1 **Formation and stabilization of nanoemulsions using**
2 **biosurfactants: Rhamnolipids**

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12 **Journal:** Journal of Colloid and Interface Science

13 **Submitted:** May 2016

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Abstract

Nanoemulsions are used in the food, cosmetics, personal care and pharmaceutical industries to provide desirable optical, textural, stability, and delivery characteristics. In many industrial applications, it is desirable to formulate nanoemulsions using natural ingredients so as to develop label-friendly products. Rhamnolipids are biosurfactants isolated from certain microorganisms using fermentation processes. They are glycolipids that have a polar head consisting of rhamnose units and a non-polar tail consisting of a hydrocarbon chain. In this study, the interfacial characteristics of this natural surfactant at medium chain triglyceride (MCT) oil-water interfaces were characterized, and its ability to form nanoemulsions was compared to that of another natural surfactant (quillaja saponins). The influence of rhamnolipid concentration, homogenization pressure, and oil type on the mean droplet diameter of emulsions produced by microfluidization was determined. Rhamnolipids were highly effective at forming small droplets ($d_{32} < 0.15 \mu\text{m}$) at low surfactant-to-oil ratios ($\text{SOR} < 1:10$) for MCT oil. Rhamnolipids could also be used to form small droplets using long chain triglyceride oils, such as corn and fish oil. Rhamnolipid-coated droplets were stable to aggregation over a range of pH values (5 to 9), salt concentrations ($< 100 \text{ mM NaCl}$) and temperatures (20-90 °C). However, droplet aggregation was observed at highly acidic (pH 2 to 4) and high ionic strength (200-500 mM NaCl) conditions. These effects were attributed to a reduction in electrostatic repulsion at low pH and high salt levels. Rhamnolipid-coated droplets had a high negative charge at neutral pH that decreased in magnitude with decreasing pH. These results indicate that rhamnolipids are effective natural surfactants that may be able to replace synthetic surfactants in certain commercial applications.

Keywords: Rhamnolipids; biosurfactants; natural surfactants; nanoemulsions; stability

1. Introduction

Emulsions and nanoemulsions are widely used in commercial products such as those produced by the food, beverage, cosmetics, personal care, and pharmaceutical industries [1]. These materials may be used to provide desirable physicochemical, sensory, or biological attributes to products, such as appearance, texture, stability, or delivery [2, 3]. Oil-in-water emulsions ($d > 200$ nm) and nanoemulsions ($d < 200$ nm) are both non-equilibrium systems that contain oil droplets dispersed within an aqueous medium [4]. The successful formulation of commercial emulsion-based products therefore relies on the identification of effective strategies to ensure their long-term kinetic stability. Surfactants are used to facilitate emulsion formation by rapidly adsorbing to oil droplet surfaces during homogenization and reducing the interfacial tension thereby facilitating droplet disruption [5, 6]. Surfactants also protect the droplets from aggregation during storage by generating repulsive interactions between them [7]. Various kinds of surfactants are available for forming and stabilizing emulsions that can be used in commercial products [8]. These surfactants differ in their functional performances due to differences in their molecular structures, physicochemical properties, and functional attributes, and they also differ in their ease of utilization, legal status, and cost [6, 9, 10]. Consequently, it is important for manufacturers of emulsion-based products to understand how different surfactants behave under different conditions so that they can select the most appropriate one for a particular application [11].

There has been a growing demand from consumers for commercial products that contain “label friendly” and sustainable ingredients [12, 13]. Biopolymer-based surfactants, such as proteins (whey, casein, soy, and egg) and polysaccharides (gum arabic and pectin), are promising natural candidates to form and stabilize emulsions [14, 15]. Nevertheless, there are important limitations to using biopolymers as surfactants in certain applications. Protein-stabilized emulsions are sensitive to aggregation at pH

values around their isoelectric point, at high ionic strengths, and at elevated temperatures [16]. For polysaccharide-based surfactants, it is often necessary to use high surfactant-to-oil ratios and it is sometimes difficult to produce emulsions with small droplet sizes ($d < 200$ nm) [14, 17]. Natural low molecular-weight surfactants, such as saponins and lecithins, have also been shown to be effective at forming and stabilizing emulsions [18, 19]. However, natural lecithin often has to be physically, chemically, or enzymatically modified to improve its emulsifying ability [20]. On the other hand, saponins are isolated from the bark of the *Quillaja saponaria* Molina tree (an evergreen native to Chile and Peru), which may limit the availability of the raw material and lead to appreciable batch-to-batch variations in its performance. In this study, we investigated the suitability of a biosurfactant (rhamnolipid) that has recently been developed for commercial utilization to form and stabilize oil-in-water emulsions.

Rhamnolipids are a type of glycolipid that can be isolated from certain microorganisms (e.g., *Pseudomonas aeruginosa*) using fermentation processes [21]. The composition and properties of rhamnolipids depend on microbial strain, fermentation conditions, and the substrates used to produce them. When these conditions are carefully controlled it is possible to produce rhamnolipids with well-defined and consistent properties. Rhamnolipids can also be chemically synthesized from natural materials, but the final ingredient products may not then be considered to be natural. Rhamnolipids are generally composed of one or two polar rhamnose units attached to a non-polar fatty acid chain containing a β -hydroxylalkanote (Fig. 1) [22, 23]. Rhamnolipids have a carboxylic acid group as part of the hydrophilic head group, which gives the rhamnolipids an anionic character under appropriate pH conditions. Rhamnolipids are surface active because they have both polar and non-polar regions on the same molecule. There have been relatively few studies on the formation and stability of emulsions using rhamnolipids. One study examined the influence of pH on the formation and stability of oil-in-water emulsions prepared using a rhamnolipid [24]. However, the emulsions used in this study were

100 fabricated using a simple shaking method and contained droplets that were too large for
101 most commercial applications. Other studies have also reported that rhamnolipids can be
102 used to form oil-in-water emulsions from a variety of different types of oils, but again the
103 methods used to prepare the emulsions only involved simple shearing methods that led to
104 the formation of large oil droplets [25-27]. One recent study showed that oil-in-water
105 emulsions containing relatively small droplets ($d < 700$ nm) could be produced from
106 rhamnolipids using a high pressure homogenizer [28] .

107 In the current study, we characterized the interfacial properties of rhamnolipids at an
108 oil-water interface, and then studied the influence of rhamnolipid concentration, oil type,
109 and homogenization conditions on the formation of oil-in-water nanoemulsions by
110 microfluidization. In addition, the influence of environmental stresses (pH, ionic strength,
111 temperature, and storage) on the stability of the nanoemulsions to aggregation and
112 creaming was explored. The performance of rhamnolipid-stabilized droplets was also
113 compared to that of another biosurfactant (quillaja saponin) that is already used in
114 industry to formulate emulsion-based products.

118 **Fig.1.** Molecular structures of the two most common rhamnolipids available as biosurfactants:
119 mono- and di-rhamnolipids.

2. Experimental

2.1. Materials

Medium chain triglycerides (MCT, Miglyol 812N) were purchased from the Warner Graham Co. (Cockeysville, MD). Fish oil was kindly provided by DSM Inc. (Columbia MD). Lemon oils (3-fold and 10-fold) were kindly provided by International Flavors and Fragrances (Union Beach, NJ). Corn oil (Mazola) was purchased from a local grocery store. Rhamnolipids (R90) were purchased from Sigma-Aldrich Co. (St. Louis, MO). This material consists of >90% rhamnolipids consisting of a mixture of di- and mono-rhamnolipids. Quillaja saponin (Q-Naturale[®] 200), which is an extract from the *Quillaja saponaria* tree, was kindly provided by Ingredion Inc. (Westchester, IL). Sodium phosphate monobasic was purchased from Sigma-Aldrich Co. (St. Louis, MO). Sodium chloride was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Double distilled water (Milli-Q) was used to prepare all solutions and nanoemulsions.

2.2. Emulsion preparation

Oil-in-water nanoemulsions were prepared by homogenizing 10 wt% oil phase (MCT, fish oil, corn oil or lemon oil) with 90 wt% aqueous phase (0.1-3 wt% biosurfactant). The aqueous phase consisted of biosurfactants (rhamnolipids or Q-Naturale) and buffer solution (10 mM sodium phosphate, pH 7.0). The commercial Q-Naturale ingredient contained 14 wt% active saponins (with the remainder being mainly water), and so the concentration of this surfactant is reported on an active ingredient basis (rather than total mass basis). Initially, coarse emulsions were prepared by blending the oil and aqueous phases together using a high speed mixer (Bamix, Biospec Products, Bartlesville, OK) for 2 min at ambient temperature. Fine nanoemulsions were then formed by passing the coarse emulsions through an air-driven microfluidizer equipped

with a Y- and Z-type interaction chamber (Microfluidics PureNano, Newton, MA, USA). The coarse emulsions were fed into the microfluidizer through a 100 ml glass reservoir (pressurized by an intensifier pump, flow rate 500 ml per minute), and were passed through the homogenization unit three times at different homogenization pressures (9 to 19 kpsi).

2.3. Emulsion stability testing

Freshly prepared MCT nanoemulsions were subjected to a series of environmental stresses that might be encountered in commercial applications, and then stored for 24 h at ambient temperature prior to analysis:

Thermal processing: Nanoemulsions were transferred into glass test tubes that were then incubated in water baths set at temperatures ranging from 30 to 90 °C for 30 min, and then cooled to room temperature.

pH: Nanoemulsions were placed in 20 ml beakers, adjusted to different pH values (2.0-9.0) using NaOH and/or HCl solutions, and then transferred into glass test tubes.

Ionic strength: Nanoemulsions were placed in 20 ml beakers, adjusted to different salt levels (100 to 500 mM NaCl) by adding NaCl, and then transferred into glass test tubes.

Storage stability: Nanoemulsions were placed in glass test tubes and then incubated at ambient temperature for at least two weeks prior to analysis.

2.4. Determination of particle size

The particle size distribution and mean particle diameter of diluted nanoemulsions were measured using a static light scattering instrument (Mastersizer 2000, Malvern Instruments, Malvern, UK). The refractive indices (RI) of the aqueous and oil phases used by the instrument to calculate the particle size are listed in **Table 1**. The droplet diameter of each sample was represented as the surface-weighted mean diameter (d_{32}),

which was calculated from the full particle size distribution.

Table 1 The refractive indices (RI) of water and different oil type used in this study. ^a

Phase	RI
Water	1.330
MCT	1.445
Corn oil	1.472
Fish oil	1.481
Lemon oil (10-fold)	1.487
Lemon oil (3-fold)	1.475

^a The RI of the oils was measured at ambient temperature (25 °C).

2.5. Determination of ζ -potential

The droplet charge (ζ -potential) of the nanoemulsions was measured using particle microelectrophoresis (Zetasizer Nano ZS-90, Malvern Instruments, Worcestershire, UK). The samples were diluted with corresponding buffer solutions prior to measurements in order to avoid multiple scattering effects.

2.6. Interfacial tension measurements

The interfacial tension *versus* rhamnolipid concentration profile was measured at a MCT oil-water interface using a drop shape analysis instrument (DSA 100, Kruss GmbH, Hamburg Germany). Initial experiments were carried out to determine the time at which the interfacial tension reached a constant value over a limited timescale (less than 15 min) or the drop completely detached from the needle. We then used an incubation time of 15 min or complete detachment of oil drop for all of the subsequent experiments. The biosurfactant concentration range used was from 0.0005% to 2.0% since over 2.0% the rhamnolipids solutions were not sufficiently transparent to make reliable measurements. The surface pressure (π) was calculated from the interfacial tension (γ) data using the

following equation: $\pi = \gamma_0 - \gamma$, where γ and γ_0 are the interfacial tensions measured in the presence and absence of biosurfactant.

2.7. Statistical analysis

All measurements were performed on at least two freshly prepared samples with three measurements per sample and are reported as means and standard deviations. Statistical analysis was performed through subsection of the data to analysis of variance (ANOVA) using Duncan's new multiple range test (Minitab 16.2.4, Minitab Inc., State College, PA). A p-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Interfacial characteristics of rhamnolipid

The interfacial characteristics of surfactants play an important role in determining their ability to form and stabilize emulsions [29]. We therefore measured the interfacial tension *versus* rhamnolipids concentration profile to better understand its interfacial behavior (Fig. 2). A representative image of one of the oil droplets generated during the measurement is shown in Fig. 2a. The interfacial tension was calculated by the instrument using drop shape analysis.

The interfacial tension decreased with increasing rhamnolipid concentration (Fig. 2b), which indicated that rhamnolipid molecules adsorbed to the MCT oil-water interfaces. The interfacial tension initially decreased steeply with increasing rhamnolipid concentration, showing that the rhamnolipids were highly effective at reducing the interfacial tension even at lower levels. A fairly constant interfacial tension was reached at high rhamnolipids levels ($\gamma_{\infty} \approx 5 \text{ mNm}^{-1}$), which indicated that the oil-water interface had become saturated with biosurfactant molecules [30]. The apparent critical micelle concentration (CMC) was determined from the interfacial tension *versus* logarithm of

surfactant concentration profile using a method described previously [31]. We estimated the CMC from the data shown in Fig. 2b to be around 0.014 wt% (140 mg/L) for rhamnolipids, which is in reasonable agreement with reported literature values (10 to 200 mg/L) of rhamnolipids obtained from different sources [32-34]. The CMC value was estimated by finding the intersection point of linear lines of interfacial tension versus emulsifier concentration extrapolated from low and high rhamnolipid concentrations (see Supplementary material Figure S1). The CMC value for the rhamnolipids was comparable to the value of 0.01 wt% reported for Q-Naturale [9], another natural small molecule surfactant, indicating that both of them have fairly similar surface activities.

Fig.2. (a) The morphology of MCT oil droplets used for interfacial tension measurements. (b) Influence of rhamnolipid concentration on the interfacial tension measured at the MCT oil-water interface. *Note:* The upper line in (a) represents the start point for measurement, while the lower lines define the reference of drop shape based on the width of the needle.

The surface activity (K) of a surfactant can be estimated using the following relationship [35]:

$$K = \frac{1}{c_{1/2}} = \exp \left(-\frac{\Delta G_{ads}}{RT} \right) \quad (1)$$

Here, $c_{1/2}$ is the surfactant concentration where half of the available adsorption sites on

the oil-water interface are covered by surfactants, and ΔG_{ads} is the free energy change associated with surfactant adsorption. To a rough approximation, $c_{1/2}$ can be taken to be equal to the surfactant concentration where the surface pressure is half its maximum value (π_{∞}). In this study, we calculated the following parameters from the interfacial pressure *versus* rhamnolipids concentration profile (Fig. S1): $c_{1/2} = 0.0063$ wt% (109 μM), $K = 9210 \text{ M}^{-1}$, and $\Delta G_{ads} = -9.13RT$. The molecular weights of rhamnolipids were 504.7 and 650.4 g/mol for mono- and di-rhamnolipids respectively, which were obtained from its molecular structure (Fig. 1). An average molecular weight of 578 g/mol was therefore used in the calculations since the rhamnolipid ingredient used in this study was a mixture of mono- and di-rhamnolipids. Consequently, the reported CMC, K , surface activity, and free energy values reported in this study should only be treated as rough estimates rather than accurate values. Overall, our measurements show that the rhamnolipids were highly effective at reducing the interfacial tension at the MCT oil-water interface, and exhibited fairly similar interfacial characteristics compared with another biosurfactant (Q-Naturale).

3.2. Formation of rhamnolipids-stabilized nanoemulsions

3.2.1 Influence of rhamnolipid concentration on nanoemulsion formation

Initially, the influence of rhamnolipid concentration on the formation of MCT oil-in-water nanoemulsions fabricated using fixed homogenization conditions was investigated. The emulsifying properties of the rhamnolipids were compared to that of another natural surfactant (quillaja saponins) already successfully used in the food industry. Overall, there was a decrease in mean particle diameter (d_{32}) with increasing surfactant concentration for both the rhamnolipids and the saponins. The droplet diameter *versus* surfactant concentration profiles followed the two-regime profile typically seen in homogenization (Fig. 3): a steep decrease at low surfactant concentrations, followed by a

relatively constant value at high surfactant levels [35, 36]. This type of behavior can be attributed to the fact that the droplet size is limited by the amount of surfactant present at low surfactant concentrations, but by the disruptive energy of the homogenizer at high surfactant levels [7, 37].

Fine droplets could be produced at high surfactant concentrations for both the rhamnolipids ($d_{32} \approx 110$ nm) and the saponins ($d_{32} \approx 120$ nm). In addition, a relatively low surfactant concentration ($\approx 0.2\%$) was required to produce small droplets ($d_{32} < 200$ nm) for both biosurfactants. These experiments indicate that both types of biosurfactants were effective at producing small droplets, and they have similar efficiency in forming nanoemulsions. Knowledge of the surface load of a surfactant at saturation (Γ) is important because it provides valuable information about interfacial properties, and about the minimum amount of surfactant required to form a given emulsion. When the droplet diameter is limited by the amount of surfactant present, the minimum surfactant concentration required to cover all of the droplets in an emulsion is given by [11]:

$$C_s = \frac{6 \times \Gamma \times \varphi}{d_{32}} \quad (2)$$

where C_s is the concentration of surfactant in the emulsion (kg/m^3), Γ is the surface load of the surfactant at saturation (kg/m^2), φ is the dispersed phase volume fraction (dimensionless), and d_{32} is the mean droplet diameter (m). This equation was used to calculate the surface load of the biosurfactants from knowledge of the disperse phase volume fraction ($\varphi = 0.1$), surfactant concentration (0.1 to 3 wt% or 1 to 30 kg/m^3), and the measured mean droplet diameters (d_{32}). The calculated surface loads of the rhamnolipids and the saponins were 2.02 and 2.03 mg/m^2 , respectively, which are in agreement with those reported in previous publications [9]. These calculations indicate that the surface loads of rhamnolipids and the saponins are very similar, which suggests that similar levels should be required to form emulsions. This equation assumes that all of the surfactant molecules are adsorbed to the droplet surfaces, and that adsorption occurs extremely quickly during homogenization, which may not be strictly true in practice.

Fig. 3. Influence of rhamnolipids and quillaja saponins concentration on the mean particle diameter (d_{32}) of 10 wt% MCT oil-in-water nanoemulsions produced using a microfluidizer under standardized homogenization conditions (13 kpsi, three cycles).

The nature of the particle size distribution is an important factor for practical applications of emulsion products. We therefore plotted the particle size distribution of nanoemulsions fabricated using rhamnolipids or saponins using standardized homogenization conditions (Fig. 4). The particle size distribution was bimodal at lower rhamnolipid levels (0.1-0.5 wt%), but monomodal at higher levels (Fig. 4a). On the other hand, the particle size distribution was monomodal at all saponin concentrations used (Fig. 4b). Overall, these results demonstrate that fine nanoemulsions with narrow particle size distributions can be produced from rhamnolipids using microfluidization.

Fig. 4. Particle size distributions of 10 wt% MCT oil-in-water nanoemulsions containing different concentrations of (a) rhamnolipids and (b) saponins produced by standardized homogenization conditions (13 kpsi, three cycles).

3.2.2 Influence of oil type on nanoemulsion formation using rhamnolipids

In commercial applications, a variety of different oils may be used to produce emulsion-based products. In this section, we therefore examined the influence of oil type on the formation of nanoemulsions using rhamnolipids (Fig. 5). Nanoemulsions ($d_{32} < 200$ nm) could be successfully produced using MCT, corn oil, fish oil, and lemon oil (10 $\times$) using 1 wt% rhamnolipids. The mean droplet diameter of MCT, corn oil and fish oil nanoemulsions were fairly similar (130 nm), while that of the lemon oil (10 $\times$) nanoemulsions was a little larger (167 nm). Emulsions containing relatively large droplets (316 nm) could only be produced using the lower-fold lemon oil (3 $\times$). This difference in the formation of different kinds of emulsions stabilized by rhamnolipids could be attributed to the nature of different oil types, such as viscosity, polarity, molar volume, *etc.* [38-40]. In particular, lower-fold lemon oils are known to be highly susceptible to Ostwald ripening due to the relatively high polarity of these oils [41].

After storage at ambient temperature for 7 days, the overall appearance of the nanoemulsions fabricated from MCT, corn oil, fish oil and lemon oil remained fairly similar to that of the initial systems (Fig. S2). The mean droplet diameters of the MCT,

corn oil, and fish oil nanoemulsions were also fairly similar to those of the initial systems (Fig. 5). On the other hand, the mean droplet diameter of the lemon oil emulsions increased appreciably after storage, *i.e.*, to 250 and 500 nm for the 10× and 3× lemon oils, respectively. The instability of these systems is most likely a result of droplet growth through Oswald ripening during storage [42]. The water-solubilities of lemon oils are known to be appreciably higher than those of medium and long chain triglycerides [38], and 3-fold lemon oil is known to be more soluble than 10-fold lemon oil [41]. This kind of instability can often be inhibited by adding a ripening inhibitor to the oil phase of the emulsions [38]. These results indicate that rhamnolipids are able to form stable nanoemulsions from various types of oil that may be commercially important.

Fig. 5. Influence of 1 wt% rhamnolipids on the mean particle diameter (d_{32}) of 10% oil-in-water nanoemulsions with different oil types produced using standardized homogenization condition (13 kpsi, three cycles). Nanoemulsions were stored at room temperature for 0 or 7 days before particle size analysis. Different letters (a to d) mean significant differences ($p < 0.05$) in the mean droplet diameter at a given oil concentration (*i.e.*, the effect of oil type).

3.3. Influence of homogenization pressure on nanoemulsion formation

The size of the droplets produced using high-pressure homogenization is known to depend on homogenization conditions, such as operating pressure and number of passes [43, 44]. We therefore examined the influence of homogenization pressure on the droplet diameter of nanoemulsions stabilized by 1 wt% rhamnolipids (Fig. 6). Overall, there was a decrease in mean droplet diameter with increasing operating pressure, which can be attributed to an increase in the magnitude of disruptive energy generated by the homogenizer [43]. Over the range of pressures used (9 to 19 kpsi), the mean droplet diameter decreased from 145 to 114 nm. There was a linear decrease in the logarithm of the mean droplet diameter with the logarithm of the homogenization pressure for nanoemulsions stabilized by rhamnolipids (Fig. 6a), which is in line with previous studies [45]. The slope determined from the $\log_{10}(d_{32})$ versus $\log_{10}(P)$ plots was around -0.31 (Fig. 6), which is lower than the values of -0.6 to -0.8 reported for high pressure homogenizers [46, 47], but similar to the value of -0.3 to -0.4 reported for microfluidizers [37, 48].

The particle size distribution of rhamnolipid-stabilized nanoemulsions produced at different pressures were monomodal at all homogenization pressures used (Fig. 6b), although the width of the distribution was much larger at 9 kpsi than at other pressures. These results again indicate that rhamnolipids are efficient biosurfactants for producing nanoemulsions, and that particle size can be modulated by altering the homogenization pressure.

Fig. 6. Influence of homogenization pressure on (a) the mean particle diameter (d_{32}) and (b) particle size distributions of 10 wt% MCT oil-in-water nanoemulsions containing 1 wt% rhamnolipids (three cycles at different pressures).

3.4. Influence of environmental stresses on emulsion stability

Practically, an emulsion-based commercial product should remain stable when exposed to different processing, storage, transportation, and utilization conditions. Emulsions may be exposed to a range of environmental conditions (e.g., pH, ionic strength, or temperature changes) throughout their lifetime, which may lead to instability through various mechanisms (e.g., flocculation, coalescence, Ostwald ripening, and gravitational separation) [35]. Therefore, it is important to establish the influence of these environmental stresses on the stability of nanoemulsions containing rhamnolipid-coated droplets.

3.4.1 Thermal processing

The influence of thermal processing on the stability of the nanoemulsions was assessed by placing them in water baths set at different temperatures (30-90 °C) for 30 min, and then cooling them to ambient temperature (Fig. 7). Statistical difference analysis of the temperature effects are reported in Table S1. Nanoemulsions stabilized by 1 wt%

rhamnolipids were stable to thermal processing across the range of temperatures studied,
i.e., there was little change in mean particle diameter (around 130 nm) and no visible
 creaming or oiling off (Fig. 7a). Rhamnolipids are relatively low molecular weight
 molecules that would be expected to form thin interfacial layers. Consequently, the range
 of the steric repulsion generated by the biosurfactant head groups would not be expected
 to be long enough (compared to the Van der Waals attraction) to prevent droplet
 flocculation. Presumably, the droplets were therefore stabilized against aggregation by
 electrostatic repulsion. Indeed, the measured ζ -potential of the droplets was relatively
 high and similar across the entire temperature range studied (around -76 mV), suggesting
 that there would be a strong electrostatic repulsion between them (Fig. 7b). The
 rhamnolipids therefore have a similar thermal stability to quillaja saponins that have also
 been shown to be stable to droplet aggregation over a similar temperature range [9].

Fig. 7. Influence of thermal processing (30-90 $^{\circ}\text{C}$, 30 min) on (a) the mean particle diameter (d_{32})
 and (b) the ζ -potential of oil-in-water nanoemulsions (1 rhamnolipid, 10% MCT) produced using
 standardized homogenization conditions (13 kpsi, three cycles) and stored at room temperature
 for 24 h before analysis. The photograph of the nanoemulsions in (a) was taken after 24 h storage.

3.4.2 pH

The influence of storage pH on the mean droplet diameter, ζ -potential, and physical

stability of the rhamnolipid-stabilized nanoemulsions was examined (Fig. 8). Statistical difference analysis of the pH effects are reported in Table S2. At pH 5 to 9, the nanoemulsions had relatively small droplets and were visibly stable to creaming and oiling off (Fig. 8a). At pH 4, a thin layer of oil was observed on top of the nanoemulsions after storage (Fig. 8a), indicating that droplet coalescence and oiling off had occurred [49]. Interestingly, the mean particle diameter at pH 4 was still relatively small (< 300 nm), despite the fact that some coalescence had occurred. At pH 2 to 3, the nanoemulsions were highly unstable to phase separation (Fig. 8a). The rhamnolipid-coated droplets therefore have a narrower range of pH stability (pH 5 to 9) than the quillaja saponin-coated droplets (pH 3 to 8) measured in a previous study [9].

The ζ -potential of the rhamnolipid-coated droplets was measured as a function of pH to provide some insight into the origin of the pH-instability of the nanoemulsions (Fig. 8b). The electrical charge went from highly negative (around -83 mV) at pH 9 to slightly positive at pH 2 (around $+6$ mV). The good stability of the nanoemulsions at high pH values can therefore be attributed to strong electrostatic repulsion between the highly charged droplets. At pH 2 and 3, the electrostatic repulsion is not strong enough to overcome any attractive interactions (e.g., van der Waals) acting between the droplets, thereby leading to droplet aggregation. The origin of the negative charge on the rhamnolipid-coated droplets can be attributed to the presence of a carboxylic acid group within the molecular structure of rhamnolipids (Fig. 1). Typically, the carboxylic acid groups of rhamnolipid have pK_a values around pH 4.28-5.50 [50], which would account for the steep decrease in ζ -potential observed around these values (Fig. 8b). At high pH values ($pH \gg pK_a$) the carboxylic acid groups are charged ($-\text{COO}^-$) whereas at low pH values ($pH \ll pK_a$) they are neutral ($-\text{COOH}$).

Fig.8. Influence of pH on (a) the mean particle diameter (d_{32}) and (b) the ζ -potential of oil-in-water nanoemulsions (1% rhamnolipids, 10% MCT) produced using standardized homogenization conditions (13 kpsi, three cycles) and stored at room temperature for 24 h before analysis. The insert photograph of the nanoemulsions in (a) was taken after 24 h storage.

3.4.3 Ionic strength

The influence of ionic strength on the stability of rhamnolipid-coated droplets was assessed by including different levels of salt (0-500 mM NaCl) in the nanoemulsions (Fig. 9). Statistical difference analysis of the ionic strength effects are reported in Table S3. Visible observation of the nanoemulsions indicated that phase separation occurred at ≥ 200 mM NaCl, with a yellowish oil layer being observed at the top (Fig. 9a). In addition, there was a large increase in the mean droplet diameter measured by light scattering at all salt levels. Interestingly, no visible phase separation was observed in the nanoemulsions containing 100 mM NaCl, but there was still an appreciable increase in mean particle diameter. These results indicate that the rhamnolipid-coated droplets were highly prone to salt-induced coalescence. Conversely, nanoemulsions prepared using quillaja saponins have been shown to be stable to droplet coalescence at all salt concentrations studied (0 to 500 mM), with no evidence of an increase in particle size measured by light scattering [9]. However, a cream layer was visually observed at higher salt concentrations (≥ 300

mM NaCl) for saponin-coated droplets, indicating that droplet aggregation was primarily due to flocculation rather than coalescence for this type of surfactant. In summary, rhamnolipids-coated droplets have much worse salt stability than quillaja saponin-coated droplets, which may limit their commercial application in some products.

The tendency for droplet aggregation of rhamnolipid-stabilized nanoemulsions to occur at high ionic strengths can be attributed to electrostatic screening effects, *i.e.*, accumulation of counter-ions around the droplet surfaces reducing electrostatic repulsion [51]. This effect was confirmed by ζ -potential measurements, which showed a steep decrease in the magnitude of the droplet charge with increasing salt concentration (from around -80 to -18 mV) (Fig. 9b). These results also suggest that electrostatic repulsion is the main stabilization mechanism for rhamnolipid-coated oil droplets. At low salt concentrations, the long-range electrostatic repulsion between the rhamnolipid-coated droplets is strong enough to overcome the long-range van der Waals attraction, and thereby prevent aggregation. However, as the salt concentration is increased the strength and range of the electrostatic repulsion decreases. Above a critical salt concentration the repulsive interactions are no longer stronger than the attractive interactions, which leads to droplet aggregation. The fact that saponin-coated droplets are stable to coalescence at high salt concentrations whereas the rhamnolipid-coated droplets are not, suggests that the rhamnolipids form an interfacial layer that is less resistant to rupture when the droplets are in close proximity [9, 18].

Fig.9. Influence of ionic strength on (a) the mean particle diameter (d_{32}) and (b) the ζ -potential of oil-in-water nanoemulsions (1% rhamnolipids, 10% MCT) produced by standardized homogenization conditions (13 kpsi, three cycles) and stored at room temperature for 24 h before analysis. The insert photograph in (a) was taken after 24 h storage.

3.4.4. Storage stability

The stability of 10 wt% MCT nanoemulsions prepared using different rhamnolipid concentrations was investigated after they were stored at room temperature for two weeks. These experiments were carried out using nanoemulsions at neutral pH in the absence of added salt. No change in appearance of nanoemulsions containing high rhamnolipid levels (≥ 0.5 wt%) was observed after storage, but some creaming was observed at lower rhamnolipid levels (0.1-0.3 wt%) (Fig. S3). Similarly, there was little change in the mean droplet diameter of the nanoemulsions containing high rhamnolipid levels, but a slight increase in droplet size at lower rhamnolipid levels (Fig. S4). These results indicate that the nanoemulsions were relatively stable to storage, provided a sufficient amount of biosurfactant was used to stabilize the droplets.

4. Conclusions

There is considerable interest in replacing synthetic emulsifiers with natural

alternatives in many industrial applications, such as foods, supplements, cosmetics, and personal care products. However, there is currently a lack of systematic information about the ability of many natural emulsifiers to form and stabilize emulsions. To the authors knowledge, this is the first detailed study of the interfacial characteristics, emulsion forming, and emulsion stabilizing properties of rhamnolipids that is carried out using commercially realistic homogenization conditions, i.e., high pressure homogenization. Rhamnolipids were found to be highly surface active, and have similar interfacial properties as a commercially available natural surfactant (quillaja saponin). The electrical charge on rhamnolipid-coated droplets went from highly negative at high pH values to slightly positive at low pH values, which was attributed to the presence of carboxylic acid groups on the rhamnolipids.

Rhamnolipids were able to form oil-in-water nanoemulsions with relatively small droplet diameters ($d_{32} < 150$ nm) at low surfactant-to-oil ratios (SOR < 1:10). Nanoemulsions could be formed from a number of different oil types, including medium chain triglycerides, long chain triglycerides, and flavor oils. The rhamnolipid-coated droplets were stable to a range of thermal treatments (30-90 °C), salt concentrations (< 100 mM NaCl), and pH conditions (pH 5-9). In addition, they had good stability to droplet aggregation and creaming when stored at room temperatures for at least two weeks. Conversely, the nanoemulsions were unstable to coalescence when stored under highly acidic (pH 2-4) or high ionic strength (200-500 mM NaCl) conditions. These effects were attributed to a reduction in the range and magnitude of the electrostatic repulsion between the droplets at low pH and high salt levels, and the fact that the steric repulsion was relatively short range. Overall, our results show that rhamnolipids are effective natural surfactants under certain environmental conditions, and may therefore be suitable for utilization in a variety of commercial emulsion-based products. Nevertheless, their successful utilization in commercial applications will also depend on other factors, such as their ease of utilization, potential toxicity, reliability of supply, and cost.

5. Acknowledgements

This material was partly based upon work supported by the Cooperative State Research, Extension, Education Service, USDA, Massachusetts Agricultural Experiment Station (MAS00491) and USDA, NRI Grants (2013-03795). Long Bai would like to thank the Chinese Scholarship Council for support.

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Graphical Abstract