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Emulsions stabilized by mini cyclic proteins for bioactive compound delivery†

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Mini cyclic proteins, members of the family of cyclotides, can stabilize oil-in-water emulsions by forming a single layer assembly at the oil–water interface. Such emulsions can be potentially employed to deliver hydrophobic bioactive cargos.

Emulsions are an efficient and economical method to prepare hierarchically structured functional materials on the micro- or nano-scale, which have been used in daily life applications, including food, medicine delivery, cosmetics, ink, *etc.*[1,2] To keep droplets stable from coalescence, detergents (or other amphiphilic molecules) are used in most cases. However, the toxic risk of most detergents to human health and other disadvantages such as foaming activity restrict the usage of detergent in certain situations and hold back further functional development.[2]

Protein stabilized emulsions, have attracted great attention because of the advantages of good bio-compatibility, no chemical products involved, *etc.* A great deal of research has revealed a better understanding of the properties of proteins in emulsions, such as the processes of production, storage and utilization of the emulsion stabilized by protein.[3–15] These results indicate that the processes are regulated by a variety of different solution conditions like the concentration of protein in the solution, pH, ionic strength, surfactants and so on. Although those exciting progresses have been achieved, it is quite challenge to generate and investigate the interfacial assembly of small molecular weight (*i.e.* <10 kDa) biomolecules. In this paper, we report on a family of mini proteins, named cyclotides, with an average size of ~2 nm in diameter, can assemble at the oil–water interface and prevent the resulted droplets from coalescence. Such droplets can be further cross-linked to form relatively stable emulsions, and used for the delivery of fluorescent hydrophobic compounds.

Cyclotides belong to an interesting family of natural proteins that can be isolated from plants and have ~30 amino acid residues.[16,17] Moreover, cyclotides are divided into 3 classes, *i.e.* möbius, bracelet, and squashed trypsin inhibitors on the base of the sequence differences, which differs. All cyclotides share a highly conserved core motif which is referred to as the cyclic cysteine knot (CCK).[18] The CCK motif makes the backbone so rigid that the side chains of hydrophobic residues are presented at the surface instead of being buried to form a hydrophobic core as in normal protein. This structural feature protects cyclotides from aggregation and denaturation in organic solvents, and also makes cyclotides more amphiphilic. Together with the CCK motif, the cyclic backbone makes cyclotides resistant to exopeptidase, heat and extreme pH intrinsically.[19–21] Therefore we assume that cyclotides can serve as ideal candidates for interfacial assembly applications. MCTI-I, a typical squashed trypsin inhibitor,[22] is employed as a model cyclotide in this study. As shown in Fig. 1, although the sequence of MCTI-I is different from other cyclotides, the conserved cysteine residues and CCK motif make the 3D structure of MCTI-I recognizable to other cyclotides.

The purity of the MCTI-I was confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) (ESI Fig. S1†). In order to observe final assemblies with a laser scanning confocal microscopy (LSCM), MCTI-I was labelled with fluorescein isothiocyanate (FITC) which presents $\lambda_{ex}/\lambda_{em}$ at 494/521 nm. The covalent ligation was confirmed by fast protein liquid chromatogram (FPLC) and tris-tricine SDS polyacrylamide gel electrophoresis (PAGE). FPLC shows that the retention volume of FITC-MCTI-I is identified with MCTI-I; meanwhile, a significantly enhanced absorbance

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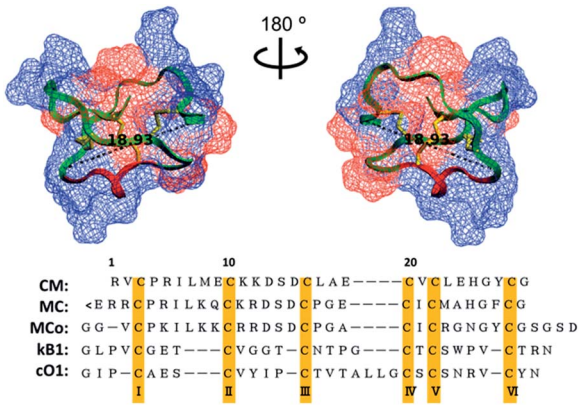

Fig. 1 Structure and sequence of cyclotide. 3D structure of MCTI-I is reconstructed with PyMOL. Loops are presented in green, helices in red, and disulfide bonds in orange. Hydrophilic surface is blue and hydrophobic surface is orange. The sequence of MCTI-I is presented at bottom, which is compared with other typical cyclotides. All residues are numbered in Arabic numbers, and cysteine is highlighted in yellow and marked in Roman numerals. Abbreviations: CM, CMTI-I; MC, MCTI-I; MCo, MCoTI-II; kB1, Kalata B1; cO1, Cycloviolacin O1. <E means pyroglutamic acid.

at 494 nm is observed due to the co-elution of FITC with MCTI-I (ESI Fig. S2A, B†). The SDS-PAGE shows the fluorescent labelling of MCTI-I. Unmodified MCTI-I samples give two bands which correspond to the fully denatured and the partially refolded MCTI-I, which is very common for the cyclotides samples (ESI Fig. S2C and S2D,† lane S). Interestingly, the fluorescein modification can prohibit the re-folding process, thus afford one single-band on SDS-PAGE (ESI Fig. S2C and S2D,† lane 1, marked by arrow).

The assembly experiments were conducted using fluorescently tagged MCTI-I. As a general protocol,[23] MCTI-I stock solution (2 mL in 100 mM K-phosphate buffer, pH 7.8, with 2 mg mL^{-1} sucrose) was mixed with 0.2 mL perfluorodecalin and the mixture was then shaken vigorously by vortexing. The transparent solution changed to opaque during the vortexing, however, a rapid sedimentation was observed after letting the emulsions rest. The process of sedimentation and coalescence was detected with a UV-vis spectrometer[24] (ESI Fig. S3†). The absorbance drops quickly in the first few minutes for both concentrations at pH 7.8, and then reaches a plateau. This analysis indicates that it was a fast process for the droplets to merge each other, thus resulting in the formation of big droplets and the occurrence of the sedimentation. After that, the absorbance reaches a plateau and a relatively stable emulsion formed. In other words, a relatively slow coalescence appeared following the rapid sedimentation. Meanwhile, concentration and pH effects on the stability of the emulsion were systematically investigated (ESI Fig. S4†). The emulsion was more stable at high pH and concentration independent, indicating the stability of emulsion is related to the electrostatic repulsion. More surface charges were detected for MCTI-I in the solution at higher pH by the investigation of zeta-potential (data not shown).

The emulsion prepared at pH 7.8 was allowed to equilibrate for 1 h, then 10 μL 50% glutaraldehyde in water was added and the mixture was shaken vigorously by vortexing. Interestingly, a well dispersed and relatively stable emulsion was observed, and the sedimentation as well as coalescence were prevented for at least 24 h (ESI Fig. S7†). This is similar to those observed for surfactant and protein-stabilized emulsions. When droplets may break apart or merge together during vortexing.[25] Meanwhile, the glutaraldehyde as a cross-linker could stabilize the protein-protected emulsions, which prevents particle desorption during thin-film drainage and provides a steric barrier to coalescence with neighboring droplets.[23,26–28] This is similar to literature reports that colloidosomes can be prepared using the cross-linking technique.[29,30] AFM revealed a spherical morphology upon drying the droplets on silica after cross-linking. The thickness of the dried capsule was about 4 nm (Fig. 2D). Considering the diameter of MCTI-I is about 2 nm, this result implies that MCTI-I close-packed and formed in a single molecular layer at the interface.

The size of droplets at different concentrations was investigated, showing that with a 0.1 mg mL^{-1} solution of MCTI-I, the diameter of droplets was around 95.6 ± 31.7 μm (Fig. 2A and ESI Fig. S5C†), while the diameter was about 80.0 ± 28.6 nm with a 1.0 mg mL^{-1} solution of MCTI-I (Fig. 2C and ESI Fig. S5C‡). Unlike at a low concentration of 0.1 mg mL^{-1}, at the high concentration 1.0 mg mL^{-1}, more protein particles could be driven to the oil–water interface, and these particles could stabilize the droplets by an effective cross-linking. At the low concentration, there are not enough protein particles to stabilize droplets, which results in the droplets merged together and

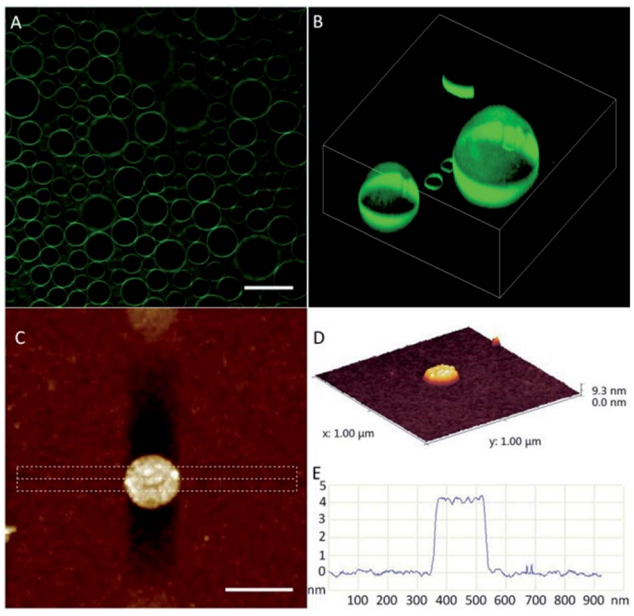

Fig. 2 (A) LSCM image of MCTI-I emulsions. (B) 3D view of MCTI-I emulsions reconstructed from LSCM. (C) Topological image and (D) 3D reconstruction of dried capsule visualized with AFM. (E) Height profile of an individual capsule shown in (C). Scale bar is 200 μm for (A) and 200 nm for (C).

formed big droplets to reach the equilibrium. Therefore, the size of the droplets is dependent on the concentrations of MCTI-I solution. These results are consistent with above discussion that the big droplet size results in the lowest minimum absorbance (ESI Fig. S3†). As mentioned above, besides the cross-linking, the electrostatic repulsion between the proteins also contributes to the stabilization. Furthermore, some literatures reported that the adsorption of soft materials such as protein or microgels to the oil–water interface leads to a stronger reduction of the interfacial tension compared to rigid particles, which may attribute to the deformation of soft materials at the interface.[31]

Using another cyclotides, MCoTI-II was prepared oil-in-water emulsions following the same protocol. The backbone of MCoTI-II is head-to-tail cyclized,[32] thus the extending loop VI of MCoTI-II presents more flexibility as shown in its crystal structure. Analyses show that MCoTI-II also formed a single molecular layer at the oil–water interface, similar to MCTI-I (ESI Fig. S6†).

To evaluate potential application of the emulsion, coumarin-6 was loaded in the droplet and the droplet was incubated with cells for the fluorescent imaging of living cells. Coumarin-6 emits strong fluorescence under UV light; however it has very low solubility in water which limits its application in bioimaging. In our experiments, 1.0 mg mL^{-1} of MCoTI-II was used in the assembly experiments which resulted in emulsions with a diameter of ~80 nm. The coumarin-6 was encapsulated stably in the emulsions (it is termed as Coumarin-6-MCoTI-II) and emitted green fluorescence under UV irradiation (ESI Fig. S7†). Using mouse fibroblast L929 cell line as a model, LSCM images show that cells treated by the emulsions loading with coumarin-6 could readily uptake coumarin-6 without any visible cell damage (Fig. 3A). DMSO was chosen as the co-solvent in control experiments to dissolve the water-insoluble coumarin-6. As shown in Fig. 3B, the DAPI signal defuses all over the cytoplasm, indicating serious nucleus damage. MTT assay also confirmed that the Coumarin-6-MCoTI-II emulsion presented much lower cytotoxicity than free coumarin-6 (ESI Fig. S8†). This result suggests that emulsion prepared with cyclotides can effectively encapsulate hydrophobic cargos for cell delivery purpose.

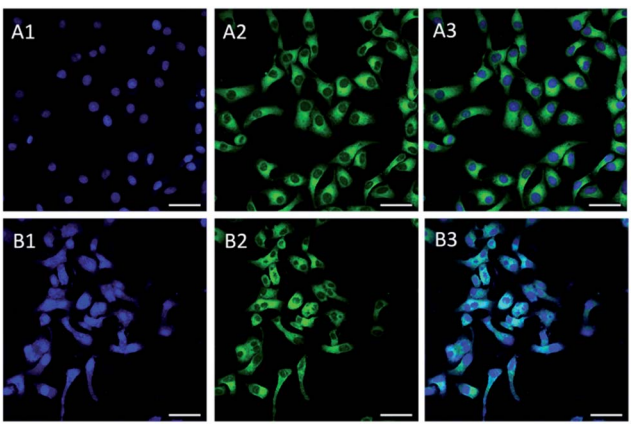

Fig. 3 Fluorescent images of L929 cells. (A and B) L929 cells incubated with Coumarin-6-MCoTI-II and Coumarin-6 for 24 h, respectively. 1, 2, 3 indicated the DAPI, Coumarin-6 and merged channels, respectively. The scale bars represent 50 μm.

In summary, we have successfully demonstrated that cyclotides could adsorb to the liquid–liquid interface and stabilize emulsions despite of the smaller size of cyclotides, *i.e.* ~2 nm. The cyclotides form a single molecular layer capsules which prevents loaded substrates from leakage. Considering the diversified biological properties of cyclotides and other mini-proteins, our discovery will potentially lead to novel approaches in controlled drug delivery or cell imaging applications.

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