Interfacial Films Formed by a Biosurfactant Modularized with a Silken Tail

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ABSTRACT

This paper reports the dynamic interfacial behavior of a new interfacially-active peptide AM-S which was designed based on a peptide surfactant AM1 modularized with an additional silk-derived hydrophobic tail to enhance anchoring to air–water interfaces. AM-S peptide shows a random coil conformation in bulk solution similar to AM1 as determined by circular dichroism spectroscopy, which facilitates rapid adsorption at the air–water interface, reducing interfacial tension from 72 to 52 mN/m within 300 s at a low concentration of 10 μ M. Although the interfacial films formed by AM-S demonstrated low tensile stress as compared to AM1, the AM-S films in the presence of Zn(II), but not in its absence, show significant resistance against compression, as peptides were unable to desorb quickly under the compression conditions imposed by the Cambridge interfacial tensiometer (CIT). These results indicate that AM-S peptides tend to undergo a multilayer adsorption at the interfaces, in contrast to AM1 peptide that only forms an interfacial monolayer, demonstrating a distinct physical effect of the silk tail. The multilayer structure of AM-S in the presence of Zn(II) was also apparent on a thin-film pressure balance experiment. The thin films formed by AM-S peptide were thicker than the films formed by AM1 peptide, thus enabling stabilization of the films against increased critical air pressure as well as the self-assembly of the AM-S peptides. Also, AM-S peptide was shown capable of forming dense foams with small bubble size and maintained foam stability comparable with AM1 peptide. This study demonstrated that addition of a silk tail peptide to a biosurfactant can significantly modify interfacial adsorption behavior at a fluid–fluid interface, which may guide further molecular design strategies.

INTRODUCTION

Biomolecular self-assembly has emerged as an attractive bottom-up approach to develop nanostructured materials with novel functional properties.¹⁻³ Peptides are short informational polymers made up of amino acids, and are increasingly viewed as the key building blocks for such self-assembled materials⁴⁻⁷ owing to their biocompatibility, sustainability, and ease of functionalization, coupled with facile methods for their economic manufacture.⁸ Peptide self-assembly has been extensively explored in bulk solutions to synthesize various supramolecular structures including nanotubes, nanovesicles, and nanofibers.⁹⁻¹² Other studies have aimed at self-assembly of peptides at the boundary between solid and liquid for various applications including as nanosensors and nanocircuits.¹³⁻¹⁵ However, there has to date been limited research into the control of peptide self-assembly at fluid–fluid interfaces despite the huge interest in controlling emulsions and foams for application in pharmaceuticals, foods and beverages, personal care, oils and minerals.¹⁶⁻¹⁸ Enabled by the diversity of the twenty naturally-occurring amino acids, there is a large sequence and structural solution space for design, yet the dynamic behaviors of soft interfaces covered with peptides remains relatively less-well studied.

Interfacial self-assembly of peptides is dictated by their amphiphilic nature and is driven by non-covalent interactions, including electrostatic and hydrophobic interactions, van der Waals forces, hydrogen bonds, π – π stacking, etc. Such peptides can originate from nature, for example, bacterial lipopeptides and partially digested protein hydrolysates such as surfactin¹⁹ and casein.²⁰ *De novo* design of peptides is widely used as a versatile approach to produce amphiphilic peptides as it enables fine tuning of hydrophobicity and hydrophilicity, and allows for distinct molecular structure based on the knowledge of individual amino acid secondary structure propensities. One class of such peptides is the surfactant-like peptides that are designed based on conventional head–

tail surfactants.²¹ The hydrophilic-head groups can be negatively or positively charged amino acids, while the hydrophobic-tail groups can comprise either hydrophobic amino acids²²⁻²³ or hydrocarbon alkyl groups²⁴⁻²⁷ having a specific chain length. Interfacial studies involving surfactant-like peptides have mainly focused on monolayers and Langmuir–Blodgett films. Recently, the utility of self-assembled peptide surfactants has been expanded to involve stabilization of emulsions.²⁸ The peptides stabilizing emulsions were designed to have a β -strand structure, and the length was kept short (9 amino acid residues) to avoid the formation of self-assembled β -sheet complexes in bulk solutions that decelerate their adsorption at soft interfaces.

A 21-residue peptide AM1 (Ac-MKQLADS LHQLARQ VSRLEHA-CONH₂) was designed capable of self-assembly at oil–water and air–water interfaces hence enabling stabilization of emulsions and foams, respectively, through the formation of a strong interfacial film.²⁹ Different from the peptide amphiphiles aforementioned, hydrophobic amino acid residues within AM1 (methionine (M), valine (V) and leucine (L)) are spatially separated by an alternate three- and four-residue to allow the formation of α -helical (3.6 residue per turn) peptide structures having distinct hydrophobic and hydrophilic faces at the interfaces. In this way, no distinct block co-polymer type structure is evident in the primary (linear) sequence, yet folding of the structure at the interface reveals the encoded amphiphilicity by allowing for distinct partitioning of the hydrophilic and hydrophobic residues onto different faces of the helix. This approach confers excellent properties of inhibited self-assembly in bulk (allowing for high diffusional rates) while also ensuring high interfacial activity. The facial amphiphilicity of AM1 at the soft interfaces can be significantly enhanced by the addition of transition metal ions such as Zn(II) which bind to histidine (H) residues forming intermolecular cross-linking and hence cohesive interfacial films.³⁰ Such strong interfacial films are also known to form upon adsorption of surface-active macromolecules such

as proteins³¹⁻³³ and polymers³⁴ at fluid–fluid interfaces. Moreover, the peptide film of AM1 can be destabilized in a stimuli-responsive manner by the addition of either a chelating agent or a pH change to break the interfacial peptide networks³⁵ which, in turn, causes emulsion coalescence or foam collapse within seconds.²⁹ Another advantage offered by the designed peptide surfactant relative to some other classes of peptide surfactants is the ability to be produced renewably and cost-effectively through integrated synthetic biology and bioprocess engineering.³⁶⁻³⁸

In this study, AM1 peptide was used as a basis for the design of a new peptide surfactant AM-S by adding a silken-tail peptide³⁹ derived from *Bombyx mori* silkworm fibroin⁴⁰ at the C-terminal AM1 that was linked through proline–serine residues. Thus, AM-S peptide surfactant was expected to synergistically combine the aforementioned merits of its parent sequence AM1 (i.e., high interfacial activity and controllable interfacial mechanics through metal-ion mediated cross-linking between histidine residues) and strong anchoring capacity of the silken tail at air–water interfaces, while its overall short peptide-chain would ensure high diffusional rate towards the interfaces. These combined properties would enable AM-S peptide surfactant to rapidly adsorb onto air–water interfaces with its hydrophobic residues at both AM1 and silken-tail modules strongly anchoring to the air phase and its hydrophilic residues projecting toward the bulk aqueous phase, providing interfacial stability. Self-assembly of the peptides at the air–water interface was compared to provide a better understanding of the link between peptide interfacial behavior and molecular design. A unique apparatus, the Cambridge Interfacial Tensiometer (CIT),^{31-33,41} was used to directly investigate the response of interfacial peptide films at the air–water interface to uniaxial extension and compression cycles, thereby measuring the force transmitted laterally in the plane of the interface. Furthermore, dynamic interfacial tension and thin film pressure balance were used to provide an overview of peptide self-assembly at the macroscopic interface. Finally,

practical application of the peptides was tested for the effective formation and stabilization of foams.

EXPERIMENTAL SECTION

Materials. All reagents and chemicals were of analytical grade purchased from either Sigma-Aldrich (Castle Hill, Australia) or Merck (Bayswater, Australia) and used as received unless otherwise stated. Water with $>18.2 \text{ M}\Omega \text{ cm}$ resistivity was obtained from a Milli-Q system with a $0.22 \mu\text{m}$ filter (Merck Millipore, Merck KGaA, Darmstadt, Germany). All glassware used to hold peptide solution was (i) soaked in detergent solution (1% (v/v) Decon-90, Decon Laboratories, Hove, UK); (ii) rinsed with 6 volumes of water; (iii) soaked for 15 mins in freshly prepared piranha solution (equal parts of 30% (v/v) H_2O_2 (Rowe Scientific, Sumner, Australia) and 98% (v/v) H_2SO_4 (Chem-Supply, Gillman, Australia); and (iv) rinsed with 10 volumes of water. Peptides AM1 (M 2473 Da, pI 8.54) and AM-S (M 3262 Da, pI 8.38) with a purity of $>95\%$ were synthesized by GenScript Corporation (Piscataway, NJ). According to the manufacturer, individual peptides were prepared from 2-chlorotrityl chloride resin by well-established solid-phase peptide synthesis protocols using Fmoc chemistry.⁴²⁻⁴⁵ Coupling reactions were performed using N,N' -diisopropylcarbodiimide/1-hydroxybenzotriazole in dichloromethane. Fmoc deprotection processes were carried out at N -terminal Fmoc-group, and subsequently at side-chain protection group, using piperidine in dimethylformamide. To purify peptides, a preparative reversed-phase high-performance liquid chromatography (RP-HPLC) equipped with an AlltimaTM C18 column ($4.6 \text{ mm} \times 250 \text{ mm}$) with mobile phase A, 0.065% v/v trifluoroacetic acid (TFA) in water, and mobile phase B, 0.05% v/v TFA in acetonitrile, was used. Elution gradient was increased from 5 to 65% B in 60 min, and a monitoring wavelength was set at 220 nm. At these conditions, AM1 or AM-S peptide molecules were eluted from RP-HPLC at retention time 20.8

or 21.1 min, respectively. Bulk peptide was dissolved in Milli-Q water at a known weight concentration, aliquoted into acid-cleaned vials, and lyophilized prior to storage at -20°C . The lyophilized samples were subjected to a quantitative amino acid analysis assay (Australian Proteome Analysis Facility, North Ryde, Australia) to determine the peptide content before generating a standard peptide-concentration curve *via* a reversed-phase high-performance liquid chromatography. HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid) was used as the buffer due to its low binding affinity to metal ions.

Circular Dichroism (CD) Spectroscopy. CD spectroscopy (Jasco 810, Easton, MD) was performed for peptides AM-S and AM1 (100 μM in 1 mM HEPES buffer pH 7) filled in a 1 cm-path-length quartz cell at 20°C . Far-UV CD spectra were recorded from 260 to 190 nm using 0.1 nm data pitch, 50 nm/s scan speed, 2 s response time, 1 nm bandwidth, and 10 accumulations. The obtained raw ellipticity was converted to molar ellipticity:⁴⁶

$$[\theta] = \frac{\theta \times M_{\text{mrm}}}{10 \times c \times l}$$

where $[\theta]$ is molar ellipticity ($\text{deg} \cdot \text{cm}^2/\text{dmol}$), θ is raw ellipticity (mdeg), c is peptide concentration (mg/mL), l is cell path length (cm), and M_{mrm} is mean residue molecular mass (mg/mmol) i.e., peptide molecular mass/number of residues.

Air-water interfacial tension. Air–water interfacial tension was measured using a Krüss Drop Shape Analysis System DSA-10 (Krüss GmbH, Hamburg, Germany). A peptide solution (8 mL) in HEPES buffer (25 mM, pH 7) in the absence or presence of ZnCl_2 , which was added at twice the molar concentration of the peptide, was filled in an 8-mL quartz cuvette (Hellma GmbH, Müllheim, Germany). An air bubble (ca. 10 μL) was formed manually *via* a glass syringe

connected to a U-shaped stainless steel capillary ($d = 1.507$ mm) submerged in the peptide solution. Measurements of the interfacial tension were made automatically over 300 s based on the changes in the bubble shape following initial air bubble formation. Prior to measurement, the interfacial tension of an air bubble in the buffer was measured at approximately 72 mN/m and remained constant for at least 10 min to ensure correct functioning, focusing, and cleanliness of the instrument.

Cambridge Interfacial Tensiometry (CIT). The tests for mechanical properties of peptide films were performed at the air–water interfaces as previously described^{29,47-48} using the CIT.^{31-33,41} Peptide solution (6.5 mL) in HEPES buffer (25 mM, pH 7) in the absence or presence of ZnCl₂, which was added at twice the molar concentration of peptide, was filled into a poly(tetrafluoroethylene) trough ($80 \times 20 \times 3$ mm³) (Nima Technology, Coventry, UK) to form a meniscus (ca. 1 mm) above the trough edge on which two identical fiber optic T-bars were placed at the air–water interface ($d = 0.25$ mm, $l = 50$ mm, separation = 1 mm). The peptide was allowed to stand undisturbed for 1 h to permit self-assembly of a cohesive peptide film. To measure the forces transmitted laterally through the film in a minimally invasive manner, the CIT was operated in a cyclic mode. One T-bar attached to a piezoelectric motor was moved at a constant displacement speed to strain the film to 5% and subsequently to 300%, and the force transmitted laterally through the interfacial film was detected by a highly sensitive force transducer connected to the second T-bar. The direction of travel was then immediately reversed to perform the compressive section of the test. The slope of the stress–strain curve from 0 to 1% strain was used to determine a two-dimensional interfacial modulus of elasticity, while the maximum interfacial stress was registered from the 300% strain cycle.^{31-33,41}

Thin Film Pressure Balance. The thin film pressure balance technique⁴⁹ was used to determine the surface forces of a free-standing horizontal liquid foam film. A peptide solution in HEPES buffer (25 mM, pH 7) in the absence or presence of ZnCl₂ at twice the molar concentration of the peptide, was used to stabilize the films. The films were formed using a microfabricated bike-wheel microcell, designed for uniform radial drainage, with a diameter of the central hole inside the microcell of 0.75 mm. A vapor-saturated chamber inserted with the film holder was placed on an inverted Olympus IX51 microscopic stage (Olympus Australia Pty Ltd, Notting Hill, Australia). Temperature was maintained at 22 ± 0.1 °C using a water-jacket, and the air pressure inside the chamber was controlled by using a 100 mL SGE gas-tight syringe driven by a M4454 micropositioner (Olympus Controls, New York, NY) as described by Wang and Yoon.⁵⁰ To record the images of the films during the increased pressure, a digital video camera was used at a rate of 30 frames per second. Upon film rupture, a critical air pressure for film rupture was reported, which despite being instrument dependent, serves the purpose of allowing comparison between the systems reported here.

Foam Formation. Foams were formed using custom-built glass-foaming apparatus as previously described.^{47,51} The apparatus consists of a glass tube ($l = 10$ cm, $d = 6$ cm), open at the top, and fitted with a porous glass frit at the bottom. Below the glass frit is an air inlet connection and a valve for draining liquid from the tube. The air inlet was connected by plastic tubing to an air filled 60 mL-syringe mounted on a syringe pump (Pump 11 Plus, Harvard Apparatus, Holliston, MA). To form a foam, 1 mL of 100 μ M solution of the peptide in HEPES buffer (25 mM, pH 7) with or without 200 μ M ZnCl₂ was pipetted onto the glass frit through the top opening in the tube. A total of 7 mL of air was bubbled through the liquid at a rate of 20 mL/min. Photographs of the foam formed in the apparatus were taken at timed intervals to monitor foam quality and stability.

RESULTS AND DISCUSSION

Peptide Design. In this study, peptide AM-S (Ac-MKQLADS LHQLARQ VSRLEHA PS GAGAGAGY-CONH₂) was designed by combining the sequence of the peptide surfactant AM1 (MKQLADS LHQLARQ VSRLEHA)²⁹ with the octapeptide (GAGAGAGY) derived from *Bombyx mori* silkworm fibroin known for its high structural integrity³⁹⁻⁴⁰ through a linker (PS). The self-assembly behavior of peptide AM-S at air–water interface was tested and compared to peptide AM1. Both AM1 and AM-S peptides were acetylated and amidated at the amino- and carboxy-termini to minimize charge–charge interactions between adjacent peptide termini. Peptide AM1 was previously designed in our group to comprise three heptads in which each heptad comprises two hydrophobic (at position 3 and 4) and five hydrophilic residues. This classic motif is known to form a peptide secondary structure of an α -helix that is stabilized upon interfacial adsorption and hence is energetically favorable.^{14,52-54} Neutron reflectometry study confirmed that AM1 molecules undergo self-assembly to a monolayer at the fluid–fluid interfaces, enabling the hydrophobic residues to orient toward hydrophobic phase (e.g., air, oil) and the hydrophilic residues to face the aqueous phase.³⁵ In the presence of transition metal ions (e.g., Zn(II), Ni(II)), two histidine (H) residues within an AM1 peptide molecule at the interfaces form metal-binding coordination, thus cross-linking, laterally to histidine residues from a neighboring adsorbed AM1 peptide molecule.³⁰ This bridging allows formation of a peptide-film network surrounding the interfaces enabling the stabilization of either emulsion droplets or foam bubbles.²⁹

The AM1 sequence was included in AM-S to impart facial amphiphilicity and hence interfacial activity.²⁹ The octapeptide composed of an alternate hydrophobic amino acid residue alanine (A) in the AM-S peptide was incorporated aiming to create a hydrophobic amino acid tail, in addition to the hydrophobic residues of AM1 within AM-S, enabling the peptide AM-S to further strengthen

its interfacial affinity toward a hydrophobic phase. The linker that connects the AM1 sequence and the octapeptide comprises proline (P) and serine (S) residues ensuring helix termination whilst providing rotational freedom.⁵⁵⁻⁵⁶

Peptide Conformations in Bulk Solution. Circular dichroism (CD) spectroscopy was carried out to determine the secondary structure of the peptides AM1 and AM-S in bulk solution (Figure 1). Low buffer concentration (1 mM) was employed to improve the signal-to-noise ratio. CD spectra of AM1 in the absence and presence of Zn(II) are dominated by a negative peak at 200 nm with a slight negative peak at 225 nm typical of a mainly random coil conformation (Figure 1A). Although AM1 sequence contains an α -helical characteristic with its 4-3 hydrophobic repeat,⁵⁷⁻⁵⁸ AM1 peptides at low bulk concentration were in the form of random coil monomers which undergo self-assembly to α -helical coiled-coil tetramers upon increasing its concentration, with a 50% α -helical content of AM1 achieved at a bulk concentration of 793 μ M.³⁰ Note that this random coil conformation converts to helical following adsorption at the interface.^{14,59} CD spectra of AM-S show a dominant negative peak at 200 nm and a slight negative 225 nm-peak suggesting a random coil conformation similar to AM1 (Figure 1B). The bulk conformation of AM-S was not significantly influenced by the presence of Zn(II).

Figure 1. Circular dichroism spectra of peptides (A) AM1 and (B) AM-S. Spectra were recorded for 100 μM peptide in 1 mM HEPES buffer pH 7 in the absence or presence of 200 μM ZnCl_2 .

Peptide Adsorption at Air–Water Interface. Air–water dynamic interfacial tension data were recorded using a Drop Shape Analysis tensiometer at low concentrations of peptides, i.e., 10 and 20 μM in HEPES buffer (25 mM, pH 7) in the absence and presence of ZnCl_2 (Figure 2). The rapid adsorption of AM1 resulted in the quick drop of the interfacial tensions within the first 2 s, and reached at a plateau of 46.4 ± 0.4 and 49.4 ± 0.3 mN/m by 300 s for 10 and 20 μM , respectively, in the absence of Zn(II) (Figure 2A). The adsorption kinetics were not significantly affected by the presence of Zn(II) as equivalent final values of interfacial tensions of 47.9 ± 0.6 and 48.8 ± 0.3 mN/m were attained for 10 and 20 μM AM1 peptide, respectively (Figure 2A). The rapid

interfacial adsorption of AM1 was due to the unstructured nature of the AM1 monomer in bulk (Figure 1A) hence unconstrained to conformational barriers to adsorption. Middelberg et al. showed that the interfacial adsorption of tetrameric peptide such as Lac28 was slower than AM1 because of an additional energy barrier to adsorption related to the dissociation of Lac28 tetramers prior to adsorption.⁵²

AM-S at 10 and 20 μ M in the absence of Zn(II) achieved slightly higher interfacial tensions of 51.9 ± 1.1 and 51.9 ± 0.7 mN/m, respectively, at 300 s (Figure 2B). Similar to AM1, the presence of Zn(II) did not have a substantial effect on the interfacial tensions of AM-S. In comparison with AM1, the decrease of AM-S's interfacial tension at an earlier time point is slower indicating a higher energy barrier to adsorption. As AM1 and AM-S possess similar charge at neutral pH, the energy barrier is probably caused by the higher molecular weight of AM-S than AM1 which slowed down the AM-S diffusion from bulk to the interface and its following adsorption.

Figure 2. Air–water interfacial tension of peptides (A) AM1 and (B) AM-S in 25 mM HEPES pH 7 with different concentrations in the absence and presence of ZnCl_2 .

Mechanical Properties of Interfacially-Adsorbed Peptide. The mechanical properties of interfacial layers of peptides formed at the air–water interface in the absence or presence of ZnCl_2 were determined by the CIT that measured the force transmission within the plane of the interface as a function of strain. The interfacial elastic moduli as a function of time for the adsorbed peptides AM1 and AM-S were obtained as the gradient of the line of best fit through measurement of the interfacial stress response over the first 1% strain (Figure 3). The interfacial elastic modulus for 10 μM AM1 in the absence of Zn(II) was 27 mN/m; doubling the concentration of AM1 did not significantly increase film elasticity as it only reached 30.5 mN/m (Figure 3A). However, the

addition of Zn(II) in AM1 solution (20 μ M) increased the interfacial elastic modulus dramatically by approximately 544% to 166.1 mN/m. This substantial increase demonstrates the formation of metal-mediated lateral structuring of AM1 peptides at the interfacial layer which enabled the peptide interfacial network to transmit a significant lateral force between the T-pieces of the CIT on movement of the motor T-piece. The ability to respond to the CIT kinetic movement reflects the high degree of AM1 peptide organization and local concentration at the interface, generated through favorable histidine–Zn(II) binding complexes between the interfacially-adsorbed AM1 peptide molecules. On the other hand, the interfacial elastic moduli of AM-S peptide layers increased slowly over the aging period with readings of 10.5 and 10.8 mN/m for AM-S concentrations of 10 and 20 μ M, respectively. Supplementing Zn(II) in the bulk solution of AM-S peptide only increased the interfacial peptide moduli to 32.2 and 35.7 mN/m for 10 and 20 μ M AM-S, respectively. The interfacial layers formed by AM-S in the presence of Zn(II) yields interfacial elastic moduli close to AM1 without Zn(II), suggesting that the interfacial layers of AM-S peptides in the presence of Zn(II) at the concentration tested lack the ability to transmit a laterally applied force due presumably to a lack of long-range connectivity between the adsorbed AM-S molecules at the interface.

Figure 3. Dynamic interfacial elastic modulus of peptides (A) AM1 and (B) AM-S at different peptide concentrations in 25 mM HEPES pH 7 in the absence and presence of ZnCl₂ as determined by the Cambridge Interfacial Tensiometry (CIT).

Figure 4 shows the stress–strain curves of interfacial peptide films after self-assembly for 1 h using different peptide concentrations in the absence or presence of ZnCl₂. In the presence of Zn(II), the maximum stress transmitted through the interfacial film of AM1 20 μ M increases from 0.8 to 4.1 mN/m (Figure 4A), demonstrating the effect of Zn(II) on the increased level of AM1–AM1 interfacial network during the tensile phase of the stress–strain test. Interestingly, the interfacial stress of 20 μ M AM1 peptide in the presence of Zn(II), but not in its absence, presents an increased sharp peak at 6%-strain followed by a region where the interfacial stress decreased

rapidly. Such behavior suggests brittle fracture and subsequent reforming, by re-adsorption of peptides from the bulk solution, of the highly cross-linked network.⁴¹ On the other hand, the maximum interfacial stress of AM-S 20 μ M increases from 0.5 to 1.8 mN/m when Zn(II) was added (Figure 4B). Moreover, AM-S–Zn(II) films showed high values of the minimum interfacial stress as judged from the negative (i.e., compressive) stress registered as the motor T-piece returned to its starting position (Figure 4B). In the presence of Zn(II), the minimum interfacial stress of AM-S peptide was –8.3 mN/m for 10 μ M AM-S which was almost double the minimum interfacial stress of AM1 at the same peptide molar concentration. Increasing the AM-S concentration to 20 μ M in the presence of Zn(II) increased the minimum interfacial stress to –11.1 mN/m (Figure 4B) which was comparable to the minimum interfacial stress of AM1 20 μ M in the presence of Zn(II) i.e., –11.4 mN/m (Figure 4A). It appears that AM-S peptide demonstrated a high degree of resistance to compression indicating a multilayer adsorption that causes the peptide unable to desorb quickly under the compression conditions. Unlike AM1 which tends to undergo monolayer adsorption,³⁵ the multilayer adsorption of AM-S peptide may be driven by the intermolecular hydrophobic interactions within AM-S. Once the AM-S peptides are in close proximity at the interface, it is also possible that Zn(II) promotes intermolecular bonding between adjacent AM-S peptides through formation of metal–histidine complexes, although the low level of interfacial stress transmission suggests that such complex formation may not be extensive.

Figure 4. Interfacial stress–strain curves for peptides (A) AM1 and (B) AM-S after self-assembly for 1 h at different peptide concentrations in 25 mM HEPES buffer pH 7 in the absence or presence of ZnCl₂ as determined by the Cambridge Interfacial Tensiometry (CIT). Arrows show the direction of strain.

Thin Liquid Foam Films. To understand the stability of a foam formed by the designed peptide surfactants, it is important to understand the surface forces of building block of the foam i.e., a single thin liquid foam film that separates the dispersed phase (air) in a continuous phase (peptide solution). A thin film pressure balance⁴⁹ was used to study free-standing horizontal liquid foam films formed by 100 μ M peptide AM1 or AM-S in the presence or absence of 200 μ M Zn(II) in HEPES buffer (25 mM, pH 7). The system in this technique is enclosed within a pressure-controlled cell and connected to the outer atmospheric pressure. Upon increasing the pressure, the

liquid within the films undergo drainage hence film thinning which eventually triggers rupture of the films. The film thickness was measured interferometrically as a function of a pressure within the cell (Figure 5). As can be seen in Figure 5, the film thickness of either AM1 or AM-S decreases when the pressure increases against the atmospheric pressure from 300 to 670 Pa. Comparing the thickness of the films formed by AM1 and AM-S, it is clear that the thickness of AM1 film is thinner than that of AM-S film irrespective of the pressure applied (Figure 5). The thicknesses of AM1 and AM-S films formed at 670 Pa were 3.3 and 12.1 nm, respectively. The thicker AM-S film as compared to the AM1 film is due to the tendency of AM-S peptide to undergo multilayer adsorption creating multiple layers of AM-S peptides at the film surface as previously explained.

Figure 5. Film thickness of 100 μ M peptides AM1 and AM-S in the presence of 200 μ M ZnCl₂ as a function of pressure.

Surface visualization of the foam films formed by AM1 and AM-S is presented in Figure 6. The AM1 films in the absence and presence of Zn(II) show homogeneous regions within the individual films suggesting homogenous thickness. The AM1 film in the absence of Zn(II) was unstable as it easily ruptured at low pressure i.e., 293 Pa. In the presence of Zn(II), the AM1 film

remained stable at pressures over 670 Pa, indicating a significant barrier to foam film rupture. Similar to AM1, the critical air pressure for AM-S film rupture increased from 269 to over 670 Pa when Zn(II) was added. The ability of the peptide films formed by either AM1 or AM-S in the presence of Zn(II) to maintain their stability at high critical pressure were in part due to the film elasticity (Figure 4). In addition, either AM1 or AM-S peptide was positively charged at the pH used in the film-thinning process which created electrostatic repulsion in the film surface and hence stabilized the film. Interestingly, AM-S peptide film in the presence of Zn(II) formed a snow-flake structure at the critical air pressure of 670 Pa. At lower pressure (i.e., 550 Pa) the self-assembly of the foam film formed by AM-S to snow-flake structure started 10-min after the film was formed. The film shows dark and bright phase regions suggesting the areas of different thicknesses within the individual film⁶⁰⁻⁶¹ in which the dark area corresponds to a domain with a reduced thickness, while the bright area indicates a thicker surface.

Figure 6. Surface visualization of the corresponding liquid foam films of (A, B) AM1 and (C, D) AM-S in the (A, C) absence or (B, D) presence of 200 μM ZnCl_2 at a critical air pressure of 670 Pa. Peptides were at a concentration of 100 μM in 25 mM HEPES buffer pH 7.

The formation of the snow-flake structure of AM-S–Zn(II) films at 670 Pa is influenced by the solution compositions (Figure 7). Increasing the concentrations of AM-S peptide from 100 to 200 μM significantly increased self-assembly process from 220 to 50 s as the higher peptide concentration increases its adsorption kinetic to the film surface (Figure 7A). The addition of NaCl (100 mM) into the continuous phase triggered formation of a dark hole at the edge of the film, which was an indicative of the initiation of film rupture (Figure 7B). The presence of NaCl apparently screened the positive charge of AM-S and thus destabilized the films. Changing Zn(II) into Ni(II) also induced film rupture over a period of time (Figure 7C). This is probably due to the differences in the strength and/or geometry of binding of Ni(II) to the AM-S peptide as compared to Zn(II) which may lower the mechanical elasticity of AM-S film. The importance of suitable

metal ions in interfacial cross-linking between adjacent peptide surfactants to increase peptide film strength was also observed by Dexter and Middelberg.³⁰

Figure 7. Effect of bulk compositions on the interfacial self-assembly behaviors of AM-S peptide in HEPES buffer (25 mM, pH 7) at a critical air pressure of 670 Pa in the presence of: (A) AM-S (200 μ M), ZnCl_2 (400 μ M); (B) AM-S (100 μ M), NaCl (100 mM); and (C) AM-S (100 μ M), $\text{Ni}(\text{NO}_3)_2$ (200 μ M).

Foam Formation and Stability. The foaming capacity of AM-S was tested and compared to AM1. Foams were formed by sparging aqueous solutions of 100 μ M peptides in the presence or absence of 200 μ M Zn(II) using air (7 mL) at a controlled air flow-rate (20 mL/min) through a sintered frit (Figure 8). The foams were imaged immediately after the air flow stopped and subsequently 2 min after foam formation. Visual inspection of the foam columns revealed that the peptide AM-S has the ability to form a dense foam of similar quality to AM1. Liquid drainage through AM-S and AM1 foams is essentially complete at 2 min of foam formation as can be seen

at the bottom column, indicating that both AM-S and AM1 foams are metastable. In the absence of Zn(II), the foams formed by AM-S and AM1 after 2 min qualitatively indicates that the thin liquid films surrounding the air bubble are ruptured, hence, resulting in an increase in the bubble size (Figure 8, top panel). In contrast, the AM-S and AM1 foams formed in the presence of Zn(II) were still intact with no indication of increased bubble-size after 2-min aging (Figure 8, bottom panel). These results indicate that the ability of AM-S peptide with or without Zn(II) to undergo spontaneous adsorption at air–water interfaces hence reducing the interfacial tension (Figure 2) facilitates foam formation similarly to AM1.⁴⁷ However, peptides surface activity is not sufficient to sustain the metastable foams at the concentration tested as the foams formed by the peptides with Zn(II) are more stable than the peptide foams without Zn(II). Zn(II) contributes significantly to the peptide film elasticity which influences foam stability by enabling the film interfaces to resist tangential stresses from the adjoining flowing liquids.

Figure 8. Comparative foamability and foam stability of 100 μM solutions of (A) AM1 and (B) AM-S peptides in the absence (top panel) and presence (bottom panel) of 200 μM ZnCl_2 in 25 mM HEPES buffer pH 7. Each panel shows two photographs; the first image was taken immediately after the air flow was stopped, while the second image was taken two minutes later.

Overall, the results show that two peptide surfactants AM1 and AM-S (which was designed by adding a small silk tail to AM1) differ significantly in their interfacial behavior (Figure 9). AM1 peptides tend to undergo monolayer adsorption at the interfaces and laterally cross-link between histidine residues through Zn(II) .^{29-30,35} The cohesive peptide network of AM1 in the presence of Zn(II) has been demonstrated to result in high interfacial stress and hence stable foams. In case of AM-S peptides, the presence of silk tail in AM-S results in the multilayer adsorption at the interfaces, instead of monolayer adsorption as in AM1. This is likely due to hydrophobic

interactions as well as Zn(II) binding between adjacent adsorbed peptide molecules. As a result, interfacial film of AM-S was found to be thicker than AM1 film with interfacial stress comparable to AM1, and the interfacial activity of AM-S in the presence of Zn(II), but not in its absence, enables the formation of stable foams.

Figure 9. Illustration of the interfacial behaviors of peptide surfactants AM1 and AM-S in the presence and absence of Zn(II).

CONCLUSIONS

AM-S peptide was designed in this work by combining a sequence of AM1 peptide with an octapeptide containing an alternate glycine and alanine residues derived from *Bombyx mori* silkworm fibroin. Both AM1 and AM-S were tested and compared to provide a better understanding of dynamic interfacial behaviors of the peptides having different sequences. The CD spectra revealed that both AM1 and AM-S have a random coil conformation in bulk solutions which facilitates their rapid adsorption to the air–water interfaces thus reducing the interfacial tension effectively. In contrast to the monolayer adsorption of AM1 peptides at air–water

interfaces, AM-S peptides promote multilayer adsorption of AM-S at the interfaces driven by intermolecular hydrophobic interactions. The multilayering of AM-S peptide at the interfaces was observed to result in high interfacial stress comparable to the cohesive film of AM1 peptide under the compression conditions of Cambridge interfacial tensiometer (CIT). Thin film pressure balance technique also showed that the foam films formed by AM-S peptide were thicker than the films formed by AM1 peptide, which enable the AM-S films to maintain stability against high critical air pressure for film rupture. Moreover, the AM-S foam film was able to undergo self-assembly during the film-thinning process, leading to the formation of a snow-flake structure depending on the solution compositions. AM-S was shown capable of forming a dense foam comprised of small bubbles and was able to maintain foam stability comparable with AM1 peptide. The ability of AM-S peptide surfactant in the presence of Zn(II) to form stable foams may find potential applications in fields of pharmaceutical, food and beverages, laundry detergents, and personal care. The benefits of using peptide surfactants as compared to their petrochemical-based counterparts lie in its biocompatibility⁶² as well as its sustainability as they can be produced in recombinant microbial cell factories using engineered bioprocess unit operations,³⁶⁻³⁸ thus paving the way for large-scale economic manufacture and hence utilities. More detailed studies of AM-S film using, for example, direct neutron reflectivity will be required in order to study AM-S interfacial architectures self-assembled in the presence of Zn(II). The results of this study show how significantly addition of a small silk tail can modify interfacial behavior, suggesting a significant design space requiring further fundamental understanding to guide design.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

A, alanine; D, aspartic acid; E, glutamic acid; G, glycine; H, histidine; K, lysine; L, leucine; M, methionine; P, proline; Q, glutamine; R, arginine; S, serine; V, valine; Y, tyrosine; *d*, diameter; CIT, Cambridge interfacial tensiometer; Da, dalton; HEPES, sodium 4-(2-hydroxyethyl)-1-piperazine ethanesulfonate; *l*, length; *M*, molecular mass; pI, isoelectric point.

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TABLE OF CONTENTS (TOC) GRAPHIC