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Effect of Supramolecular Peptide Hydrogel Scaffold Charge on HepG2 Viability and Spheroid Formation.

Supplementary Information

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Supplementary Figures:

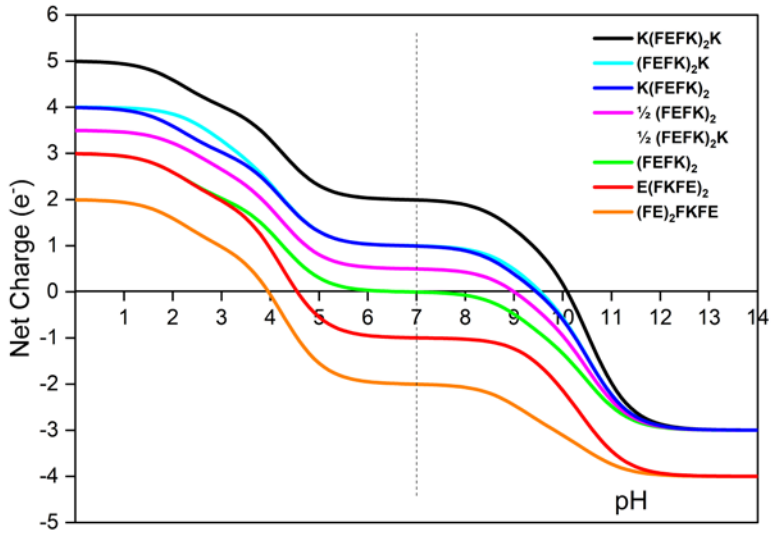

Figure S1*. The overall charge carried by the peptides was calculated using the following equation:*

$$Z = \sum_i N_i \frac{10^{pKa_i}}{10^{pH} + 10^{pKa_i}} - \sum_j N_j \frac{10^{pH}}{10^{pH} + 10^{pKa_j}}$$

where $N_{i/j}$ are the numbers and pKa i/j the pKa values of the basic (i – pKa > 7) and acidic (j – pKa < 7) groups present respectively. The ionic groups present on the peptides are carboxylic acid (COOH / COO^-) at the C-terminus (theoretical pKa 2.18 and 2.19 on K and E side, respectively) and on the glutamic acid side chains (theoretical pKa 4.25), and amine (NH_3^+ / NH_2) at the N-terminus (theoretical pKa 8.95 and 9.97 on K and E side, respectively) and on the lysine side chains (theoretical pKa 10.53).

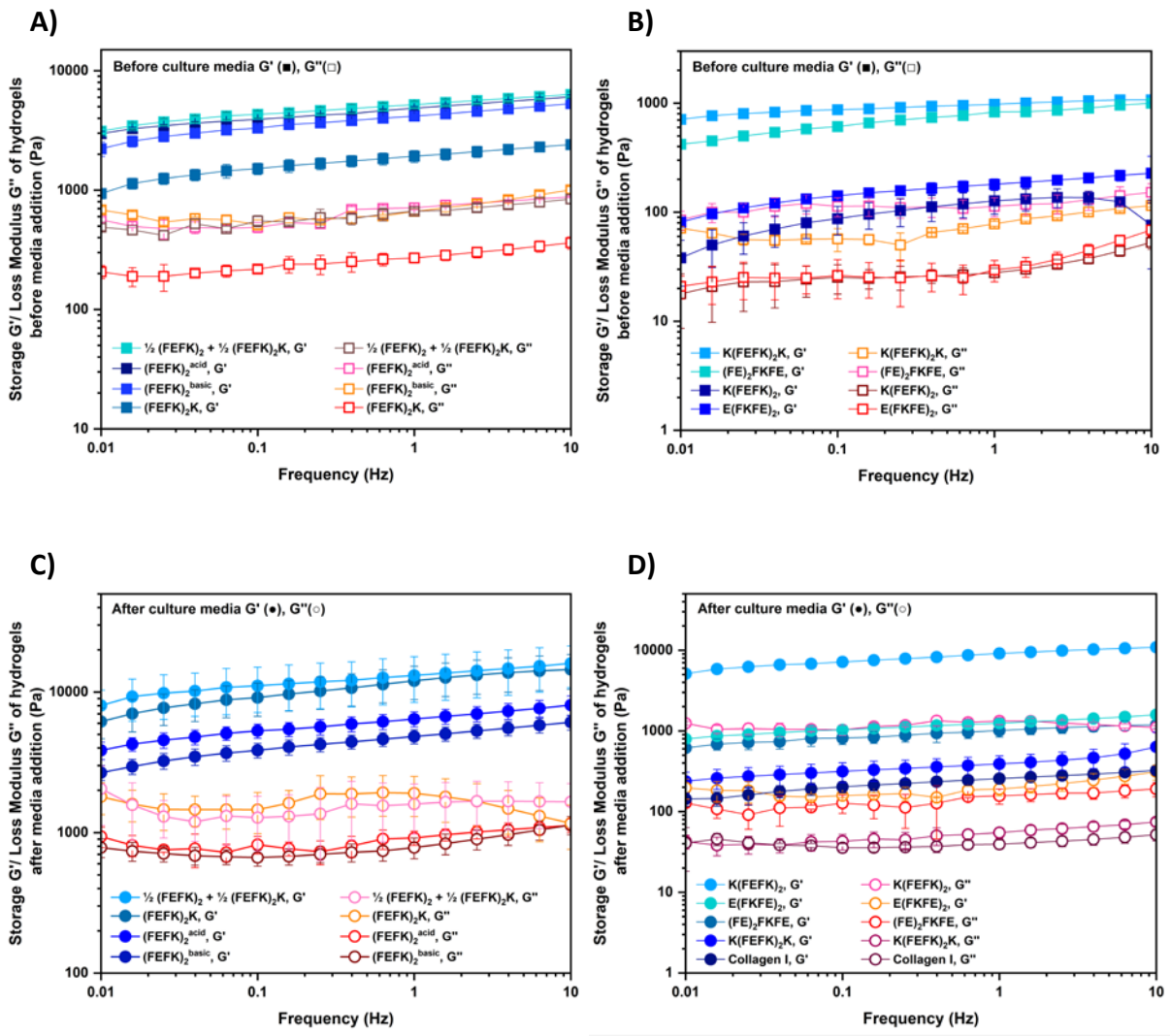

Figure S2. *Storage (G') and loss (G") moduli vs frequency (0.001 to 10 Hz) were obtained for SAPHs and collagen gel at 0.2 % strain.* ***A)*** *and* ***B)*** *at 25°C before cell culture media conditioning,* ***C)*** *and* ***D)*** *at 37°C after 24-hour cell culture media conditioning. Data are presented as mean ± SD; n=3.*

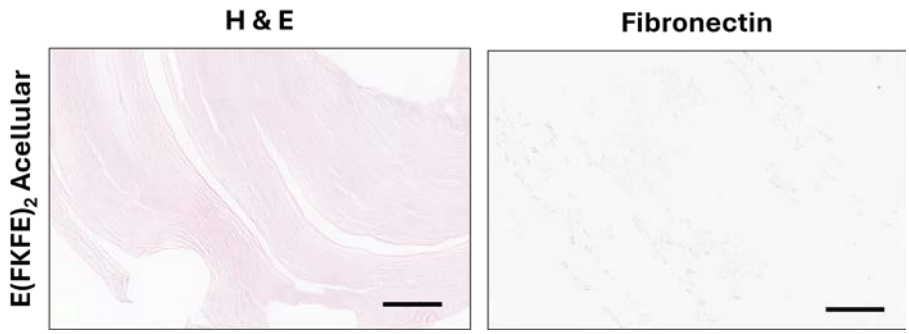

Figure S3**:** *Negative histological staining control images of acellular E(FKFE)$_2$. A) Haematoxylin and Eosin (H&E) staining. Blue = nuclei; pink = cytoplasm and hydrogel; scale bar = 200 µm; n = 2. B) Immunohistochemical fibronectin staining. Brown = fibronectin; blue = nuclei.; scale bar = 100 µm; n = 2.*

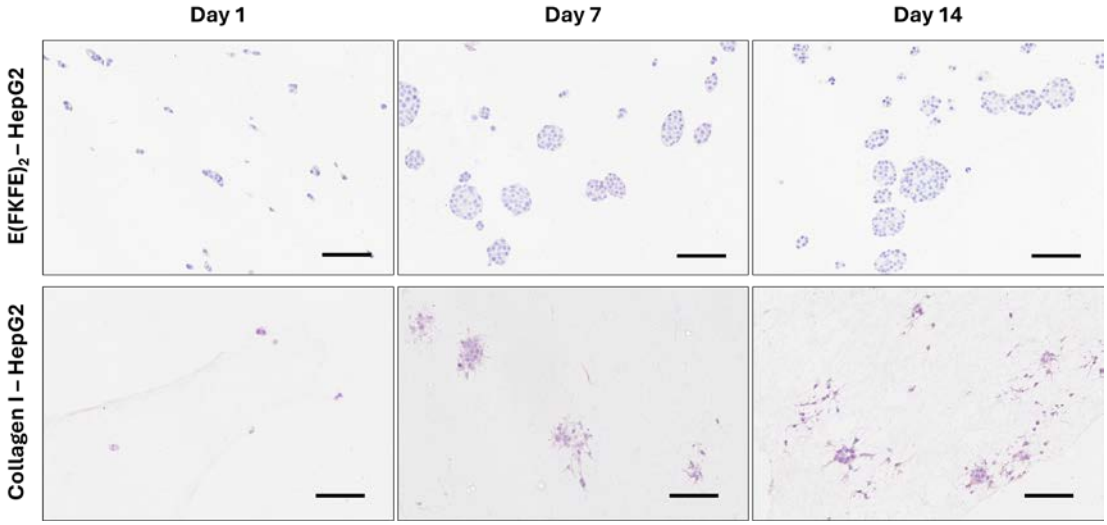

Figure S4**:** *Negative immunohistochemical staining control images (without primary antibody treatments) for E-cadherin, Laminin, Fibronectin, Collagen I) for HepG2 loaded E(FKFE)$_2$ and collagen hydrogels at day 1, 7 and 14. Brown = positive staining; blue = nuclei; scale bar = 100 µm; n = 2.*

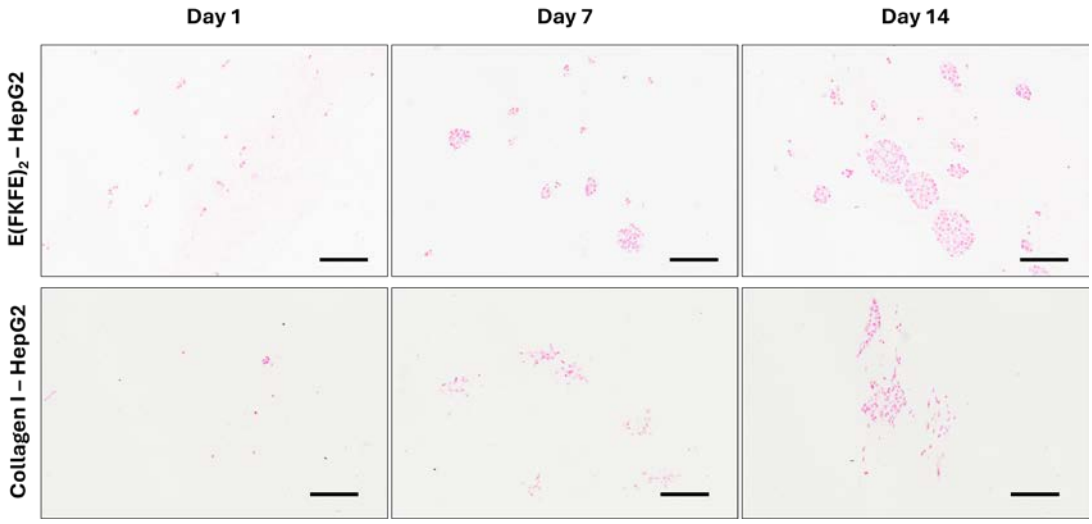

Figure S5: *Negative albumin immunohistochemical staining control images (without albumin primary antibody treatment) for HepG2 loaded E(FKFE)$_2$ and collagen hydrogels at day 1, 7, and 14. Brown = positive staining; pink = nuclei; scale bar = 100 µm; n = 2.*

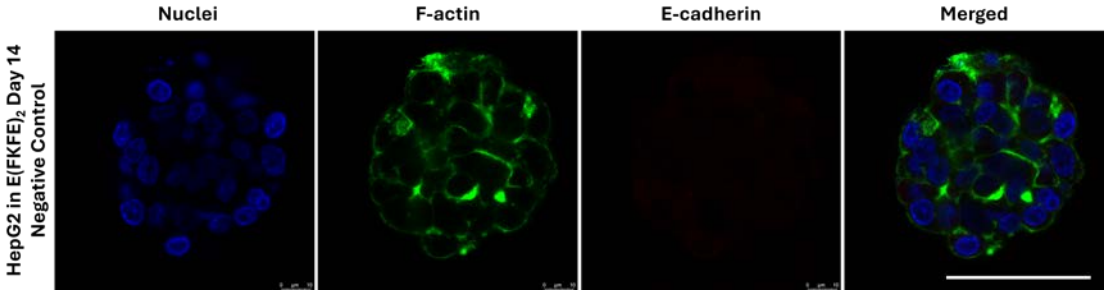

Figure S6: *Negative E-cadherin staining (without primary antibody treatment) confocal images. Blue = Nuclei; green = F-actin, red = E-cadherin; Scale bar = 50 µm; n=2.*

Supplementary Methods:

Histological staining sample preparation: At timepoints of day1, day7 and day14 post-seeding, 3D encapsulated samples were fixed in 10% (v/v) neutral buffered formalin for 1 hour. Samples were subsequently processed overnight using a Vacuum Infiltration Processor 2000 (Miles Scientific, USA). Samples were embedded in paraffin wax and were sectioned into 5 µm-thick slices using a Leica RM2145 microtome and deposited on SuperFrostPlus™ glass slides (Thermo Scientific, UK). The slides were left to dry at 60℃ for 30 minutes. Samples were stained using Haematoxylin and Eosin (H&E).

Immunofluorescent and immunocytochemical staining: Samples were collected at day1, day7 and day14, and were washed in PBS threefold. The samples were fixed in 10% (v/v) formalin for 1 hour at room temperature. Samples were washed in three times with PBS for 5 mins and then permeabilized with 1ml of 0.1% Triton X-100 in PBS solution for 15 minutes. Samples were washed using PBS for 5 minutes three times. 1ml of 1% (w/v) Bovine serum albumin (BSA) in PBS was subsequently added to block the sample for 30 minutes. The working solution was prepared by diluting the primary antibody of E-cadherin (1:250) in 1% BSA with PBS. 700 µl of working solution were added to the positive samples; and an equivalent volume of PBS was added to the negative controls, and all samples were incubated at 4℃ overnight, protected from the light. Samples were washed in three times with PBS and Alexa Fluor™ 488 Phalloidin (1:50, A12379) and secondary antibody Alexa Fluor™ 546 (1:200) were mixed in 1% BSA with PBS, samples were incubated with the mixed working solution overnight at 4℃ in dark. Samples were washed in PBS for 5 minutes three times. Samples were incubated in 300 nM DAPI solution at 4℃ overnight and protected from light,

followed by washing in PBS. Imaging was carried out using a Leica TCS SP8 Confocal Laser Scanning Microscope.

Immunohistochemistry: Slides were stained using UltraVisionTM Quanto detection System HRP (Epredia, UK) and counterstained using Hematoxylin. For intercellular albumin IHC staining, a biotinylated secondary antibody and VECTASTAIN Elite ABC kit were added for 30 minutes and washed again in PBS for 5 minutes. DAB reagent was used followed by nuclear fast red counterstaining. Sections which were not exposed to the primary antibody were used as negative controls for all IHC staining experiments. Positive tissue controls were also used for all antibodies. Cell-free hydrogel controls were also prepared for all stains. All histological staining results were recorded using the brightfield Leica DM2700 Microscope.

Table S1: *Information of antibodies used for immunohistochemical staining of HepG2-hydrogels samples.*

Marker	**Primary antibody**	**Dilution**	**Secondary antibody**	**Positive control**
Laminin	Mouse Anti-Laminin Antibody [LAM-89] (ab49726, Abcam), reacts with human, pig and cat	1:200	HRP Polymer Quanto (TL-060-QPH, Epredia)	Pig kidney
Fibronectin	Rabbit Anti-Fibronectin Antibody (F3648, Sigma), reacts with human	1:1000	HRP Polymer Quanto (TL-060-QPH, Epredia)	Pig Skin
Collagen I	Rabbit Anti-Collagen I Antibody [EPR7785] (ab138492, Abcam), reacts with human	1:1000	HRP Polymer Quanto (TL-060-QPH, Epredia)	Pig lung
E-Cadherin	Rabbit Anti-E Cadherin Antibody [24E10] (3195, Cell Signaling), reacts with human and mouse	1:500	HRP Polymer Quanto (TL-060-QPH, Epredia)	LNcap cell pellet

Albumin	Chicken Anti-Albumin antibody (ab106582, Abcam), reacts with human and mouse	1:50	Rabbit Anti-Chicken IgY H&L (ab6752, Abcam)	Rat liver

Gene expression of CYP450 enzymes using qPCR: Cell-laden hydrogels were digested in 10 mg/mL of Pronase E solution from Streptomyces griseus (Sigma Aldrich, UK) at 37°C for 5 mins, then disrupted in 1ml of TRIzol®. RNA samples were extracted according to the manufacturer's instructions (Geno Technology, US). The cDNA samples were reacted with Fast SYBR Green Master Mix (Applied Biosystems, UK) and DNA Oligo Primers (Sigma-Aldrich, UK). Reacted sample was prepared in triplicate. The gene expression values of CYP450 enzymes, β-Actin and albumin were determined using a StepOnePlusTM Real-Time PCR system (Applied Biosystems, UK). Data were analyzed according to the $2^{-\Delta Ct}$ method, with gene expression normalized to the pre-validated β-Actin reference gene.

Table S2: *Genes and primers used for the qRT-PCR analysis of 3D cultured hepatocytes.*

Gene Name	**Primer Forward**	**Primer Reverse**	**Ref.**
CYP1A2	CTGGAGACCTTCCGACACT	ACCTGCCACTGGTTTACG	[1]
CYP3A4	GCTGTCTCCAACCTTCACCAG	GGCTTGCCTGTCTCTGCTTC	1
CYP2C9	TGTGCTCCCTGCAATGTGAT	CCAGGGGCTGCTCAAAATCT	1
CYP2E1	TTCAGCGGTTCATCACCCT	GAGGTATCCTCTGAAAATGGTGTC	2
β-Actin	GAGAAAATCTGGCACCACACC	GATAGCACAGCCTGGATAGCAA	1
Albumin	CCCCAAGTGTCAACTCCAAC	GGTTCAGGACCACGGATAGA	1

1. M. Zhou, L. Shen, Y. Qiao and Z. Sun, *J Cell Biochem*, 2020, **121**, 566-573.

2. S. C. Ramaiahgari, M. W. den Braver, B. Herpers, V. Terpstra, J. N. Commandeur, B. van de Water and L. S. Price, *Arch Toxicol*, 2014, **88**, 1083-1095.