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Effect of lipophilization on the distribution and reactivity of ingredients in emulsions

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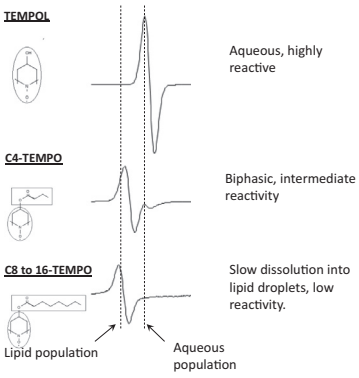
HIGHLIGHTS

- A homologous series of lipophilized spin probes were synthesized.
- Probes distributed between phases of an emulsion according to their lipophilicity.
- The rate of reduction by ascorbate was greater for more hydrophilic probes.
- Larger probes precipitated when added to the emulsion as ethanolic solution.
- Precipitated probes slowly diffused to lipid droplets.

GRAPHICAL ABSTRACT



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ABSTRACT

Hypothesis: The reactivity of small molecules in emulsions is believed to depend on their partitioning between phases, yet this is hard to verify experimentally *in situ*. In the present work, we use electron paramagnetic resonance (EPR) spectroscopy to simultaneously measure the distribution and reactivity of a homologous series of lipophilized spin probes in an emulsion.
Experiments: 4-Hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) was derivatized with saturated fatty acids to create a series of spin probes with increasing lipophilicity (C4-, C8-, C12-, and C16-TEMPO). The probes were added to a 10 wt.% tetradecane-in water emulsions ($d_{32} \sim 190$ nm) stabilized with sodium caseinate (1 wt.% in the aqueous phase, pH 7). The distribution of the probes between phases was measured by electron paramagnetic resonance (EPR) spectroscopy.
Findings: TEMPOL partitioned into the aqueous phase, C4-TEMPO distributed between the lipid and aqueous phases (69% and 31% respectively) while the more lipophilic probes dissolved exclusively within the lipid droplets. Interestingly, the more lipophilic probes initially precipitated upon their addition to the emulsion, and only slowly redistributed to the droplets over hours or days, the rate of which was dependent on their carbon chain length. The reactivity of the probes with aqueous an aqueous phase reductant (ascorbate) generally depended on the proportion in the aqueous phase (i.e., TEMPOL > C4-TEMPO > C8-TEMPO ~ C12-TEMPO ~ C16-TEMPO).

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1. Introduction

Oil-in-water (O/W) emulsions can be used to deliver small hydrophobic molecules in a variety of products (e.g., flavors in foods, drugs in pharmaceuticals, perfumes in personal care products) and the phase behavior of the small molecule ingredients affects their chemical reactivity and functionality [1]. This is particularly important in understanding the capacity of antioxidants to delay rancidity in food emulsions, where nonpolar antioxidants tend to be more effective than polar antioxidants because they accumulate in the lipid phase where the oxidation is occurring (i.e., the so-called "polar paradox") [2,3]. However studies using antioxidants lipophilized with homologous series of fatty acids have revealed a limit to this rule; antioxidant effectiveness increases with increasing lipophilicity to a critical point but then abruptly decreases (i.e., the cut off effect) [2–6].

If the polar paradox can be explained using a two-phase partitioning model, then any model to explain the cut off effect must require a third factor [7]. Laguerre and others [3] argued that beyond the critical limit the antioxidant may (i) have lowered mobility and be unable to diffuse to react with radicals, (ii) be internalized in the lipid phase away from the interface where many oxidations are believed to occur, or (iii) self-aggregate, perhaps as micelles, as a separate phase suspended in the aqueous phase. Very recently Aliaga and others [8] offered a novel fourth possibility based on a study of a homologous series of lipophilized spin probes in a micellar solution. Using molecular dynamics simulation they showed the reduced partition coefficient for highly lipophilic molecules could be explained by a change of orientation with the micelle.

A full understanding of the mechanism of the cut off effect in any microheterogeneous system depends on a simultaneous determination of antioxidant localization and reactivity. Most attempts involving analysis of antioxidants in emulsions have required phase separation e.g., [9], while other workers used a pseudophase kinetic model to estimate the antioxidant partitioning and reactivity different emulsion phases [10,11]. Phase separation may disrupt delicate structures and phase equilibria while the pseudophase model assumes, reasonably in most cases, that rapid diffusion means measurements made in a coarse mixture apply to submicron emulsions of the same overall composition. Furthermore, the pseudophase model has not yet been applied to protein-stabilized systems.

One promising approach to the challenge of measuring small molecules in opaque heterogeneous media is electron paramagnetic resonance (EPR) spectroscopy [12]. This technique is sensitive only to unpaired electrons, so it can be used to detect either radicals formed during oxidation or the properties of molecules tagged with stable radicals (i.e., spin probes). Deconvolution of the resulting EPR spectra allows determination of the concentration of molecules in phases of different polarity as well as their mobility without physically breaking the emulsion [13,14]. The EPR deconvolution method has been used to measure spin probe distribution in a micellar solution [15–18]. An alternative EPR method was used recently by Aliaga and co-workers [8] to study the distribution of a homologous series of lipophilized spin probes in a surfactant solution. Their method [described in more detail in [19]] was based upon accurately measuring the *g*-factor for the spin probe as a function of surfactant concentration and calculating the partition coefficient from a two-state binding model and was supported by pyrene florescence quenching measurements. Reduction of the spin probes with ascorbate can be used to measure their accessibility to the aqueous phase [17,18,20,21]. More details of the spin probe-ascorbate reaction are available in the literature [22–24].

Previously, we have used EPR to study the effects of droplet size, interfacial composition and crystallinity on the localization of a spin labeled nitroxide probe [14,25,26] and also showed that probes with very different structures displayed different partitioning behavior between the phases of an emulsion [17,27,28]. In all of these studies, the greater the hydrophobicity of the probe, the higher proportion that partitioned into the lipid phase, and the more slowly it was reduced by aqueous ascorbate.

In the present work, we esterify spin probes with a series of fatty acids with variable chain lengths (C4, C8, C12, C16) and use them as model molecules to test the localization argument behind the polar paradox theory and cut off effect. Similar molecules were recently used by Aliaga and co-workers [8] in a micellar solution who showed maximum binding coefficient with micelles at C8 and a cut off beyond that point. We use an EPR spectroscopy-deconvolution method to measure the distribution of the antioxidants between phases in an intact protein-stabilized O/W emulsion as well as their reactivity with aqueous ascorbate.

2. Materials and methods

2.1. Materials

Sodium caseinate from bovine milk, sodium L-ascorbate and sodium azide were purchased from Sigma Chemical Company (St. Louis, MO, USA). 4-hydroxy-TEMPO (97%), octanoyl chloride (99%), triethylamine and solvents of analytical grade were purchased from Sigma Aldrich (Saint Quentin Falavier, France). Butanoyl chloride (98%), dodecanoyl chloride (98%) and hexadecanoyl chloride (98%) were from Alfa Aesar (Karlsruhe, Germany). Silica gel 60 (60–200 μm, pore size 60 Å) for column chromatography, pre-coated silica gel 60 F_{254} plates (10 × 20 cm glass; 0.25 mm thickness) for TLC, and pre-coated silica gel 60 F_{254} plates (10 × 20 cm glass; 0.5 mm thickness) for preparative TLC were purchased from Merck (Darmstadt, Germany). n-Tetradecane (C14) was purchased from Acros Organics (Geel, Belgium) and ferric chloride 6-hexahydrate was obtained from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). Sodium phosphate monobasic monohydrate and sodium phosphate dibasic heptahydrate was obtained from Fisher Scientific (Pittsburgh, PA). Water was purified through a Milli-Q System (Millipore Corp., Bedford, MA).

2.2. Methods

2.2.1. Synthesis of TEMPO alkyl esters

In a 100 mL capacity 2-neck round-bottom flask, 2.90 mmol of 4-hydroxy-TEMPO (TEMPOL) and 2.90 mmol of triethylamine were dissolved in 20 mL anhydrous degassed toluene under stirring at room temperature. Then, 2.85 mmol of acyl chloride in 5 mL anhydrous degassed toluene were added dropwise and the reaction progress was monitored by thin layer chromatography (TLC) coupled with densitometry at 250 nm. The reaction was stopped by quenching with methanol at equilibrium (4–5 h, 70–85% TEMPOL conversion). The mixture was then vacuum-dried and washed with hexane. The hexane extract was dried over anhydrous sodium sulfate, filtered (0.45 μm), concentrated to 2–3 mL, separated chromatographically on a 25 g silica gel column with hexane/ethyl acetate (85:15; *v*/*v*) and finally highly purified by preparative TLC with hexane/acetone/formic acid mixtures. TEMPO alkyl esters (>98% purity, TLC, 250 nm) were recovered as a red viscous liquid. Fig. 1 shows the reaction scheme for the synthesis of TEMPO alkyl esters. The molecular weight of TEMPOL, C4-TEMPO, C8-TEMPO, C12-TEMPO and C16-TEMPO are 172.24 g mol^{-1}, 242.33 g mol^{-1}, 298.44 g mol^{-1}, 354.55 g mol^{-1} and 410.65 g mol^{-1} respectively.