Supporting Information for

A 3D-Bioprinted Hydrogel Platform with Tunable Matrix Stiffness Reveals Mechanical Adaptation and Doxorubicin Resistance in Triple-Negative Breast Cancer

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Experimental methods:

1.1 Print fidelity ratio

The designed angles for each of the eight circular sectors in the STL file (Figure S1) were used as reference values to determine the print fidelity ratio. The actual angles of each sector in the printed scaffolds were then measured using ImageJ software. The print fidelity ratio was calculated for each sector by dividing the actual angle by the corresponding designed angle, allowing a quantitative assessment of how closely the printed scaffolds matched the original design using the following equation.

$$Print\ fidelity\ ratio = \frac{Actual\ angle}{Designed\ angle} \times \ 100$$

1.2 Immunofluorescence staining for Ki-67

3D-bioprinted scaffolds were first fixed in 3.7% formalin solution for 30 min, then washed thrice with PBS. The scaffolds were permeabilized by incubating in 0.1% Triton X-100 in PBS for 30 min under gentle rocking, followed by three additional PBS washes. To block non-specific binding, scaffolds were incubated with 1% BSA in PBS for 1 h. After blocking, a 1:500 dilution of anti-Ki-67 antibody (Thermo, specific for human) was added to each scaffold and incubated at room temperature with rocking for 3 h. After three washes with PBS containing 0.1% Triton-X (PBST), scaffolds were incubated with Alexa Fluor 488-conjugated secondary antibody diluted in 1% BSA in PBS for 1 h under rocking. After incubation, scaffolds were washed thrice with 1× PBST. Nuclear staining was then performed by adding 2 µg/ml Hoechst 33342 and incubating for 30 min at 25 °C with rocking. Finally, the scaffolds were washed twice with PBS before proceeding to fluorescence imaging.

1.3 Release study of BSA from printed scaffolds

To investigate molecular diffusion constraints in bioprinted constructs, cylindrical scaffolds (10.4 mm diameter, 1.2 mm thickness) were fabricated via 3D printing using G7.5 and G12.5 bioink formulations, each supplemented with 70 mg/mL BSA. Individual scaffolds were immersed in 1 mL phosphate-buffered saline (PBS, pH 7.4) and incubated at 37 °C. At defined intervals within 12 h, 120 µL aliquots of the release medium (V*t*) were collected for BSA quantification by UV–vis spectrophotometry (Implen GmbH) at 280 nm wavelength [1]. The cumulative amount of BSA released at each time point (M_t) was determined using Equation S1:

$$M_t = C_t V + \sum C_{t-1} V_t \quad \text{(S1)}$$

Where C_t is the BSA concentration at time t, V is the total volume of release medium (1000 µL), and V_t corresponds to the volume (120 µL) sampled for analysis [2]. The experiment was performed in triplicate. The initial BSA content loaded into each scaffold (M_O) was used to calculate the release ratio at each time point, as per Equation S2.

$$Release\ ratio\ \% = \frac{M_t}{M_0} \times 100 \quad \text{(S2)}$$

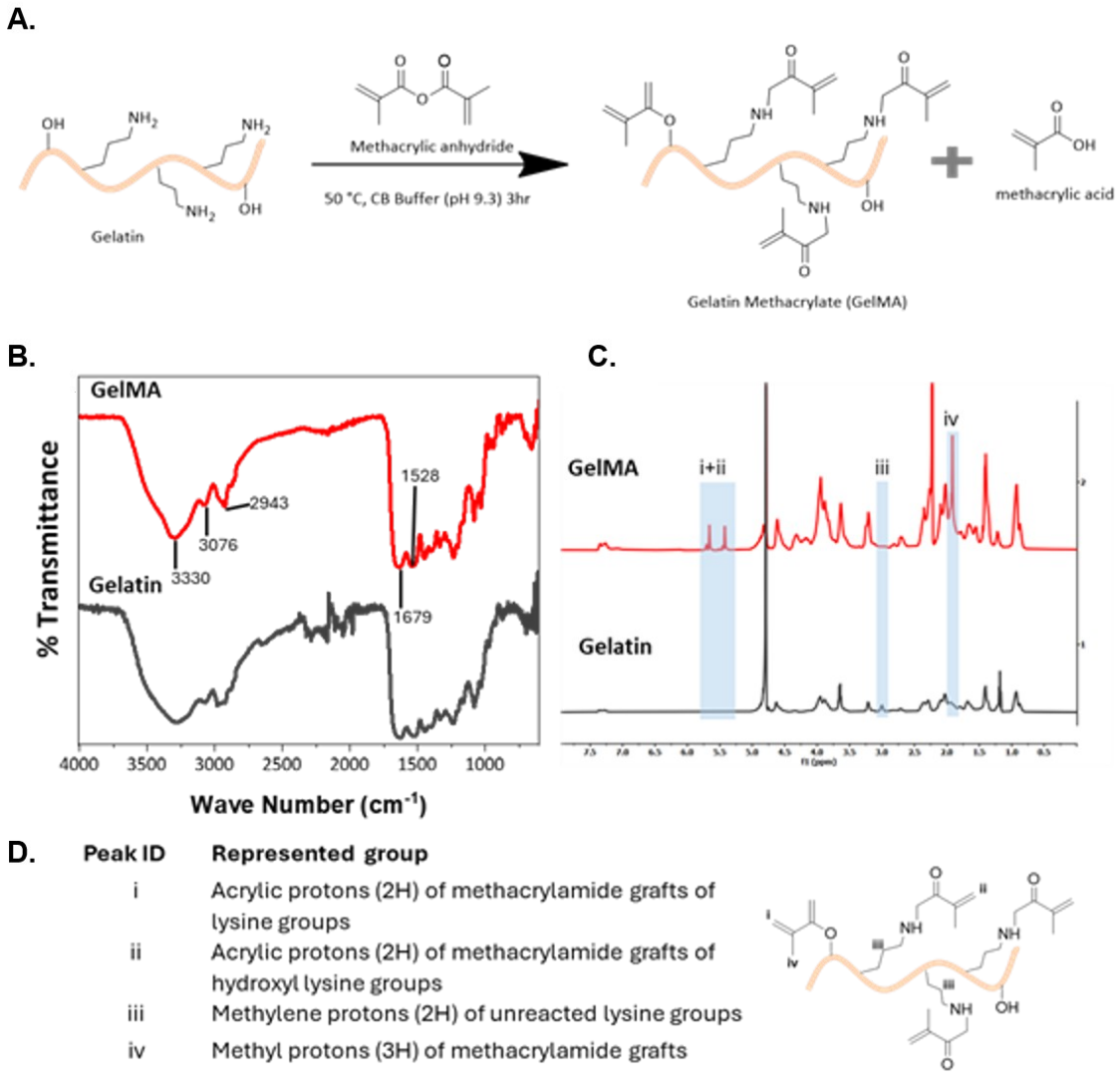

Figure S1: **Schematic representation of GelMA synthesis and characterization via FTIR and ^{1}H NMR.** A. Reaction scheme for methacrylation of gelatin. B. FTIR spectra of gelatin and GelMA with characteristic functional group peaks. C. ^{1}H NMR spectra of gelatin and GelMA. D. Protons specific to functional groups of gelatin and GelMA.

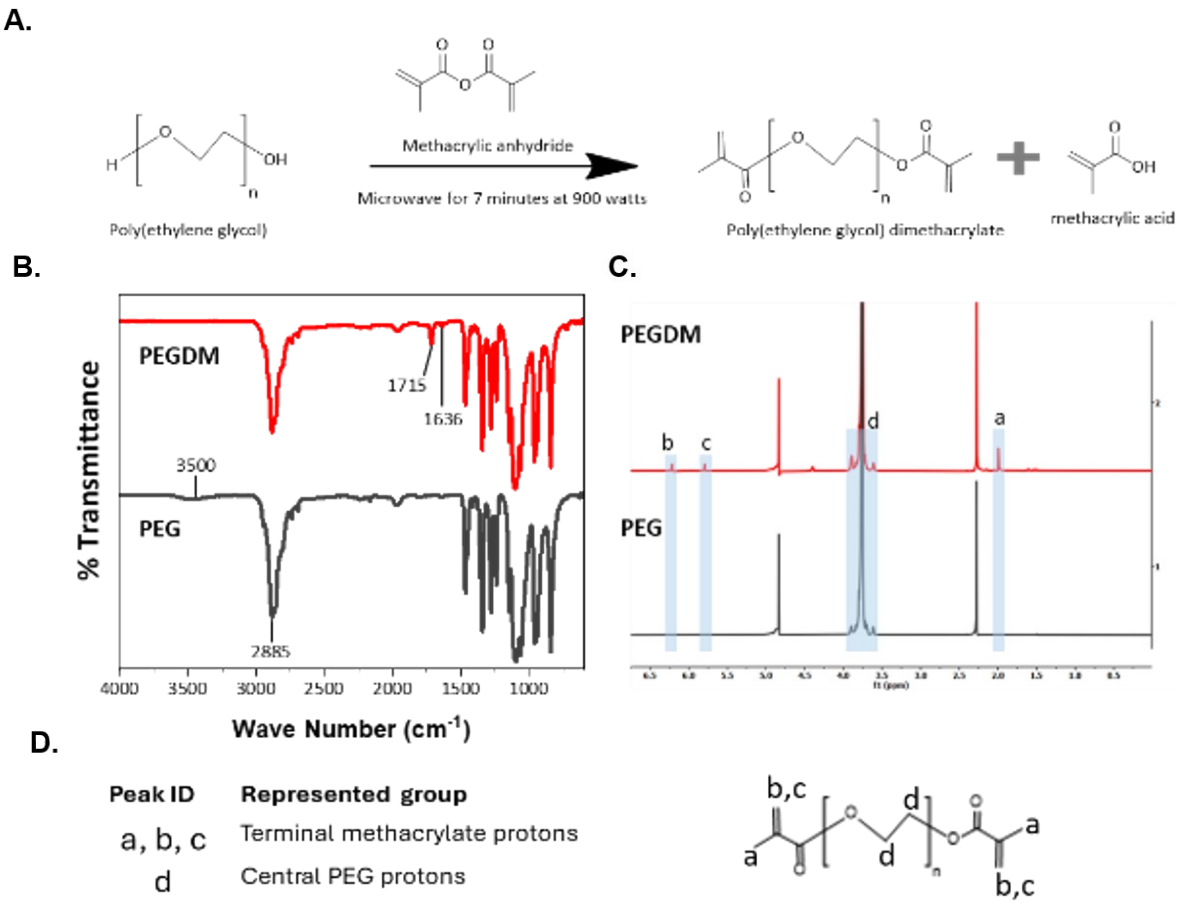

Figure S2: **Schematic representation of PEGDM synthesis and characterization via FTIR and ^{1}H NMR.** A. Reaction scheme for methacrylation of PEG. B. FTIR spectra of PEGDM and PEG with characteristic functional group peaks relevant to the polymer. C. ^{1}H NMR spectra of PEGDM and PEG. D Protons specific to functional groups of PEG and PEGDM.

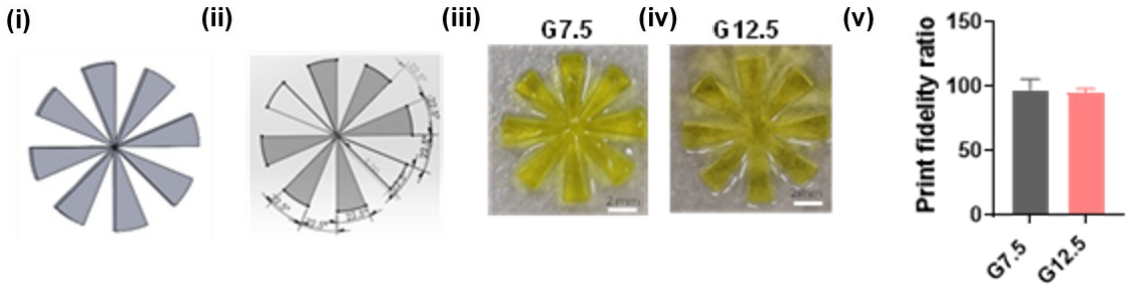

Figure S3: **Quantification of printing fidelity ratio.** i. CAD designs of the 3D printed model with the eight angles labeled. ii. CAD drawing for angles and dimensions of the STL file. 3D printed constructs for G7.5 (iii) and G12.5 (iv) (scale bar: 2 mm), and graph for printing fidelity (v) quantification from 8 angles. n = 3, mean ± S.D.

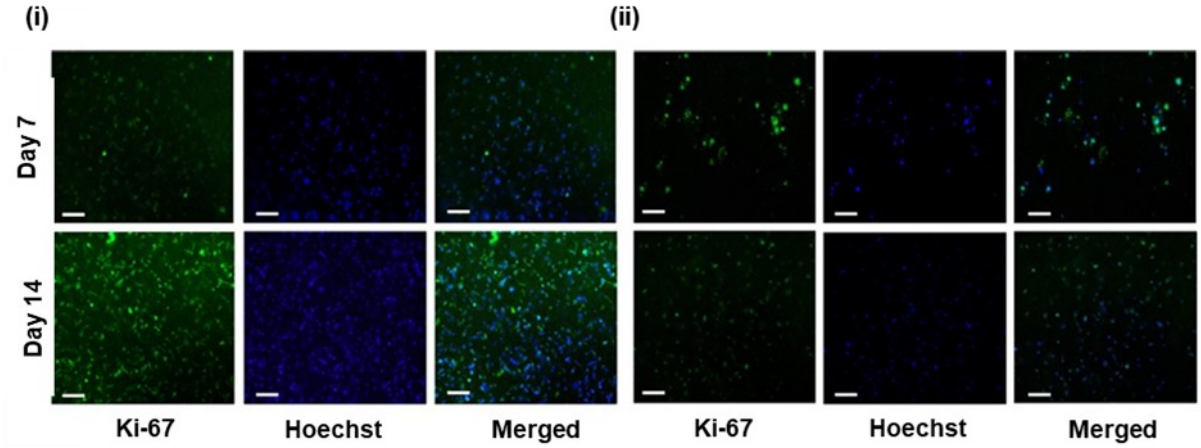

Figure S4: **Ki-67 immunofluorescence staining for 3D bioprinted scaffolds.** Epifluorescence micrographs for Ki-67 (green) and nuclear (blue) staining of MDA-MB-231 in G7.5 (i), G12.5 (ii) 3D-bioprinted scaffolds at Days 7 and 14 (scale bar: 100 µm).

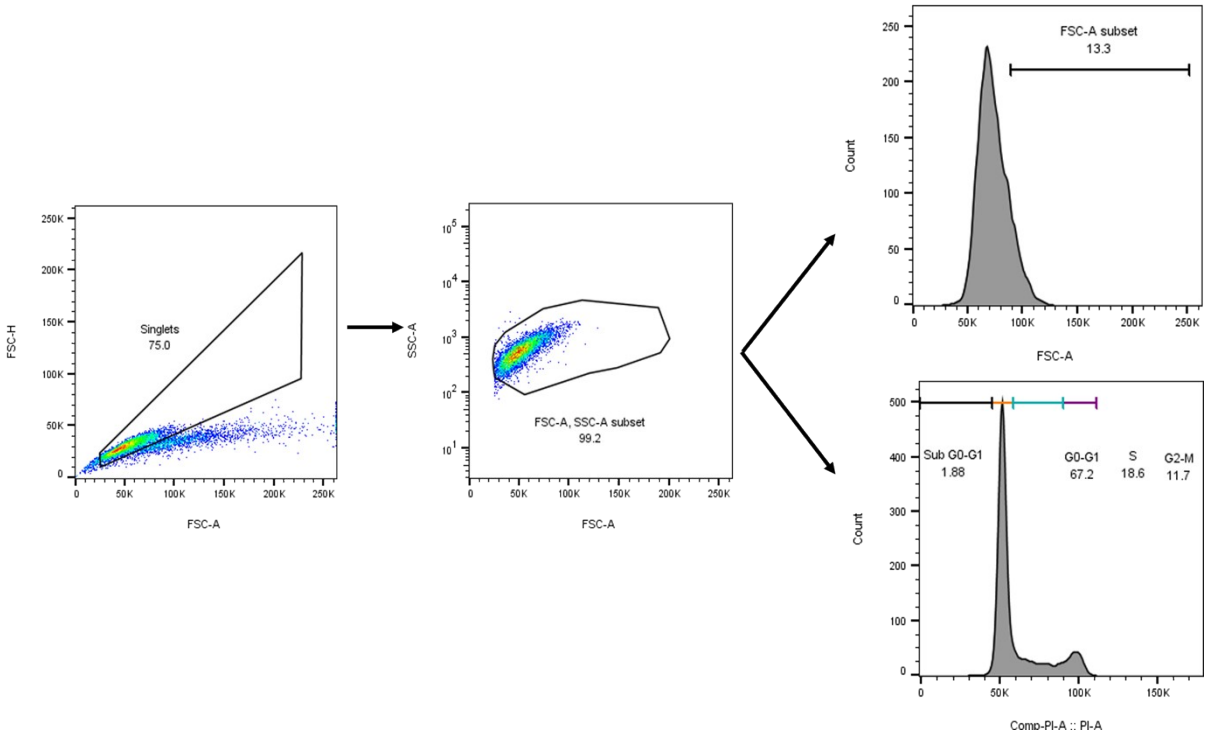

Figure S5: Gating strategy for cell cycling and size analysis of cells isolated from 2D culture conditions and 3D-bioprinted scaffolds.

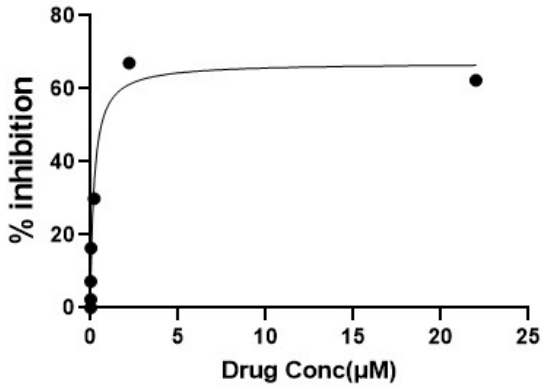

Figure S6: Dose-response curve for doxorubicin cytotoxicity in MDA-MB-231 cells cultured in 2D, as measured by the alamar blue reduction assay.

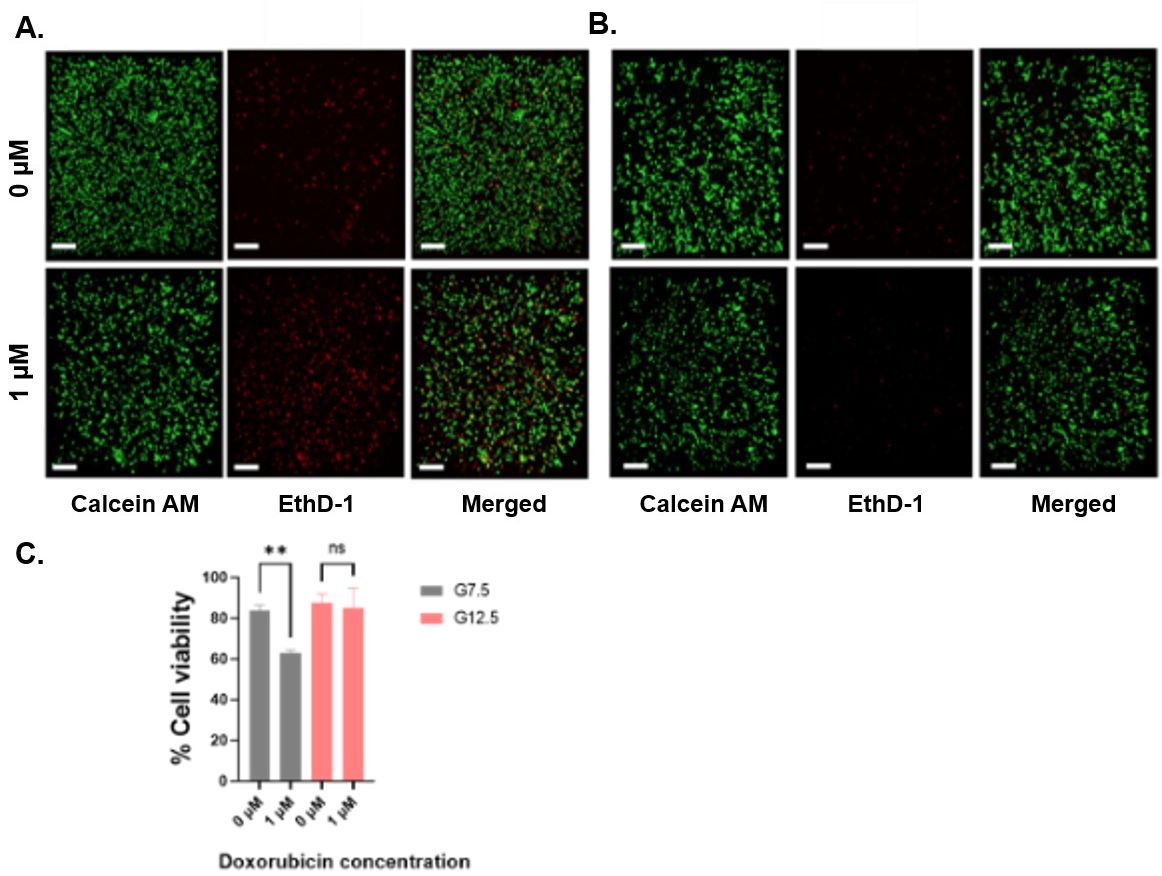

Figure S7: Drug screening in 3D bioprinted scaffolds cultured for 7 days. Fluorescence micrographs of cells treated with 0 or 1 µM Doxorubicin in G7.5(A) and G12.5(B) 3D-bioprinted scaffolds using calcein AM (green/ live) and EthD1 (red/ dead) (scale bar: 200 µm). C. Plot of fractions of viable cells calculated from live/dead staining images (n=3) after doxorubicin treatment in 3D-bioprinted scaffolds. Data are presented as mean ± S.D. from three independent experiments. A one-way

ANOVA was performed to evaluate the statistical significance of differences both within and between the groups. Statistical significance is indicated as *$p < 0.05$ and ***$p < 0.0001$.

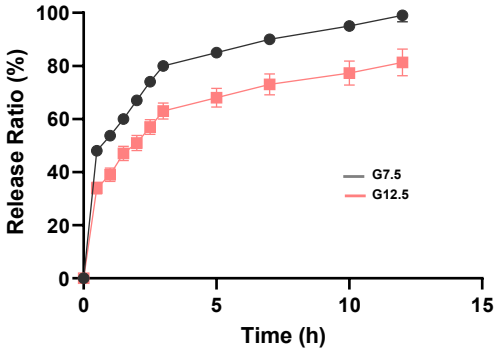

Figure S8: BSA release profile from 3D printed scaffolds.

References

1. Miri, A.K., et al., *Permeability mapping of gelatin methacryloyl hydrogels.* Acta Biomater, 2018. **77**: p. 38-47.
2. Indrakumar, S., et al., *Silk Composite-Based Multifunctional Pellets for Controlled Release.* Macromol Biosci, 2025. **25**(2): p. e2400410.