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Surface and self-assembling properties of cyclic lipopeptide surfactin produced by *Bacillus subtilis*

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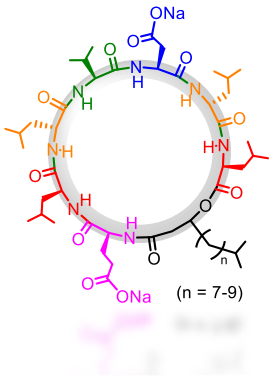
Peptides produced from amino acids are versatile oligomers. When amino acids are organized into oligomers with specific sequences, they provide folded and flexible structures, resulting in wide-ranging functionalities including molecular recognition, self-assembly, catalysis, and more. This review describes surface and self-assembling properties of cyclic lipopeptide (CLP) of surfactin (SF) produced by *Bacillus subtilis* (*B. subtilis*). The sodium salt of SF (SFNa) is regarded as one of the promising surface-active CLPs and exhibits remarkable self-assembly behavior under considerably low concentration. SFNa also have several advantages over conventional petroleum-derived surfactants such as high biodegradability. With regard to its numerous attractive characters, it is presumed that these are owing to the hydrogen bond interactions between the cyclic peptide moieties to form secondary structures.



- *Low CAC*
- *Remarkable self-assembly behavior*
- *Excellent surface tension-lowering ability*
- *Encapsulation of cations*
- *High biodegradability*
- *Low toxicity*

Keyword: *cyclic lipopeptide, surfactin, biosurfactant, self-assembly, micelles*

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Surface and self-assembling properties of cyclic lipopeptide surfactin produced by *Bacillus subtilis*

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1. Introduction

Peptides produced from amino acids are versatile oligomers[1]. When amino acids are organized into oligomers with specific sequences, they provide folded and flexible structures, resulting in wide-ranging functionalities including molecular recognition, self-assembly, catalysis, and more[2,3]. Recent progress in peptide libraries encouraged by both genetic and synthetic methods enabled their application as drugs as well as supramolecular structures in material sciences[3,4]. In this review, recent research findings of surface and self-assembling properties of cyclic lipopeptide (CLP) of surfactin (SF) produced by *Bacillus subtilis* (*B. subtilis*) are presented.

2. Cyclic lipopeptides produced by *Bacillus subtilis*

It is well known that *B. subtilis* produces cyclic lipopeptides (CLPs) as secondary metabolites[5]. The series of CLPs generally consists of the cyclic peptides with different amino acid sequences together with a long alkyl chain. They are produced as a mixture of analogues, and the composition varies depending on conditions, such as strain species and medium compositions[6]. We investigated sixty *B. subtilis* strains for their ability to produce CLPs and found that *B. subtilis* NBRC 109107 produced at least three types of CLPs[7]. High-performance liquid chromatography (HPLC) analysis suggested that these CLPs were thought to be SF, iturin A, and fengycin. They are usually synthetized through a non-ribosomal peptide synthetases (NRPSs). Among them, SF is one of the promising surface-active CLPs (Figure 1)[8-11]. SF consists of a peptide loop, which contains both L- and D-amino acids (L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu), and a fatty acid side chain. There is a commercially available sodium salt of SF (sodium surfactin, SFNa) which is produced by Kaneka Corporation.

3. Surface activities of SFNa

The surface-active CLP of SFNa is regarded as one of the promising biosurfactants[8-11]. SFNa has several advantages over conventional

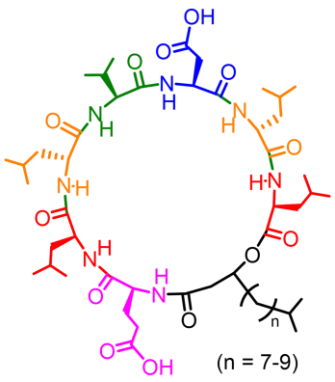

Figure 1. Structure of surfactin (SF).

petroleum-derived counterparts such as high biodegradability. We isolated one strain of *Pseudomonas putida* as a SFNa-degrading bacterium[12]. This strain degraded 1 g/L SFNa below the detectable level of HPLC analysis within 14 days. The high biodegradability of SFNa hold promise for the potential application in microbial enhanced oil recovery[13] or bioremediation[14].

Moreover, owing to its unique molecular structure including a bulky head group and chiral centers, they show remarkable self-assembly. We measured surface tension lowering ability of SFNa by Wilhelmy plate method[15]. The critical aggregation concentration (CAC) of SFNa was estimated to be 2.7×10^{-5} M (Figure 2), whose value is significantly lower than that of sodium dodecyl sulfate (SDS). SFNa can reduce surface tension of water from 72 to 27 mN/m, whose surface tension-lowering ability is significantly higher than that of SDS. Its numerous attractive characters are presumably owing to the hydrogen bond interactions between the cyclic peptide moieties to form secondary structures. When the cyclic peptide of SFNa adopts a horse saddle shape via intramolecular hydrogen bonds, two carboxy groups of L-Glu and L-Asp on the same side of the ring form "claw" configuration[16]. We found that the secondary structures of SFNa are significantly enhanced through increased dissociation of L-Asp and L-Glu as revealed by a titration experiment of SF solution to estimate apparent pK_1 and pK_2 values together with circular dichroism (CD) spectroscopy[17]. The apparent pK_1 and

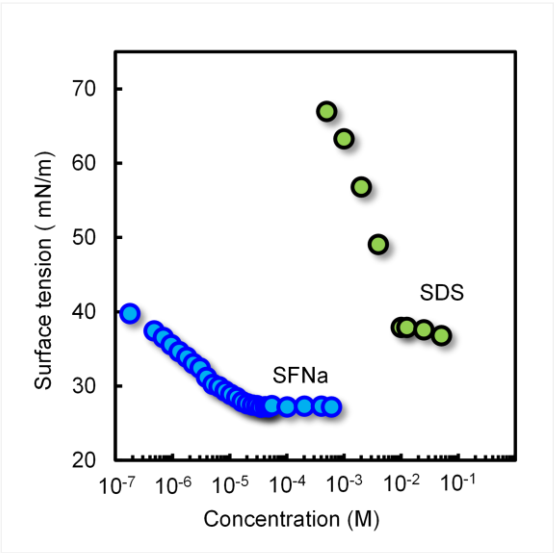

Figure 2. Relationship between surface tension and concentration of SFNa and SDS at 25 °C.

pK_2 of SF were estimated to be 5.8 and 6.8, respectively. Additionally, we conducted a titration in water; complete dissolution of SF was observed when 1.5–2.0 equivalents of NaOH were added (pH > 6.9). The apparent pK_a values were higher than those of related amino acids (pK_a for Asp and Glu are expected to be around 3.4 and 4.2, respectively) as well as β-peptides (pK_a for Asp and Glu are expected to be around 4.3 and 4.5, respectively)[18]; however, they are similar to that of SF at an air–water interface (pK_a around 6)[19]. These results indicate that the dissociation of the two acidic amino acids of SF is suppressed because of their incorporation into the cyclic peptide and/or location at the air–solvent interface. The secondary structure formation enhanced with increasing pH as well as counter cations, obviously because of enhancement of intermolecular hydrogen bond interactions.

The critical aggregation concentrations (CACs) of SF with different counter cations

such as potassium (SFK), monoethanolamine (SFMEA), diethanolamine (SFDEA), and triethanolamine (SFTEA) were 2.8–4 times higher than that of SFNa[20]. Furthermore, the CACs increased as the organic counter-cations became less hydrophobic (SFTEA (8.7×10^{-5} M) < SFDEA (9.8×10^{-5} M) < SFMEA (1.1×10^{-4} M)) as shown in Figure 3(a) and (b). In addition, CD measurements revealed that a negative band corresponding to β-sheets increased with increasing organic counter cation hydrophobicity (SFMEA < SFDEA < SFTEA) (Figure 3(c)). These results clearly indicate that intermolecular hydrogen bond interaction promote self-assembly of SF in aqueous solution.

4. Self-assembling properties of SFNa

Dynamic light scattering (DLS) measurement of an aqueous solution of SFNa (1 mM) revealed that SFNa spontaneously forms giant micelles[15]. Based on the histogram, the size distribution of SFNa micelles shows a peak top around 300–500 nm. The giant micelles of SFNa can be separated by ultrafiltration technique. The amount of SFNa in both retentates and permeates were measured using HPLC. The giant micelles of SFNa were recovered quantitatively and detected mostly in the retentate (> 99%). Knoblich et al. reported that the morphology of surfactin micelles is significantly affected by external conditions such as pH, metal ions, and temperature by cryo-transmission electron microscopy (cryo-TEM)[21]. Ishigami et al. reported the formation of

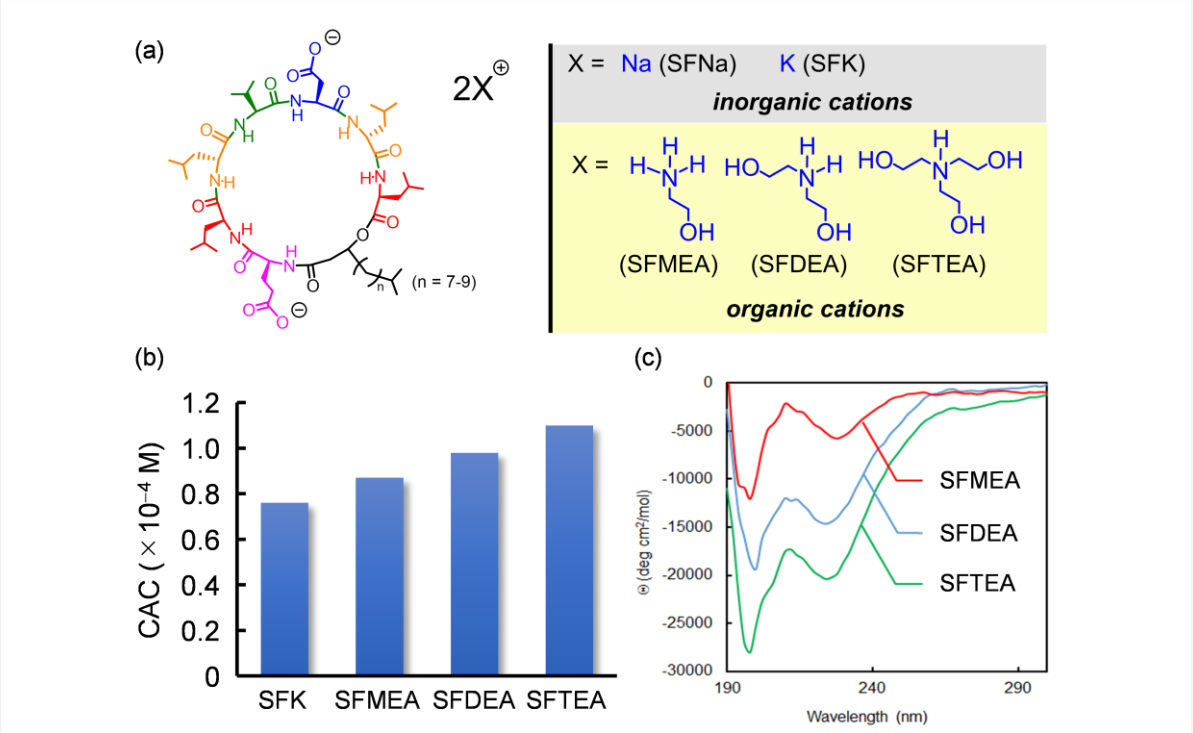

Figure 3. (a) Structures of SF salts with inorganic and organic counter-cations. (b) CAC values of SFK, SFMEA, SFDEA, and SFTEA. (c) CD spectra of SF-MEA, SF-DEA, and SF-TEA in water (0.25 mM, 25 °C).

large rod-shaped micelles[22]. The small-angle x-ray scattering (SAXS) measurement revealed that presence of small micelles in water[23]. Consequently, morphologies of SF micelles change depending on the external conditions, which are different from those of synthetic surfactants.

Another unique feature of SFNa is to form various lyotropic liquid crystals[24]. Such self-assembling behavior in aqueous solution with relatively high concentrations is also crucial not only for the application of SFNa in various fields, but also to understand its role in a biological system. Bellow 50 wt%, SFNa provided transparent and less-viscous aqueous solutions, indicating the formation of micellar phases (Figure 4). By polarized light microscopy, the aqueous solutions of SFNa at the concentrations above 50 wt% were optically anisotropic and gave mosaic textures (Inset of Figure 4). SAXS diffractograms of the SFNa solution above 50 wt% clearly gave the three peaks whose spacing ratio of 1:2:3, indicating the presence of the lamellar phase.

We reported the molecular interactions of monolayers composed of cyclic and linear forms of SF, which have the same peptide sequence and alkyl chain, but different topology[25]. Atomic force microscopic (AFM) images of the monolayers revealed phase-separated microdomain structures, indicating that the cyclic and linear SFs are nearly immiscible. These results indicated that topological features of SF are crucially important for self-assembling systems: the restriction of conformational freedom of cyclic forms of

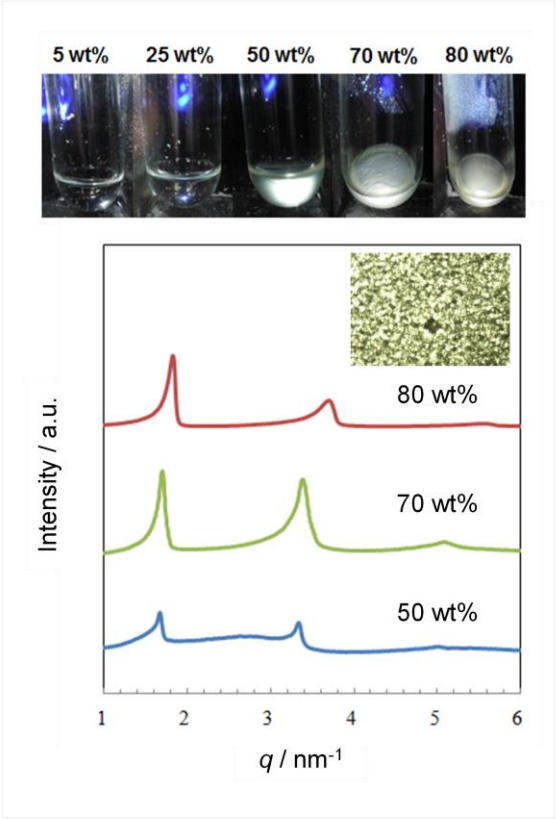

Figure 4. (top) Visual observation of aqueous solutions of SFNa at different concentrations under cross polarized light. (bottom) SAXS patterns of lyotropic liquid crystal of SFNa. Inset represents a mosaic texture of 80 wt% of SFNa.

SF allows the molecules to specifically recognize each other.

5. Encapsulation properties of SFNa

Cyclic peptides are known to act as ionophores. SF is expected to be a useful as a novel sorbent for large monovalent Cs^+ because the heptapeptide moiety of SF provides an ideal coordination number for Cs^+ (between 6 and 8). The cavity diameter (4–7 Å) is larger than that of the ion (3.38 Å), making it suitable for Cs^+ encapsulation. SF also has two carboxylate groups ($R\text{-}COO^-$) that bind Cs^+ to give a neutral salt (RCOOCs) through a

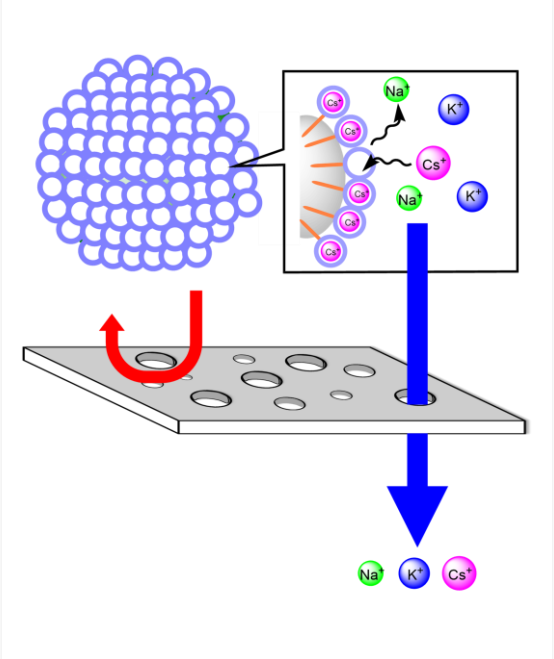

Figure 5. Cartoon representation of Cs^+ removal from water using the micellar system of SF.

similar mechanism as polyether ionophores. Moreover, SF forms giant micelles in aqueous solution, with the heptapeptide moieties aligned on the surface. High binding affinity and effective removal of Cs^+ can thus be achieved through micellar-enhanced ultrafiltration (MEUF), a low-energy separation process combing entrapment by surfactant micelles and ultrafiltration (Figure 5)[15]. We found that SF micelles selectively encapsulate Cs^+, which was suggested by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS), together with nuclear magnetic resonance (NMR) and Fourier transform infrared (FT-IR) spectroscopy. A highly effective separation of Cs^+ immobilized on the surface of the SF micelles was also achieved through facile centrifugal ultrafiltration in 91% even in coexisting with other alkali metal ions such as Na^+ and K^+. Thus, the use of the giant micellar system of SF with its high Cs^+ affinity and distinctive assembling properties would be a new approach for the treatment of contaminated soil and water.

Mixing SFNa with alkylammonium bromides with different alkyl chain lengths leads to form vesicles and provides stable colloidal suspensions[20]. When the approximate effective ionic radii of the cyclic peptide moiety of SF and the ammonium moiety are considered, SFNa and didodecyldimethylammonium bromide (C_{12}DMA-Br) can be regarded as having cone and inverse truncated cone shapes, respectively. In the case of SFNa alone, which is a single-chain anionic surfactant with a large head group (cone shape), a transparent micellar solution was formed. In contrast, as an inverse truncated cone-shaped surfactant with a small head

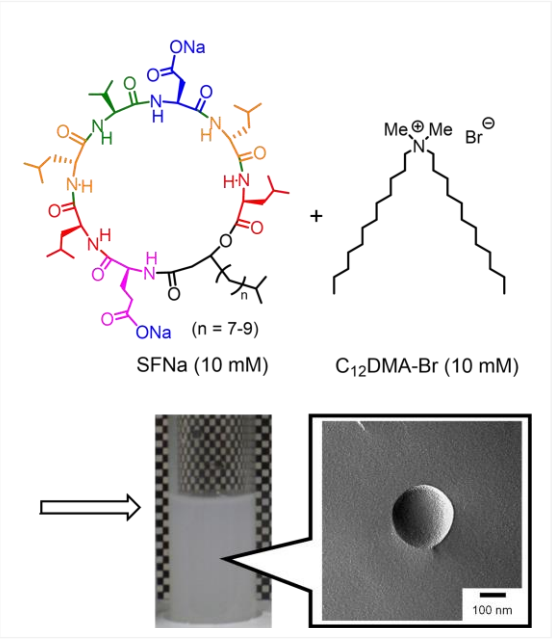

Figure 6. Photograph of the colloidal suspension of the mixture of SF-Na and C_{12}DMA-Br. freeze-fracture transmission electron microscopy (FF-TEM) images of the colloidal solution are also shown.

group and a double chain, C_{12}DMA-Br gave a colloidal suspension. Generally, anionic surfactants with steric bulky organic counter-cations exhibit decreased water solubility and form precipitates, which can hamper industrial applications. In contrast, SFNa, which has a bulky cyclic peptide moiety, provides open space at the air/water interface, which can mitigate the negative impact of organic counter-cations including bulky double-tailed cationic surfactants that give vesicles in water without any precipitation. The combination of SFNa as an environmentally friendly anionic surfactant with organic counter-cations may provide ideal formulations for cosmetic and pharmaceutical applications with reduced irritant potential and enhanced mildness and comfort.

6. Applications

As described above, changes in the secondary structure of SFNa induced by increased pH. We found that the secondary structure formation of SF is significantly enhanced through increased dissociation of L-Asp and L-Glu. We also studied the activity of the common detergent enzyme subtilisin in SFNa solution at above its pK_2 (pH 7.6) to understand the role of the dissociation degree in the interaction with the protein[17]. The mixing of SFNa with subtilisin suppressed the decrease in protease activity observed in the presence of synthetic surfactants such as SDS and polyoxyethylene alkyl ether. Thus, SF has great potential for use in laundry detergent formulations, to improve the stability and reliability of detergent enzymes.

SFNa also shows remarkabke emulsifying activities against cyclosiloxane oils as octamethylcyclotetrasiloxane (D4) and decamethylcyclopentasiloxane (D5) which are regarded as causative agents for pollution from silicon sealants[26]. Generally, removing cyclosiloxanes is troublesome due to their super-hydrophobic characters. Thus, SFNa would contribute to application for the environmentally friendly surfactant for various wastes in industry.

7. Conclusions

In this review, the surface and self-assembling properties of SFNa produced by *B. subtilis* are described. SFNa having a cyclic heptapeptide moiety allows the molecules to recognize each other specifically, via hydrogen bond interactions. Thus, SFNa exhibits remarkable self-assembly behavior under considerably low concentration. SFNa self-assembled to form giant micelles which can be separated by ultrafiltration technique. Another unique feature of SFNa is formation of lamellar liquid crystals at high concentrations. Together with the numerous attractive characters, inner space of the cyclic peptide can be applicable for encapsulation of cations such as Cs^+.

SFNa has also several advantages over typically used synthetic surfactants, such as low toxicity and high biodegradability. Because of the environmental compatibility, SFNa can be applicable for various industrial applications, e.g., in cosmetics, food additives, enhancement of oil recovery, and detergent formulations.

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