Supplementary Information

Injectable Self-Assembled Peptide Hydrogels with Programmable Sequences for Cell Culture

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1. Materials

Peptide sequences C_{16}-AAGGEE-COOH (purity >98%) and C_{16}-AAGGEESS-NH_2 (purity >98%) were synthesized and purified by Beyonpep Biotechnology Co., Ltd. (Tianjin, China). Sodium chloride (NaCl, 99%), potassium chloride (KCl, 99%), sodium hydroxide, anhydrous calcium chloride ($CaCl_2$), glucono-δ-lactone (GDL, 98%), and calcium carbonate ($CaCO_3$, 99%) were all purchased from Tianjin Heowns Biochem Technologies Co., Ltd (Tianjin, China). MEM medium (BR), DMEM medium (BR), penicillin-streptomycin, and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Inc. (USA). The CCK-8 assay kit was obtained from Tianjin YiBoHengTai Biotechnology Co., Ltd. (Tianjin, China). All chemicals were of analytical grade unless stated otherwise.

2. Preparation of peptide hydrogels

Peptide powders were dissolved in a solution containing 150 mM NaCl and 3 mM KCl to prepare peptide solutions at 1.0% (w/v), 0.5% (w/v), and 0.2% (w/v). The pH of the solution was adjusted to 7 using 1 M NaOH. Subsequently, different crosslinkers (25 mM $CaCl_2$, 25 mM GDL, or 10 mM $CaCO_3$-20 mM GDL) were added to the solutions, which were then left to stand at room temperature for 12 h.

3. Characterization of peptide hydrogels

3.1 Transmission Electron Microscopy (TEM)

The peptide hydrogel was diluted to 0.1 wt% and sonicated for 5 minutes until fully dissolved. A 7 μL aliquot of the sample solution was dropped onto a carbon-coated copper grid, left to stand for 5 minutes and the excess solution was removed with filter paper before air drying. A small amount of filtered 1 wt% phosphotungstic acid solution was applied for negative staining. Excess stain was removed with filter paper, and the grid was air-dried before imaging with a transmission electron microscope (JEM-2100F, JEOL, Japan) at an accelerating voltage of 120 kV.

3.2 Scanning Electron Microscopy (SEM)

The lyophilized peptide hydrogel samples were mounted onto conductive adhesive tape and coated with a platinum layer using a sputter coater (15 mA, 100 s) to enhance conductivity. The microstructure of the hydrogel samples was examined using a SEM (S-4800, Hitachi Limited, Japan).

3.3 Circular Dichroism (CD) Spectroscopy

The hydrogel samples were diluted to 0.1 wt% using a buffer containing 150 mM NaCl and 3 mM KCl and secondary structure analysis was performed using a circular dichroism spectrometer (J-1700, JASCO, Japan). The measurement parameters were configured with a 0.1 mm pathlength cuvette, 190-500 nm wavelength range, 200 nm/min scan rate, and 2 nm bandwidth.

3.4 Rheological Properties

A rheometer (MCR-302, Anton Paar, Austria) was used to investigate the stability and mechanical properties of the peptide hydrogels. 500 μL of hydrogel sample was placed on the cone-plate geometry and equilibrated at 0.1% shear strain and 1 Hz frequency for 2-10 minutes. The storage modulus (G') and loss modulus (G'') were recorded as a function of time. The self-healing and recovery behavior of the hydrogels after shear was also investigated using the rheometer. For strain sweep measurements, shear strain was increased from 0.1% to 100% at a constant frequency of 1 Hz. For the thixotropic test, the hydrogel was sheared at 11% strain for 5 minutes, then allowed to

recover at 0.1% strain for 20 minutes. G' and G'' were recorded and the cycle was repeated three times.

3.5 Cytotoxicity

293T cells were plated in 96-well plates with DMEM medium (3×10^4 cells/well) and incubated statically at 37°C in 5% CO_2 for 24 hours. After cell adhesion, the culture medium was removed and replaced with 100 μL of fresh medium containing 0.1-0.5% (w/v) peptide samples. Cells were incubated for another 24 hours. Blank (medium only) and control (medium with cells) groups were also set up. After 24 hours, the medium was discarded and replaced with 100 μL of fresh medium and 10 μL of CCK-8 reagent per well. Plates were incubated in the dark for 1-4 hours. Absorbance at 450 nm was measured by a microplate reader.

3.6 Application of Peptide Hydrogels in 3D Cell Culture

HepG2 cells were thawed and cultured in complete MEM medium at 37°C with 5% CO_2. After reaching the third passage, the cells were used for hydrogel-based cell culture experiments. The cells were digested with trypsin and counted using a hemocytometer. Cells were encapsulated in sterilized hydrogel at a density of 3000 cells per well. 100 μL of cell-laden hydrogel was dispensed into each well of a 96-well plate. After settling for 10 min, 200 μL of complete MEM medium was slowly added to each well. On days 3, 5, 7, and 10, the old medium in each well was removed. A mixture of 100 μL fresh complete MEM and 20 μL CCK-8 solution was added to each well. The plate was incubated in the dark for 5 h. The supernatant was then transferred to a new 96-well plate. Absorbance at 450 nm was measured using a microplate reader (EnVision 2105, PerkinElmer, USA). The OD value of the control group (OD_n) was obtained from hydrogel extracts without cells. The OD value of the experimental group (OD_t) was obtained from hydrogel extracts containing encapsulated cells. Cell viability (CV) was calculated using the following formula:

$$CV = (OD_t - OD_n) / OD_n \times 100\% \quad (1)$$

4. Supporting tables

Table S1. Comparison and summary of preparation conditions of peptide hydrogels

Peptides	Crosslinker	Photographs	Gelation properties				
			Time	Color	pH	Stability	Injectability
1.0% (w/v) AGE	25 mM $CaCl_2$		Instant	off-white	Neutral	Yes	No
	25 mM GDL		Instant	off-white	Acidic	Yes	No
	50 mM $CaCO_3$-100 mM GDL		2 h	off-white	Neutral	Yes	No
	25 mM $CaCO_3$-50 mM GDL		2 h	off-white	Neutral	Yes	No
0.5% (w/v) AGE	25 mM $CaCO_3$-50 mM GDL		2 h	Semi-transparent	Neutral	Yes	No
	10 mM $CaCO_3$-20 mM GDL	 Optimal	2 h	Semi-transparent	Neutral	Yes	Yes
	5 mM $CaCO_3$-10 mM GDL		No gelation				
	25 mM GDL		20 min	Semi-transparent	Acidic	Yes	Yes
0.2% (w/v) AGE	25 mM $CaCO_3$-50 mM GDL		Poor gelation				
1.0% (w/v) AGES	25 mM $CaCl_2$		Poor gelation				
	25 mM GDL		Instant	off-white	Acidic	Yes	No
	50 mM $CaCO_3$-100 mM GDL		2 h	off-white	Neutral	Yes	No
0.5% (w/v) AGES	25 mM GDL		Poor gelation				
	5 mM $CaCO_3$-10 mM GDL		No gelation				
	10 mM $CaCO_3$-20 mM GDL		No gelation				
	15 mM $CaCO_3$-30 mM GDL		No gelation				

20 mM $CaCO_3$-40 mM GDL		No gelation

Table S2. Rheological properties of different hydrogels

Hydrogels	G' (Pa)	G'' (Pa)	τ_{yield} (%)	τ_{flow} (%)
1.0% AGE/25 mM GDL	1604.5	331.1	0.9	18.1
1.0% AGES/25 mM GDL	255.8	62.8	0.9	35.5
1.0% AGE/50 mM $CaCO_3$-100 mM GDL	11226.5	4544.6	0.6	8.2
1.0% AGES/50 mM $CaCO_3$-100 mM GDL	1028.3	445.1	-	130.3
0.5% AGE/10 mM $CaCO_3$-20 mM GDL	4181.2	1193.8	1.4	18.0

Note: G' and G" values were measured at a shear strain of 1%

5. Supporting figures

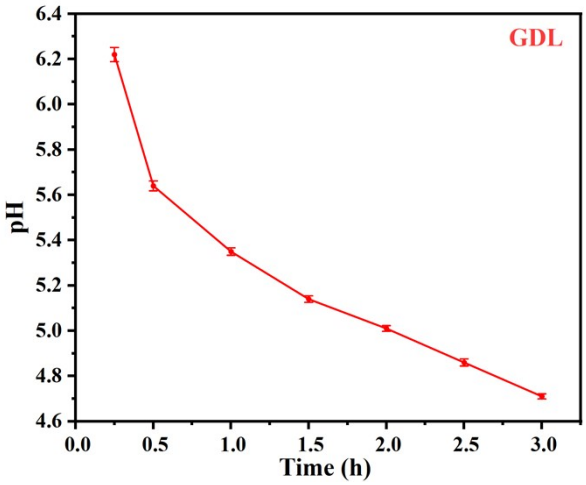

Fig. S1 Time-dependent pH profile of the GDL system.

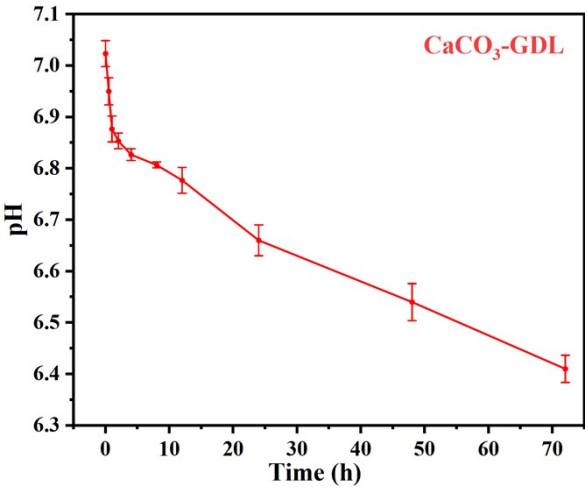

Fig. S2 Time-dependent pH profile of the $CaCO_3$-GDL system.