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**Stability and Interfacial Viscoelasticity of Oil-Water Nanoemulsions Stabilized by Soy
Lecithin and Tween 20 for the Encapsulation of Bioactive Carvacrol**

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rheometry

ABSTRACT

The rheology of oil-in-water (O/W) droplet interfaces stabilized by food-grade emulsifiers (soy lecithin or Tween 20) under controlled aqueous conditions was investigated to elucidate its contribution in the kinetic stabilization of nanoemulsion-based delivery systems containing carvacrol, a naturally-derived antimicrobial compound. Dilational rheology of surfactant-laden O/W interfaces was measured using axisymmetric drop shape analysis. The kinetic stability of corresponding nanoemulsions (containing mixtures of carvacrol and medium-chain triglyceride (MCT) oil dispersed in water (pH 7)) was characterized using dynamic light scattering. Zwitterionic lecithin molecules adsorbed to the O/W interface for 24 hours formed a notably viscoelastic layer, compared to nonionic Tween 20 molecules. The kinetic stability within the first 24 hours for each nanoemulsion was strongly dependent upon encapsulated carvacrol concentration, with higher carvacrol concentrations leading to lower kinetic stability. Lecithin-stabilized nanoemulsions (pH 7) were highly stable, yielding monodispersed droplet size distributions and high resistance to increases in droplet size over 30 days. Contrarily, corresponding Tween 20-stabilized nanoemulsions showed large increases in the droplet size and

developed a bimodal droplet size distribution over time. The initial size of oil droplets stabilized by lecithin was slightly dependent on pH, yielding smaller droplets at pH 7 and larger droplets at pH 3; however, the extended kinetic stability was not greatly impacted by pH modulation. Determining a positive association between interfacial viscoelasticity and nanoemulsion stability may potentially be very useful for food manufacturers seeking to optimize the encapsulation and delivery of lipophilic antimicrobial molecules using food-grade emulsifiers.

1. INTRODUCTION

In recent years, the use of essential oils as natural antimicrobial molecules in food products has attracted growing interest to meet consumers' desires in terms of food quality and safety. These bioactive molecules are used for microbial disinfection during food processing, as well as to ensure a certain shelf-life of the food product without negatively affecting the organoleptic properties of food (*e.g.*, taste, texture).[1,2] Carvacrol (2-methyl-5-isopropylphenol) is an antimicrobial, lipophilic compound that is primarily derived from the essential oil fractions of oregano (ca. 60-75%) and thyme (ca. 45%).[3–5] Carvacrol is added to many food products including baked goods and nonalcoholic beverages due to its antimicrobial activity against several strains of harmful foodborne bacteria (*i.e.* *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, etc.).[4–8] Unfortunately, carvacrol (like most lipophilic bioactive compounds) has a limited solubility in water (0.83 mg/L),[9] where microorganisms are most likely to grow and proliferate.[10,11] Thus, more advanced delivery methods are required to incorporate lipophilic bioactive molecules into the water-rich phases within food products, such as encapsulating the bioactive molecules in oil-based emulsion droplets.[12,13] Encapsulation of essential oils into nanoscale delivery systems is known to increase their physical stability by protecting these compounds from undesirable chemical interactions with food ingredients.[14]

Also, the encapsulation of the essential oil compounds carvacrol and eugenol into nanoscale micelles has been shown to increase their antimicrobial activity due to improved physicochemical interactions of the antimicrobials with the outer membranes of microorganisms.[15,16]

Oil-in-water (O/W) nanoemulsions containing carvacrol have been recently created by high-energy homogenization,[1] as well as low-energy spontaneous emulsification.[17] In the latter study, initial droplet size was sensitive to the concentration of carvacrol in the carrier oil (medium chain triglyceride, MCT), and over 30 days, the droplets increased in size with the greatest increases observed for droplets containing the greatest amount of carvacrol (30, 40 wt.% carvacrol in MCT).[17] The authors speculate that the increased polarity of the oil phase resulting from the increased concentration of carvacrol contributed to the accelerated droplet coalescence due to weakened adsorption of the surfactant (Tween 20) to the O/W interface.[18] Indeed, the adsorption strength and level of intermolecular interactions of molecules adsorbed to fluid interfaces will have a strong effect on the overall kinetic stability of nanoemulsions, as well as the measured rheological response of the interface. Although, many past studies have concluded that there is not always a direct correlation between the interfacial rheological response and nanoemulsion stability.[19,20]

Here, to directly quantify the molecular-level interactions of surfactant-stabilized O/W nanoemulsions containing carvacrol, we investigated O/W droplet interfaces containing zwitterionic (soy lecithin) or non-ionic (Tween 20) molecules using axisymmetric drop shape analysis. Lecithin is a naturally derived mixture of zwitterionic phospholipid molecules widely used as an emulsifying agent in the food industry. Its major component, phosphatidylcholine, is composed of a polar head group containing phosphocholine and glycerol residue.

Phosphatidylcholine also contains a non-polar region composed of two hydrocarbon fatty acid chains. The effectiveness of lecithin as a nanoemulsion stabilizer is attributed to the self-assembly of the molecule at the O/W interface and subsequent formation of a thick viscoelastic film strengthened by hydrogen bonding between phosphate groups on neighboring molecules.[21] Tween 20 is a food-grade, non-ionic surfactant containing a polyoxyethylene head group and fatty acid tail linked together by sorbitol. Recent investigations have attributed the protection against oil droplet coalescence in O/W emulsions stabilized by Tween 20 to the high surface activity and steric interactions of Tween 20 molecules at oil droplet interfaces.[18,22] These surfactants were chosen to due to their widespread use as food grade emulsifiers.

This study seeks to elucidate the contribution of the rheological properties of surfactant-laden O/W interfaces in the stabilization of nanoemulsions containing carvacrol. The dynamic interfacial tension isotherms for soy lecithin or Tween 20 adsorbed O/W interfaces were measured by static pendant drop tensiometry. The development of viscoelasticity in these interfaces within the first 24 hours of formation was quantified by oscillating pendant drop tensiometry. Nanoemulsions containing these surfactants were created by ultrasonication. The Z-Average (Z-Ave) diameter and zeta (ζ) potential of oil droplets were measured 24 hours after formulation. The Z-Ave diameter and ζ potential of nanoemulsions were monitored over 30 days at 23 °C under standard gravity and comparisons were made between initial and final values to quantify the extended stabilization behavior.

2. EXPERIMENTAL METHODS

2.1. Materials and Reagents

For both axisymmetric drop shape analysis and nanoemulsion preparation, the aqueous phase was a citrate-phosphate buffer (5 mmol citric acid and 10 mmol dibasic sodium phosphate) mixed in various ratios to obtain pH 7 and 3 buffers. These aqueous phase pH values were selected to mimic the neutral and acidic environments of common commercial beverages, such as milk and soft drinks. Deionized water was obtained from a Barnsted Nanopure™ system with a 0.2 µm filter at 18 MΩ resistivity. The emulsifying agents investigated were Ultralec® P soy lecithin (Archer Daniels Midland Specialty Products, stored at 4 °C) and Tween® 20 (Sigma-Aldrich, stored at 23 °C) and were used as received. The manufacturer reported lecithin's major phospholipid content as 23 wt.% phosphatidylcholine, 18 wt.% phosphatidylethanolamine, 15 wt.% phosphatidylinositol, and 6 wt.% phosphatidic acid.

For nanoemulsion preparation, the oil phase comprised 5 wt.% of the total 20 g nanoemulsion and the concentrations of carvacrol (≥99.8% pure, Sigma-Aldrich) in the oil phase were 0 (control), 5, 10, 20, 40, 60 and 80 wt.% carvacrol in Neobee® 1053 medium-chain triglyceride (MCT) oil (Stepan Specialty Products). Each oil phase mixture was passed through an alumina column three times to remove surface-active trace impurities prior to use. For axisymmetric drop shape analysis, the oil phase contained 10 wt.% carvacrol in MCT oil.

2.2. Axisymmetric Drop Shape Analysis

Details on these measurement techniques are described in the literature.[23–26] In brief, the interfacial tension of the O/W interface was determined by fitting the Laplace equation to the profile of an axisymmetric oil droplet ($V \approx 30 \mu\text{L}$ to $32 \mu\text{L}$) formed at the tip of an inverted 20-gauge stainless steel needle (0.584 mm i.d.). The oil droplet was immersed in 5 mL of the aqueous surfactant solution in a rectangular glass cuvette. The dimensionless shape factor, β , exceeded the minimum value ($|\beta| > 0.1$) for accurate measurement of the interfacial tension for

each system ($|\beta| > 0.18$).^[27] This experimental setup was used for both static and oscillating pendant drop tensiometry and included a contact angle goniometer/tensiometer with DROPImage Advanced software (Model 500), automated drop volume dispensing system, motor-driven oscillator, and halogen fiber optic illuminator manufactured by Ramé-Hart, Inc. (Succasunna, New Jersey, USA).

2.3. Viscoelasticity of Surfactant-Adsorbed Interfaces

The apparent viscoelasticity of O/W interfaces was measured by harmonically oscillating the initial interfacial area (A_0) at a small dilational strain ($\Delta A/A_0 = 2.5\%$) and a low frequency ($\omega = 0.01$ Hz) by increasing and decreasing the droplet volume using an in-line mechanical plunger. The interfacial strain and oscillation frequency used in this analysis fall below critical limits defined in the literature and the interface may be considered to be at mechanical equilibrium.^[28] Frequently referred to as oscillating pendant drop tensiometry, this technique has been applied to study the conformation of proteins,^[29–31] as well as the viscoelasticity of phospholipids,^[32] low molecular weight surfactants,^[27,33–36] and mixed surfactant/polymer solutions^[37] at oil-water and air-water interfaces. In this technique, the apparent complex viscoelastic modulus (E^*) is determined by measuring the response in interfacial tension variation ($\Delta\gamma$) resulting from a small, harmonically applied strain ($\Delta A/A_0$), as well as the phase angle (δ) between the periodic interfacial tension and strain curves of an interfacial element^[25]:

$$E^*(\omega) = E'(\omega) + iE''(\omega) = |E| \cos \delta + i|E| \sin \delta \quad (1)$$

where $|E| = \frac{\Delta\gamma}{\Delta A/A_0} \quad (2)$

The apparent complex modulus is primarily a measure of the system's response to dilational deformation (although, there is some evidence to suggest that shear contributions may be present

in mm-scale oscillating droplet measurements as well[38,39]) and quantifies the elastic storage modulus (E') and the viscous loss modulus (E''), both dependent on the oscillation frequency (ω). The rheological behavior of surfactant adsorbed interfaces was monitored over 24 hours and area oscillations were imposed every 60 minutes. The first oscillatory measurement occurred 1 hour after the formation of a fresh oil drop at the tip of the inverted needle. Experiments were performed in a standard laboratory environment ($T = 23^\circ\text{C}$).

2.4. Determination of Minimum Nanoemulsion Surfactant Concentration

In the process of nanoemulsion formulation, very large surface to volume ratios of oil droplet interfaces are generated. Therefore, the concentration of emulsifier (lecithin or Tween 20) necessary to accommodate the large interfacial areas generated were determined prior to emulsion formulation. The minimum surfactant concentration (C_a) necessary for nanoemulsion formation is given by Equation 1,[40–42]

$$C_a = \frac{6\phi\Gamma_s}{d} \quad (3)$$

where ϕ is the volume fraction of the disperse phase, d is the mean droplet diameter, and Γ_s is the surface load. For our nanoemulsions, the dispersed oil phase volume fraction was $\phi = 0.05$ and the desired droplet diameter was $d = 150$ nm. The surface load Γ_s in Equation 1 was be estimated by assuming that the required surfactant concentration at the O/W interfaces was equal to the saturated interface concentration, Γ_∞ , where the O/W interface is saturated with surfactant molecules.[43] The saturated interface concentration is given by Gibbs adsorption isotherm,

$$\Gamma_\infty = -\frac{1}{nRT} \frac{d\gamma}{d \ln C} \quad (4)$$

where γ is the interfacial tension (mN/m), C is the bulk concentration (mol/L), R is the gas constant, T is the temperature (K), and the integer, n , accounts for the charge interactions within the polar head group of the surfactant. For non-ionic surfactants (Tween 20) the value for $n = 1$.^[44,45] Likewise, the value for n may be taken as 1 for zwitterionic surfactants (lecithin) due to the zero-net ionization of the polar head group.^[46,47] To estimate Γ_{∞} , the diffusion-limited equilibrium adsorption was quantified for each surfactant using static pendant drop tensiometry. Dynamic O/W interfacial tension isotherms were measured for each surfactant at various concentrations until a plateau value was reached and are provided in supplemental information. Plateau values obtained from O/W interfacial tension isotherms were plotted as a function of surfactant concentration in Figure 1 to estimate Γ_{∞} . Furthermore, the data in Figure 1 yielded approximations for the critical aggregation concentrations (CAC) of lecithin and Tween 20. The CAC values were determined to be 0.08 g/L (0.008 wt.%) for lecithin and 0.1 g/L (0.01 wt.%) for Tween 20.

The slopes obtained from the semi-log plot of interfacial tension versus surfactant concentration, at concentrations near the point where the interfacial tension approaches a constant value, were used in Equation 4.^[44,45] Values for Γ_{∞} were determined to be 2.0 $\mu\text{mol}/\text{m}^2$ for lecithin and 3.1 $\mu\text{mol}/\text{m}^2$ for Tween 20. Substituting these values of Γ_{∞} for the values of Γ_s in Equation 3, C_a values were quantified for 150 nm diameter spherical oil droplets and an oil volume fraction, $\phi = 0.05$, which yielded approximate minimum nanoemulsion surfactant concentrations of 3.1 mg/mL (0.31 wt.%) for lecithin and 7.6 mg/mL (0.76 wt.%) for Tween 20. In order to quantify the measurable effects that surfactant concentration had on the interfacial rheology and kinetic stability of nanoemulsions, concentrations below C_a (0.25 wt.%)

lecithin and 0.25 wt.% Tween 20) and above C_a (0.35 wt.% lecithin and 1 wt.% Tween 20) were investigated.

2.5. Nanoemulsion Preparation

Nanoemulsions were prepared using a BransonTM digital sonifier. The sonifier was operated at 40% maximum intensity and sonics were applied for 2 second pulses for a total processing time of 3 minutes. Nanoemulsions were cooled during ultrasonic processing using an ice bath at 4 °C. A duplicate of each nanoemulsion was formulated to ensure reproducibility.

Each nanoemulsion consisted of 5 wt.% dispersed oil phase in a citrate-phosphate buffer continuous phase, for a total emulsion mass of 20 g. Initially, we examined how the composition of the oil phase affected the phase separation behavior of bulk nanoemulsions. The oil phase composition of bulk nanoemulsions was varied by combining different mass ratios of our bioactive compound (carvacrol) and carrier oil (MCT) prior to emulsification (0, 5, 10, 20, 40, 60 and 80 wt.% carvacrol in MCT oil). Otherwise, a standardized oil phase composition (10 wt.% Carvacrol in MCT oil) was used in order to quantify variation in the viscoelasticity and long-term stabilization behavior of nanoemulsions containing lecithin to those containing Tween 20. Details on the determination of C_a for lecithin and Tween 20 are provided in Section 2.4. The nanoemulsion surfactant concentrations less than C_a were 0.25 wt.% lecithin or 0.25 wt.% Tween 20 and the concentrations greater than C_a were 0.35 wt.% lecithin or 1 wt.% Tween 20.

2.6. Nanoemulsion Droplet Size Measurements

The droplet properties analyzed in this study were average oil droplet size ($Z\text{-Ave}$) and ζ potential, obtained after storage at 23 °C for 1, 7, 15, and 30 days using a Malvern[®] Zetasizer

199 Nano ZS under standard operating conditions. Values are reported as averages $\pm$ 1 standard
200 deviation (for $n > 3$ measurements).

201 **3. RESULTS AND DISCUSSION**

202 **3.1. Effect of Oil Phase Composition and Surfactant Concentration on Initial** 203 **Nanoemulsion Stability**

204 The composition of the oil phase and concentration of the surfactant in the aqueous phase
205 were major contributors to the initial stabilization behavior of nanoemulsions that contained
206 carvacrol. Nanoemulsion instability manifested as the formation of a distinct lipid layer at the top
207 of the container and a relatively clear, droplet-depleted aqueous layer at the bottom of the
208 container. Nanoemulsions stabilized by 0.25 wt.% Tween 20, a concentration below its C_a , were
209 highly unstable within the first 24 hours. When the concentration of carvacrol in the oil phase
210 exceeded 10 wt.%, the formation of two distinct oil and aqueous phases begins to occur (Figure
211 2a). Nanoemulsions containing 1 wt.% Tween 20, a concentration above its C_a , appeared to be
212 slightly more stable (Figure 2b). For these nanoemulsions, a distinct lipid layer at the top of the
213 container also began to form when the concentration of carvacrol exceeded 10 wt.%; however,
214 the bottom layer of the nanoemulsions remained relatively turbid until the concentration of
215 carvacrol in the oil phase exceeded 60 wt.%. Very low kinetic stability of nanoemulsions
216 stabilized by Tween 20 has also been observed in the literature.[22]

217 Contrarily, nanoemulsions containing 0.25 wt. % lecithin, a concentration below its C_a ,
218 were notably more stable than Tween 20 nanoemulsions on all accounts. Nanoemulsions
219 stabilized by lecithin did not form a visible lipid layer at the top of the container after 24 hours
220 until the concentration of carvacrol in the oil phase exceeded 20 wt.%. Also, the bottom layer of

these nanoemulsions remained turbid for each carvacrol concentrations investigated (Figure 2c). Furthermore, when the nanoemulsions contained 0.35 wt.% lecithin (above its C_a), the stability of carvacrol containing nanoemulsions further increased. Nanoemulsions remained turbid for all carvacrol concentrations investigated and no visible lipid layer formed until the concentration of carvacrol exceeded 40 wt.% of the oil phase (Figure 2d).

The differences in kinetic stability seen between nanoemulsions containing surfactant concentrations below C_a to those above C_a may in part be due to a deficiency of surfactant in the aqueous phase that could adsorb to the large O/W interfacial areas generated during emulsification at surfactant concentrations below C_a . Generally, an excess of emulsifier is necessary because there is an equilibrium between emulsifier at the droplet surface and that in the continuous phase that must be achieved for adequate kinetic stability.[41]

A recent investigation of O/W nanoemulsions containing carvacrol attributed the rapid O/W phase separation to coalescence and Ostwald ripening mechanisms resulting from the addition of carvacrol.[17] Several studies have suggested that the high polarity and low interfacial tension of carvacrol (and similar compounds) accelerates the rate of emulsion droplet coalescence,[17,18] the process where colliding droplets merge together.[42,48] Likewise, nanoemulsions containing oils with some limited water solubility (*i.e.* carvacrol) have also been shown to be susceptible to Ostwald ripening,[17,49] the process where large droplets grow at the expense of smaller droplets due to the transport of mass through the aqueous continuous medium.[13,17,49,50] It has therefore been suggested that the presence of a non-polar, insoluble oil (*i.e.* MCT) decreases the rate of coalescence by decreasing the oil phase polarity and increasing the O/W interfacial tension[17] and inhibits Ostwald ripening due to an entropy of mixing effect.[49,51–53]

Indeed, the concentration of surfactant in the aqueous phase, relatively high polarity, low O/W interfacial tension, and limited water solubility of carvacrol molecules are likely attributable factors in the coalescence and Ostwald ripening of oil droplets and ultimately the macroscopic kinetic destabilization seen in these nanoemulsions. However, our interfacial rheometry results described in the following section indicate that the viscoelasticity of the surfactant-laden interfacial layer may also play a crucial role in the stabilization of nanoemulsion oil droplets.

3.2. Effect of Surfactant Concentration on Interfacial Viscoelasticity

The apparent viscoelastic moduli, E' and E'' , were measured for an oil droplet in aqueous surfactant solutions at pH 7 and 3. The oil droplet was a mixture of carvacrol and MCT oil (10 wt.% carvacrol in MCT oil). The aqueous phase contained lecithin or Tween 20 at concentrations below and above their corresponding C_a values. It is important to note that all aqueous phase surfactant concentrations used in oscillating pendant drop experiments (0.25, 0.35 wt.% lecithin, or 0.25, 1 wt.% Tween 20) were much higher than the experimentally determined critical aggregation concentrations (CACs) for lecithin (≈ 0.008 wt.%) and Tween 20 (≈ 0.01 wt.%).

The concentration of the surfactant in the aqueous phase appeared to slightly influence the developed viscoelastic response of O/W interfaces containing lecithin after 24 hours. When the concentration of surfactant in the aqueous phase (pH 7) was increased from 0.25 wt.% lecithin (Figure 3a) to 0.35 wt.% lecithin (Figure 4a), the apparent E' value after 24 hours increased by ca. 13%. Similar viscoelastic behavior was seen for aqueous lecithin solutions at pH 3. When the concentration of lecithin was increased from 0.25 wt.% lecithin (Figure 3b) to 0.35 wt.% lecithin (Figure 4b), the developed E' value increased by ca. 28%. Conversely, the developed viscoelastic response for O/W interfaces containing Tween 20 appeared to be

insensitive to surfactant concentration in the aqueous phase (at pH 7 and 3). Values for E' and E'' remained unchanged when the concentration was increased from 0.25 wt.% Tween 20 to 1 wt.% Tween 20.

In all interfacial rheological measurements of surfactant-laden interfaces, the applied strain was small (2 %) and rate of interfacial area deformation was low (0.01 Hz). Also, the concentrations of surfactant in the aqueous phase relative to the CACs for lecithin and Tween 20 were several orders of magnitude higher. Therefore, the variations in the viscoelastic response (or lack thereof) resulting from increases in surfactant concentration in the aqueous phase were believed not to be a result of Marangoni stresses, where surfactant concentration gradients in the interface lead to non-uniform interfacial tension contributions. The slight increase in the elastic storage modulus, E' , when the concentration of lecithin was increased from 0.25 to 0.35 wt.% was presumably due to accumulation of lecithin molecules near the O/W interface and the further development of a rigid, two-dimensional layer of lecithin molecules in the aqueous sub-phase. As stated in the literature, lecithin is believed to form a thick viscoelastic film that is strengthened by hydrogen bonding between phosphate groups on neighboring molecules.[21] On the other hand, Tween 20 molecules are non-ionic, and thus lack the ability to form a strongly associated network with neighboring molecules. Therefore, a very low resistance to interfacial deformation (i.e. a low viscoelastic response) was seen for each O/W interface containing 0.25 and 1 wt. % Tween 20.

3.3. Effect of pH on Interfacial Viscoelasticity

The pH of the aqueous phase markedly contributed to the apparent viscoelastic response developed after 24 hours for O/W interfaces containing lecithin. For aqueous solutions containing 0.25 wt.% lecithin, shifting the pH from 7 to 3 decreased E' by ca. -28% (Figure 3).

Similarly, for aqueous solutions containing 0.35 wt.% lecithin, shifting the aqueous phase pH from 7 to 3 decreased E' by ca. -19% (Figure 4).

In previous studies, [54,55] the surface mechanics of insoluble monolayers formed by phosphatidylcholine molecules at the air-water interface were investigated at pH 9, 7, and 5. The explicit underlying assumption in these studies was that all spread phospholipid molecules remained at the air–water interface during relaxation experiments. The authors created uniform phospholipid monolayers by direct spreading of phosphatidylcholine molecules at the air-water interface using a volatile solvent. With this process, the concentration of phosphatidylcholine molecules in the aqueous sub-phase was essentially zero, as all spread surfactant molecules adsorbed to and strongly remained at the air-water interface during rheological investigation. The authors stated that the presence of surfactant aggregates in the aqueous sub-phase may lead to desorption/readsorption of surfactant molecules from/to the interface during relaxation experiments, which would have a noticeable impact on the observed interfacial viscoelastic behavior.[54] In the current study, the interfacial systems investigated contained aqueous phases with high surfactant concentrations (and thus surfactant aggregates) in the bulk aqueous phase. The presence of surfactant aggregates in the aqueous sub-phase allows the surfactant molecules to readily adsorb/desorb to accommodate interfacial dilational fluctuations. It is believed that at pH 7, the tendency for desorption/readsorption of phospholipids from/to the oil-water interface is relatively low due to strong attractive van der Waals interactions (hydrogen-bonding) between phospholipids and surrounding molecules provided by the ionized phosphate moieties within phospholipid head-groups, and thus the measured elastic response of the interface is high. However, at pH 3, it is believed the desorption/readsorption tendency is slightly more pronounced due to a net increase in the repulsive electrostatic interactions between

phospholipids. Presumably, this behavior is due to the protonation of the negatively charged phosphate moieties (which begins to occur near pH 3 [56]) and the domination of repulsive interactions originating from the positively charged choline and ethanolamine moieties of PC and PE, respectively. Thus, surfactant desorption/adsorption would occur more easily at low pH for these systems and would in turn lead to the decrease in the elastic response of the surfactant stabilized interface observed in this study.

Contrarily, the developed viscoelastic response after 24 hours for Tween 20 monolayers was very low overall and less sensitive to the pH of the aqueous phase. Adjusting the aqueous phase pH from 7 to 3 slightly decreased the O/W interface's (very low) viscoelastic response. For aqueous solutions containing 0.25 wt.% Tween 20, adjusting the pH from 7 to 3 decreased E' by ca. -11% (Figure 3). Likewise, when the aqueous phase contained 0.35 wt.% Tween 20, shifting the pH from 7 to 3 decreased E' by ca. -7% (Figure 4).

The very low viscoelastic response overall for interfaces stabilized by Tween 20 may be due to the fact that these systems also contained high concentrations of surfactant in the bulk aqueous phase which would allow desorption/readsorption of surfactant molecules from/to the interface during relaxation experiments. Furthermore, the tendency for desorption/readsorption would be magnified by the nonionic nature of Tween 20 molecules and the lack of a strongly associated surfactant network to act as a barrier for surfactant desorption/readsorption from/to the interface in response to imposed dilational strains for each pH investigated.

In general, shifting the pH of the aqueous phase from neutral to acidic notably decreased the interfacial elasticity of O/W interfaces containing zwitterionic lecithin molecules, and to a much lesser extent for O/W interfaces containing non-ionic Tween 20 molecules. However, upon analysis of the evolution of nanoemulsion size distributions and change in Z-Ave droplet

diameter over 30 days (discussed in Section 3.4), there was no apparent correlation between the pH of the aqueous phase and the extended stability of the bulk surfactant-stabilized nanoemulsions. Therefore, the pH of the aqueous was determined to not be a primary contributor to the extended stability of bulk nanoemulsions.

3.4. Observed Correlations Between Interfacial Dilational Viscoelasticity and the Extended Stability of Surfactant-Stabilized Nanoemulsions

The viscoelastic response of O/W interfaces after 24 hours was shown to correlate with the extended kinetic stability of bulk nanoemulsions. For the O/W interface containing 0.25 wt.% Tween 20, a concentration below its C_a (Figure 3), a very low viscoelastic response was observed. Similarly, a low viscoelastic response was also observed for the O/W interface containing 1 wt.% Tween 20, a concentration above its C_a (Figure 4). Previous research on foam bubbles[57] and emulsion droplets[58] has indicated that interfacial films with low elasticity and electrical potential are susceptible to the development of bimodal droplet size distributions over time through the mechanism of Ostwald ripening.[57,58]

This behavior was evident in the droplet size distributions of O/W nanoemulsions containing 0.25 wt.% Tween 20 at pH 7 (Figure 5a) and pH 3 (Figure 6a), as well as those containing 1 wt.% Tween 20 at pH 7 (Figure 5b) and pH 3 (Figure 6b). Over 30 days, the nanoemulsion droplet size distributions consistently transitioned from monomodal to biomodal. Furthermore, for all nanoemulsions containing Tween 20, the Z-Ave droplet diameters of oil droplets increased by a least 140% over 30 days of storage (Figure 7). Analysis of the droplet ζ potential 24 hours after formulation (Table 2) for pH 7 nanoemulsions containing 0.25 wt.% Tween 20 yielded near zero values of ca. +1.1 mV and those at a concentration of 1 wt.% Tween

20 also yielded relatively low values of ca. +1.3 mV. Very similar ζ potential values were also observed when the pH was shifted from 7 to 3 (Table 2).

Contrary to the interfacial layer formed by non-ionic Tween 20 molecules, the layer formed by lecithin molecules was highly viscoelastic. This translated to greatly improved extended stability of nanoemulsions stabilized by lecithin. For nanoemulsions containing 0.25 wt.% lecithin at pH 7, the increase in droplet diameter was relatively small over the course of 30 days, increasing by 23% (Figure 7). Shifting the aqueous phase pH down to 3 reduced the change in oil droplet diameter over time, yielding increases of only ca. 6% after 30 days. Changing the pH of the aqueous phase also appeared to slightly affect the initial Z-Ave diameter of nanoemulsions containing 0.35 wt.% lecithin. At pH 7, the initial diameter of oil droplets was ca. 142 nm; however, when the aqueous phase pH was decreased to pH 3 at this surfactant concentration, the initial diameter of oil droplets increased to ca. 196 nm. Increases in droplet diameter over time remained small, however, increasing by ca. 8% after 30 days.

The dependence of initial droplet diameter on pH may be due to a change in the optimum curvature of the surfactant at the O/W interface as a result of a change in the sub-phase pH, as discussed in the literature.[41,59,60] This is supported by the behavior seen in the ζ potential measurements of lecithin-stabilized nanoemulsions over 30 days. Lecithin molecules adsorbed to O/W interface formed monolayers with relatively high ζ potential. For the nanoemulsions containing 0.25 wt.% lecithin at pH 7, the initial ζ potential values were ca. -34 mV. Likewise, for nanoemulsions containing 0.35 wt.% lecithin at pH 7, the ζ potential values were ca. -37 mV after 24 hours, comparable to the values found by other researchers.[61] When the pH of the aqueous phase was shifted to 3 for nanoemulsions containing 0.25 wt.% lecithin, there was a noticeable increase in the magnitude of the initial ζ potential to ca. -52 mV. Likewise, when the

pH was shifted to 3 for nanoemulsions containing 0.35 wt. % lecithin, the magnitude of the initial ζ potential increased to ca. -49 mV. This correlated well with observed increase in initial Z-Ave droplet diameter of lecithin-stabilized nanoemulsions under acidic (pH 3) conditions, compared to those in a neutral environment (pH 7). Presumably, protonation of the phosphate ion in zwitterionic lecithin molecules would increase the overall ionization of these molecules and simultaneously change the optimum curvature of these molecules at the O/W interface. An increase in the number of adsorbed negative ions at the O/W interface would cause the increase the magnitude of measured ζ potential. Likewise, the change in optimum curvature of lecithin molecules at the interface would result in the observed increase in initial oil droplet diameter.

Generally, the nanoemulsions stabilized by Tween 20 were anticipated to have poor extended stability due to the absence of a strongly associated viscoelastic network of surfactant molecules at the O/W interface and low electrostatic stabilization between oil droplets. These properties likely led to the high susceptibility of Tween 20 stabilized nanoemulsions to destabilization mechanisms involving interfacial deformation (i.e. coalescence). This behavior was supported by data showing the consistent formation of a bimodal droplet size distribution in Tween 20 stabilized nanoemulsion over 30 days. However, nanoemulsions stabilized by lecithin were anticipated to be relatively more stable due to the formation of oil droplets with high interfacial viscoelasticity and electrical potential. These predictions were confirmed in the analysis of the oil droplet size for these nanoemulsions, which revealed the continuation of monomial size distributions over the storage time investigated, agreeing well with other researchers.[58] This information potentially illustrates that the extended kinetic stabilization of nanoemulsion delivery systems is closely related to the physicochemical properties of the bulk nanoemulsion, as well as the mechanical properties of the interfacial layer.

CONCLUSIONS

This investigation indicates that the formation of surfactant-laden interfacial layer with high viscoelasticity may provide additional benefits in food-based O/W nanoemulsion delivery systems. This analysis also supports the use of oscillating pendant drop tensiometry as a viable metric for kinetic stability of nanoemulsions, to be used in parallel with other nanoemulsion characterization analyses. Tensiometry results indicated that the O/W interface containing lecithin displayed much higher viscoelasticity than interfaces containing Tween 20, possibly due to strong chemical association between lecithin and adjacent molecules. This phenomenon correlated well with kinetic stabilization properties of nanoemulsions containing carvacrol, such as the nanoemulsion's resistance to Z-Ave diameter increases and the formation of a bimodal droplet size distribution over time. Past studies have ultimately determined that there is not always a direct correlation between measured shear or dilational interfacial rheology and emulsion stability.[19,20] It has been found, however, that interfaces with highly elastic or gelled structures reduce the overall magnitude of deformation that a droplet encounters due to external forces.[62,63] The results in this work indicate that the resistance to interfacial deformation displayed by lecithin-stabilized interface may contribute to the highly stable bulk nanoemulsion characteristics, in terms of resistance to increases in Z-Ave droplet diameter and the formation of a bimodal size distribution. This information is potentially very useful for the fabrication of stable nanoemulsion-based delivery systems containing other antimicrobial and flavor compounds with similar chemical architectures to carvacrol (*i.e.*, thymol and menthol); however, due to the differences in physicochemical properties of these compounds, detailed analyses of such systems would be required for specific determination of the extended nanoemulsion stability. The techniques provided in this work also give insight into the interfacial criteria for

selecting appropriate emulsifiers to use in nanoemulsion-based delivery systems containing food grade compounds.

SUPPORTING INFORMATION

Numerical data sets for the interfacial tension isotherms for lecithin and Tween 20 at the O/W interface (oil phase: 10 wt.% carvacrol in MCT oil) are available online.

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- 615

617

618 **Figure 1.** Interfacial tension as a function of bulk concentration (mol/L) for lecithin

619 (blue/circles) and Tween 20 (green/triangles). Error bars represent ± 1 standard deviation.

620 Surfactants were dispersed in a pH 7 citrate-phosphate buffer solution and the oil phase consisted

621 of 10% (v/v) carvacrol in MCT oil. The lines are best fits of equation 3.

Figure 2. Photographs taken 24 hours after emulsification for oil-in-water nanoemulsions at pH 7 stabilized by (a) 0.25 wt.% Tween 20, (b) 1 wt.% Tween 20, (c) 0.25 wt.% lecithin or (d) 0.25 wt.% lecithin. Each emulsion contains 5 wt.% dispersed oil phase, with increasing amounts of carvacrol in MCT oil from left to right (0 (control), 5, 10, 20, 40, 60, 80 wt.% carvacrol).

630

631 **Figure 3.** Evolution of the viscoelastic moduli, E' and E'' , over 24 hours for 0.25 wt.% Lecithin
 632 and 0.25 wt.% Tween 20 stabilized O/W interfaces at (a) pH 7 and (b) pH 3. The aqueous phase
 633 contained the emulsifier, while the oil phase had a composition of 10 wt.% carvacrol in MCT oil.
 634 Oscillations were conducted at a frequency of 0.01 Hz. Lines simply connect data points to aid
 635 the eye.

636

(a)

(b)

Figure 4. Evolution of the viscoelastic moduli, E' and E'' , over 24 hours for 0.35 wt.% Lecithin and 1 wt.% Tween 20 stabilized O/W interfaces at (a) pH 7 and (b) pH 3. The aqueous phase contained the emulsifier, while the oil phase had a composition of 10 wt.% carvacrol in MCT oil. Oscillations were conducted at a frequency of 0.01 Hz. Lines simply connect data points to aid the eye.

644

645 **Table 1.** Measurements of Z-Ave oil droplet diameter collected on Day 1 and Day 30 for lecithin
 646 and Tween 20 nanoemulsions at pH 7 and 3. Each system had a dispersed oil phase that
 647 contained 10 wt.% carvacrol in MCT oil (0.05 wt.% carvacrol overall). Data are reported for
 648 nanoemulsions with lecithin or Tween 20 concentrations less than and greater than C_a . Values
 649 are reported as averages $\pm$ 1 standard deviation ($n > 3$).

	Nanoemulsions containing Tween 20					Nanoemulsions containing Lecithin				
	Concentration	pH	Day 1	Day 30	% Increase (time)	Concentration	pH	Day 1	Day 30	% Increase (time)
Z-Ave Diameter (nm)	0.25 wt.%	7	159 $\pm$ 5	956 $\pm$ 73	500	0.25 wt.%	7	201 $\pm$ 3	248 $\pm$ 5	23
		3	241 $\pm$ 3	584 $\pm$ 76	142		3	205 $\pm$ 2	218 $\pm$ 4	6
	1 wt.%	7	232 $\pm$ 2	569 $\pm$ 81	145	0.35 wt.%	7	142 $\pm$ 2	151 $\pm$ 1	6
		3	228 $\pm$ 2	558 $\pm$ 69	145		3	196 $\pm$ 1	212 $\pm$ 2	8

650

651

652 **Table 2.** Zeta (ζ) potential measurements collected on Day 1 and Day 30 for lecithin and Tween
 653 20 nanoemulsions at pH 7 and 3. Each system had a dispersed oil phase that contained 10 wt.%
 654 carvacrol in MCT oil (0.05 wt.% carvacrol overall). Data are reported for nanoemulsions with
 655 lecithin or Tween 20 concentrations less than and greater than C_a . Values are reported as
 656 averages $\pm$ 1 standard deviation ($n > 3$).

	Nanoemulsions containing Tween 20				Nanoemulsions containing Lecithin			
	Concentration	pH	Day 1	Day 30	Concentration	pH	Day 1	Day 30
ζ potential (mV)	0.25 wt.%	7	1.1 $\pm$ 0.1	1.2 $\pm$ 0.1	0.25 wt.%	7	-33.9 $\pm$ 0.4	-32.4 $\pm$ 1.9
		3	1.0 $\pm$ 0.2	0.8 $\pm$ 0.3		3	-51.8 $\pm$ 0.1	-51.9 $\pm$ 0.3
	1 wt.%	7	1.3 $\pm$ 0.2	1.4 $\pm$ 0.1	0.35 wt.%	7	-36.9 $\pm$ 0.9	-38.9 $\pm$ 0.2
		3	0.9 $\pm$ 0.1	0.9 $\pm$ 0.2		3	-48.9 $\pm$ 0.4	-56.9 $\pm$ 0.5

657

Figure 5. Intensity size distributions over 30 days for oil-in-water emulsions that contained 5 wt.% oil (10 wt.% carvacrol in MCT oil) stabilized by (a) 0.25 wt.% Tween 20, (b) 1 wt.% Tween 20, (c) 0.25 wt.% lecithin or (d) 0.35 wt.% lecithin at pH 7. Data were offset from zero to illustrate size distribution changes over time.

Figure 6. Intensity size distributions over 30 days for oil-in-water emulsions that contained 5 wt.% oil (10 wt.% carvacrol in MCT oil) stabilized by (a) 0.25 wt.% Tween 20, (b) 1 wt.% Tween 20, (c) 0.25 wt.% lecithin or (d) 0.35 wt.% lecithin at pH 3. Data were offset from zero to illustrate size distribution changes over time.

(a)

(b)

Figure 7. Visualization of the % change in droplet Z-Ave droplet diameter over 30 days for Tween 20 and lecithin stabilized nanoemulsions at surfactant concentrations (a) below and (b) above their corresponding C_a values. The oil phase made up 5 wt.% of the total nanoemulsion (10 wt.% carvacrol in MCT oil). Lines simply connect data points to aid the eye.