Emulsions Stabilize by Mini Cyclic Proteins for Bioactive Compound Delivery

Wenyan Xu,[‡,a] Bo Wang,[‡,a,b] Yuan Lin,[*a] Yuhua Li,[b] Zhaohui Su,[a] Wenjun He,[c] Ninghua Tan,[*c] and Qian Wang[a,d]

[a] *State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun, 130022, P.R. China. linyuan@ciac.ac.cn;*
[b] *College of Life Science, Northeast Forestry University, Harbin 150040, China*
[c] *State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201. P.R. China. nhtan@mail.kib.ac.cn*
[d] *Department of Chemistry and Biochemistry, University of South Carolina, Columbia 29208, USA.*

[‡]These authors contribute equally.
*Corresponding authors

Electronic Supplementary Information

CONTENTS:

1. Materials

MCTI-I was isolated from seeds of Momordica cochinchinensis and further purified with HPLC following reference.[1] Fluorescein isothiocyanate (FITC) and 95% Perfluorodecalin were obtained from Sigma-Aldrich, and other reagents were purchased from Beijing Chemical Works. All the chemicals were used directly without any further purification. Ultrapure water was obtained from UNIQUE-R20 system (18.2 MΩ/cm).

2. Measurements

The structure of MCTI-I was confirmed by MALDI-TOF and MALDI-TOF-TOF comparing the mass spectrum with fragments data of cyclotides in the Cybase.[2] UV-Vis spectra were acquired on a TU-1901 spectrometer (Beijing Purkinje General Instrument Co., Ltd.). ζ-potential was investigated by Zetasizer Nano ZS90 (Malvern Instruments Ltd.). Electrophoresis system was SE 250 Minivertical

Unit coupled with EPS-301 Power Supply from GE healthcare life sciences. Fast Protein Liquid Chromatography (FPLC) analysis was performed on an AKTA explorer (GE Biotech) instrument. Transmission electron microscopy (TEM) measurements were carried out on a JEOL JEM-1011 microscope operating at an accelerating voltage of 100 kV. Laser scanning confocal microscopy (LSCM) were obtained on Carl Zeiss LSM 700 LSCM. Atom force microscopy (AFM) investigations were carried out on Agilent 5500 AFM with tapping mode. Silicon tips with a resonance frequency of ~300 Hz, a spring constant of about 2 $N \cdot m^{-1}$ and a scan rate of 0.6 Hz were used.

3. Modification of MCTI-I with FITC

5.1 mg MCTI-I was dissolved in 900 μL carbonate sodium buffer (0.5 mM, pH 9.5). Then 180 μL FITC (3.44 mg) in DMSO was added and the mixture was shaken for 17 h at 4 °C. After that the mixture was eluted through Hiprep^{TM} 10/26 desalting column with FPLC system. All samples were analyzed in Kphos buffer (100 mM, pH 8, with 2 mg/mL sucrose) at a flow rate of 3 mL/min. The eluted fractions were collected and used in the next experiments. The amount of FITC-MCTI-I was investigated by UV absorbance. Covalent ligation was confirmed by Tris-tricine SDS PAGE. Fluorescent modified MCoTI-II was obtained using the same method.

4. Emulsion Preparation

FITC-MCTI-I stock solution (1.4 mg/mL) was diluted with Kphos buffer, then 200 μL perfluorodecalin (ρ=1.9 g/mL) was added into 2 mL FITC-MCTI-I solution, and then the mixture was shaken by vortexing for 10 min. The emulsion was kept at 4 °C for 1 h, so that the interfacial aggregation could be equilibrated. After the equilibration, 10 μL 50 % glutraraldehyde in water was added and the mixture was shaken vigorously by vortexing for 10 min. The emulsion was kept in dark until further investigation and experiment. The concentration of MCTI-I was 0.1 mg/mL for LSCM and 1.0 mg/mL at pH 7.8 for AFM and TEM investigation in final aqueous solution. Control was prepared with free FITC. The transmittance at 400 nm of perfluorodecalin was set as 100%. MCTI-I stock solution was diluted to final concentration of 0.1 mg/mL or 1.0 mg/mL with Kphos buffer (pH 7.8). The diluted solution (500 μL) was moved into a UV-Vis curette, and then 50 μL perfluorodecalin was added. The mixture was vortexed thoroughly and was immediately placed into spectrometer, and then the transmittances at 400 nm were recorded every 2 min.

5. Coumarin-6 loading and cell assay

Coumarin-6 (200 μL, 10 mg/mL) was mixed with 2 mL of MCoTI-II in PBS buffer (1 mg/mL) . The mixture was vortexed thoroughly, then the emulsion was kept at 4 °C for 1 h. After the equilibration, glutaraldehyde in water (10 μL, 50 %) was added, then the mixture was vortexed again. The emulsion was kept in dark until further investigation and experiment.

Cellular cytotoxicity in vitro was investigated as follows: L929 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. L929 (6 × 105 cells/mL) cells were seeded in 96-well plates and cultured for 24 h. The medium was then replaced by the emulsion of MCoTI-II loading coumarin-6 or free coumarin-6 dissolved and diluted in DMEM at various dye concentrations of 1, 2, 4, 8, 16 μg/mL. After incubation for 2 and 24 h, 20 μL of MTT solution in PBS (5 mg/mL) was added per well and the plates were incubated for another 4 h at 37 °C. After that, the medium containing MTT was replaced with DMSO to dissolve the MTT formazan crystals. Finally, the plates were shaken for 10 min before the UV measurement at 570 nm.

Cell imaging was investigated as follows: coverslips were sterilized with 75% ethanol, and washed with PBS buffer before putting into 24-well culture plates for cell culture. L929 cells were seeded at a density of 3 × 104 cells/well and incubated at 37 °C with 5% CO_2 for 24 h. Then the cells were treated with the emulsion of MCoTI-II loading coumarin-6 or free coumarin-6 (equivalent coumarin-6 concentration: 1 μg/mL) at 37 °C with 5% CO_2 for 2 h. After incubation, cells were washed twice with PBS. Subsequently the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed three times with PBS again. Later, cell nucleus was stained with DAPI (1.25 μg/mL) for 10 min followed by washing with PBS. Samples were examined by confocal scanning microscope (LSCM, LSM 700). Coumarin-6 was excited at 466 nm with emissions at 504 nm.

6. References

(1) Hara, S.; Makino, J.; Ikenaka, T. *Journal of Biochemistry* **1989**, *105*, 88.

(2) Wang, C. K. L.; Kaas, Q.; Chiche, L.; Craik, D. J. *Nucleic Acids Research* **2008**, *36*, D206.

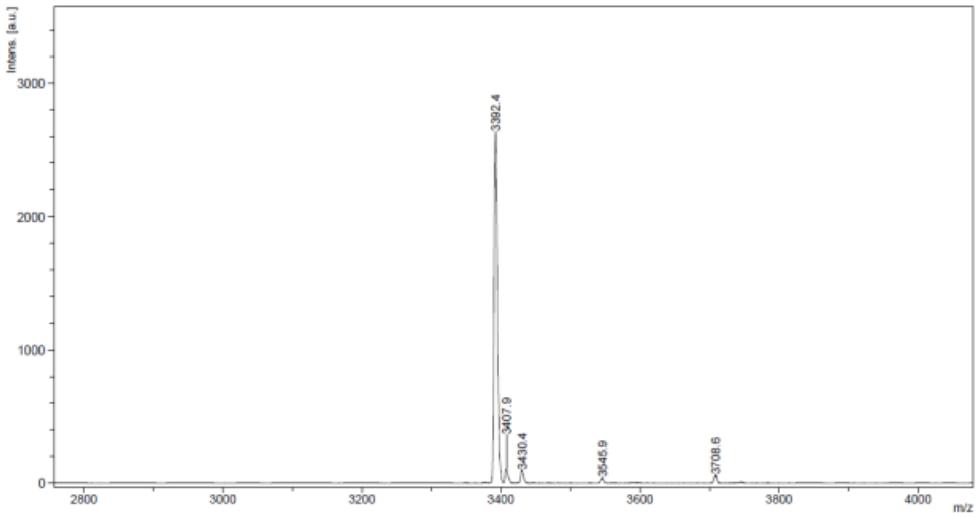

Fig. S1 MALDI-TOF spectrum for the standard of MCTI-I.

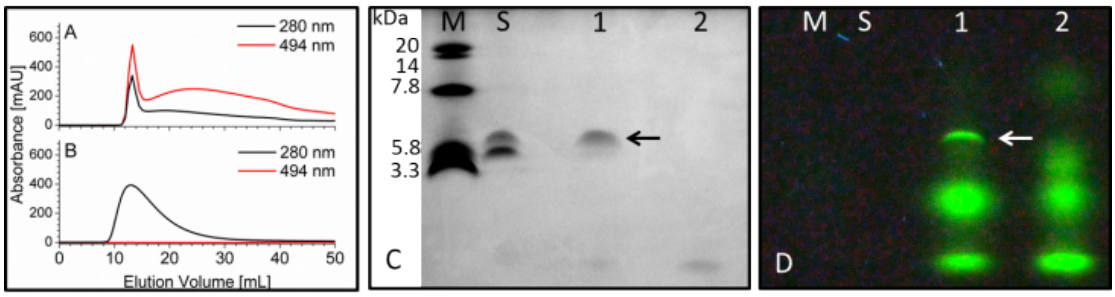

Fig. S2 FPLC and SDS-PAGE analysis of fluorescently labelled MCTI-I. (A) FPLC curves for FITC-MCTI-I. (B) FPLC curve for MCTI-I. (C) Tris-tricine SDS PAGE gel stained and under white light; M, Marker 20.1, 14.4, 7.8, 5.8, 3.3 kDa; S, standard of MCTI-I; 1, mixture of fluorescent modification of MCTI-I with FITC; 2, free FITC. (D) The same gel as C but under UV at 365 nm. FITC-MCTI-I is marked by arrow.

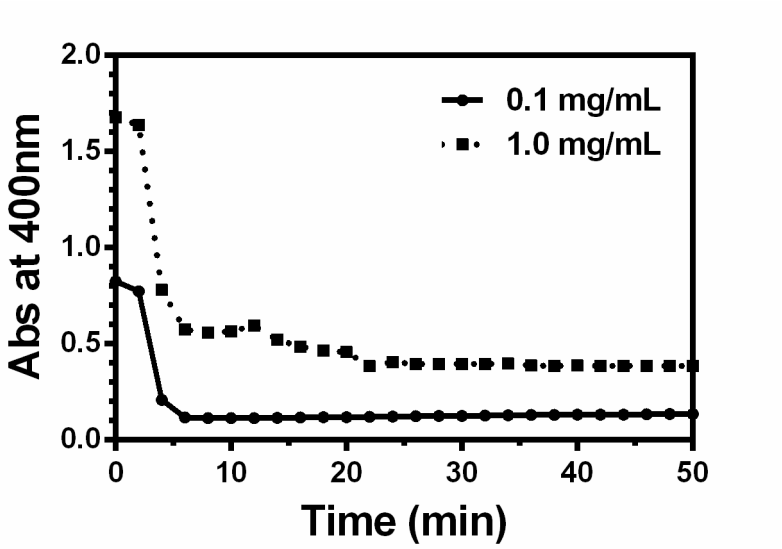

Fig. S3 Tendency of absorbance at 400 nm vs time for the emulsions prepared with solutions 0.1 mg/mL and 1.0 mg/mL at pH 7.8. The emulsions were prepared by vortexing for 10 min following the method mentioned previously, and then the samples were moved into cuvette immediately after the vortex. The time point "0" was set as the moment when the cuvette was put into the cabinet of spectrometer. The cuvette was sat in the spectrometer all through the investigation and the absorbance was recorded every 2 minutes.

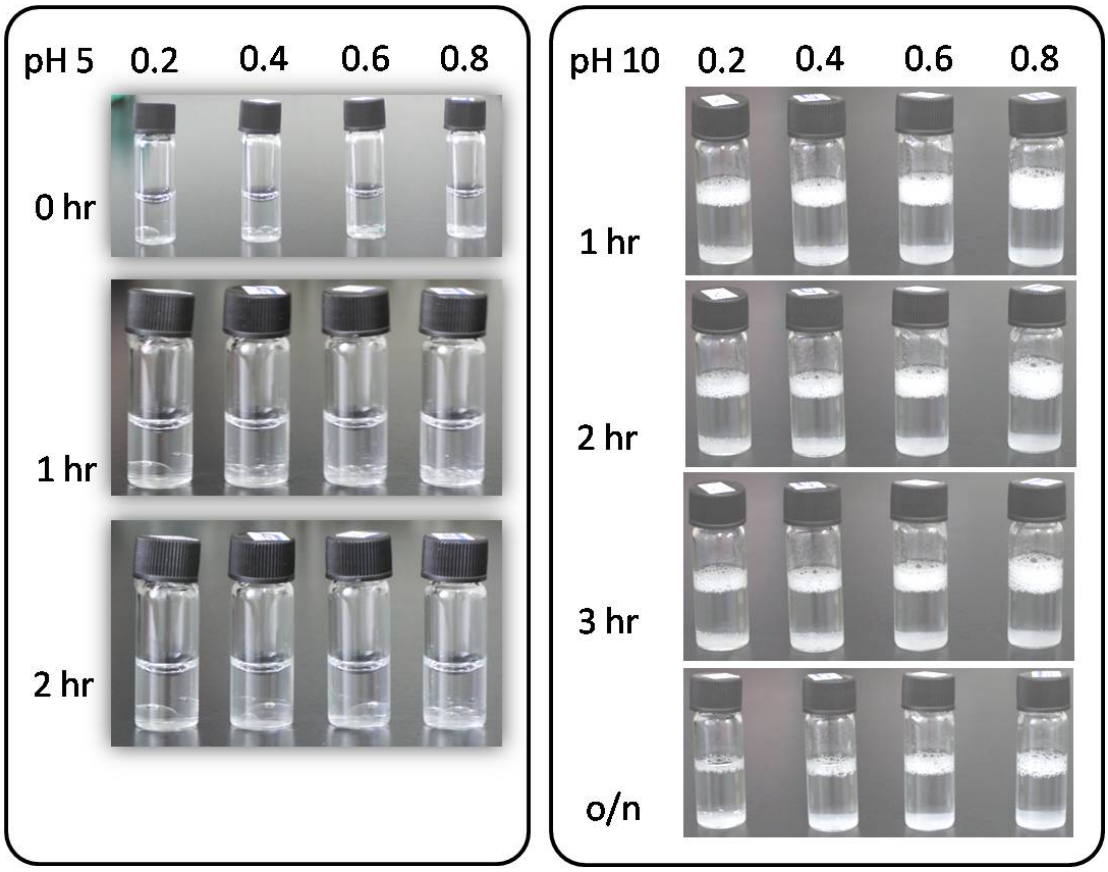

Fig. S4 Stability of coalescence without crosslink prepared with MCTI-I at different pH and concentration of MCTI-I. The concentration (mg/mL) is marked on the top, and the pH is marked at top on left in each frame. (o/n indicates over night) No obvious emulsions were observed in the solution at pH 5 except for few huge drops of oil at the bottom. However, the white emulsified oil phase is quite stable although the sedimentation has completed.

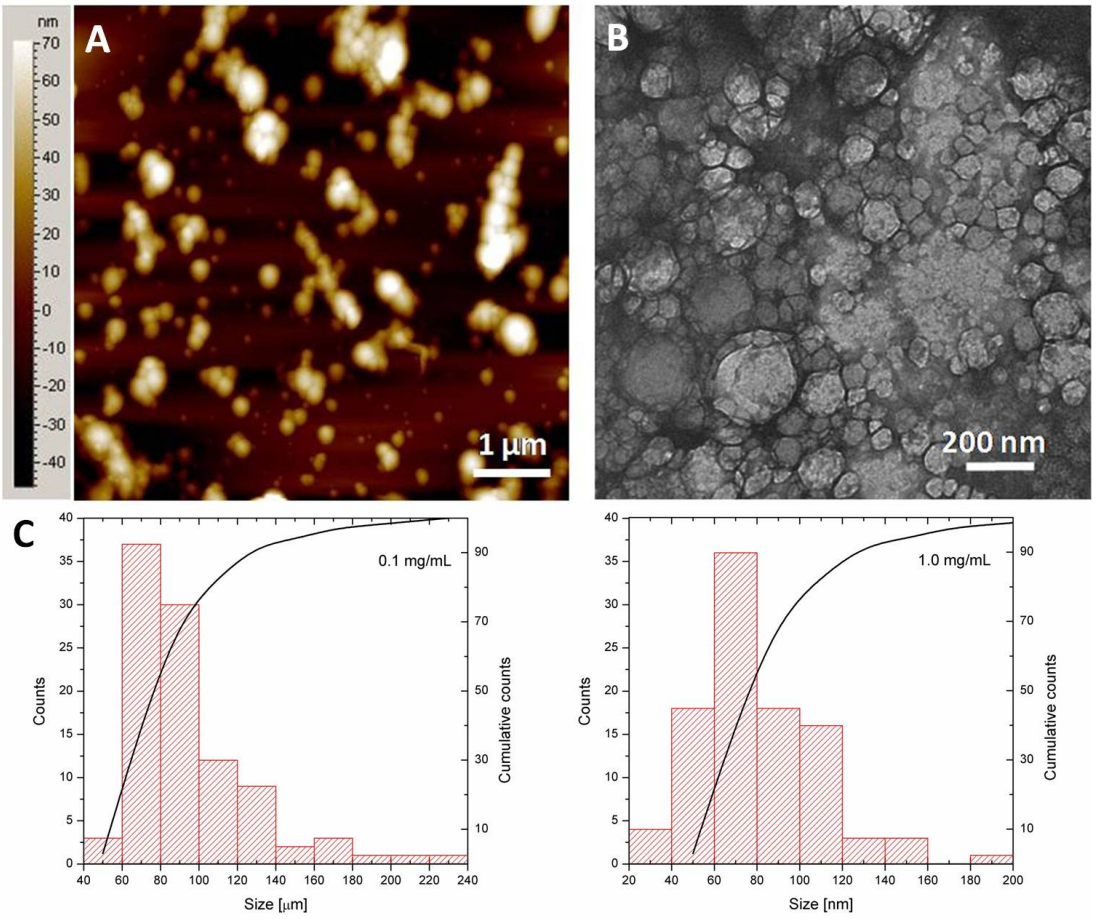

Fig. S5 (A) AFM image of crosslinked capsules after dried 3 hours with MCTI-I 1.0 mg/mL concentration., (B) TEM image of crosslinked capsules with MCTI-I 1.0 mg/mL concentration, (C) Distribution of size measured for crosslinked droplets prepared with MCTI-I in 0.1 mg/mL and 1.0 mg/mL concentrations.

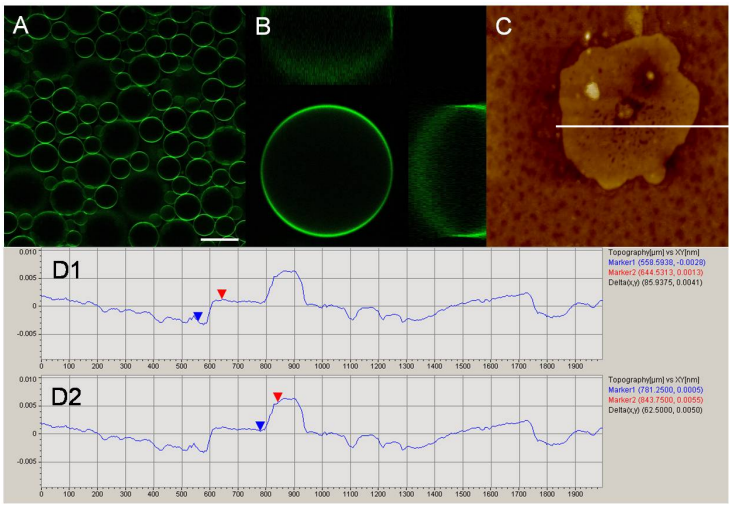

Fig. S6 Images of the crosslinked capsules of MCoTI-II. (A) Fluorescent image investigated with LSCM with MCTI-I 0.1 mg/mL concentration; (B) Fluorescent image of LSCM in orthotic model; (C) Topography image investigated with AFM after totally dried with MCTI-I 1.0 mg/mL concentration; D1 and D2, Height analysis of the capsule in C. Scale bar is 200 µm for A. The width of C is equal to 2 µm.

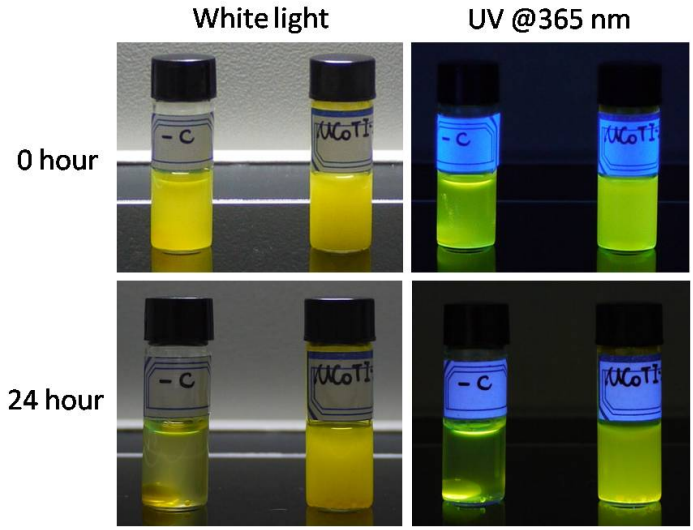

Fig. S7 Optical images and fluorescence of emulsion prepared at 1.0 mg/mL of MCoTI-II pH 7.8 loading coumarine-6 and control mixture without MCoTI-II

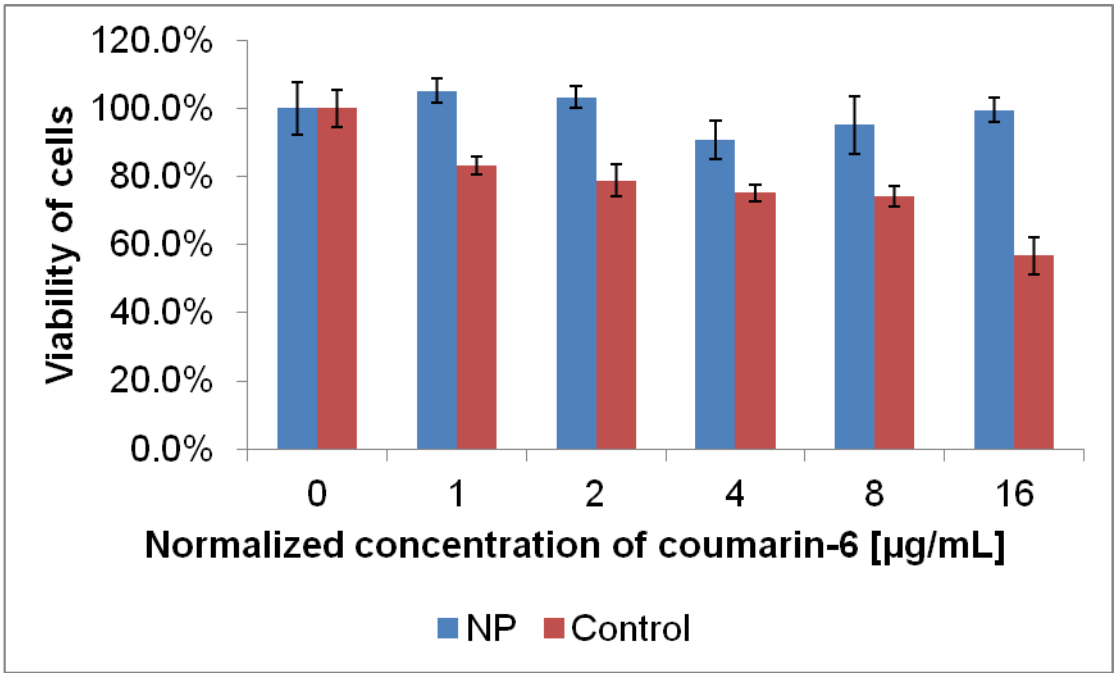

Fig. S8 Viability of cells investigated with MTT assay. NP is for the cells treated with emulsion. Control is for the cells treated with coumarin-6 in DMSO.