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Micelle morphology observation method of lipopeptide by negative-staining-based transmission electron microscopy

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ABSTRACT

Lipopeptides, novel biosurfactants showing versatile promising applications in enhanced oil recovery, textile industry, agriculture and daily chemical products, etc., are profoundly highlighted recently. Surfactin is one of the most typical representatives of lipopetide family. The critical micelle concentration (CMC) of surfactin is as low as 10–20 mg/L. When its concentration reaches above the CMC, different micelle structure will be formed and the surface-active performances might be changed with varied micelle morphologies. Thus, observation of the changes of surfactin micellar form at different concentrations is of great significance for its new applications. But so far, the micelle structure of surfactin (and also other lipopeptide molecules) is not reported yet, and the method for effectively observing the micelle morphology is limited as well. Here, we developed a method based on transmission electron microscopy combined with negative staining to observe the morphology of surfactin micelles, with which we can clearly observe the changes of micelle morphology of surfactin (or other lipopeptides) at different concentrations. Spherical micelles only form when the concentration of surfactin is low. With the increase in concentration, rod-shaped micelles of surfactin can form. Furthermore, complex rod-shaped-micelle-layer and big ring structure will form when the concentration of surfactin is very high.

1. Method details

1.1. Method summary

It is very important to observe the micelle morphology of surfactants such as surfactin in lipopeptide family. New observation method is urgently required. Here we developed a useful transmission electron microscopy method based on negative staining that can successfully observe the micelle structures of surfactin with diverse morphologies. With this method, we clearly observed the micelle morphology of surfactin samples with different concentrations.

1.2. Method description

Surfactin is a lipopeptide family biosurfactant that can spontaneously aggregate and assemble at liquid-solid, oil-water or gas-liquid surfaces or interfaces, thereby reducing their boundary (surface) tension. Due to their good surface/interfacial activity, emulsification and solubilization, surfactin has a wide range of applications in retrieving petrochemicals, the textile industry, the food industry and the production of household chemicals.[1,5–7] When a low concentration of surfactin is dissolved in the system, it is adsorbed on the surface of the solution as single molecules, but as its concentration increases, the surface (interface) is saturated with surfactin and the molecules begin to move toward the main phase inside the solution. Due to the different affinity for water of its polar and nonpolar groups, the nonpolar groups of the molecules attract each other and form micelles.[2] There are various forms of micelles, including spherical, rod-like, and lamellar, etc.

For a surfactant, no matter bio-based or not, the micelle structure is always important for its applications in different fields. For example, spherical or elongated peptide amphiphile (PA) micelles show the potential for targeted diagnostic applications with protein-mimicking functions, while other PAs which form fiber and gels show promise for regenerative medicine applications.[8] Meanwhile, multimicelle aggregates (MMAs) show potential applications in numerous fields such as light-emitting materials and nanoporous materials.[9]

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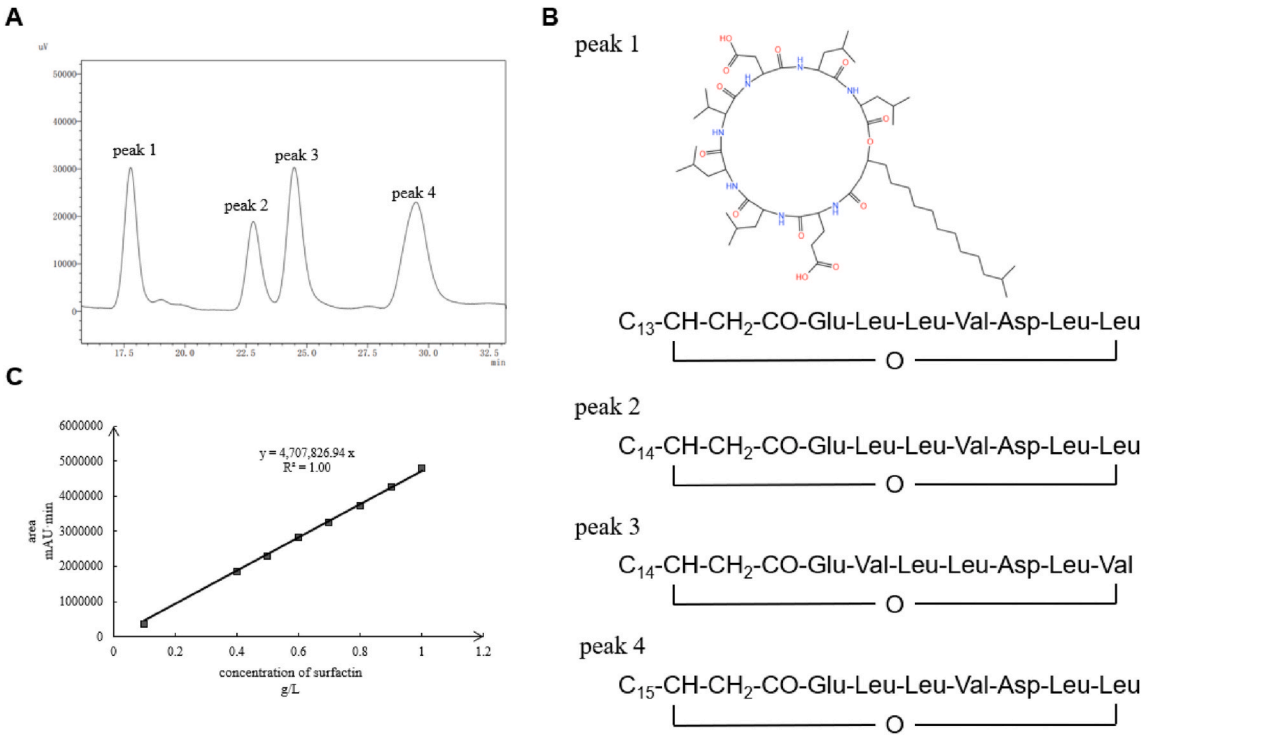

Fig. 1. (A) HPLC analysis of representative surfactin samples containing 4 main components corresponding to the four peaks. A mixture of methanol: H_2O: trifluoroacetic acid = 85: 15: 0.1 used as the mobile phase, and the flow rate is 1 mL/min. The retention time was 40 min, and surfactin was detected under the wavelength of 205 nm. (B) Molecular structure of the 4 main components of sufactin. (C) Standard curve of HPLC analysis. y, surfactin concentration (g/L); x, area (mAU·min).

Several studies have investigated the micelle morphology of surfactin. For example, Knoblich, etc[10] observed micelles of surfactin by an ice-embedding technique and transmission electron cryo-microscopy. Li, etc[11] investigated micelle size distribution and morphology of surfactin by freeze-fracture transmission electron microscopy. Arutchelvi, etc[12] observed the presence of vesicles and large aggregate of surfactin samples by transmission electron microscopy. However, the procedures in these methods are relatively complex in general, not easy to be used and the fine-structures of surfactin micelles can't be not clearly observed as well.

Here we developed a novel transmission electron microscopy (TEM) method based on negative staining for surfactin micelles observation for the first time. Negative staining is used to show the profile of biological samples by placing them in an environment where the electron density is much greater than themselves, which creates a strong contrast by imaging brighter on the photograph due to stronger electron scattering from the environment.[3]

To sum up, the novel TEM observation based on negative staining can clearly show the changes of micelle structure at different surfactin concentrations and provide guidance for the subsequent evaluation of surfactin properties. Here we describe the process for the preparation of surfactin samples with different concentrations and transmission electron microscopy observation based on negative staining.

1.3. Detailed protocol

Step1: preparation of surfactin samples with different concentrations.

1.3.1. Material

- LB medium: tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L
- Fermentation medium: brown sugar 70 g/L, yeast paste 1 g/L, $NaNO_3$ 25 g/L, KH_2PO_4 0.333 g/L, Na_2HPO_4–$12H_2O$ 1 g/L, $MgSO_4$–$7H_2O$ 0.15 g/L, $CaCl_2$ 75 mg/L, $MnSO_4$–H_2O 6 mg/L, $FeSO_4$–$7H_2O$ 6 mg/L, with NaOH to adjust pH to 7.0
- Concentrated medium: brown sugar 350 g/L, $NaNO_3$ 125 g/L
- 1 M isopropyl β-d-1-thiogalactopyranoside (IPTG) stock solution (Solarbio, Beijing, China)
- 50 mg/mL chloramphenicol stock solution (Solarbio, Beijing, China)
- Leucine (Solarbio, Beijing, China)
- Shaking incubator (ZWYR-D2402, ZHICHENG, Shanghai, China)
- Shaking flask
- *Bacillus subtilis* TS1726 that can produce surfactin with high titer (as previously reported)[4,15,16]

1.3.2. Procedure

Note: All operations need to be sterile to prevent contamination.

1. Single colony is picked from LB solid plate and inoculated in LB liquid medium containing 5 mg/L chloramphenicol (10 mL in a 50 mL shaking flasks). Then it is incubated in a shaking incubator at 37 °C and 200 rpm for 12 h culture.
2. The seed is inoculated into the fermentation medium (50 mL in a 500 mL baffle shaking flasks) until OD_{600} is 0.2. Then it is incubated in a shaking incubator at 37 °C and 200 rpm.

For preparation of surfactin in different concentrations.

3. 50 μL of 1 M IPTG solution is added into the flasks at 3 h after the start of the incubation,
4. 0, 0.2, 0.3 or 0.5 g/L of leucine (UV light irradiation for 20 min) is added at the same time (different surfactin concentrations will be obtained with the varied leucine concentration).
5. Continue to incubate in a shaking incubator at 37 °C and 200 rpm for 21 h.
6. 5 mL concentrated medium along with leucine (the titer of which is the same as in procedure 4) is supplemented at 24 h.

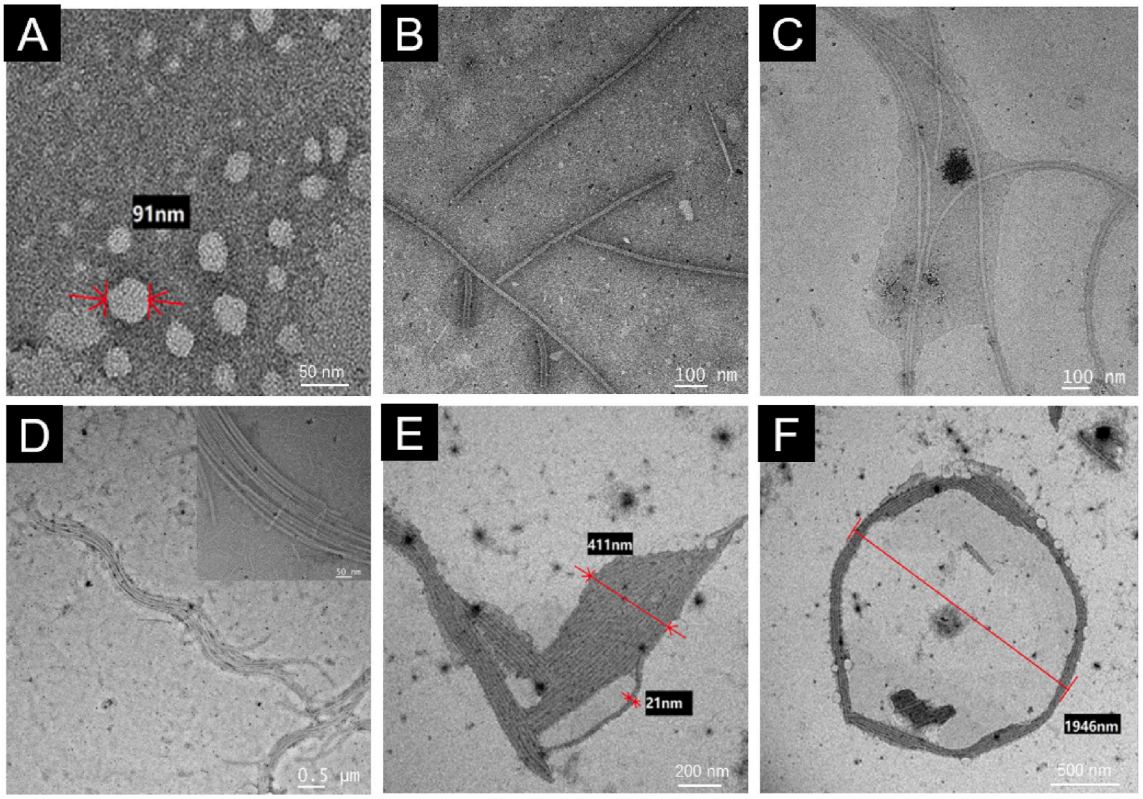

Fig. 2. Images of surfactin at different concentrations. (A) Spherical micelles in 350 mg/L surfactin solution (B) single rod-shaped micelles in 2.9 g/L surfactin solution (C) aggregated rod-shaped micelles in 6.6 g/L surfactin solution (D) composite rod-shaped micelle structure in 18.9 g/L surfactin fermentation solution (E) complex multilayer structure in 27.6 g/L surfactin fermentation solution (F) ring-like structure in 27.6 g/L surfactin fermentation solution.

7. Continue to incubate in the incubator at 37 °C and 200 rpm and for another 24 h.

Step2: measurement of surfactin concentration.

1.3.3. Material

- Different concentrations of surfactin fermentation broth
- Deionized water
- Methanol
- Trifluoroacetic acid
- Sodium Carbonate solution with a mass fraction of 1%
- Surfactin powder with a purity of 85.56% (prepared previously)
- Cryogenic centrifuge (3–18K, Sigma, Shanghai, China)
- LC-20A high performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan)
- ODS-SP column (250 × 4.6 mm, 5 mm; GL Sciences, Kyoto, Japan)
- PES filter membrane (JIN TENG, Tianjin, China)

1.3.4. Procedure

1. Dissolve the surfactin powder in methanol to configure 1 g/L of surfactin standard solution.
2. Dilute the standard solution to concentration gradient of 0.1–1 g/L with sodium carbonate solution to draw the standard curve.
3. The fermentation broth is placed in a 50 mL centrifuge tube, and then centrifuged at 4 °C, 13000 rpm for 10 min and the supernatant is collected.
4. Dilute the supernatant 20-fold with deionized water.
5. The standard solution as well as diluted supernatant is filtrated through PES filter membrane and injected into the HPLC for analysis.[13,14]
6. Determine the concentration of surfactin by the peak area according to the standard curve. The results were illustrated in Fig. 1.

Step3: negative-staining-based TEM observation of surfactin samples.

1.3.5. Material

- Different concentrations of surfactin fermentation broth
- Deionized water
- Sodium phosphotungstate solution with a mass fraction of 1% (Macklin, Shanghai, China)
- Pipette
- Carbon-coated copper grid with carbon support film (Zhongjingkeyi, Beijing, China)
- Filter paper
- Transmission electron microscope
- 50 mL centrifuge tube

1.3.6. Procedure

1. Pipette 1 μL of supernatant collected previously (without dilution) and drop it on the carbon-coated copper grid with a pipette. Then leave it for 2 min.
2. Use filter paper to absorb excess liquid droplets.
3. Pipette 1 μL of sodium phosphotungstate solution and drop it onto the surfactin sample with a pipette, and then stain it for 1.5 min.
4. Use filter paper to absorb excess stain solution.
5. Take 1 μL of deionized water and drop it on the sample with a pipette, and leave it for 1.5 min.
6. Use filter paper to absorb excess liquid droplets to remove the stain.
7. Repeat procedure 5–6 once more.

8. Store the copper grid (loaded with sample) in the dryer overnight at room temperature.
9. Observe the sample with transmission electron microscope.

1.4. Method validation

Negative staining is a common technique when observing biological samples by electron microscopy. Stains containing high atomic number elements (e.g., heavy metals) are wrapped around the sample, and due to the difference in their electron density, the stain is darker in the image while the sample is brighter, thus creating a strong contrast and allowing the morphological features of the sample to be clearly seen.

In this study, we obtained 27.6 g/L, 18.9 g/L, 6.6 g/L, and 2.9 g/L of surfactin fermentation broths by different fermentation strategies, and prepared 350 mg/L standard solution with surfactin powder.

At first, the structure of surfactin micelles in 350 mg/L standard solution was observed. Similar with the previous study,[10] spherical morphology was clearly shown (Fig. 2 (A)), demonstrating the validity of the negative-staining-based TEM.

Furthermore, we observed whether the micelle structures of surfactin in solutions with different concentrations would change. Interestingly, we found that single rod-shaped micelles were mainly present in solutions with relatively low surfactin concentrations (2.9 g/L), as shown in Fig. 2 (B). Two or more (Fig. 2 (C) (D)) rods aggregated to form a composite rod-shaped micelle structure as the concentration increased (6.6 g/L). When the surfactin concentration continued to increase, more rod-shaped micelles aggregated and formed the complex multilayer structures (18.9 g/L, Fig. 2 (E)). Particularly, at a high concentration of 27.6 g/L of surfactin solution, we observed multiple rod-shaped micelles arranged in parallel and forming rings with a diameter of several microns (Fig. 2 (F)). It is an innovative finding that the surfactin micelles form a rod-shaped structure at enhanced concentration, and will further aggregate to compose multi-layer rod-shaped micelles at even-higher concentrations. It's also first to know that clusters of micelles can be further arranged into rings sometimes when the surfactin concentration is extremely high (such as 27.6 g/L).

To sum up, the structure of surfactin micelles was successfully observed using a TEM observation method based on negative staining technique in this paper. We can clearly observe different morphologies of surfactin (lipopeptide) micelles from spherical, single-rod, multi-layer rods to unique-ring structures. We found that only low concentration of surfactin form spherical micelles; and when the concentration increased, the rod-shaped micelles of surfactin can form with several micrometers in length, tens of nanometers in diameter. That is, the structures of surfactin micelles are varied with concentrations. We also found the interesting phenomenon that a complex rod-shaped-micelle-layer or a big ring (in size of 1946 nm) can form when the concentration of surfactin is very high. These micelle structures and variations we observed will provide significant guidance for exploring the performances of surfactin (lipopeptide) for future applications.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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