber Profile 2, as indicated in **Panel A**.

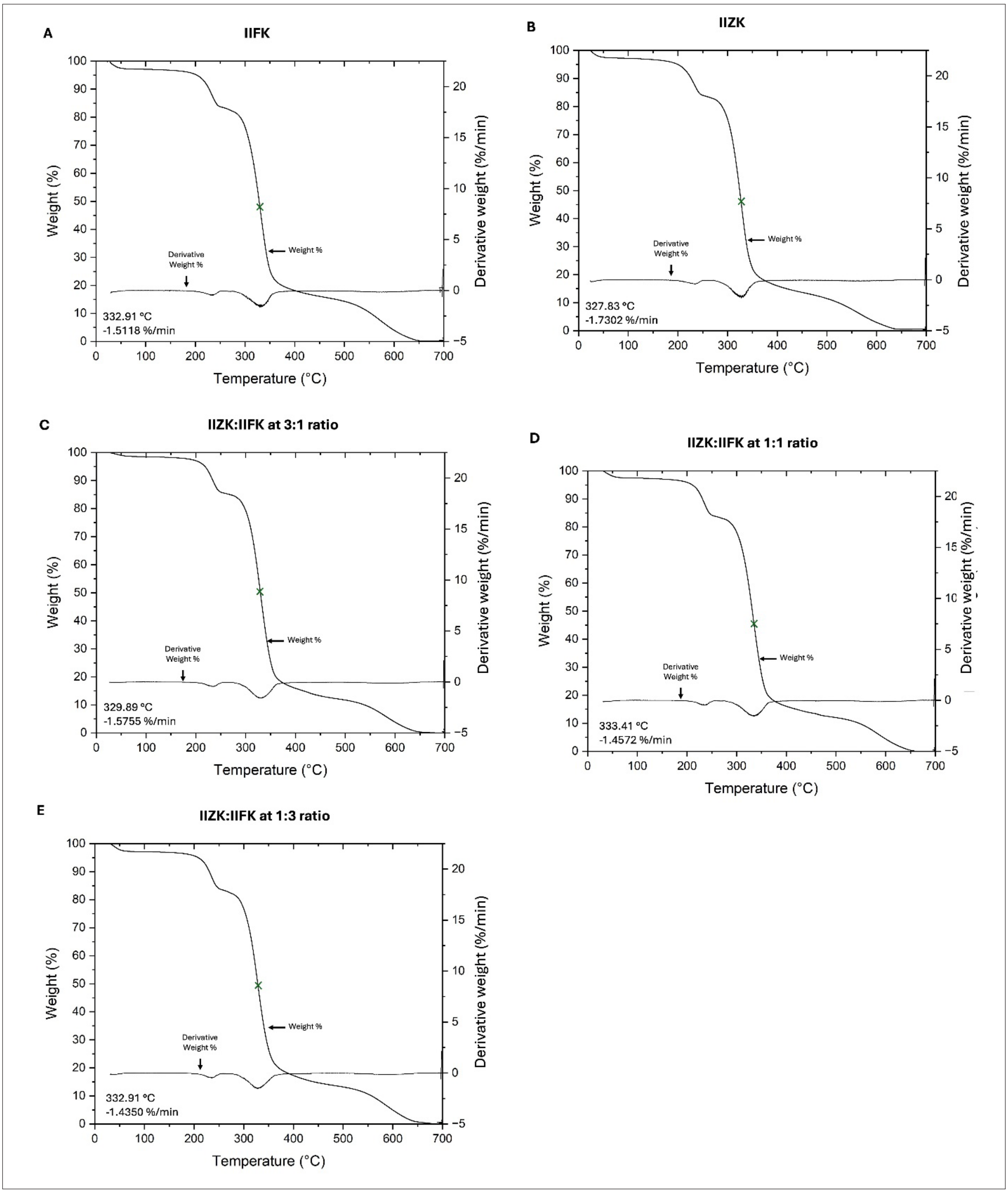


Figure S15. Thermogravimetric analyses of peptide bioink formulations with varying peptide ratios: (A) pure IIFK, (B) pure IIZK, (C) 3:1 IIZK:IIFK, (D) 1:1 IIZK:IIFK, and (E) 1:3 IIZK:IIFK.

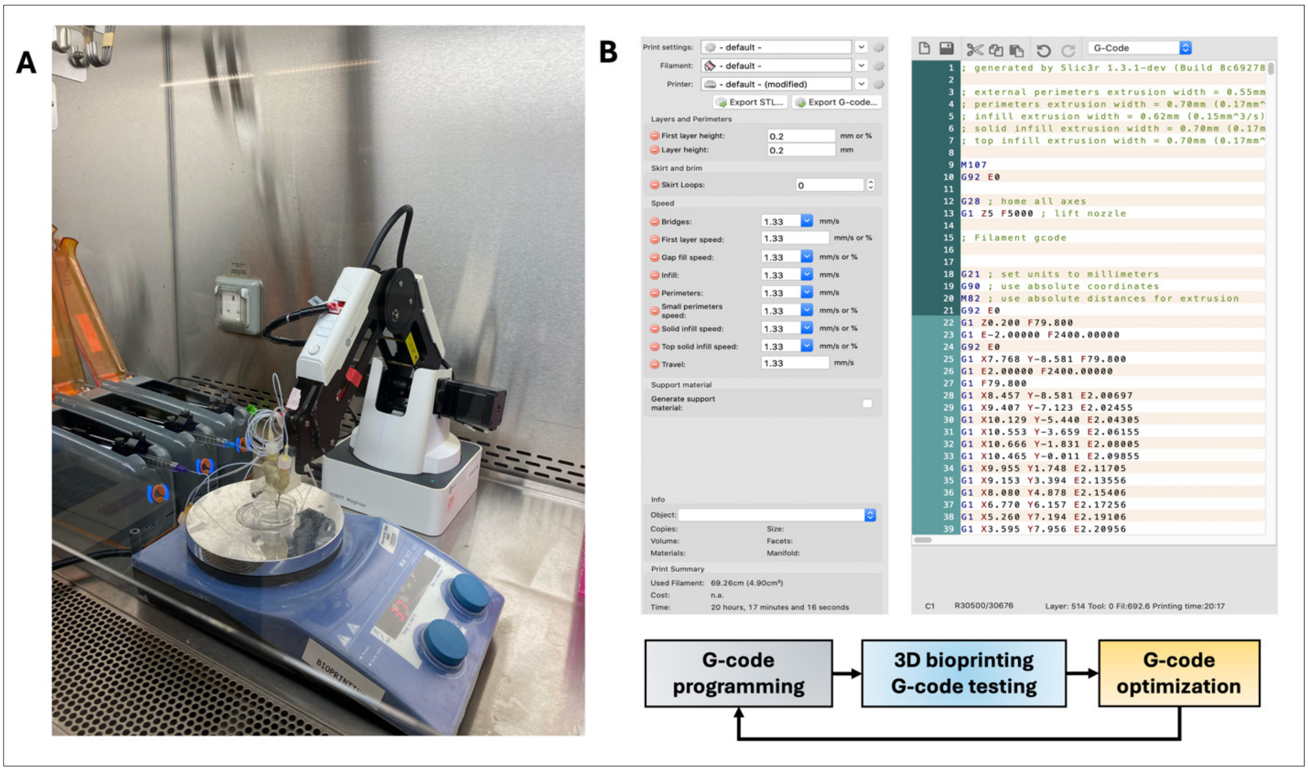


Figure S16. Robotic arm-assisted 3D bioprinting system for the fabrication of a trachea-like model. (A) 3D bioprinter system. (B) Flowchart for G-code optimization for printing.

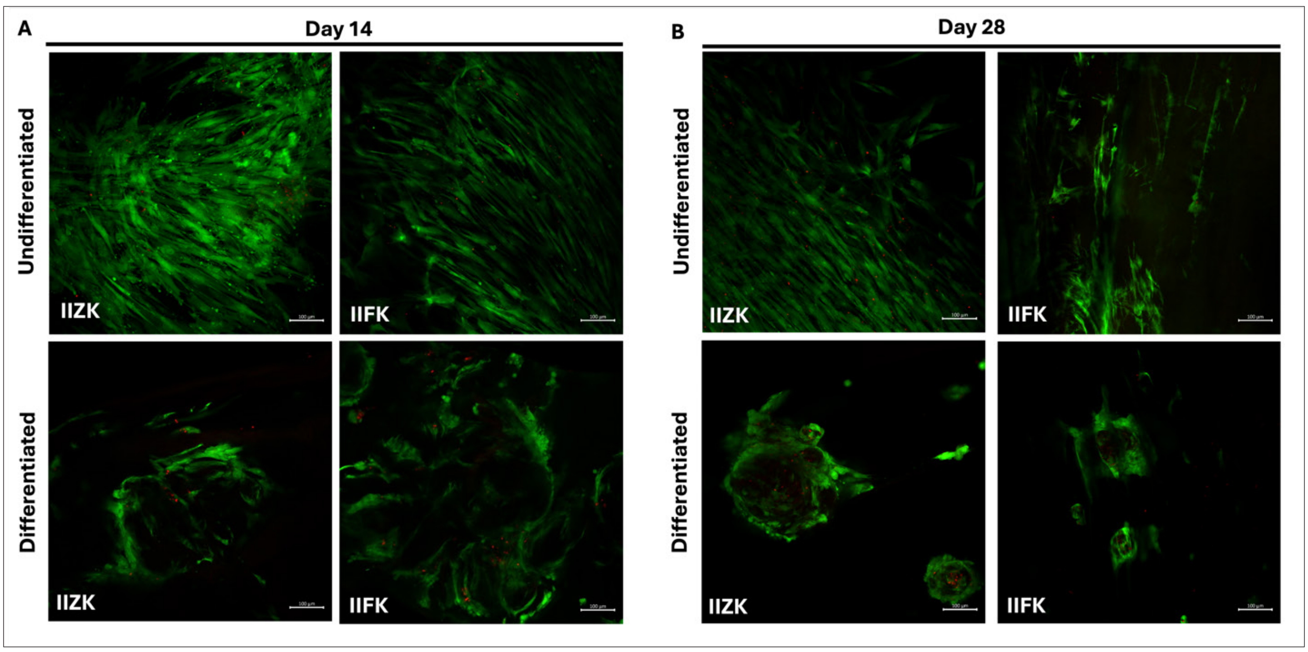


Figure S17. The individual IIZK or IIFK peptide is used as a scaffold for the fabrication of 3D bioprinted trachea-like constructs. Viability assessment of the 3D bioprinted cells is shown on (A) day 14 and (B) day 28. The undifferentiated cells refer to bone marrow-derived mesenchymal stem cells, while the differentiated cells refer to chondrocytes. Scale bars: 100 μm; magnifications: 20×.

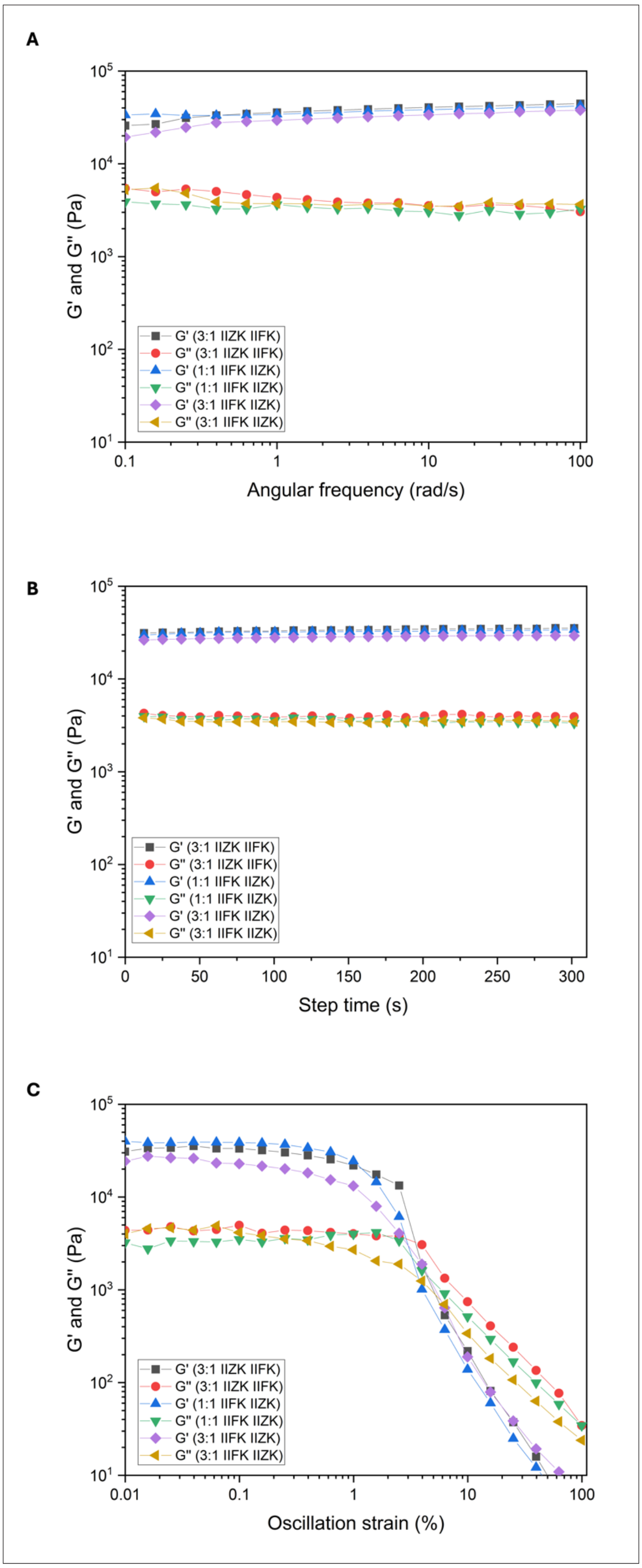


Figure S18. Viscoelastic properties of the formulated bioinks. The storage modulus G' and loss modulus G" are shown for sweeps of (A) angular frequency, (B) step time, and (C) oscillation time.

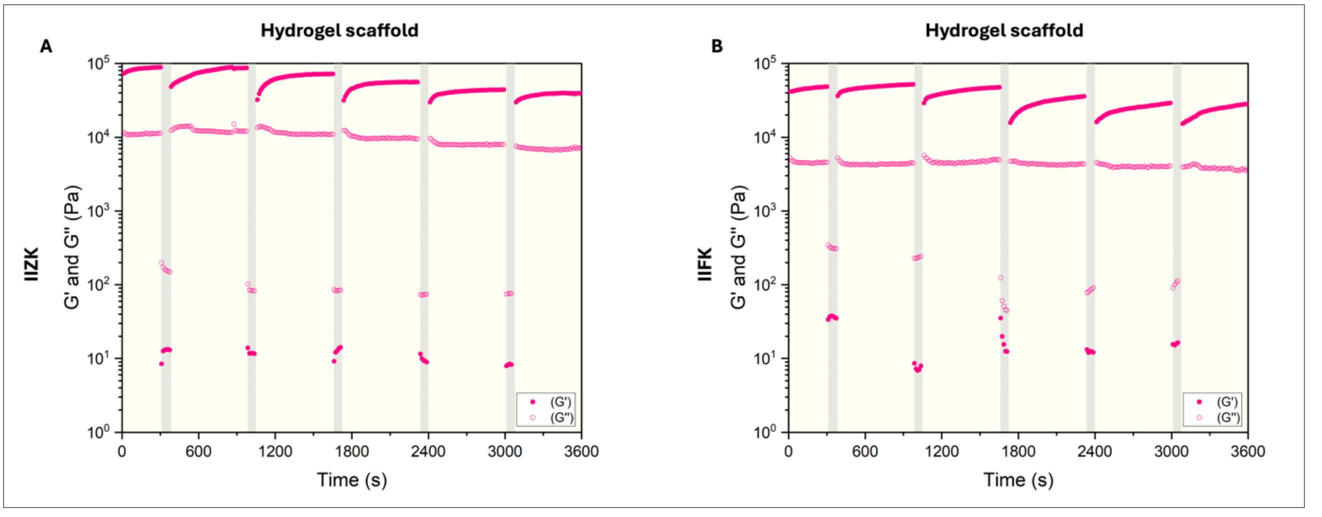


Figure S19. Thixotropic behaviors of individual peptide (A) IIZK and (B) IIFK over 60 min (3600 s).

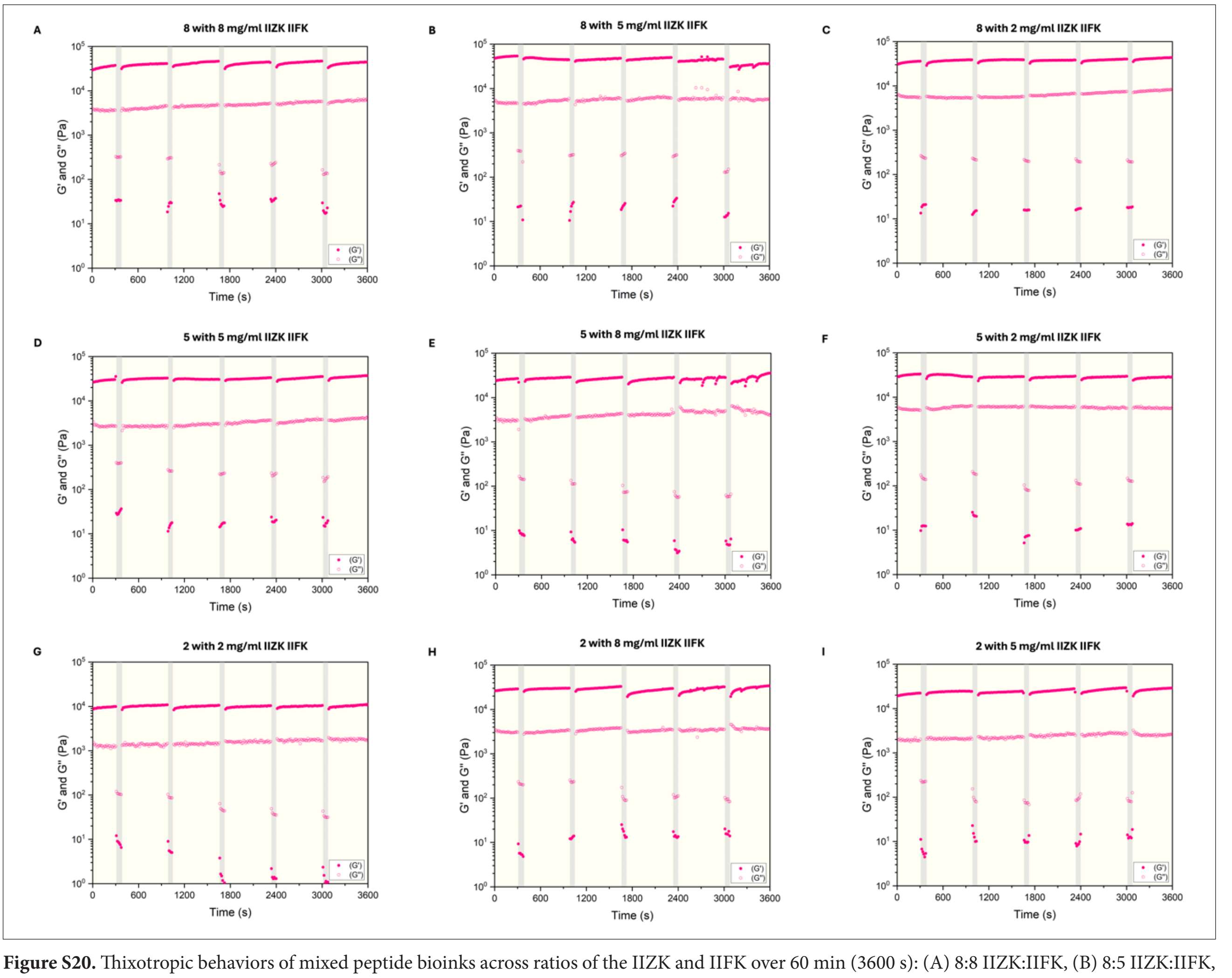
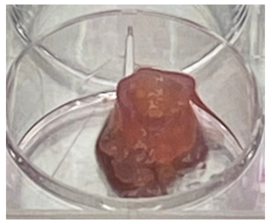
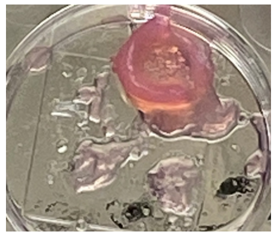
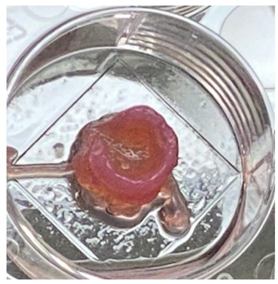
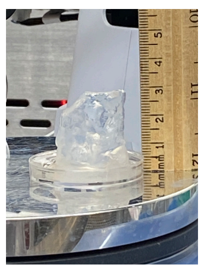
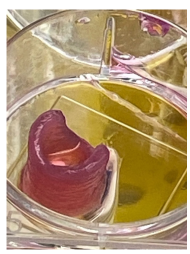


Figure S20. Thixotropic behaviors of mixed peptide bioinks across ratios of the IIZK and IIFK over 60 min (3600 s): (A) 8:8 IIZK:IIFK, (B) 8:5 IIZK:IIFK, (C) 8:2 IIZK:IIFK, (D) 5:5 IIZK:IIFK, (E) 5:8 IIZK:IIFK, (F) 5:2 IIZK:IIFK, (G) 2:2 IIZK:IIFK, (H) 2:8 IIZK:IIFK, and (I) 2:5 IIZK:IIFK.

Table S1. 3D bioprinted trachea-like structures using the formulated mixed peptide bioinks

Formulation	Dimension (cm)	Construct
1:3 IIZK:IIFK	H: 1.4 D: 0.5	
	H: 1.2 D: 0.5	
1:1 IIZK:IIFK	H: 1.3 D: 0.5	
	H: 1.4 D: 0.5	
3:1 IIZK:IIFK	H: 3.1 D: 2.2	 
	H: 1.5 D: 0.5	

Notes: H refers to height; D refers to diameter.

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Table S2. Statistical model of the response surface

Model	Equation
Full factorial	$\gamma = \delta_0 \pm \delta_1\alpha \pm \delta_2\beta \pm \delta_3\alpha^2 \pm \delta_4\beta^2 \pm \delta_5\alpha\beta + \varepsilon$
Linear with interactions	$\gamma = \delta_0 \pm \delta_1\alpha \pm \delta_2\beta \pm \delta_5\alpha\beta + \varepsilon$
Linear with squared terms	$\gamma = \delta_0 \pm \delta_1\alpha \pm \delta_2\beta \pm \delta_3\alpha^2 \pm \delta_4\beta^2 + \varepsilon$
Linear	$\gamma = \delta_0 \pm \delta_1\alpha \pm \delta_2\beta + \varepsilon$

Note: In the equations, γ represents the storage modulus (Pa), δ_0, δ_1, δ_2, δ_3, δ_4, and δ_5 are the obtained constants from the statistical equation from the fit of the experimental data, α represents peptide 1 (factor 1 in mg/mL), β represents peptide 2 (factor 2 in mg/mL), and ε represents the total error from the model.

Table S3. Parameters of the response surface equations

Model	S	R^2	R^2 (adjusted)	R^2 (predicted)
Full factorial	4654.09	71.37	70.42	69.05
Linear with interactions	5620.77	58.01	56.86	55.30
Linear with squared terms	4648.62	71.28	70.49	69.30
Linear	5729.30	55.89	55.18	54.18

Table S4. Coded coefficients of the stiffness peptide map model from the design of experiments

Term	Coefficient	SE coefficient	*t*-value	*p*-value	VIF
Constant	34,417	813	42.34	<0.001	–
Blocks: 1	3510	381	9.21	<0.001	1.25
IIZK	3617	415	8.72	<0.001	1.00
IIFK	6402	415	15.44	<0.001	1.00
IIZK×IIZK	−1388	762	−1.82	0.070	1.12
IIFK×IIFK	−7512	762	−9.86	<0.001	1.13
IIZK×IIFK	383	508	0.76	0.451	1.00

Abbreviation:VIF, variance inflation factor.

Table S5. Analysis of variance of the stiffness peptide map

Source	DF	Adjusted SS	Adjusted MS	*F*-value	*p*-value
Model	6	9,825,053,097	1,637,508,850	75.60	<0.001
Blocks	1	1,839,324,583	1,839,324,583	84.92	<0.001
Linear	2	6,811,962,102	3,405,981,051	157.24	<0.001
IIZK	1	1,648,073,047	1,648,073,047	76.09	<0.001
IIFK	1	5,163,889,055	5,163,889,055	238.40	<0.001
Square	2	2,118,048,102	1,059,024,051	48.89	<0.001
IIZK×IIZK	1	71,961,636	71,961,636	3.32	0.070
IIFK×IIFK	1	2,106,457,290	2,106,457,290	97.25	<0.001
2-Way interaction	1	12,348,010	12,348,010	0.57	0.451
IIZK×IIFK	1	12,348,010	12,348,010	0.57	0.451
Error	182	3,942,213,715	21,660,515		
Lack-of-fit	2	1,468,047,226	734,023,613	53.40	<0.001
Pure error	180	2,474,166,489	13,745,369	Pure error	180
Total	188	13,767,266,812			

Abbreviations: DF, degree of freedom; MS, mean square; SS, sum of squares.

Table S6. Mean absolute deviation test to identify outliers

Mixed peptide bioinks	Median	Mean absolute deviation	Minimum threshold limit	Maximum threshold limit
2 mg IIZK with 2 mg IIFK	−0.24086	0.83208	−2.7371	2.25538
2 mg IIZK with 8 mg IIFK	−0.05869	0.78174	−2.4039	2.28653
5 mg IIZK with 5 mg IIFK	−0.09868	0.75222	−2.3553	2.15798
8 mg IIZK with 2 mg IIFK	−0.18787	0.80515	−2.6033	2.22758
8 mg IIZK with 8 mg IIFK	−0.34282	0.80856	−2.7685	2.08286
2 mg IIZK with 5 mg IIFK	−0.23819	0.88470	−2.4159	2.89229
5 mg IIZK with 2 mg IIFK	−0.36740	0.76816	−2.6719	1.93708
5 mg IIZK with 8 mg IIFK	−0.15409	0.73460	−2.3579	2.04971
8 mg IIZK with 5 mg IIFK	−0.36078	0.72165	−2.5257	1.80417

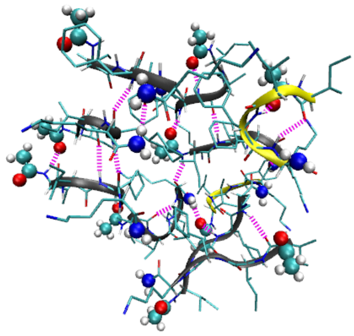
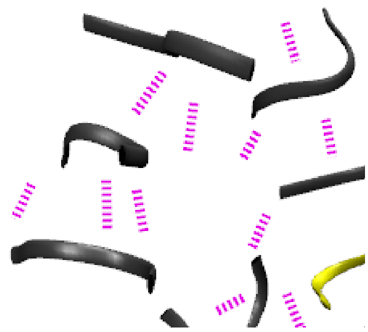
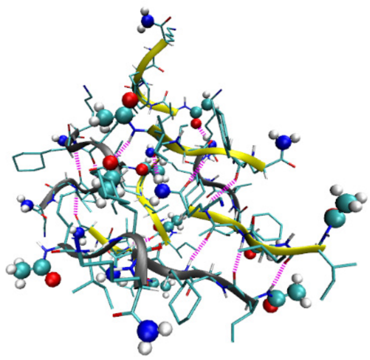
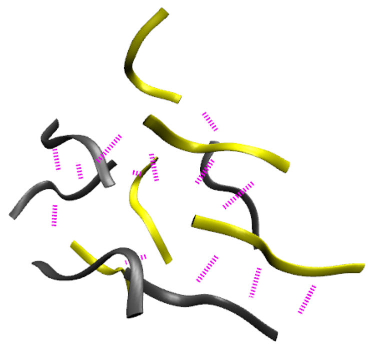
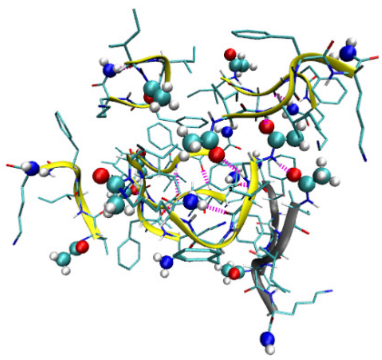
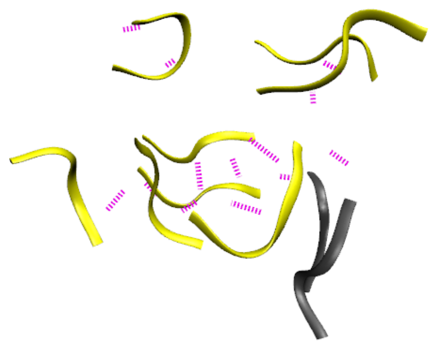
Table S7. The predicted values of stiffness versus the empirical stiffness of the stiffness peptide map

IIFK (mg)	IIZK (mg)	Predicted (Pa)	Measured (Pa)	Difference (%)
2.909	2.909	23,300	19,590.99	15.92
2.909	7.091	31,850	30,670.23	3.70
7.091	2.909	27,960	30,405.76	8.75
7.030	7.030	37,290	42,715.73	14.55

Table S8. Peptide simulation parameters

Peptide	Experimental concentration (mg/mL)			Ratio in simulation								
IIFK	2	5	8	1	2	4	2	5	8	12	30	48
IIZK	8	5	2	4	2	1	8	5	2	48	30	12

Table S9. Final assembled structures at the end of a 100 ns trajectory

IIFK:IIZK ratio	Simulation	
1:4		
2:2		
4:1		

Notes: Yellow represents IIFK; black represents IIZK.

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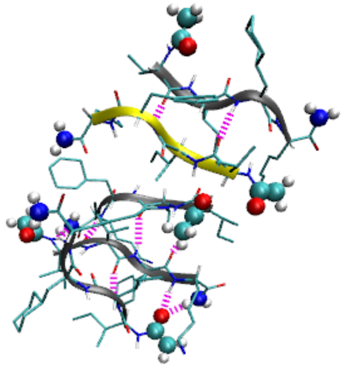
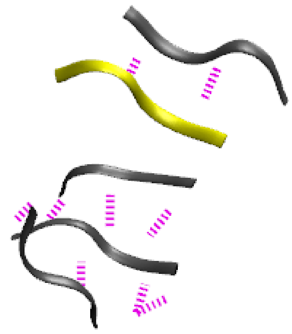
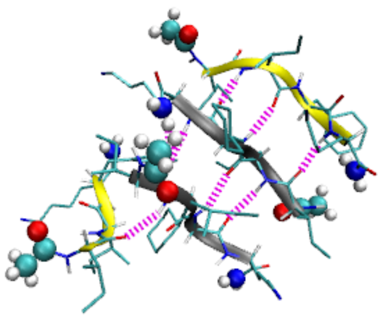
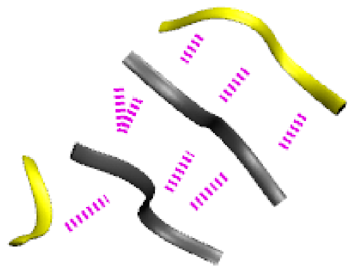
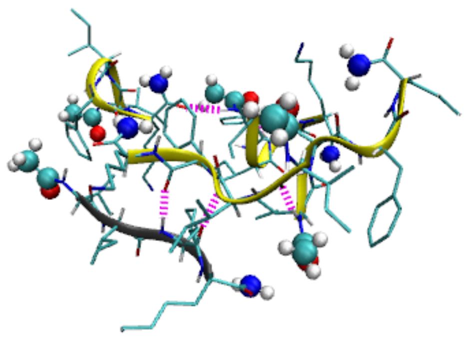
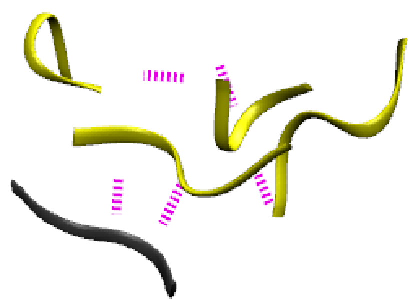
Table S10. Final assembled structure at the end of a 100 ns trajectory with higher ratios

IIFK:IIZK ratio	Simulation	
2:8		
5:5		
8:2		

Notes: Yellow represents IIFK; black represents IIZK.

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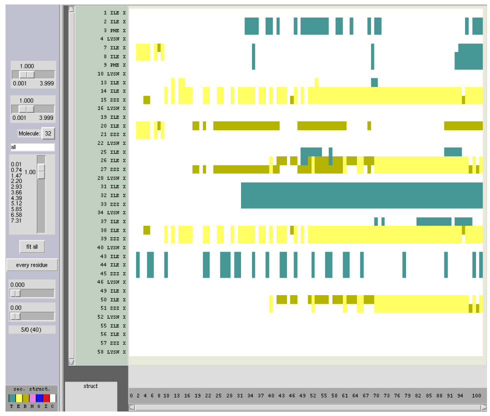
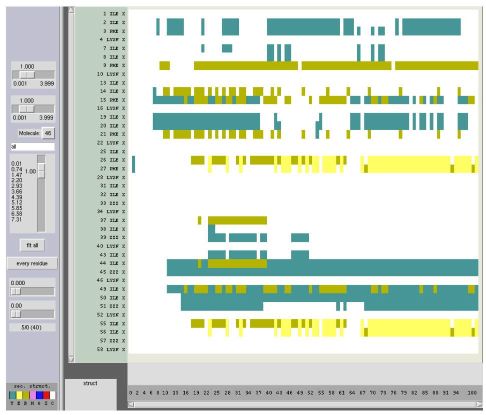
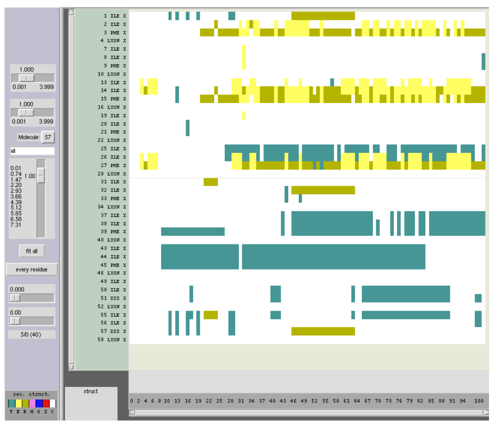
Table S11. Peptides' secondary structure during the trajectory at higher amounts of peptide molecules (IIZK and IIFK)

IIFK:IIZK ratio	Secondary structure
2:8	
5:5	
8:2	

Note: Color key for secondary structure plots. Visual Molecular Dynamics (VMD) https://www.ks.uiuc.edu/Training/Tutorials/science/timeline/tutorial_timeline-html/node4.html)

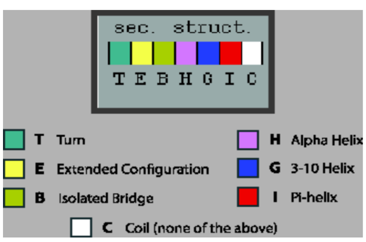
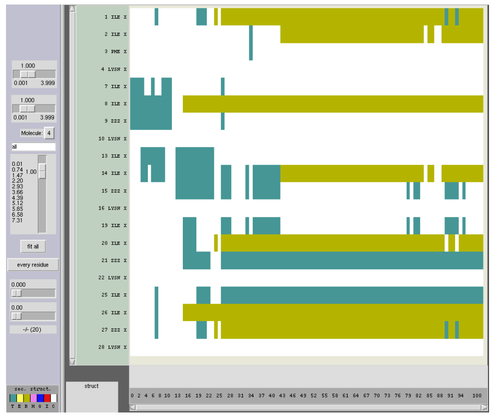
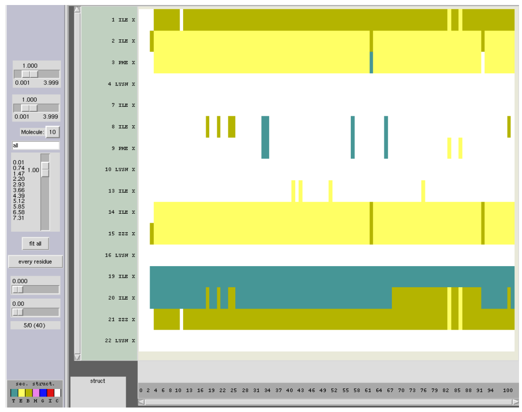
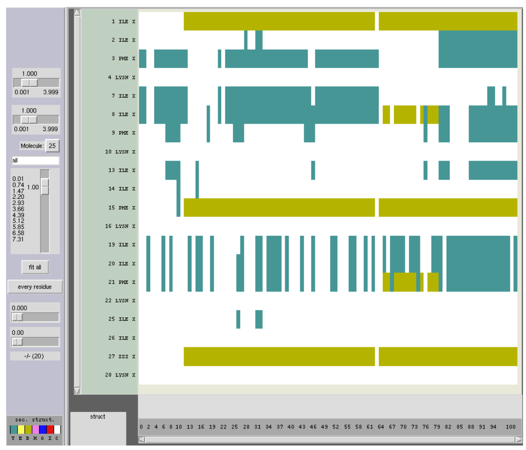


Table S12. Peptides' secondary structure during the trajectory at lower amounts of peptide molecules (IIZK and IIFK)

IIFK:IIZK ratio	Secondary structure
1:4	
2:2	
4:1	

Note: Visual Molecular Dynamics (VMD) https://www.ks.uiuc.edu/Training/Tutorials/science/timeline/tutorial_timeline-html/node4.html

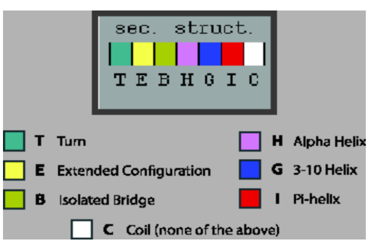
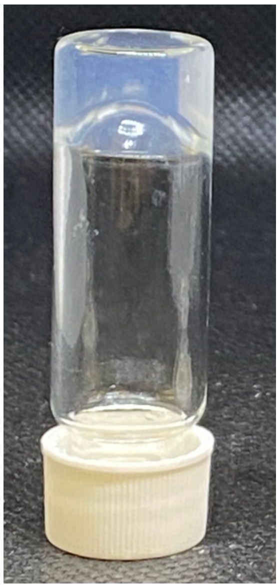


Table S13. Overview of gelation and solubility across mixed peptide bioinks

Bioink	**1**	**2**	**3**
Concentration (w/v) ratio	3:1 IIZK:IIFK	1:1 IIZK:IIFK	3:1 IIFK:IIZK
Gelation speed with PBS (min)	<1	<2	<2.5
Solubility in water	Low	High	High
Results			

Abbreviation: PBS, phosphate-buffered saline.

Supplementary videos

Video S1. 3D bioprinting of a trachea model structure.

Video S2. 3D bioprinted tracheal-like model elasticity (part 1). The trachea model's elasticity was evaluated after 28 days of culture.

Video S3. 3D bioprinted tracheal-like model elasticity (part 2). The trachea model's elasticity was evaluated after 100 days of culture.

Video S4. 3D bioprinted tracheal-like structure printed at large-scale dimensions.

References

1. Visual Molecular Dynamics (VMD). https://www.ks.uiuc.edu/Training/Tutorials/science/timeline/tutorial_timeline-html/node4.html