A versatile family of sophorolipid esters: engineering surfactant structure for stabilization of lemon oil-water interfaces

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Abstract

Sophorolipids (SLs) are biosurfactants produced by *Candida bombicola* from renewable feedstocks in yields > 400 g/L. Molecular editing of natural SLs gave a series of *n*-alkyl SL-esters that, along with natural SLs, were interrogated to determine how structural changes alter SL interfacial tension (IFT), diffusion, adsorption, and emulsification at the lemon oil/water interface. SL-ethyl ester (SL-EE) reduced the IFT by 95.1% and has a critical aggregation concentration (CAC) of 0.026mg/mL while SL-hexyl ester (SL-HE) had a lower CAC, 0.02mg/mL, but a lower IFT reduction (87.5%). Adsorption behavior further highlighted differences between the SL-esters as SL-decyl ester (SL-DE) had the lowest surface excess concentration while SL-HE had the highest adsorption coefficient. The competing effects of these interfacial parameters were manifested in the relative performance of SL-esters in forming lemon oil-in-water emulsions. SL-EE had the largest average emulsion droplet size but showed no oil separation up to 200:1 wt/wt oil/surfactant. Microscopy provided information on macroscopic emulsion morphologies. For example, flocculation was observed for all 1wt% SL 20wt% lemon oil emulsions. These results were corroborated by shear thinning rheological behavior. Studies were conducted with a natural SL mixture consisting of 1:1 wt/wt lactonic and

acidic SLs (LSL+ASL). LSL+ASL has similar emulsion properties to SL-EE at low SL concentrations while emulsions collapsed at higher SL concentrations.

Keywords: sophorolipid, emulsion, interfacial tension, critical aggregation concentration, flocculation, lemon oil¹

1. Introduction

Emulsions are kinetically stable mixtures of immiscible liquids generally stabilized by a surfactant.[1] They are found in products from almost every industry, from food to pharmaceuticals to cosmetics. However, work to develop, optimize and characterize emulsion performance renews as the requirements for the product evolves.[2,3] Significant advances have been made in understanding surfactant performance with different immiscible phases by analysis of interfacial tension, rheology (bulk and interfacial) and interfacial elasticity under various conditions (e.g. salinity, temperature).[4,5] One goal of this work is to assess how experimentally determined interfacial characteristics can be used to predict surfactant performance as an emulsifier. This requires a thorough understanding of the effect of surfactant structure on interactions at the interface for a variety of immiscible mixtures.

Interest in microbially produced biosurfactants continues to evolve due to their sustainability and low environmental impact. However, limited adoption of biosurfactants is due in part to their cost-performance relative to existing commercial products.[6] Sophorolipids (SLs), the glycolipid product of *Candida bombicola* fermentation, has proven to be one of the most viable biosurfactant targets due to high yields (>400 g/L) and safe for production strains.[7]

¹ Abbreviations: SL: sophorolipid, LSL: lactonic sophorolipid, ASL: acidic sophorolipid, SL-EE: SL-ethyl ester, SL-HE: SL-hexyl ester, SL-DE:SL-decyl ester, IFT: interfacial tension, CAC: critical aggregation concentration, max%-IFT: maximum percentage interfacial tension decrease.

Natural SLs, as shown in Scheme 1, are generally produced as mixtures of lactonic (LSL) ring-closed and acidic (ASL) ring-opened forms. The SL structure consists of sophorose (2-*O*- β -D-glucopyranosyl-D-glucose or β -D-Glc-(1 $\rightarrow$ 2)-D-Glc) linked to a hydroxyl fatty acid (e.g. 17-hydroxy-9-*cis*-octadecenoic acid, 17-HOC18:1 Δ^9) through a glycosidic bond. Sophorose head groups may be acetylated at one, both, or neither of the 6' and 6'' positions.[8] Natural SLs are already being produced commercially by Ecover (Belgium), Soliance/WheatOleo (France), Saraya (Japan), SyntheZyme (US), MG Intobio (South Korea), and Allied Carbon Solutions (ACS, Japan). SL mixtures were reported to have critical micelle concentrations (CMC) from 20-130 mg/L and minimum surface tensions (mSFT) between 32 and 42 mN/m.[9-11]

While a few studies have evaluated the properties of SLs for emulsification, recent work by the Gross laboratory has described the connection between interfacial tension and emulsification performance of natural SLs as well as a homologous series of modified SLs.[12,13] The later resulted from molecular editing of SLs by esterification of the lipid tail that gave a family of SL-esters whose hydrophobic moiety is extended from 18 carbons (17-HOC18:1 Δ^9) to 20, 24, or 28 carbons, respectively (Scheme 2).[14] These studies focused on almond oil, a C18 fatty-acid based triglyceride, and liquid paraffin that consists of a mixture of alkanes generally ranging from C16 to C50. The results revealed that the critical aggregation concentration of SL-esters is predictive of its emulsification performance for a given oil phase. Furthermore, important improvement in interfacial performance of SL-esters relative to natural SLs was achieved by 'tuning' the length of the *n*-alkyl ester moieties. Moreover, the performance of SL-ester emulsifiers is generally superior to comparative surfactants such as polysorbates and dodecyl maltoside.[12]

The preferred length of the SL-ester *n*-alkyl moiety differed for the stabilization of almond oil and paraffin oil/water interfaces. To further our understanding of SL interfacial properties while building on our predictive capabilities for determining the preferred SL structure for stabilization of a given oil/water interface, it is important that we expand the oil phases studied to those that significantly differ from paraffin and almond oil. Paraffin and almond oil both consist of long hydrocarbon chains, although, almond oil is significantly more hydrophilic and has a higher unsaturated oil character.

This work focuses on lemon oil as the hydrophobic phase for interfacial and emulsification studies. Lemon oil is a mixture of a wide variety of terpenes, although limonene predominates for single-fold oils.[15] Other terpene-based oils include orange oil, lemongrass oil, and rose oil. These oils are often used as fragrances, flavors, antimicrobial, and pesticide ingredients.[16-18] Unlike almond and paraffin oils, lemon oil components are largely small (<10 carbons) molecules, often cyclic, that may have significant water-solubility.[15] Thus, lemon oil presents a very different oil/water interface to SLs than those previously studied. As such, this work with lemon oil is important to enhance our fundamental and predictive understanding of natural and molecularly edited SL interfacial and emulsification performance.

2. Experimental

2.1. Materials

Lemon oil, cold-pressed originating from California, was purchased from Sigma-Aldrich and used without purification. All lemon oil samples used herein are from a single batch that was stored in sealed amber glass bottles to prevent degradation and loss of volatile components. Compositional analysis of a similar lemon oil is given in the literature.[15] All water used herein

was Millipore de-ionized. Dodecyl maltoside, sodium dodecyl sulfate, polysorbate 80 (Tween 80) and Nile Red were all purchased from Sigma-Aldrich in the highest available purity and used as received.

2.2. *Sophorolipid (SL) production via fermentation*

Sophorolipids (SLs) were synthesized through fermentation of *Candida bombicola* as reported in previous published work using high oleic acid sunflower oil as the lipid source.[19] The resulting solid was primarily a mixture of lactonic SL (LSL) and acidic sophorolipid (ASL) with varying degrees of acetylation as described in Scheme 1. Analysis of the final mixture by an LC-MS method exactly as is described in the literature shows the major component (approx. 95 mol%) is di-acetylated LSL.[20]

2.3. *SL-Ester Synthesis*

SL modification to produce SL-ethyl ester (SL-EE), SL-hexyl ester (SL-HE), and SL-decyl ester followed previously reported protocols.[14] The structure of purified SL-ester products was confirmed by Liquid Chromatography-Mass Spectrometry (LC-MS), ^1H NMR, and ^{13}C NMR which are identical to those previously reported.[12-14]

2.4. *Interfacial Tension (IFT)*

IFT was measured with a Krüss (Hamburg, Germany) DSA100 Drop Shape Analyzer at sophorolipid derivative concentrations from 0.001 mg/mL to 50 mg/mL for SL-EE, SL-HE, SL-DE, ASL, and a 1:1 wt/wt LSL+ASL mixture. Upper concentrations for IFT measurements was limited by SL solubility. Dynamic IFT measurements of each SL-ester were used to calculate

diffusion coefficients using the short-time approximation (Equation 2) of the Ward and Tordai diffusion controlled adsorption relationship (Equation 1).

$$M = 2 \sqrt{\frac{D}{\pi}} \int_0^{\sqrt{t}} \varphi(t) d\sqrt{t - \tau} \quad (1)$$

$$\frac{\gamma(t) - \gamma_0}{\sqrt{t}} = -2RTC \sqrt{\frac{D_{ap}}{\pi}} \quad (2)$$

M is surfactant concentration, D is diffusion coefficient, φ is the interfacial surfactant concentration, and τ is a dummy integration variable. In Equation 2, $\gamma(t)$ is IFT at time t, γ_0 is the pure oil/water IFT (16.4 mN/m for lemon oil used), C is the bulk surfactant concentration, and D_{ap} is the apparent diffusion coefficient.[21,22]

Equilibrium IFT, γ_{eq} , was used in Equation 3 to calculate the corresponding percentage IFT reduction at each SL-ester concentration.

$$\% \text{IFT Reduction} = \frac{\gamma_0 - \gamma_{eq}}{\gamma_0} * 100 \quad (3)$$

%IFT reduction vs. concentration data was used to calculate a critical aggregation concentration (CAC), maximum interfacial reduction (max %-IFT), surface excess concentration (Γ), and the Langmuir adsorption coefficient for each SL. Max %-IFT is calculated by averaging at least three %-IFT reduction values in the concentration-independent regime of the IFT vs. concentration curve. CAC is calculated as the intersection of a line drawn through at least three %-IFT reduction values in the concentration dependent regime and three %-IFT reduction values in the concentration-independent regime of the IFT vs. concentration curve. This is identical to the method of calculating a critical micelle concentration but is named to indicate the presence of two self-interacting phases. Γ , the surface excess concentration, is calculated by the Gibbs adsorption equation:

$$\Gamma = \frac{-1}{RT} \frac{d\gamma_{eq}}{d \ln c} \quad (4)$$

where R is the gas constant, T is the measurement temperature, γ_{eq} is the equilibrium IFT, and c is the bulk surfactant concentration.[23] The adsorption coefficient K_L is calculated by fitting the IFT vs. concentration curve using the Szyszkowski equation:

$$\Pi = nRT\Gamma_m \ln(1 + K_L c) \quad (5)$$

where Π is the surface pressure, R is the gas constant, T is the measurement temperature, Γ_m is the maximum surface excess concentration and c is the bulk surfactant concentration.[23]

2.5. Emulsification

Emulsions of lemon oil were formed by high shear homogenization using an IKA T25 shear homogenizer mixing at 24,000 rpm for 60 seconds. Emulsions were made with oil concentrations of 1, 10, or 20wt% and SL concentrations of 0.1 or 1wt%. All weight percentages are based on total emulsion weight.

Average emulsion droplet sizes were measured immediately after homogenization or after aging at 25°C for 24 hours or 1 week and are reported as the emulsion z-average diameter in nanometers. Droplet sizes were measured using a Malvern Zetasizer ZSP (Worcestershire, England) with a 173° backscatter angle detector. Samples were diluted 100 times in de-ionized water to minimize multiple scattering effects. Emulsion physical stability was recorded at 24 hours and 1 week of aging using a Nikon D5200 camera with a Nikon AF-S Micro Nikkor 60 mm lens.

Emulsion rheology was analyzed using an Anton Paar MCR 301 Rheometer (Graz, Austria) with a cone-plate geometry. The cone used had a diameter of 24.975mm and an angle of 1.002°. A glass plate was used to minimize sample spreading. Viscosity measurements for each sample were performed by successively ramping the shear rate from 10 s⁻¹ to 5000 s⁻¹, 5000

s^{-1} to 10 s^{-1} , and then 10 s^{-1} to 5000 s^{-1} . Consistent with above, emulsions analyzed for rheology were prepared with 1, 10, and 20wt% lemon oil and either 0.1 or 1 wt% SL. Measurements for triplicate samples were made immediately after homogenization or after aging for 24 hours at 25°C .

Confocal microscopy was performed on a Zeiss LSM 510 with a META I detector. Emulsion samples analyzed with confocal microscopy were made using lemon oil stained with $15 \mu\text{g/mL}$ Nile Red, which has a maximum absorption at 553nm. Optical microscopy was performed using a Motic BA210 digital microscope. Samples were not diluted before confocal or optical microscopy analyses.

3. Results & Discussion

3.1. *Effect of SL structure and concentration on IFT*

Surfactants decrease the system interfacial energy by preferentially adsorbing to immiscible phase interfaces with their hydrophilic region interacting with one phase and their lipophilic region interacting with the other.[1] As the bulk concentration of surfactant is increased, the surfactant adsorbs to the interface until it is saturated, reaching a minimum IFT. The concentration at which saturation occurs is referred to herein as the critical aggregation concentration (CAC). The CAC is identical in meaning to the critical micelle concentration (CMC) measured at air/water interfaces, but connotes two self-interacting phases rather than one. Above the CAC, in the concentration independent regime of IFT vs. concentration curve, micellar aggregates form in the bulk solution.[24]

Figure 1 shows %-IFT drop vs. concentration plots for SL-EE, SL-HE, SL-DE, LSL+ASL (1:1 w/w) and ASL at the lemon oil/water interface. The relative magnitude of

corresponding CAC values is $SL-HE < SL-EE < SL-DE \approx LSL+ASL < ASL$ (Table 1). By comparison of CAC values we learn, for example, that a higher concentration of SL-EE relative to SL-HE is required to saturate the oil/water interface. SL-DE and LSL+ASL have CAC values about 20 times that of SL-HE and SL-EE. This indicates SL-DE and LSL+ASL at concentrations below their CAC should have significantly less stable and/or larger-sized emulsions than SL-EE and SL-HE. For max %-IFT, the trend is different where SL-EE has a max %-IFT drop of 95.1% that is significantly higher than that for the other SLs (Table 1). SL-HE, SL-DE, and LSL+ASL have similar max %-IFT decreases (87.5%, 85.1%, and 86.8%, respectively) whereas that for ASL was not reached by 50mg/mL. While CAC is a good measure of the concentration, per volume, based efficiency of SLs to stabilize oil/water interfaces, a higher max %-IFT drop should correlate for concentrations above the CAC with improved long-term emulsion stability and resistance to coalescence.

While considerable work is available in the literature on characterizing lemon oil and the lemon oil/water interface, few studies discuss the influence of surfactants on the interfacial tension. [15,25-27] The reduction in the lemon oil/water interfacial tension by dodecyl maltoside and sodium dodecyl sulfate (SDS), evaluated in our lab on the Krüss DSA100 at 0.5mg/mL, is 94.4 and 43.3%, respectively. Pérez-Mosqueda et al. reported that Pluronic PE9400 reduced the limonene-water interfacial tension to 16 mN/m (from 44 mN/m, a 63.6% decrease) and has a CAC of 5×10^{-5} mol/L.[28] In contrast, SL-EE has a CAC of 4×10^{-5} mol/L and reduces the lemon oil-water interfacial tension to 0.81 mN/m (95.1% decrease). Limonene is the major constituent of single-fold lemon oil.[15] Hence, surfactant performance at the limonene/water interface is a good indicator of performance at the lemon oil/water interface. Purwanti et al. found that β -lactoglobulin reduced the limonene/water interfacial tension to ~ 2

mN/m at 5 mg/mL.[29] From the discussion above and comparison of the results in Table 1 to literature reports on surfactant induced reduction of the lemon oil/water interfacial tension, we conclude that SL-esters have comparable or superior performance.

Surfactant diffusion and adsorption at the lemon oil/water interface was also characterized. Figure 2 shows diffusion coefficients for SL-esters from 0.001 to 2mg/mL as well as LSL+ASL and ASL from 0.001 to 50mg/mL. At low concentrations ($< \text{CAC}$), SL-EE has the highest diffusion coefficient of any SL-ester. This is attributed to that SL-EE is the smallest SL-ester. SL-EE and SL-HE both show a peak around their CAC values and decrease in diffusion coefficients thereafter. The decrease in the diffusion coefficient at concentrations above the CAC is attributed to crowding at the saturated oil/water interface which decreases the mobility of SLs. However, the decrease in the diffusion coefficient occurs more slowly for SL-HE than SL-EE. Indeed, at the upper concentrations measured (about 1mg/mL), the diffusion coefficient of SL-HE is almost four orders of magnitude greater than SL-EE. SL-DE and LSL+ASL show similar diffusion coefficient decreases with no distinct peak at or nearby the CAC. Instead, there is a flattening of the diffusion coefficient vs. concentration curve near the CAC. Similarly, ASL shows no peak, which is consistent with that the concentrations studied are below the CAC. Furthermore, ASL shows a regular decrease in the diffusion coefficient with increased SL concentration.

The adsorption performance of SLs was also calculated using Equations 4 and 5 (above) and values are listed in Table 1. Γ_m , a measure of the maximum adsorbed surfactant per unit interface at surface saturation, was calculated for SL-EE, SL-HE, SL-DE and LSL+ASL using the concentration dependent regime of IFT vs. concentration plots. The adsorption coefficient, K_L , which indicates a surfactants affinity to adsorb on to or desorb from an interface, was also

calculated for SL-EE, SL-HE, SL-DE, and LSL+ASL by fitting the IFT vs. concentration plots below the CAC of the respective SLs so as to fulfill the ideal dilute solution assumption. Γ_m and K_L were not calculated for ASL as no max %-IFT was reached in the concentration range measured. Γ_m tends not to vary greatly between surfactants, as shown by Chang et al.²³ However, a small difference in Γ_m values is seen between the SLs (Table 1). SL-DE has the lowest Γ_m , meaning that at the theoretical maximum saturation concentration ($1.1 \times 10^{-6} \text{ mol/m}^2$) SL-DE needs the least surfactant to stabilize the same interfacial area. SL-EE has the highest Γ_m ($2.3 \times 10^{-6} \text{ mol/m}^2$), which appears at odds with the CAC trends discussed earlier. However, this seeming contradiction is explained by that the oil/water interface is saturated at a lower bulk SL-EE concentration, but that less interfacial area is stabilized. Alternatively, SL-DE, with a high CAC but low Γ_m , requires more surfactant in the bulk phase to saturate the oil/water interface, but can stabilize more interfacial area per mole of surfactant. Based on adsorption at equilibrium at bulk concentrations below the CAC, it is expected that the lower CAC SL would have an enhanced ability to stabilize emulsions as it is closer to completely saturating the interface created. However, above the CAC, the lower Γ_m SL may better stabilize emulsions as it can stabilize more interface resulting in smaller droplets.

A high K_L indicates that a surfactant has a faster rate of adsorption to, or slower rate of desorption from the interface. It follows that a high K_L at non-equilibrium conditions such as during high shear mixing can result in better surfactant interfacial stabilization. Table 1 shows that the relative order of SL-ester K_L values are as follows: SL-HE ($710 \text{ m}^3/\text{mol}$) > SL-DE ($350 \text{ m}^3/\text{mol}$) > SL-EE ($220 \text{ m}^3/\text{mol}$). Thus, SL-HE has the strongest affinity for absorption at the lemon oil/water interface. The K_L of LSL+ASL ($190 \text{ m}^3/\text{mol}$) is lower than SL-EE.

3.2. Emulsification of lemon oil by SLs

Emulsions were prepared with 1, 10, or 20wt% lemon oil and 0.1 or 1.0wt% SL. Analysis of emulsions was performed immediately after emulsification and after aging at 25°C for 24 hours and 1 week. Figure 3 shows the average droplet sizes of emulsions at each of these three time points. Figure 4 displays the physical appearance of the emulsions after 24 hours and 1 week of aging.

Study of Figure 3 shows two obvious trends. First, for every time point and SL concentration with the exception of 0.1wt% SL-HE 20% lemon oil immediately after homogenization, the size trend is $SL-EE > SL-HE \geq SL-DE$. The second apparent trend is that, 0.1wt% and 1wt% average emulsion sizes for each formulation, again with the exception of 0.1wt% SL-HE 20% lemon oil immediately after homogenization, are very similar when comparing emulsions of the same SL-ester. The second trend would suggest that the emulsions are made in the concentration independent regime of Figure 1, and thus the surfactant concentrations are above the CAC. If the effective interfacial concentration is above the CAC then all the available oil/water interface is saturated with the surfactant. This would explain why the trend in emulsion sizes does not match the trend in CACs of the SL-esters.

From Figure 4, it is seen that 0.1 and 1.0wt% SL-DE at 20wt% lemon oil and 0.1wt% SL-HE at 20wt% lemon oil undergo oil separation by 24 hours. Oil separation will result in smaller emulsion droplet sizes, as the largest of the emulsion droplets will have coalesced and formed the bulk oil phase. The significant decrease in droplet size for the 0.1wt% SL-HE 20wt% lemon oil emulsion that occurs at some time between immediately after emulsification and 24 hours is due to oil separation. That there is no significant decrease in the SL-DE 20wt% lemon oil droplet sizes may indicate that the oil separation occurred immediately after emulsification,

which was confirmed through visual inspection. Figure 4 also shows that SL-EE is the only SL-ester that precipitated at 1wt% SL 1wt% oil within 24 hours. After 1 week, all 1wt% SL-EE emulsions showed precipitation. All emulsions without oil separation, with the exception of 1wt% SL-EE or SL-HE and 20wt% oil, show multiple emulsion phases, indicating high polydispersity in emulsion sizes. The difference in physical stability between the SL-ester emulsions suggests that SL-esters perform differently despite being above their respective CACs.

Figure 4 also shows the physical stability of emulsions with LSL+ASL at aging for 24 hours at 25°C. Emulsions of 0.1wt% LSL+ASL look similar to those of SL-EE with no oil separation and no precipitation. However, at 1wt% LSL+ASL, all emulsions show oil separation, but no precipitation. Figure 5, which shows average droplet sizes of LSL+ASL stabilized emulsions, confirms the similarity between SL-EE emulsions at 0.1wt%, with little change over time. Consistent with visual observations, at 1wt% LSL+ASL, oil separation leads to large decreases in oil droplet sizes between immediate emulsion formation and 24 hours aging at all oil concentrations.

During shear, the bulk oil phase is broken up into smaller units of oil, increasing the interfacial area of the system. While the bulk oil phase is dispersed, SLs must diffuse to and adsorb onto the new oil/water interface. The SL stabilized interface must then resist further coalescence during and after shear. The more interfacial area is stabilized and the better that area resists coalescence, the smaller the final emulsion droplets. As such, at effective interfacial concentrations above the CAC, the SL performance will be dictated by the diffusion and adsorption related to saturation of the interface as well as the max %-IFT drop. A discussion of how these factors relate to the emulsion performance studies herein is discussed below.

From Figure 2, the general trend in diffusion coefficients above 0.5mg/mL is SL-HE>SL-DE≥SL-EE. However, it is unlikely that, in a high shear environment, the diffusion coefficient under static conditions will be the dominant driver of emulsion performance. The diffusion coefficient would be expected to be a more important predictor of performance at lower mixing rates. For Γ_m and K_L the trends are SL-EE>SL-HE>SL-DE and SL-HE>SL-DE>SL-EE, respectively. For SL-DE, a low (1.1×10^{-6} mol/m²) surface excess surfactant concentration suggests that, above interfacial saturation, a larger amount of interface can be stabilized as compared to SL-EE. SL-HE, with the highest adsorption constant and intermediate surface excess concentration relative to the SL-ester series (Table 1) should also be able to stabilize more interface than SL-EE above interfacial saturation. However, SL-EE has the highest max %-IFT drop of all three SL-esters and shows no oil separation at any concentration. These comparisons assume that all the SL-esters efficiently adsorb to and stabilize the oil/water interface (e.g. no precipitation) and that Γ_m is an accurate indicator of how much SL is at the interface.

The theoretical maximum of surfactant adsorbed to the total emulsion surface area for each SL-ester emulsion was calculated based on Equation 6 from knowledge of Γ_m , the average emulsion size immediately after formation and the emulsion oil concentration.

$$Max\ SL\ Adsorbed = \frac{Volume\ Oil\ Used}{\frac{4}{3}\pi r^3} * 4\pi r^2 * \Gamma_m \quad (6)$$

By subtracting the maximum total adsorbed surfactant from the total surfactant added to emulsions, values of the %-total surfactant used to stabilize emulsion oil droplets were determined and plotted in Figure S1, included in supplementary information. The highest %-of-total SL-ester in emulsions that resides at oil droplet surfaces was 65% for 0.1% SL-HE and 10% oil. Generally, high fractions of SL-HE at oil droplet surfaces are consistent with that SL-HE has the highest K_L of the SL-ester series. Nevertheless, more than 35% of SL-esters in emulsions do

not reside at oil droplet surfaces. In the case of 1% SL-EE 1% oil, 99% of the surfactant resides at locations other than oil droplet surfaces. One potential fate for remaining SL-ester in the bulk phase is precipitation. Visual observation of SL-ester precipitation only occurred for 1 wt% SL-EE emulsions. However, it may be that other SL-esters in the bulk phase precipitate, due to their low water solubility, but form relatively small particles that remain suspended and, therefore, are not detected by visual inspection. Furthermore, SL solubility may be enhanced by water-soluble lemon oil components. The availability of SL in the bulk phase is an important factor as it may be involved with other interfacial phenomena such as flocculation (see below).

3.3. SL-Ester Emulsion Microscopy

Confocal microscopy was used to investigate the SL-ester emulsion morphology. Emulsions were analyzed simultaneously by optical microscopy, and no precipitation was seen beyond that observed visually in Figure 4. Figure 6 shows optical and confocal microscopy images of 20wt% lemon oil emulsions with 0.1 and 1.0 wt% SL-EE, SL-HE and SL-DE aged for 24 h. At 0.1wt% SL-EE and SL-HE, emulsions appear as disperse, or non-interacting, emulsion droplets. However, at 1wt% SL-EE and HE with 10 and 20 wt% lemon oil and 24 hour aging flocculation is observed. While confocal microscopy images of 1wt% SL-EE emulsions clearly show significant flocculation, flocculation by optical microscopy of the same emulsion is viewed as a shadow over other emulsion droplets. In contrast, confocal and optical microscopy images of 1wt% SL-HE emulsions both show flocculation with little evidence of individual droplets indicating tight droplet packing. For emulsions of SL-DE at both 0.1 and 1.0wt%, flocculation into non-disperse structures is observed by both optical and confocal microscopy.

3.4. *SL-Ester Emulsion Flocculation and Rheology*

To quantify flocculation as a function of emulsion composition, viscosities of SL-ester emulsions was measured consecutively at shear rates from 100 to 5000s⁻¹, 5000 to 100s⁻¹, and 100 to 5000s⁻¹. Figure 7 shows the resulting viscosity vs. shear rate for SL-ester emulsions after 24 hours of aging at 25°C. Neat lemon oil, measured under the same conditions, was Newtonian and had a viscosity of 0.56 mPa·s.

Viscosity vs shear rate plots of 0.1wt% SL with 1 and 10wt% oil as well as 1wt% SL with 1 wt% oil (Figures 7a, b, and d, respectively) show for all SL-esters that there is no shear thinning and little hysteresis. Emulsions prepared using 0.1wt% SL-EE and SL-HE with 20 wt% lemon oil (Figure 7c) show little shear thinning while the viscosity of the SL-HE emulsion is significantly below that of the other SL-esters due to oil phase separation (see above). Emulsions consisting of 1 wt% SL-EE or SL-HE with 10 or 20wt% oil (Figures 7e and f, respectively) exhibit shear thinning that intensifies for the 20 wt% oil emulsions. Shear thinning indicates flocculation, as increased shear overcomes the attractive forces between droplets resulting in a decrease in solution viscosity. The visualization by confocal and optical microscopy that flocculation occurs for 1wt% SL-HE and SL-EE is in excellent agreement with the observed shear thinning behavior of these emulsions. The viscosity behavior of SL-DE is unlike that of SL-EE and SL-HE at 1wt% with 10 and 20wt% oil (Figures 7e and f, respectively) as well as 0.1wt% with 20wt% oil (Figure 7c). All three of these SL-DE emulsion systems have much higher viscosity than SL-EE and SL-HE. Plots of these SL-DE emulsions at low shear rates start with either a shear thickening or shear thinning behavior. For 1wt% SL-DE with 10wt% lemon oil, increasing shear above 294s⁻¹ caused a decrease in viscosity corresponding to shear thinning behavior. For 0.1 wt% SL-DE with 20 wt% lemon oil, shear thickening is observed during the

first sweep at shear rates of 10 to 337 s⁻¹ after which, the viscosity showed little change up to 2230 s⁻¹ and, thereafter decreased. On the second and third shear rate sweeps shear thinning with little hysteresis is observed. At 1wt% SL-DE with 20wt% lemon oil, little shear thinning was observed on the first shear rate sweep, while an increase in viscosity and shear thinning were observed on the second and third sweeps. The induced shear thinning and thickening indicates a system that reversibly disperses and flocculates as shear is increased and subsequently decreased. Since flocculated emulsions are more prone to coalescence, flocculation observed in Figure 6 at 0.1 and 1.0wt% SL-DE with 20wt% lemon oil is consistent with the observation that these emulsions undergo oil separation. Furthermore, the fact that shear thinning was not observed for 0.1wt% SL-DE with 10wt% oil (Figure 8b) is consistent with that this emulsion does not tend to flocculate and, corresponding does not undergo oil separation.

Depletion flocculation, where a depletion force results in attractive forces between colloidal particles, is believed to be responsible for flocculation observed herein. This phenomenon is generally seen at concentrations above interfacial saturation, i.e. above the CAC, when there is a large excess of surfactant remaining in bulk solution.[30-32] That flocculation occurs without hysteresis or interfacial surfactant depletion is consistent with the formation of flocs that are held together by these weak reversible interactions. SL-EE and SL-HE show weaker flocculation than SL-DE, which enhances the ability of SL-EE and SL-HE emulsions to resist coalescence. This ability to resist coalescence is greater for SL-EE due to its high max %-IFT reduction. The strong interaction between SL-DE-lemon oil emulsion droplets results in increased viscosity. At 0.1wt% SL-DE with 10wt% oil, there is less SL-DE remaining in the bulk (54.7% of added SL, Figure S1) that leads to stronger depletion forces between emulsion droplets that flocculate than is observed at 1.0wt% SL-DE with 10wt% oil (94.1% of added, see

Figure S1). However, shear weakens the interactions such that shear thinning to a lower zero-shear viscosity is observed. For 1.0wt% SL-DE with 20wt% lemon oil, emulsion droplets are larger and the kinetic repulsion between them is lower. Consequently, SL-DE emulsion interfacial interactions are strengthened by shear, potentially allowing for further depletion of the SL-DE micelles between emulsion interfaces, causing shear thinning but an increase in zero-shear viscosity after the first viscosity sweep (Figure 7f). This tighter flocculation, along with SL-DE's low max %-IFT drop, results in coalescence and oil separation.

Figure 8 displays plots of viscosity vs. shear rate for LSL+ASL emulsions aged for 24 hours at 25°C. At 0.1wt% LSL+ASL, the rheological behavior is similar to SL-EE with little to no shear thinning and a slight increase in viscosity as oil concentration increased. Emulsions of 1.0 wt% LSL+ASL all showed low viscosity relative to 0.1wt% LSL+ASL, which is explained by oil separation that was visually observed for all of these emulsions. The viscosity of emulsions of 20wt% lemon oil and 0.1 or 1.0 wt% Tween 80 were also analyzed. These emulsions were found to show no shear thinning with viscosities of 1.51 ± 0.07 and 1.57 ± 0.04 mPa·s, respectively (time-dependent data not shown here), which is slightly higher than non-flocculated SL emulsions. Tween 80's Newtonian rheological behavior indicates that not all water-soluble, non-ionic surfactants flocculate at these formulation conditions.

Emulsions formed with 0.1 wt% LSL+ASL have similar adsorption behavior to SL-EE and do not flocculate, as seen from their rheology in Figure 8. However, at 1wt% LSL+ASL, oil separation occurs most likely because of repulsive interactions between the ASL carboxylic groups at hydrophobic tails. Furthermore, it is likely that the water-soluble components of lemon oil enhance LSL+ASL solubility. This would explain why almost complete oil separation occurs for 1wt% LSL+ASL emulsions without precipitation of the LSL that has low water solubility.⁷

This enhanced water solubility may also allow for the SL-ester flocculation, as the SLs remain in solution rather than precipitating. Indeed, flocculation was not observed in previous SL emulsification studies with almond and paraffin oils.

4. Conclusions

The paper interrogates effects of SL structure, particularly the lengthening of the *n*-alkyl ester moiety on the hydrophobic tail, on interfacial performance. SL-EE has the highest max %-IFT reduction (95.1%) while SL-HE has the lowest CAC (0.02 mg/mL). Surface excess concentration and the adsorption coefficient of the SL-esters each showed a different trend with SL-DE having the lowest Γ_m and SL-HE having the highest K_L . Emulsification with SL-EE, SL-HE, and SL-DE resulted in surfactant concentration-independent average droplet sizes, indicating an interfacial concentration above the CAC for all three SLs. SL-EE emulsions consistently have larger droplet sizes than those with SL-HE and SL-DE. Furthermore, SL-EE emulsions prepared with up to 200:1 wt:wt oil:surfactant aged for 1 week did not undergo oil separation. Emulsions prepared with SL-HE and SL-DE have similar emulsion sizes, and both showed oil separation after 24 hours at 20wt% oil 0.1wt% SL. SL-DE also showed oil separation at 20wt% oil and 1.0wt% SL.

The relatively smaller droplet sizes of lemon oil emulsions with SL-HE and SL-DE is attributed to their higher adsorption coefficient. However, oil separation of for SL-HE and SL-DE emulsions is likely due to a combination of their low max%-IFT (87.5 and 85.1%, respectively) and flocculation due to interfacial depletion from SLs that reside in the bulk solution. Microscopy and rheology confirmed that flocculation is present for all SL-esters at

1wt% with 20wt% lemon oil as well as for 0.1wt% SL-DE 20wt% lemon oil and 1wt% SL-DE 10wt% lemon oil emulsions.

LSL+ASL was also examined for interfacial performance at the lemon oil/water interface. This combination had the highest CAC (0.442 mg/mL), a max %-IFT comparable to SL-HE and SL-DE (86.8%), and adsorption parameters comparable to SL-EE. Emulsions of 0.1wt% LSL+ASL showed no flocculation and have emulsion droplet sizes similar to SL-EE. However, at 1.0wt% LSL+ASL, all emulsions showed complete oil separation by 24 hours. This is attributed to repulsion between the anionic carboxyl groups on ASL hydrophobic tails.

This work focused on SL interactions at the lemon oil-water interface. Previously our research team described SL interactions at the almond and paraffin oil/water interfaces. The goal in every case was not only to understand the performance of SLs to stabilize the oil/water interface, but also to reveal how extension of the hydrophobic tail of SL-esters alters the surfactants interfacial tension, diffusion, adsorption, and emulsification properties. Furthermore, the cumulative results of this and two previous publications provides information on how the same set of SL-esters and natural SLs behave at three very different oil phase/water interfaces.

Paraffin oil is a largely alkane based oil whereas almond oil consists of triglycerides with primarily oleic and linoleic fatty acids. Lemon oil, as mentioned above, consists of a variety of small molecules that are often cyclic or partially hydrophilic terpenes. The trends in CAC, max %-IFT, and emulsion performance are significantly different between the three oil/water systems. SL-EE consistently has the highest CAC and lowest max %-IFT for paraffin oil and almond oil, while having the highest max %-IFT and the second lowest CAC for lemon oil. SL-DE is among the best performing SL-esters for almond oil and paraffin oil, but has a CAC an order of magnitude larger than SL-EE and SL-HE for lemon oil. Oil separation, with SL-HE and

SL-DE, was only seen for lemon oil while SL-ester emulsions of almond oil and paraffin oil consistently show precipitation. Emulsions of lemon oil, while using the same formulation concentrations as emulsions made with almond oil and paraffin oil, were the only ones studied above the CAC and were the only ones found to flocculate. LSL+ASL showed poor interfacial performance for all three oil/water systems studied. This later point attests to the importance of molecular editing (e.g. chemical modification) of natural structures to enhance their performance.

The diverse properties of SLs at each oil/water interface demonstrate the importance of understanding the influences of both surfactant and hydrophobic phase structure. Taken together, these three studies are the first in depth analysis of a surfactant series with a variety of oil structures. Future work will begin to develop models that will enable the prediction of SL performance as a function of its structure (e.g. ester chain length) and various characteristics of the oil phase under study.

5. Supplemental Information

- Figure S1 - Values of SL remaining in bulk after emulsification.

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Scheme 1. Structures of lactonic and acidic sophorolipids which are the natural products formed by fermentation of *Candida bombicola* on high oleic acid carbon sources. 6' and 6'' carbons are highlighted as the positions of potential acetylation.[12]

Scheme 2. Structures of SL-EE, SL-HE, and SL-DE.

	CAC		Max %-IFT	Minimum IFT (mN/m)	Γ_m (mol/m ²)	K_L (m ³ /mol)
	mg/mL	mol/L				
SL-EE	0.026	4.0E-05	95.1 ± 0.1%	0.81 ± 0.01	2.3E-06	2.2E+02
SL-HE	0.020	2.9E-05	87.5 ± 0.4%	2.05 ± 0.07	1.8E-06	7.1E+02
SL-DE	0.465	6.1E-04	85.1 ± 0.9%	2.45 ± 0.14	1.1E-06	3.5E+02
LSL + ASL	0.442		86.8 ± 1.8%	2.16 ± 0.29	1.2E-06	1.9E+02
ASL	>50	>0.08	n.d.	n.d.	n.d.	n.d.

Table 1. Values of critical aggregation concentration (CAC), max %-IFT, maximum surface excess concentration (Γ_m) and adsorption coefficient (K_L) for a series of SL-esters, LSL+ASL, and ASL at the lemon oil/water interface. IFT averages and standard deviations are based on at least three measurements.

Figure 1 – Lemon oil %-IFT drop across concentration from 0.001 to 50 mg/mL for SL-esters, LSL+ASL, and ASL. Average values and standard deviations from at least three measurements.

Figure 2 – Diffusion coefficients of SL-esters, LSL+ASL, and ASL at the lemon oil-water interface. Average and standard deviation values are calculated from at least three measurements.

Figure 3 – Average emulsion droplet sizes for 0.1 and 1.0wt% SL-EE, SL-HE, and SL-DE immediately after homogenization or at aging times of 24 hours and 1 week at 25°C. Average and standard deviation values are calculated from at least three measurements.

24 Hours						
% Oil	0.1 wt% SL			1wt% SL		
	1%	10%	20%	1%	10%	20%
SL-EE
SL-HE
SL-DE
LSL+ASL

Figure 4 – Photographs of SL-ester and LSL+ASL emulsions after 24 hours of aging at 25°C. Photographs outlined in red show oil separation. Photographs outlined in pink show precipitation.

Figure 5 – Average emulsion droplet sizes of 0.1 and 1.0wt% LSL+ASL stabilized emulsions. Average and standard deviation values are calculated from at least three measurements.

Figure 6 – Confocal and optical microscopy images of 20wt% lemon oil emulsions with either 0.1 or 1.0 wt% SL-EE, SL-HE, or SL-DE aged for 24 hours.

Figure 7 – Viscosity measurements of SL-ester stabilized emulsions after 24 hours of aging at shear rates sweeps from 100 to 5000 s⁻¹, then 5000 to 100 s⁻¹, and lastly 100 to 5000 s⁻¹. Values are an average of three measurements. Arrows indicate the first shear sweep.

Figure 8 - Viscosity measurements of LSL+ASL stabilized emulsions after 24 hours of aging at shear rates from 100 to 5000 s^{-1} , then 5000 to 100 s^{-1} , and lastly 100 to 5000 s^{-1} . Note that the y-axis differs from Figure 7. Values are an average of three measurements.

A versatile family of sophorolipid esters: engineering surfactant structure for stabilization of lemon oil-water interfaces

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Graphical Abstract