JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Influence of Surfactant Charge on Antimicrobial Efficacy of Surfactant-Stabilized Thyme Oil Nanoemulsions

Khalid Ziani, Yuhua Chang, Lynne McLandsborough, and David Julian McClements*

Department of Food Science, Chenoweth Laboratory, University of Massachusetts, Amherst, Massachusetts 01003, United States

ABSTRACT: Thyme oil-in-water nanoemulsions stabilized by a nonionic surfactant (Tween 80, T80) were prepared as potential antimicrobial delivery systems (pH 4). The nanoemulsions were highly unstable to droplet growth and phase separation, which was attributed to Ostwald ripening due to the relatively high water solubility of thyme oil. Ostwald ripening could be inhibited by incorporating ≥75% of corn oil (a hydrophobic material with a low water solubility) into the nanoemulsion droplets. The electrical characteristics of the droplets in the nanoemulsions were varied by incorporating ionic surfactants with different charges after homogenization: a cationic surfactant (lauric arginate, LAE) or an anionic surfactant (sodium dodecyl sulfate, SDS). The antifungal activity of nanoemulsions containing positive, negative, or neutral thymol droplets was then conducted against four strains of acid-resistant spoilage yeasts: *Zygosaccharomyces bailli*, *Saccharomyces cerevisiae*, *Brettanomyces bruxellensis*, and *Brettanomyces naardenensis*. The antifungal properties of the three surfactants (T80, LAE, SDS) were also tested in the absence of thymol droplets. Both ionic surfactants showed strong antifungal activity in the absence of thymol droplets, but no antimicrobial activity in their presence. This effect was attributed to partitioning of the antimicrobial surfactant molecules between the oil droplet and microbial surfaces, thereby reducing the effective concentration of active surfactants available to act as antimicrobials. This study shows oil droplets may decrease the efficacy of surfactant-based antimicrobials, which has important consequences for formulating effective antimicrobial agents for utilization in emulsion-based food and beverage products.

KEYWORDS: emulsions, nanoemulsions, thyme oil, stability, charge, antifungal, antimicrobial, fungi, yeast

INTRODUCTION

Emulsion-based systems are being increasingly used as delivery systems to encapsulate lipophilic bioactive components, such as antitumor agents,[1,2] anti-inflammatory agents,[2] vitamins,[3,4] and antimicrobials.[5−9] Emulsion-based systems may be divided into either conventional emulsions (radius > 100 nm) or nanoemulsions (radius < 100 nm) depending on the dimensions of the droplets they contain.[10,11] In this study, we investigated the potential of using nanoemulsions as antimicrobial delivery systems suitable for utilization within the food and other industries. Oil-in-water nanoemulsions consist of fine oil droplets dispersed within an aqueous liquid.[12,13] Like conventional emulsions, nanoemulsions are thermodynamically unstable systems that tend to break down due to a variety of physicochemical mechanisms, such as gravitational separation, flocculation, coalescence, and Ostwald ripening.[14] However, the small size of the droplets in nanoemulsions means they have physicochemical and biological properties different from those of conventional emulsions, which provides advantages for certain industrial applications.[15] The relatively small droplet size in nanoemulsions means that (i) they are more stable to gravitational separation, flocculation, and coalescence than conventional emulsions; (ii) they scatter light less efficiently than conventional emulsions and so may be transparent or only slightly turbid; (iii) they interact with biological surfaces differently from conventional emulsions, which may affect the bioactivity of any encapsulated components.[16] In general, emulsion-based systems also offer the potential of incorporating multiple active components into a single delivery system, which may increase their efficacy.

This study focuses on the development of antimicrobial nanoemulsions containing two different antimicrobial agents: essential oils and ionic surfactants. Essential oils have been shown to have antibacterial, antiviral, and antifungal activities.[17−19] Commercial essential oils contain a complex mixture of different constituents that vary in their molecular and physicochemical properties, for example, molecular weight, polarity, solubility, partitioning, and biological activity. Some of the most potent antimicrobial components in essential oils are hydrophobic compounds, such as cinnamic aldehyde, carvacrol, thymol, and eugenol.[20] Consequently, they must usually be encapsulated within colloidal delivery systems if they are going to be incorporated into aqueous-based foods and beverages.[21−24] An essential oil extracted from thyme was utilized in this study to formulate antimicrobial nanoemulsions. Thyme oil is obtained commercially by hydrodistillation of the flowering plants of common thyme (*Thymus vulgaris*).[25] It is commonly used as a flavoring agent in food products,[26] but it also has applications in cosmetics, personal care products, and pharmaceuticals.[27,28] Thyme oil has been shown to have inhibitory activities against various bacteria and yeasts.[20] Thymol, the major component of thyme oil,[29] has also been shown to exhibit antimicrobial activity against several bacteria and fungi.[30,31] The antimicrobial activity of thymol has been attributed to its ability to penetrate into and disturb the structure of microbial cell membranes, thereby leading to leakage of ions and other cell contents.[17]

Received: February 2, 2011
Revised: April 25, 2011
Accepted: April 26, 2011
Published: April 26, 2011

ACS Publications © 2011 American Chemical Society

A number of ionic surfactants have also been shown to exhibit strong antimicrobial activity, such as lauric arginate (a cationic surfactant) and sodium dodecyl sulfate (an anionic surfactant).[32−36] The antimicrobial activity of ionic surfactants has been attributed to their ability to be incorporated into the lipid membrane of microbial cells, thereby disrupting normal cellular function.[37] Ionic surfactants may also influence the antimicrobial activity of nanoemulsions by altering the electrical characteristics of the oil droplets. The surfaces of microorganisms are typically negatively charged,[38,39] and therefore positively charged droplets might be expected to be electrostatically attracted to their surfaces, whereas negatively charged droplets would be expected to be repelled.[38] Consequently, there should be a higher concentration of any active component encapsulated by lipid droplets within the immediate vicinity of the microbial cell surface for positive droplets than for negative droplets. Thus, the antimicrobial activity of any encapsulated substances might be expected to increase when they are incorporated into positively charged droplets. The electrical charge on the droplets in nanoemulsions can be manipulated in a number of different ways, for example, by using one or more emulsifiers with different charge characteristics or by adsorbing charged substances onto the droplet surfaces.[15] Consequently, controlling the electrical characteristics of nanoemulsion delivery systems may be an effective way of increasing their antimicrobial activity. Conversely, antimicrobial surfactants may remain adsorbed to the surfaces of oil droplets in nanoemulsions, which could reduce the amount present to interact with the microorganisms, thereby decreasing their efficacy. It is therefore important to establish whether nanoemulsions containing a combination of different antimicrobials are more or less effective than the individual components themselves.

The main objectives of the current research were to prepare physically stable nanoemulsions containing both thyme oil and ionic surfactants and then to test their antimicrobial activity against a number of acid-resistant yeasts. In addition, we aimed to determine whether using a combination of antimicrobial agents (essential oils and ionic surfactants) within a single delivery system had synergistic or antagonistic effects on the overall antimicrobial efficacy. The results of this study have important implications for the design and utilization of nanoemulsions as antimicrobial delivery systems in the food and other industries.

■ MATERIALS AND METHODS

Materials. Thyme oil was purchased from Optimal Health Solutions (La Pine, OR). Corn oil was obtained from a local supermarket. Both oils were used without further purification. A nonionic surfactant (Tween 80, T80) and an anionic surfactant (sodium dodecyl sulfate, SDS) were purchased from Sigma-Aldrich Co. (St. Louis, MO), and a cationic surfactant (Mirenat-N) was provided by Vedeqsa (Grupo Lamirsa Terrassa, Spain). For confocal microscopy work, technical grade Nile Red dye (CAS Registry No. 7385-67-3) was purchased from Sigma-Aldrich.

Nanoemulsion Preparation. The aqueous phase used to prepare the nanoemulsions consisted of 0.5% (w/w) T80 dispersed in an aqueous buffer solution (10 mM acetate, pH 4). Lipid phases were prepared by mixing different mass ratios of thyme oil and corn oil (from 0 to 100 wt % corn oil) prior to homogenization. The lipid phase (5% w/w) was mixed with the aqueous phase (95% w/w) using a high-speed blender for 60 s. The resulting emulsion premix was then homogenized by passing it five times through a high-pressure homogenizer at 9 kPa (Microfluidics 110 L, Microfluidics Corp., Newton, MA) to further reduce the particle size. After preparation, the nanoemulsions formed were stored at 20 °C prior to analysis. To change the interfacial charge of the previously formed droplets, different amounts of either SDS solution or lauric arginate (LAE) solution were added.

Nanoemulsion Characterization. *Particle Size Measurements.* The mean particle diameters (*Z*-averages) of the nanoemulsions were measured using a dynamic light scattering instrument (Zetasizer Nano ZS, model ZEN 3600, Malvern Instruments, Malvern, U.K.). This instrument determines the particle size from intensity−time fluctuations of a laser beam (633 nm) scattered from a sample at an angle of 173°. Each individual measurement was an average of 13 runs. The nanoemulsions were diluted using buffer solution (10 mM acetate buffer, pH 4) prior to analysis to avoid the effects of multiple scattering.

Particle Charge Measurements. The electrical charge (ζ-potential) of the droplets in the nanoemulsions was measured using a particle electrophoresis instrument (Zetasizer Nanoseries ZS, Malvern Instruments, Worcestershire, U.K.). Initially, the nanoemulsions were diluted 100-fold using buffer solution (10 mM acetate, pH 4) to avoid multiple scattering effects. Diluted samples were then placed in a disposable cuvette that acted as a measurement chamber, and the ζ-potential was determined by measuring the direction and velocity that the particles moved in the applied electric field. The Smoluchowsky model was used by the instrument's software program to convert the electrophoretic mobility measurements into ζ-potential values.

Determination of Antifungal Activity. *Minimum Inhibitory Concentration (MIC) Assay.* Four strains of acid-resistant spoilage yeasts were provided by the Pepsico R&D Culture Collection (Valhalla, NY): *Zygosaccharomyces bailli* (ZB), *Saccharomyces cerevisiae* (SC), *Brettanomyces bruxellensis* (BB), and *Brettanomyces naardenensis* (BN). Yeast cultures were kept frozen at −70 °C in 25% glycerol. The yeast strains were refreshed on malt extract agar plates (Becton Dickinson, Sparks, MD) before treatments. A single yeast colony from the plate was inoculated into 10 mL of malt extract broth (MEB) medium (Becton Dickinson, Sparks, MD), which was adjusted to pH 4.0 by citrate buffer with the final strength of 10 mM. The culture was then incubated at 25 °C under mild agitation (150 rpm in a rotary shaker) for about 2 days until the optical density at 600 nm (OD_{600}) was around 1.0. As a guideline, at an OD_{600} of 1.0, cultures of yeast strains contain approximately 5×10^6 CFU/mL. The cultures were then diluted 100-fold using fresh pH 4.0 MEB medium to determine the MIC.

The MIC was determined for each of the four yeast strains mentioned above using 96-well microtiter plates (Falcon 3075) by a 2-fold serial dilution method. The microtiter plate wells of the 1st and 12th columns were filled with 150 μL of blank MEB medium to establish potential contamination, and the rest of the wells were filled with 150 μL of 1:100 inoculated pH 4.0 MEB medium as stated above (about 5×10^4 CFU/mL inoculation level). One hundred and fifty microliters of inoculated MEB medium (pH 4.0) containing 10% of the treatment solution (Table 1) was added to each of the eight wells of the second column and mixed thoroughly; 150 μL of the mixed medium from the second column were then transferred to the wells of the third column, and this procedure was continued so as to make successive 2-fold dilutions up to the 11th column. The final concentrations of each treatment solution from the 2nd column to the 11th columns were therefore 5, 2.5, 1.25, 0.6, 0.3, 0.16, 0.8, 0.4, 0.2, and 0.1%, respectively. Plates were incubated at 25 °C for 5 days, and the MICs were assessed visually as the lowest concentration showing complete growth inhibition.

Microbial Adhesion to Hydrocarbon (MATH) Assay. The surface hydrophobicity of yeast cells after treatments with nanoemulsions was measured by a MATH assay described by Rosenberg[40] with some modifications. In these experiments, only *S. cerevisiae* (SC) cells were used as representative yeast cells. One and a half milliliters of freshly grown SC cells in MEB medium (pH 4.0) was collected by centrifugation and then resuspended in 1 mL of either 5% nonionic emulsion (oil + 0.5% T80), 5% cationic emulsion (oil + 0.5% T80 + 0.32% LAE),

Table 1. Antifungal Properties of Different Treatments against *Saccharomyces cerevisiae*, *Zygosaccharomyces bailli*, *Brettanomyces bruxellensis*, and *Brettanomyces naardenensis*[a]

initial delivery system composition (%)					final amount of delivery system added to MEB (wt %)				
oil[c]	T80	SDS	LAE	fungus name	5	2.5	1.25	0.6	0.3
0	0.5	0	0	*Zygosaccharomyces bailli*	+	+	+	+	+
0	0.5	0.8	0		−	−	+	+	+
0	0.5	0	0.32		−	−	−	+	+
5	0	0	0		+	+	+	+	+
5	0.5	0	0		+	+	+	+	+
5	0.5	0.8	0		+	+	+	+	+
5	0.5	0	0.32		+	+	+	+	+
0	0.5	0	0	*Brettanomyces naardenensis*	+	+	+	+	+
0	0.5	0.8	0		−	+	+	+	+
0	0.5	0	0.32		−	−	+	+	+
5	0	0	0		+	+	+	+	+
5	0.5	0	0		+	+	+	+	+
5	0.5	0.8	0		+	+	+	+	+
5	0.5	0	0.32		+	+	+	+	+
0	0.5	0	0	*Brettanomyces bruxellensis*	+	+	+	+	+
0	0.5	0.8	0		−	+	+	+	+
0	0.5	0	0.32		−	−	+	+	+
5	0	0	0		+	+	+	+	+
5	0.5	0	0		+	+	+	+	+
5	0.5	0.8	0		+	+	+	+	+
5	0.5	0	0.32		+	+	+	+	+
0	0.5	0	0	*Saccharomyces cerevisiae*	+	+	+	+	+
0	0.5	0.8	0		−	−	+	+	+
0	0.5	0	0.32		−	−	−	+	+
5	0	0	0		+	+	+	+	+
5	0.5	0	0		+	+	+	+	+
5	0.5	0.8	0		+	+	+	+	+
5	0.5	0	0.32		+	+	+	+	+

[a] Initial delivery systems consisted of either surfactant only or surfactant-stabilized thymol oil droplets. [b] Calculated final compositions of delivery systems in microbial broth:

concn of delivery system in MBE broth (wt %)	thyme oil (wt %)	T80 (wt %)	LAE (wt %)	SDS (wt %)
5	0.0625	0.025	0.016	0.04
2.5	0.03125	0.0125	0.008	0.02
1.2	0.016	0.00625	0.004	0.01
0.6	0.0078	0.0031	0.002	0.005
0.3	0.0039	0.0015	0.001	0.0025

[c] 25% thyme oil/75% corn oil.

5% anionic emulsion (oil + 0.5% T80 + 0.8% SDS), or acetate buffer (10 mM, pH 4.0) as a control. After 2 h of treatment at room temperature, the yeast cells were centrifuged, the treatment solutions were poured off, and the yeast cells were then washed twice with 1.2 mL of acetate buffer to remove the remaining treatment solutions. The yeast cells were then resuspended in appropriate amounts of acetate buffer to achieve an initial absorbance (A_I) of 1.0 cm^{-1} at 600 nm, using a UV−visible spectrophotometer (Ultraspec 2000, Pharmacia Biotech). In a clean borosilicate round-bottom glass tube (12 × 75 mm), 1 mL of hexadecane (Sigma, purity ≥ 99%) was added to 2 mL of yeast suspension. The tube was vortexed for 2 min and set aside to rest for 15 min to allow the hexadecane phase to fully rise and separate due to gravity. Next, the yeast suspension was collected with a clean Pasteur pipet; great care was taken to avoid collection of the hexadecane layer. The yeast suspension was then transferred to a cuvette for the final absorbance measurement (A_F) at 600 nm. Adhesion of yeasts to the hydrocarbons was evaluated as the fraction partitioned to the hydrocarbon phase, FPC, which was calculated as $FPC = 1 - A_F/A_I$. The value of FPC could be used to evaluate the surface hydrophobicity of yeast cells. The MATH assays were performed in duplicate.

Surface Charge (ζ-Potential) Measurements of Yeast Cells. Additional information about the interactions between nanoemulsion

droplets and yeast cells was determined by measuring the surface charge of mixed systems. The ζ-potential of yeast cells (SC) after treatment with different nanoemulsions (nonionic, cationic, anionic) was measured using a particle microelectrophoresis instrument (Zetasizer Nano-ZS). The yeast cells were treated exactly the same way as described above, and then they were resuspended in appropriate amounts of acetate buffer (10 mM, pH 4.0) to achieve an OD_{600} of around 1.0 cm^{-1}. The ζ-potential of the resulting yeast cell suspensions was then measured at room temperature. Each measurement was conducted in duplicate.

Confocal Fluorescence Microscopy. Interactions between nanoemulsion droplets and freshly grown yeast cells (SC) were observed using confocal fluorescence microscopy. The yeast cells (Syto 9) and droplets (Nile Red) were stained using two different fluorescent dyes so that we could distinguish them. Nile Red is a lipid soluble stain that gives a red-fluorescent color. A Nile Red solution was prepared by dissolving 0.1 mg of Nile Red in 10 mL of methanol. Syto 9 is a nucleic acid stain that dyes both live and dead cells a green-fluorescent color. A Syto 9 solution was prepared by dispersing the dye in an aqueous solution according to the manufacturer instructions (L7012; Molecular Probes, Carlsbad, CA) and then incubated with the yeast cells. The yeast cells were then washed twice with acetate buffer (10 mM, pH 4.0) to remove the residual Syto 9, and the stained yeast cells were incubated for 2 h with nanoemulsions. For the control, the yeast cells were incubated with buffer solution. Two drops of Nile Red dye solution were then added to the samples prior to transfer onto glass microscope slides. Syto 9 fluorescence was detected by excitation at 488 nm, and emission was collected with a 500−530 nm band-pass filter. Nile Red fluorescence was detected by excitation at 605 nm, and emission was collected with a 565−615 nm band-pass filter. All images were acquired and processed using the instrument's software program (EZ-CS1 version 3.8, Nikon, Melville, NY).

RESULTS

Impact of Lipid Phase Composition on Nanoemulsion Formation and Stability. If nanoemulsions are going to be used as delivery systems for antimicrobial agents, then it is important that they have good long-term stability during storage. We therefore carried out a series of preliminary experiments to establish the physical stability of the nanoemulsions to droplet growth and phase separation.

Initially, we attempted to prepare a nanoemulsion by homogenizing pure thyme oil and an aqueous nonionic surfactant solution: 5% thyme oil, 0.5% T80, pH 4.0. However, the resulting emulsions were highly unstable to droplet growth and phase separation; after 3 days of storage, the mean particle diameter was >7000 nm, and there was visible evidence of creaming and oiling off (Figure 1). The high instability of this system can be attributed to the fact that thyme oil has an appreciable water solubility (≈1 g L^{-1} for thymol at 25 °C, MSDS sheet), which means that it is highly susceptible to Ostwald ripening (OR). OR is the growth of large oil droplets at the expense of smaller oil droplets due to diffusion of oil molecules through the intervening aqueous phase.[41,42] The increase in mean droplet diameter (d) with time (t) due to Ostwald ripening of a one-component emulsified lipid in the steady state regime is given by the following equation:[43]

$$d^3 - d_0^3 = \omega t = \frac{32}{9}\alpha c D t \quad (1)$$

Here, ω represents the Ostwald ripening rate, α (= $2\gamma V_m/RT$), γ is the interfacial tension, V_m is the molar volume of the lipid, R is the gas constant, T is the absolute temperature), c is the solubility of the lipid in the aqueous phase, and D is the translational diffusion coefficient of the lipid in the aqueous phase. This equation highlights that the rate of OR is directly proportional to the solubility of the oil in the aqueous phase, which accounts for the observed increase in droplet size during storage observed in the thyme oil nanoemulsions (Figure 1).

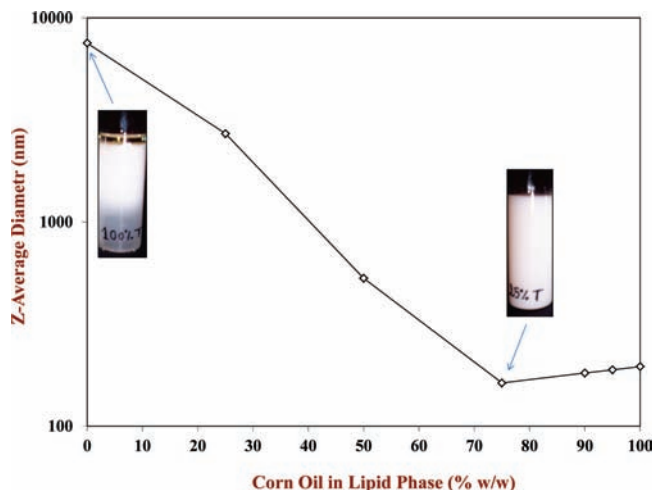

Figure 1. Dependence of mean droplet diameter after 2 days of storage at ambient temperature on oil phase composition for 5% oil-in-water emulsions containing different amounts of thyme oil and corn oil in the lipid phase (0.5% Tween 80, 10 mM acetate buffer, pH 4).

Previous studies have shown that OR can be retarded or prevented in emulsions by mixing an oil that has a relatively high water solubility with another oil that has a very low water solubility prior to homogenization.[42,44,45] This second oil can be referred to as a "ripening inhibitor", and it is usually a highly nonpolar substance with a relatively high molecular weight. The amount of ripening inhibitor required to prevent OR depends on the molecular weights and solubilities of the low and high water-soluble oils. In the current study, we examined the possibility of improving the OR stability of our emulsions by mixing thyme oil with corn oil, because this is a food-grade oil that could be used as part of an edible delivery system. Corn oil is primarily composed of highly nonpolar triacylglycerol molecules with relatively high molecular weights and low water solubilities and is therefore a highly effective ripening inhibitor.[44]

After homogenization, samples with different droplet compositions (thyme oil/corn oil) were stored for 2 days at ambient temperature, mixed to ensure they were homogeneous, and then their particle size was measured (Figure 1). As mentioned earlier, emulsions prepared using a lipid phase consisting of only thyme oil (0% corn oil) were highly unstable, being susceptible to droplet growth, creaming, and oiling off. We postulate that the droplet size increased due to OR, which led to rapid creaming because of the relatively large droplet size (d > 5000 nm) and relatively low density of thyme oil (ρ = 923 kg m^{-3}) compared to water (ρ = 1000 kg m^{-3}). As the concentration of corn oil in the lipid phase was increased, the extent of droplet growth observed after 2 days of storage decreased (Figure 1). From 0 to 75% corn oil, there was a steep decrease in mean particle diameter with increasing corn oil concentration, which can be attributed to the ability of the triacylglycerol oil to inhibit OR.[39,46] The smallest droplet diameter (163 nm) that could be produced occurred when about 75% of corn oil was present in the lipid phase prior to

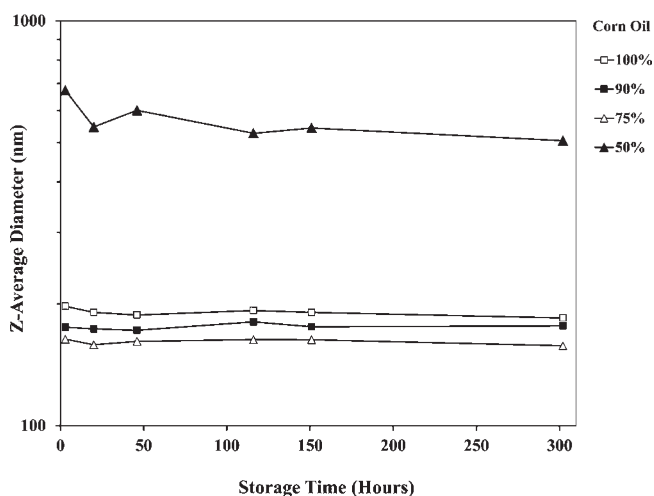

Figure 2. Evolution of mean droplet diameter with time at ambient temperature for diluted 5 wt % oil-in-water emulsions containing different ratios of thyme and corn oil in the lipid phase (0.5% Tween 80, 10 mM acetate buffer, pH 4).

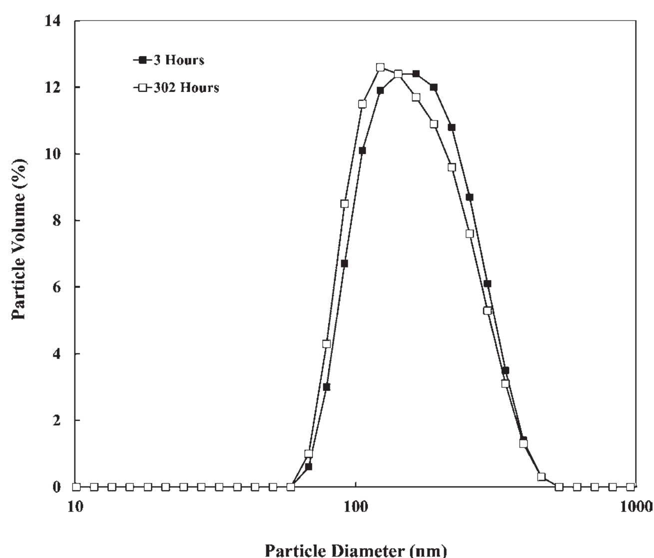

Figure 3. Dependence of particle size distributions before and after storage of diluted 5% oil-in-water emulsions containing 25% thyme oil and 75% corn oil in the lipid phase (0.5% Tween 80, 10 mM acetate buffer, pH 4).

homogenization. A further increase in corn oil concentration caused a slight increase in mean droplet diameter (196 nm for pure corn oil), which can be attributed to the increase in lipid phase viscosity and interfacial tension, both of which oppose the formation of small droplets in a high-pressure homogenizer.[47,48]

The ability of low water solubility oils to retard OR in emulsions containing high water solubility oils can be attributed to an entropy of mixing effect.[42,44,45] Consider an oil-in-water emulsion that contains droplets composed of two different lipid components: a water-insoluble component (such as corn oil) and a water-soluble component (such as thyme oil). The water-soluble oil molecules will diffuse from the small to the large droplets due to OR. Consequently, there will be a greater percentage of water-soluble oil molecules in the larger droplets than in the smaller droplets after OR occurs. Differences in the composition of emulsion droplets are thermodynamically unfavorable because of entropy of mixing: it is more favorable to have the two oils distributed evenly throughout all of the lipid droplets rather than concentrated in particular droplets. Consequently, there is a thermodynamic driving force that operates in opposition to the OR effect. The change in droplet size distribution with time then depends on the concentration and solubility of the two components within the oil droplets.

Practically, one would like to maximize the amount of an active ingredient (in this case thyme oil) present in a delivery system. Our study suggests that nanoemulsions with relatively small droplets ($r < 85$ nm) can be produced using a lipid phase of 25% corn oil and 75% thyme oil. We also measured the long-term stability of these systems to droplet growth by measuring the change in mean particle diameter with time (Figure 2) and the particle size distribution (Figure 3) before and after storage. There was no appreciable change in the mean droplet diameter or particle size distribution of the samples containing 75% or more corn oil up to 300 h (12.5 days) of storage. Samples containing 50% corn oil and 50% thyme oil had larger mean diameters, but they also appeared to remain relatively stable to droplet growth during storage, indicating that OR had been largely inhibited.

Impact of Surfactant Type and Concentration on Nanoemulsion Droplet Charge. The functional performance of nanoemulsions containing antimicrobial essential oils is likely to depend on the electrical characteristics of the oil droplets, because this would be expected to affect their interactions with the anionic surfaces of microorganisms. We therefore examined the possibility of preparing nanoemulsion droplets with different electrical characteristics by mixing the nonionic surfactant stabilized nanoemulsions with different types and concentrations of ionic surfactants after homogenization. The initial nanoemulsions prepared in these experiments consisted of 5% lipid phase (25% thyme oil/75% corn oil) stabilized by 0.5% T80. A cationic surfactant (LAE) was used to create positively charged droplets, whereas an anionic surfactant (SDS) was used to create negatively charged droplets.

The impact of adding different types and concentrations of ionic surfactants on the ζ-potentials of the nanoemulsion droplets was determined (Figure 4). The ζ-potential of the initial nanoemulsion (containing only T80) was slightly negative (≈ -4 mV), which can be attributed to the presence of some anionic impurities in the surfactant (such as free fatty acids) or adsorption of anionic species from the water (such as hydroxyl ions) to the droplet surfaces.[49] When SDS was added to the initial nanoemulsion, the droplet charge became much more strongly anionic. There was a steep increase in the negative charge on the droplets from −4 to −44 mV when the SDS concentration was increased from 0 to 0.1%, after which the charge remained relatively constant upon further addition of SDS. This suggested that the anionic SDS molecules adsorbed to the oil−water interface and displaced some or all of the nonionic T80 molecules. Indeed, the ζ-potential of the droplets in a nanoemulsion prepared using only SDS was measured to be ≈ -67 mV, which suggested that the droplets were covered predominately by SDS rather than T80 at high SDS concentrations. Conversely, when LAE was added to the initial nanoemulsion, the droplet charge became increasingly cationic. There was an appreciable increase in the positive charge on the droplets from −4 to +39 mV when the LAE concentration was increased from 0 to 0.32%. The change in droplet charge with increasing

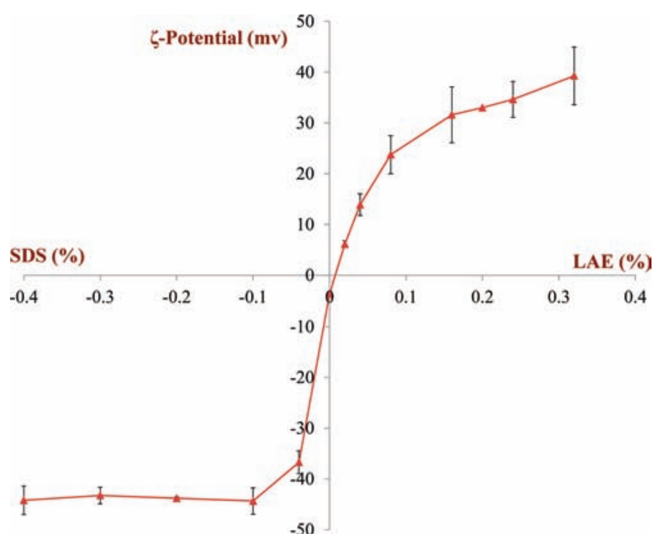

Figure 4. Effect of addition of different levels of anionic surfactant (SDS) or cationic surfactant (LAE) to T80-stabilized 5% oil-in-water nanoemulsions on the droplet ζ-potential. The lipid phase consisted of 25% thyme oil and 75% corn oil. For comparison, the ζ-potential of nanoemulsions prepared with only SDS was −67 mV, and that of those prepared with only LAE was +73 mV.

LAE concentration was steepest from 0 to 0.08%, but still increased gradually from 0.08 to 0.32%, and did not appear to reach a plateau region. This suggested that the cationic LAE molecules had adsorbed to the oil−water interface and displaced at least some of the nonionic T80 molecules. The ζ-potential on a nanoemulsion prepared using only LAE was measured to be ≈+73 mV, which suggested that the droplet surfaces were not completely saturated with LAE in the presence of T80 (Figure 4).

The initial change in particle charge with increasing ionic surfactant concentration was higher for SDS than for LAE (Figure 4), which may be attributed to differences in their molecular characteristics. The molecular weight of LAE (421.0 g mol^{-1}) is considerably higher than that of SDS (288.4 g mol^{-1}). When the results were expressed on a molar basis, rather than a mass basis, then the change in droplet charge with increasing surfactant concentration is more similar for LAE and SDS (data not shown). However, the SDS molecules still appeared to have a somewhat higher affinity for the T80-coated droplets than the LAE molecules. SDS and LAE both have similar nonpolar tails consisting of a lauric acid chain (12 carbon atoms), but they do have different head groups. The cationic headgroup (arginate) on LAE is considerably larger than the anionic headgroup (sulfate) on SDS, which suggests that the SDS molecules may be able to pack more effectively at the oil−water interface.

These results indicate that nanoemulsions with a range of different charge characteristics can be prepared by mixing nonionic coated droplets with different types and concentrations of ionic surfactant solutions. In the following sections, the antimicrobial activities of nanoemulsions with different charge characteristic were tested. For these studies we prepared three nanoemulsions with different electrical characteristics: anionic (0.5% T80 + 0.8% SDS), nonionic (0.5% T80, 0% SDS or LAE), and cationic (0.5% T80 + 0.32% LAE).

Antifungal Activity of Surfactant Solutions. Many surfactants are known to have strong antimicrobial activity,[36] and therefore we initially measured the ability of SDS, LAE, and

Table 2. Physicochemical Characteristics of the Surfactant Micelles Used in This Study

	T80	T80/SDS	T80/LAE
ζ-potential (mV)	−0.1	−18	+16.3
Z-average diameter (nm)	12.6	7.4	6.9

Tween 80 solutions to inhibit microbial growth using similar conditions as were used to test the nanoemulsions (Table 1). The antifungal activities of the three surfactants against four yeast strains, *Z. bailli* (ZB), *S. cerevisiae* (SC), *B. bruxellensis* (BB), and *B. naardenensis* (BN), were tested by measuring the MIC (Table 1). The MIC was determined by calculating the final amount of surfactant in the broth that was required to completely inhibit observable microbial growth. The nonionic surfactant (T80) did not show any antifungal activity at the levels used in this study; that is, fungal growth occurred at all concentrations used. On the other hand, both ionic surfactants were able to inhibit microbial growth above a certain concentration. For convenience, we express the surfactant concentrations as micrograms per milliliter for the microbiology experiments, which is equivalent to 0.0001%. For the anionic surfactant, the presence of 200 μg/mL (0.02%) of SDS inhibited the growth of ZB and SC, whereas the presence of 400 μg/mL (0.04%) of SDS inhibited the growth of BB and BN. For the cationic surfactant, the presence of 40 μg/mL (0.004%) of LAE inhibited the growth of ZB and SC, whereas the presence of 80 μg/mL (0.008%) of LAE inhibited the growth of BB and BN. These results show that LAE was more effective at inhibiting microbial growth than SDS and that T80 did not show any antifungal effects against all four tested yeast strains.

We also measured the characteristics of the micelles formed by the three types of surfactant (Table 2). The T80 micelles were slightly larger in diameter than the T80/SDS or T80/LAE micelles. As would be expected, the T80 micelles had an electrical charge close to zero, the T80/SDS micelles were strongly anionic, and the T80/LAE micelles were strongly cationic. It has been proposed that the antimicrobial activity of ionic surfactants is at least partly due to their ability to penetrate into microbial cell membranes, leading to membrane disruption and loss of biological function.[33] One might expect that cationic surfactants, which have an opposite charge to bacterial membranes, would adsorb more strongly and be more effective at disrupting the membranes. In addition, previous studies have shown that both anionic and cationic surfactants promote denaturation of globular proteins above a critical level, whereas nonionic surfactants are not particularly effective.[50] These surfactants may therefore have been able to interfere with functional proteins associated with the cell membranes (e.g., transport or signaling proteins). Further research is clearly required to establish the physicochemical and/or biochemical mechanisms by which surfactants inhibit microbial growth.

Antifungal Activity of Nanoemulsions. Initially, oil-in-water nanoemulsions were prepared by homogenizing 5% lipid phase (25% thyme oil; 75% corn oil) with 95% aqueous phase (0.5% T80, pH 4.0). Nanoemulsions containing either anionic or cationic droplets were then prepared by mixing the original nanoemulsion with either 0.8% SDS or 0.32% LAE, respectively.

For all three surfactants, thyme oil nanoemulsions did not show any antifungal effects, even at the highest concentrations tested (625 μg/mL (0.0625%) thyme oil in broth). This was

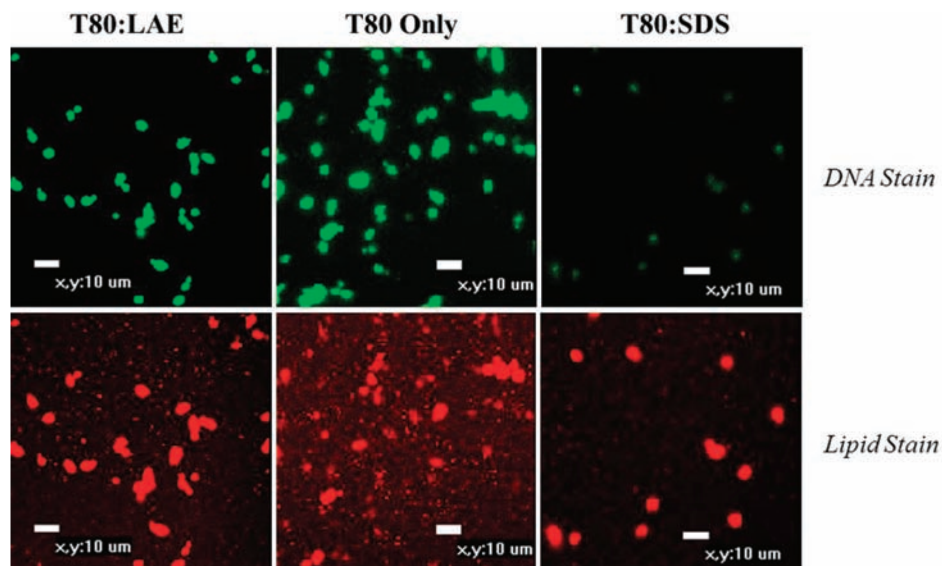

Figure 5. Confocal microscopy images of mixed systems containing nanoemulsions and yeast cell (SC) suspensions. The yeast cells were stained green with a DNA stain (Syto 9), whereas the oil droplets and yeast cell membranes were stained red with a nonpolar stain (Nile Red). The nanoemulsions consisted of 5% oil phase (25% thyme oil/75% corn oil), 0.5% nonionic surfactant (Tween 80), and additional LAE, buffer, or SDS (10 mM acetate buffer, pH 4).

probably because the highest concentration of thyme oil used was lower than the MIC. Paster et al. reported that thyme oil did not inhibit mycelial growth of *Aspergillus ochraceus* at 700 μg/mL.[51] Higher thyme oil concentrations were not investigated in this study because the yeast's growth could not be accurately determined by the MIC measurement protocol due to the fact the samples are too opaque to measure optical densities. In addition, higher thyme oil concentrations would be unacceptable for most food applications due to the distinct flavor characteristics of this essential oil. Finally, the presence of the corn oil may have affected the ability of the thyme oil to become incorporated into the microbial membranes, for example, by acting as a hydrophobic sink.

Initially, we hypothesized that when thyme oil was incorporated within positively charged (LAE) nanoemulsion particles, it would be a more effective antimicrobial because it would be attracted to the surfaces of negatively charged yeast cells. Conversely, we hypothesized that when thyme oil was incorporated into negatively charged (SDS) nanoemulsion particles, it would be less effective as an antimicrobial because it would be repelled from negatively charged yeast cell surfaces. In practice, we found that thyme oil droplets actually decreased the antimicrobial activity of both LAE and SDS, which were found to be antimicrobial when used in isolation but ineffective when used in the presence of the oil droplets (Table 1). In other words, the presence of nanosized lipid droplets actually reduced the antifungal activity of the ionic surfactants. We hypothesize that this effect was due to partitioning of the surfactants between the lipid droplet surfaces and the yeast cell surfaces. The presence of the lipid droplets would reduce the effective concentration of surfactants in the aqueous phase, thereby reducing their ability to interact with the microbial membranes. The small size of the lipid droplets in nanoemulsions means that they have a high surface area per unit mass of oil, which would exacerbate this effect. Similar results have been reported for microemulsion systems, in which the presence of milk fat droplets was found to decrease the antimicrobial efficacy of surfactant microemulsions containing an essential oil (eugenol).[52]

Nature of Interactions between Nanoemulsion Droplets and Yeast Cells. In this section, we used a number of methods to provide more information about the interaction of the various kinds of nanoemulsion droplets with the yeast cells. Because all strains of yeast behaved fairly similarly in the antimicrobial assays (Table 1), we used only SC cells as a representative strain in these studies.

Some insight into the nature of the interaction between nanoemulsion droplets and yeast cell surfaces was obtained using a MATH assay. Yeast surface hydrophobicity after nanoemulsion treatments was expressed by their FPC value: control = 0.06 $\pm$ 0.03; LAE/T80 = 0.63 $\pm$ 0.07; SDS/T80 = 0.07 $\pm$ 0.02; T80 = 0.05 $\pm$ 0.03. These results show that the yeast cells became much more hydrophobic after treatment with cationic nanoemulsions, but showed little change in hydrophobicity when treated with either nonionic or anionic nanoemulsions. It is possible that the positively charged oil droplets in the cationic nanoemulsion attached to the negatively charged yeast cell surfaces, thereby increasing their hydrophobicity. Alternatively, the yeast cell surfaces may have become more hydrophobic because the cell membrane was disrupted by the cationic surfactant.

Additional information about the interaction of nanoemulsion droplets with yeast cells was obtained using ζ-potential measurements. The yeast cells were highly anionic before treatment with nanoemulsions: -18.1 ± 1.2 mV. After treatment, the ζ-potential of the yeast cells depended on the type of surfactant initially coating the nanoemulsion droplets: LAE/T80 = 2.0 $\pm$ 0.4 mV; SDS/T80 = -18.4 ± 0.5 mV; T80 = -18.7 ± 0.5 mV. These results support the MATH data, indicating that there was a strong interaction between the cationic droplets and the yeast cells, but little interaction between the nonionic or anionic droplets and the yeast cells. The fact that the yeast cells became positively charged after being in contact with the cationic nanoemulsion suggests that at least some of the cationic surfactant adsorbed to the yeast cell surfaces. Whether the LAE was adsorbed as individual molecules, micelles, or lipid droplets is currently unknown and would be a useful topic for future study.

Finally, we used confocal microscopy to observe differences in the microstructure of suspensions containing a mixture of yeast cells (SC) and nanoemulsions (Figure 5). The yeast cells were stained green with a DNA dye (Syto 9), whereas the oil droplets were stained red with a nonpolar dye (Nile Red). The separated fluorescent images of the two stains showed that Syto 9 stained only yeast cells but that Nile Red stained both nanoemulsion droplets and yeast cells (Figure 5). The intensity of the green dye in the yeast cells was appreciably lower for the SDS-containing systems than for the other systems, which may have been because this anionic surfactant interfered with the cationic Syto 9 stain. Presumably, the Nile Red was located in the lipophilic environments of both the nanoemulsion droplet interiors and the phospholipid-rich microbial membranes. Because the Nile Red stained both the yeast cells and the nanoemulsion droplets, it was not possible to ascertain whether droplets had preferentially accumulated around the yeast cells. Nevertheless, the microscopy images indicated that there were lipid droplets dispersed within the aqueous phase surrounding the yeast cells for all three nanoemulsion types (Figure 5). However, there appeared to be somewhat fewer droplets in the aqueous phase of the samples containing LAE (lower intensity of small red dots), which may indicate that more cationic LAE-coated droplets were absorbed to the yeast cell surfaces. Nevertheless, further work would be required to confirm this. It should be noted that it is not possible to observe the individual nanoemulsion droplets in the images because their dimensions were below the resolution of optical microscopy. The fact that many of the lipid droplets were present in the aqueous phase surrounding the yeast cells may account for the fact that the ionic surfactants were less effective as antimicrobials in the presence of nanoemulsions. As mentioned earlier, some of the ionic surfactants would be located at the surfaces of the lipid droplets and would therefore be unavailable for interacting with the microbial surfaces.

In future studies, it would be useful to carry out a detailed analysis of the physicochemical and biological mechanisms by which ionic surfactants act as antimicrobial agents and to establish the impact of lipid droplets on these mechanisms. Understanding the interactions of surfactants and lipid droplets with microbial surfaces may lead to the design of more effective antimicrobial delivery systems. In this study, we have shown that physically stable thyme oil-in-water nanoemulsions containing droplets with a range of different electrical characteristics, from highly positive to highly negative, can be produced by adding different types and amounts of ionic surfactants (cationic or anionic) after homogenization. In the absence of nanoemulsion droplets, the cationic and anionic surfactants both had appreciable antimicrobial activity, which was attributed to their ability to adsorb to the surfaces of the yeast cells and disrupt their lipid membranes. However, the antimicrobial activity of the ionic surfactants was lost when they were incorporated into nanoemulsions, which was attributed to the tendency for the surfactants to partition between the oil droplet surfaces and the cell membranes. In conclusion, the combination of an antimicrobial oil (thyme oil) and antimicrobial surfactant (SDS or LAE) had an antagonist, rather than a synergistic, impact on the overall antimicrobial efficacy. The results of this study have important implications for the design and utilization of nanoemulsions as antimicrobial delivery systems in the food and other industries.

AUTHOR INFORMATION

Corresponding Author

*Phone: (413) 545-1019. Fax: (413) 545-1262. E-mail: mcclements@foodsci.umass.edu.

Funding Sources

K.Z. thanks the Department of Education of Navarra Government (Spain) and the Department of Food Science at the University of Massachusetts (Amherst) for financial support. This material is based upon work supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, and the Massachusetts Agricultural Experiment Station.

ACKNOWLEDGMENT

We thank Vedeqsa for providing us with the lauric arginate ingredient.

REFERENCES

(1) Seimon, R. V.; Wooster, T.; Otto, B.; Golding, M.; Day, L.; Little, T. J.; Horowitz, M.; Clifton, P. M.; Feinle-Bisset, C. The droplet size of intraduodenal fat emulsions influences antropyloroduodenal motility, hormone release, and appetite in healthy males. *Am. J. Clin. Nutr.* **2009**, *89* (6), 1729–1736.

(2) Huang, Q. R.; Yu, H. L.; Ru, Q. M. Bioavailability and delivery of nutraceuticals using nanotechnology. *J. Food Sci.* **2010**, *75* (1), R50–R57.

(3) Kang, H. S.; Kwon, S. S.; Kim, B. H.; Lee, B. R.; Kang, K. H.; Hong, J. E.; Han, S. H.; Chang, I. S. Nanoemulsion as a vitamin E acetate carrier to enhance infiltration into oral mucous membrane. *J. Ind. Eng. Chem.* **2002**, *8* (4), 348–353.

(4) Relkin, P.; Jung, J. M.; Ollivon, M. Factors affecting vitamin degradation in oil-in-water nano-emulsions. *J. Therm. Anal. Calorim.* **2009**, *98* (1), 13–18.

(5) Lee, V. A.; Karthikeyan, R.; Rawls, H. R.; Amaechi, B. T. Anti-cariogenic effect of a cetylpyridinium chloride-containing nanoemulsion. *J. Dentistry* **2010**, *38* (9), 742–749.

(6) Ferreira, J. P.; Alves, D.; Neves, O.; Silva, J.; Gibbs, P. A.; Teixeira, P. C. Effects of the components of two antimicrobial emulsions on food-borne pathogens. *Food Control* **2010**, *21* (3), 227–230.

(7) LiPuma, J. J.; Rathinavelu, S.; Foster, B. K.; Keoleian, J. C.; Makidon, P. E.; Kalikin, L. M.; Baker, J. R. In vitro activities of a novel nanoemulsion against *Burkholderia* and other multidrug-resistant cystic fibrosis-associated bacterial species. *Antimicrob. Agents Chemother.* **2009**, *53* (1), 249–255.

(8) Teixeira, P. C.; Leite, G. M.; Domingues, R. J.; Silva, J.; Gibbs, P. A.; Ferreira, J. P. Antimicrobial effects of a microemulsion and a nanoemulsion on enteric and other pathogens and biofilms. *Int. J. Food Microbiol.* **2007**, *118* (1), 15–19.

(9) Hamouda, T.; Myc, A.; Donovan, B.; Shih, A. Y.; Reuter, J. D.; Baker, J. R. A novel surfactant nanoemulsion with a unique non-irritant topical antimicrobial activity against bacteria, enveloped viruses and fungi. *Microbiol. Res.* **2001**, *156* (1), 1–7.

(10) McClements, D. J. Edible nanoemulsions: fabrication, properties, and functional performance. *Soft Matter* **2011**, *7* (6), 2297–2316.

(11) McClements, D. J.; Rao, J. J. Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Crit. Rev. Food Sci. Nutr.* **2011**, *51* (4), 285–330.

(12) Mason, T. G.; Wilking, J. N.; Meleson, K.; Chang, C. B.; Graves, S. M. Nanoemulsions: formation, structure, and physical properties. *J. Phys.: Condens. Matter* **2006**, *18* (41), R635–R666.

(13) Tadros, T.; Izquierdo, R.; Esquena, J.; Solans, C. Formation and stability of nano-emulsions. *Adv. Colloid Interface Sci.* **2004**, *108–09*, 303–318.

(14) Velikov, K. P.; Pelan, E. Colloidal delivery systems for micronutrients and nutraceuticals. *Soft Matter* **2008**, *4* (10), 1964–1980.

(15) McClements, D. J. Emulsion design to improve the delivery of functional lipophilic components. In *Annual Review of Food Science and Technology;* 2010; Vol. 1, pp. 241−269

(16) Acosta, E. Bioavailability of nanoparticles in nutrient and nutraceutical delivery. *Curr. Opin. Colloid Interface Sci.* **2009**, *14* (1), 3–15.

(17) Burt, S. Essential oils: their antibacterial properties and potential applications in foods − a review. *Int. J. Food Microbiol.* **2004**, *94* (3), 223–253.

(18) Giatrakou, V.; Ntzimani, A.; Savvaidis, I. N. Effect of chitosan and thyme oil on a ready to cook chicken product. *Food Microbiol.* **2010**, *27* (1), 132–136.

(19) Silva, N. C. C.; Fernandes, A. Biological properties of medicinal plants: a review of their antimicrobial activity. *J. Venomous Anim. Toxins Including Trop. Dis.* **2010**, *16* (3), 402–413.

(20) Gaysinsky, S.; Davidson, P.; McClements, D.; Weiss, J. Formulation and characterization of phytophenol-carrying antimicrobial microemulsions. *Food Biophys.* **2008**, *3* (1), 54–65.

(21) Gaysinsky, S.; Davidson, P. M.; Bruce, B. D.; Weiss, J. Growth inhibition of *Escherichia coli* O157:H7 and *Listeria monocytogenes* by carvacrol and eugenol encapsulated in surfactant micelles. *J. Food Prot.* **2005**, *68* (12), 2559–2566.

(22) Gaysinsky, S.; Davidson, P. M.; Bruce, B. D.; Weiss, J. Stability and antimicrobial efficiency of eugenol encapsulated in surfactant micelles as affected by temperature and pH. *J. Food Prot.* **2005**, *68* (7), 1359–1366.

(23) Perez-Conesa, D.; McLandsborough, L.; Weiss, J. Inhibition and inactivation of *Listeria monocytogenes* and *Escherichia coli* O157:H7 colony biofilms by micellar-encapsulated eugenol and carvacrol. *J. Food Prot.* **2006**, *69* (12), 2947–2954.

(24) Lo Cantore, P.; Shanmugaiah, V.; Iacobellis, N. S. Antibacterial activity of essential oil components and their potential use in seed disinfection. *J. Agric. Food Chem.* **2009**, *57* (20), 9454–9461.

(25) Schulz, H.; Quilitzsch, R.; Krüger, H. Rapid evaluation and quantitative analysis of thyme, origano and chamomile essential oils by ATR-IR and NIR spectroscopy. *J. Mol. Struct.* **2003**, *661−662*, 299–306.

(26) Holley, R. A.; Patel, D. Improvement in shelf-life and safety of perishable foods by plant essential oils and smoke antimicrobials. *Food Microbiol.* **2005**, *22* (4), 273–292.

(27) Burt, S. Essential oils: their antibacterial properties and potential applications in foods − a review. *Int. J. Food Microbiol.* **2004**, *94* (3), 223–253.

(28) Tiwari, B. K.; Valdramidis, V. P.; O'Donnell, C. P.; Muthukumarappan, K.; Bourke, P.; Cullen, P. J. Application of natural antimicrobials for food preservation. *J. Agric. Food Chem.* **2009**, *57* (14), 5987–6000.

(29) Gaysinsky, S. Emulsions and microemulsions as antimicrobial delivery systems. Doctorate Thesis, University of Massachusetts, Amherst, 2007.

(30) Sivropoulou, A.; Papanikolaou, E.; Nikolaou, C.; Kokkini, S.; Lanaras, T.; Arsenakis, M. Antimicrobial and cytotoxic activities of origanum essential oils. *J. Agric. Food Chem.* **1996**, *44* (5), 1202–1205.

(31) Friedman, M.; Henika, P. R.; Mandrell, R. E. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* **2002**, *65*, 1545–1560.

(32) Asker, D.; Weiss, J.; McClements, D. J. Formation and stabilization of antimicrobial delivery systems based on electrostatic complexes of cationic-non-ionic mixed micelles and anionic polysaccharides. *J. Agric. Food Chem.* **2011**, *59* (3), 1041–1049.

(33) Hamouda, T.; Baker, J. R. Antimicrobial mechanism of action of surfactant lipid preparations in enteric Gram-negative bacilli. *J. Appl. Microbiol.* **2000**, *89* (3), 397–403.

(34) Dai, Y. M.; Normand, M. D.; Weiss, J.; Peleg, M. Modeling the efficacy of triplet antimicrobial combinations: yeast suppression by lauric arginate, cinnamic acid, and sodium benzoate or potassium sorbate as a case study. *J. Food Prot.* **2010**, *73* (3), 515–523.

(35) Howett, M. K.; Kuhl, J. P. Microbicides for prevention of transmission of sexually transmitted diseases. *Curr. Pharm. Des.* **2005**, *11* (29), 3731–3746.

(36) Singh, A.; Van Hamme, J. D.; Ward, O. P. Surfactants in microbiology and biotechnology: Part 2. Application aspects. *Biotechnol. Adv.* **2007**, *25* (1), 99–121.

(37) Xia, J. D.; Xia, Y. M.; Nnanna, I. A. Structure−function relationship of acyl amino-acid surfactants − surface-activity and antimicrobial properties. *J. Agric. Food Chem.* **1995**, *43* (4), 867–871.

(38) Li, J.; McClements, D. J.; McLandsborough, L. A. Interaction between emulsion droplets and *Escherichia coli* cells. *J. Food Sci.* **2001**, *66* (4), 570–574.

(39) Naitali, M.; Dubois-Brissonnet, F.; Cuvelier, G.; Bellon-Fontaine, M. N. Effects of pH and oil-in-water emulsions on growth and physicochemical cell surface properties of *Listeria monocytogenes*: impact on tolerance to the bactericidal activity of disinfectants. *Int. J. Food Microbiol.* **2009**, *130* (2), 101–107.

(40) Rosenberg, M. G. D.; Rosenberg, E. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* **1980**, *9* (1), 29–33.

(41) Kabalnov, A. Ostwald ripening and related phenomena. *J. Dispersion Sci. Technol.* **2001**, *22* (1), 1–12.

(42) Kabalnov, A. S.; Shchukin, E. D. Ostwald ripening theory − applications to fluorocarbon emulsion stability. *Adv. Colloid Interface Sci.* **1992**, *38*, 69–97.

(43) Taylor, P. Ostwald ripening in emulsions. *Adv. Colloid Interface Sci.* **1998**, *75* (2), 107–163.

(44) Li, Y.; Le Maux, S.; Xiao, H.; McClements, D. J. Emulsion-based delivery systems for tributyrin, a potential colon cancer preventative agent. *J. Agric. Food Chem.* **2009**, *57* (19), 9243–9249.

(45) Wooster, T.; Golding, M.; Sanguansri, P. Impact of oil type on nanoemulsion formation and Ostwald ripening stability. *Langmuir* **2008**, *24* (22), 12758–12765.

(46) Coupland, J. N.; Weiss, J.; Lovy, A.; McClements, D. J. Solubilization kinetics of triacyl glycerol and hydrocarbon emulsion droplets in a micellar solution. *J. Food Sci.* **1996**, *61* (6), 1114–1117.

(47) Walstra, P. Principles of emulsion formation. *Chem. Eng. Sci.* **1993**, *48*, 333.

(48) Walstra, P. *Physical Chemistry of Foods*; Dekker: New York, 2003.

(49) McClements, D. *Food Emulsions: Principles, Practices, and Techniques*; CRC: Boca Raton, FL, 2005.

(50) Kelley, D.; McClements, D. J. Interactions of bovine serum albumin with ionic surfactants in aqueous solutions. *Food Hydrocolloids* **2003**, *17* (1), 73–85.

(51) Conner, D. E.; Beuchat, L. R. Effects of essential oils from plants on growth of food spoilage yeasts. *J. Food Sci.* **1984**, *49* (2), 429–434.

(52) Gaysinsky, S.; Taylor, T. M.; Davidson, P. M.; Bruce, B. D.; Weiss, J. Antimicrobial efficacy of eugenol microemulsions in milk against *Listeria monocytogenes* and *Escherichia coli* O157:H7. *J. Food Prot.* **2007**, *70* (11), 2631–2637.