Enzymatic Formation of a Meta-stable Supramolecular Hydrogel for 3D Cell Culture

Guoqin Chen, Jiaxin Chen, Qicai Liu, Caiwen Ou* and Jie Gao*

Supporting Information

Materials and methods:

Chemicals: Fmoc-amino acids were obtained from GL Biochem (Shanghai). Alkaline phosphatase was purchased from Sigma-Aldrich CO. LLC. 2-Cl-trityl chloride resin was obtained from Nankai University resin Co. Ltd. Commercially available reagents were used without further purification, unless noted otherwise. Nano-pure water was used for all experiments. All other chemicals were reagent grade or better.

Alkaline Phosphatase: 1U corresponds to the amount of enzyme which hydrolyzes p-nitrophenyl phosphate to form 1 μmol of p-nitrophenol at pH 8.0 and 37 °C.

General methods: The synthesized compounds were characterized using $^1$H and $^{31}$P NMR (Bruker ARX 400). The LC-MS spectrometric analyses were performed at the Thermo Finnigan LCQ AD System. HPLC was conducted at LUMTECH HPLC (Germany) system using a C$_{18}$ RP column with MeOH (0.1 % of TFA) and water (0.1 % of TFA) as the eluents. TEM was done on a Tecnai G2 F20 system, operating at 200 kV. SEM image was obtained at QUANTA 200 (America). CCK-8 data was recorded on a BioTek Synergy™ 4 Hybrid Microplate Reader. Confocal microscopy images was obtained on a Leica TCS SP5 system (Germany). The HR-MS data was obtained at Agilent 6520 Q-TOF LC/MS (America).

Peptide synthesis: The peptide derivative was synthesized by solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin, the corresponding N-Fmoc protected amino acids with side chains properly protected by different group. The first amino acid was loaded on the resin at the C-terminal with the loading efficiency about 1.0 mmol/g. 20% piperidine in anhydrous N,N’-dimethylformamide (DMF) was used during deprotection of Fmoc group. Then the next Fmoc-protected amino acid was coupled to the free amino group using O-(Benzotriazol-1-yl)-N,N’,N’-tetramethyluroniumhexafluorophosphate (HBTU) as the coupling reagent. The growth of the peptide chain was according to the established Fmoc SPPS protocol. After the last step, excessive reagents were removed by a single DMF wash for 5 minutes (5mL per gram of resin), followed by five steps of washing using DCM for 1 min (5mL per gram of...
resin). The peptide derivative was cleaved using 95% of trifluoroacetic acid with 2.5% of TMS and 2.5% of H2O for 0.5 hour. 20mL per gram of resin of ice-cold diethylether was then added to cleavage reagent. The resulting precipitate was filtrated and washed by ice-cold diethylether. The resulting solid was dried by lyophilizer and purified by HPLC.

**FEFKFEpYK**: ¹H NMR (400 MHz, DMSO) δ 8.69 (d, J = 8.2 Hz, 1H), 8.35 (s, 1H), 8.26 - 8.10 (m, 5H), 8.04 - 7.04 (m, 6H), 7.28 - 7.12 (m, 13H), 7.04 (d, J = 8.2 Hz, 2H), 4.52 (d, J = 8.0 Hz, 3H), 4.40 - 4.00 (m, 6H), 3.05 - 2.94 (m, 4H), 2.81 - 2.66 (m, 6H), 2.33 - 2.12 (m, 4H), 1.85 (s, 2H), 1.71 - 1.51 (m, 8H), 1.35 - 1.23 (m, 4H). HR-MS: Calc. M⁺ = 1216.5206, found (M+H)⁺ = 1217.5266.

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**Fig.S-1.** ¹H NMR of Compound **FEFKFEpYK**

**Fig.S-2.** HR-MS of Compound **FEFKFEpYK**

**FEFKFEpYK**: ³¹P NMR (162 MHz, DMSO) δ -6.19 (s).
Formation of the hydrogels:
3.2 mg of FEFKFEpYK was dissolved in PBS (400 mL, pH = 7.4). Then 1, 2, 5, 10 and 15 U/mL of alkaline phosphatase was added to above solution, respectively. The final concentration of FEFKFEpYK was 0.8 wt%. The hydrogels would form after about 270, 120, 40 min, 20 and 15 min at room temperature, respectively.

3D cell culture in the hydrogels: Peptide was dissolved in DMEM at the concentration of 1.6 wt% and the pH was carefully adjusted to 7.4 with the Na$_2$CO$_3$ solution. Cells were suspended in cell culture medium with the density of 2,800,000/mL. Equal volume of those two medium were quickly mixed with vortex and then phosphatase was added to the solution. The mixture was transported to plates with 96 wells immediately just before the gelation (with in 5 minutes), and then incubated in the 5% CO$_2$ incubator at 37 °C for 2 hours. 2 hours later after the gelation, cell culture medium was added to each well, on top of the cell-gel constructs.

Determination of cell proliferation rate by CCK-8: To quantify cell proliferation inside the cell-gel constructs, a CCK-8 assay was performed at a series of time points. A 3D culture standard was made by encapsulating cells into hydrogels following the above 3D-culture procedure. To perform the CCK-8
assay, each cell-gel construct was incubated with 10 μL CCK-8 in DMEM. The plates were then incubated in the 5% CO₂ incubator for 4 hours at 37 °C. The absorbance at 450 nm was determined using the microplate reader (MultiskaniMark, Bio-Rad, USA). The experiments were conducted for five times and SD was determined.

**LC-MS analysis for the conversion from FEFKFEpYK to FEFKFEYK:** The hydrogel was prepared using above mentioned procedure. At each time point, 10 μL of the gel was taken out by pipette and then dissolved in 500 μL of DMSO. The DMSO samples were directly used for LC-MS analysis. The areas of peaks in LC-MS spectra were used to determine the conversion percentage from FEFKFEpYK to FEFKFEYK during hydrogelations.