Supplementary Information

Ultrasound Accelerated Gelation of Novel L-lysine Based Hydrogelators

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1. Synthesis and Characterization

1.1 Materials and Methods

All reactions were conducted under N_2 atmospheres. 7-hydroxy coumarin were purchased from Alfa Aesar and vacuum dried at room temperature for 24h before used. N, N-diisopropylethylamine (DIEA), 1-ethyl-(3-dimethyllaminopropyl) carbodiie hydrochlide (EDC·HCl), 1-hydroxy-benzotriazole monohydrate (HOBT), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU) and L-lysine methyl ester dihydrochloride (H-Lys-OMe·2H-Cl) were purchased from GL Biochem. Ltd. (Shanghai, China). Trifluoroacetic acid (TFA), Di-tert-butyl dicarbonate and Hydrazine monohydrate were purchased from Asta Tech (Chengdu) Biopharm. Co. Ltd. (Chengdu, China). DMSO-d₆ was purchased from Sigma-Aldrich. Co (Steinheim, Germany) and used as received. Acetone, CH₂Cl₂ and N,N-Dimethylformamide (DMF) were purchased from Kelong Chemical Co. (Chengdu, China) and used without further purification.

The structure was determined by NMR (Bruker Avance II, 600MHz) and HRMS (WATERS Q-TOF Premier). Fluorescence spectra (F-7000, Hitachi, 350 nm, 10K/min) were adopted to analyze the π - π intermolecular interactions. The morphologies were performed on the transmission electron microscope (TEM, H-600 electron microscope) and the scanning electron microscope (SEM, S-4800, HITACHI). Differential scanning calorimetry (DSC) thermograms were obtained on a TA Q2000 with the heating rate of 10 °C/min. Rheology test was performed on an AR 2000ex (TA Instruments) system using 40 mm parallel plates at a gap of 56 µm.

1.2 Preparation of Hydrogel

Synthesis of DCOU-Lys-OH

7-Carboxyl methoxycoumarin was synthesized according to the procedure previously reported.^[S1] The synthesis of DCOU-Lys-OH was as follow: H-Lys-OMe·2HCl (0.319 g, 1.37 mmol), 7-Carboxyl methoxycoumarin (0.543 g, 2.6 mmol), HBTU (0.989 g, 2.6 mmol) and HOBT (0.352

g, 2.6 mmol) were dissolved in 25 mL anhydrous DMF in N₂ atmosphere. Subsequently, DIEA (1.36 g, 10.52 mol) in 20 mL anhydrous DMF was added dropwise into the solution under stirring. The reaction was carried out in an ice bath and lasted at room temperature for 40 h. The solution was distilled and 50 mL CHCl₃ was added. The solution was washed with the solutions of 1 M NaHCO₃ and 1 M NaHSO₄ each for 3 times. After the organic solution was concentrated, the product of DCOU-Lys-OCH₃ was purified by column chromatography and recrystallized in CHCl₃. The solvent for column chromatography was mixed solvent (dichloromethane: ethyl acetate: methanol = 6: 2: 1).

DCOU-Lys-OCH₃ (0.6 g) was dissolved in methanol (18 mL) in an ice bath. 1 M NaOH solution (18 mL) was added dropwise into the solution and the mixture was stirred at room temperature for 18 hours. The solution was transferred to an ice bath, 1 M HCl solution was added into the aqueous solution with stirring till the pH reached 2. The product was precipitated from the solution, separated by filtration and washed with deionized water. The product was vacuum-dried at room temperature overnight.

Synthesis of DCOU-Lys-CONH-NH₂

DCOU-Lys-OH (0.674 g, 1.22 mmol), BocNHNH₂ (0.243 g, 1.84 mmol), EDCI (0.282 g, 1.47 mmol) and HOBT (0.198 g, 1.47 mmol) were dissolved in 25 mL anhydrous DMF in N_2 atmosphere. Subsequently, DIEA (0.8g, 6.1mmol) was added dropwise into the solution under stirring in an ice bath. The reaction was lasted at room temperature for 30h. The solvent was removed and replaced with 50 mL CHCl₃, the solution was washed with 1 M NaHCO₃ and NaHSO₄ each for 3 times. The DCOU-Lys-CONH-NHBOC was received after concentration. The product was purified by column chromatography and recrystallized in CHCl₃.

DCOU-Lys-CONH-NHBOC (0.162 g,0.24 mmol) was dissolved in CH_2Cl_2 (0.3 mL), TFA (0.3 mL) was added dropwise into the solution under stirring in an ice bath. The solution was refluxed for 4h. TFA and CH_2Cl_2 were removed using rotatory evaporator. Anhydrous diethyl ether (20 mL) was poured into the product and stirred for 8h. The precipitate in the solvent was separated by filtration, washed with anhydrous diethyl ether and vacuum-dried at room temperature overnight. The product was purified by HPLC (Agilent 1260 Infinity) with the mixed solvent (acetonitrile: distilled water = 20: 80) as eluent at the flow rate of 1mL/min, the detect UV wavelength of 320 nm (The tested purity was 96.7%). The purified DCOU-Lys-CONH-NH₂ was tested by elemental analysis (theoretical: C: 59.57%; N: 9.93%; H: 5.00%, tested: C: 59.42%; N: 9.86%; H: 4.95%).

The Formation of Hydrogel

The hydrogelator was dissolved in distilled water with the concentrations of 0.25, 0.5, 0.75, 1, 1.5 and 2 wt%. The solutions were stirred for 30 minutes and then stood at room temperature for 24 hours. It was found that only the concentration higher than 1 wt% could gelated. The ultrasound treatment was that the soultion was agitated by ultrasound for 30 s after the stirring. The ultrasound treatment was performed on a Kun Shan KQ-5200 DE ultrasound cleaner (Kunshan Ultrasound Instrument Co, Kunshan China), the power was 80W and the frequency was 40 KHz. **Molecular Simulation**

Materials Studio program (MS, version 4.2 Accelrys Software Inc., United States) was used to simulate the possible conformations and optimized the simulation with Module/Discover/Minimizer under Compass field. Modules/Amorphous Cell/Construction of Materials Studio program was used for the molecular dynamic calculation within 100 molecules. NPT and NVT were used for 200ps balance and molecular dynamic calculation in 1 ns. There was no water in the simulation, the starting geometry was a cube with 5.152 nm \times 5.152 nm \times 5.152 nm. The force field was COMPASS.

1.3 Cell Culture

Mouse fibroblasts NIH 3T3 were used to evaluate the cytotoxicity of the hydrogels. The fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μ g/mL streptomycin and 100 units/mL penicillin. The suspension of cells was received via rinsing with 0.025 % tyrosine and 0.02% EDTA.

The hydrogels were prepared in 96-well cell culture plates. 100 uL of 3T3 fibroblasts (8×10^3) suspension, which was diluted with the medium, were added onto the surface of hydrogels. The extracts co-culture experiments were prepared following the International Organization for Standardization tests (ISO 10993-5). The culture medium was replaced in the next two days.

1.4 Cell Proliferation Test (CCK8 assay)

To quantify the cell proliferation and the cytotoxicity of the hydrogels, an CCK8 assay was performed. DMEM mediums contained 10 μ L of CCK-8 was prepared into each well and the 96-well plates were maintained in an incubator for 4 h. Then the absorbance was measured with a Thermo Varioskan Flash microplate reader at the wavelength of 450 nm.

2. Results and Discussion

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Scheme S1. The synthetic route of DCOU-Lys-CONH-NH₂ hydrogelator.



Figure S1. The ¹H NMR spectra of DCOU-Lys-OH (A), DCOU-Lys-CONH-NHBOC (B) and DCOU-Lys-CONH-NH₂ (C).

The 1 H NMR spectra of DCOU-Lys-OH, DCOU-Lys-CONH-NHBOC and DCOU-Lys-CONH-NH₂ were presented in Figure S2. The assignment of the protons were

presented in the spectra. All the signals in the spectra were attributed to corresponding protons. The results showed that the designed hydrogelator was synthesized.



Figure S2. ESI-MS spectrum of DCOU-Lys-CONH-NH₂ hydrogelator,

The calculated $[M]^+$ of DCOU-Lys-CONH-NH₂ was 564.1975, and the tested result was 564.195, it demonstrated that the hydrogelator was received.



Figure S3. The HPLC spectrum of DCOU-Lys-CONH-NH₂.

The purity of DCOU-Lys-CONH-NH2 was tested by HPLC and the calculated purity of the hydrogelator was 96.7%.



Figure S4. ¹H NMR spectra of DCOU-Lys-CONH-NH₂ hydrogelator in DMSO-d₆ with different concentrations at 25 $^{\circ}$ C.

The downfield shift of protons ^aH, ^bH and ^cH with increasing the concentration in DMSO- d_6 revealed the existence of hydrogen bond within the hydrogelator.



Figure S5. ¹H NMR spectra of DCOU-Lys-CONH-NH₂ (a) in DMSO-d₆ with 15wt% H₂O and (b) in D₂O with varying temperature. The concentration of hydrogelator was 0.5 wt%.

As previously reported, hydrogen-bonded supramolecular polymers were exploited in a polar solvent,^[S2] using DMSO-d₆ as solvent.^[S3] The downfield shift of amide-protons ^aH, ^bH, ^cH were detected by adding water to DMSO-d₆ solution, which indicated the existence of hydrogen bonding, moreover, a simultaneous upfield shift of aromatic - protons ¹H, ²H were also detected, which indicated the contribution of π - π stacking interaction during the process of self-assembly aggregation (Figure S4A).^[S4] It shown that the initially broad peak of aromatic - protons ¹H, ²H, ³H, ⁴H, ⁵H gradually transformed to a sharper peak broad peaks and got downfield shifted at a higher temperatures, which was attributed to the weakening of intermolecular interactions and proved the existence of π - π stacking interaction (Figure S4B).^[S5]



Figure S6. 2D NOESY spectrum of DCOU-Lys-CONH-NH₂ hydrogelator.

The 2D NOESY spectrum of DCOU-Lys-CONH- NH_2 hydrogelator was used to study the conformation of the hydrogelator molecules. The assignments of the protons were presented in the spectrum and the results confirmed the structure of the hydrogelator.



Figure S7. The simulated four conformations of DCOU-Lys-CONH- NH_2 hydrogelor, the data were the calculated energy of the conformation.

The simulation results showed four conformation types. The energies of the four conformations were calculated. As the energy of type B was the lowest, it was the most stable form. We further used Modules/Amorphous Cell/Construction of Materials Studio program for the molecular dynamic calculation within 100 molecules with type B formation in 1 ns. According to the calculated molecular dynamics, the hydrogen bond sites were C=O---H (NH₂), C--O---H (NH₂) (C-O in the ring) and C--O---H (NH₂) (C-O in the chain).



Figure S8. Energy diagram of intermolecular hydrogen bond. The hydrogen bond sites were C=O---H (NH₂), C—O---H (NH₂) (C-O in the ring) and C—O---H (NH₂) (C-O in the chain). The hydrogen bond was mainly formed between the oxygen of C=O and hydrogen in NH_2 (C=O---H (NH₂)).

The main hydrogen bond was formed between the oxygen of C=O and hydrogen in NH_2 (C=O---H (NH2)), the oxygen (C=O...H) was at 0.2 nm and the peak was at 2.6. The other two

types of oxygens appeared beyond 0.3 nm, as the length of hydrogen bond was commonly lower than 0.3 nm, thus we concluded that the hydrogen bond was mainly formed between C=O---H (NH_2) as shown in Scheme 1A.



Figure S9. TEM images of hydrogel with ultrasound treatment (A, 1 wt%) and hydrogel morphology of recovery (B, 1 wt%), the hydrogel in B was destroyed by shaking vigorously and stood at room temperature for 1 h.

The fibrils in Figure S9A were homogenous, it demonstrated that the ultrasound treatment could homogenize the self-assembly of hydrogelators into homogenous fibrils. Entangled fibrils were observed in Figure S9B, it meant that the network was formed again after destroyed and stood at room temperature for 1 h. This result exhibited the recovery of the hydrogel.



Figure S10. Rheological and thermal properties of the hydrogels with dynamic time sweep at the strain of 0.1% and frequency of 1 rad/s. Gel 1 was the hydrogel without ultrasound treatment, gel 2 was treated with ultrasound, the concentrations of the hydrogelators were 2 wt%.

Both the G' and G" of gel 2 were higher than the corresponding G' and G" of gel 1. It revealed that the ultrasound treatment increased the mechanical strength of the hydrogel.



Figure S11. Confocal microscopy images of NIH 3T3 fibroblasts in the hydrogels (with the concentration of 2 wt%). The scale bar was $75\mu m$.

The tomoscan function of confocal laser scanning microscopy (CLSM) was used to explore the condition of the cells in hydrogels. The laser was focused on different focal planes, each plane was called a layer, the thickness of a layer was 5 μ m. The cells in different layers were scanned. And it was found that the green fluorescence of the stained cells was strengthened with time increased, it implied that the cells proliferated inside the hydrogel. Cells could be observed in each ten layers, even in the deep of 200 μ m (40 layers), which was much larger than the size of a cell (the size of a fibroblast was about 20-40 μ m), plenty of cells were observed. This result indicated that the cells seeded on the hydrogel surface migrated in the hydrogel and they were alive. It implied that the hydrogel was biocompatible and nontoxic to the cells.

References

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