Quantitative Analysis of Surface-Located Triacylglycerol in Intact Emulsion Particles

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The amount of triacylglycerol (TG) in the surface monolayer of intact phospholipid-stabilized emulsions was determined using ^{13}C nuclear magnetic resonance (^{13}C NMR). ^{13}C NMR spectra of emulsions composed of bulk long-chain or medium-chain TG were prepared with [$^{13}C_3$]-carbonyl-enriched triolein, tripalmitin, or trioctanoin, and were analyzed and compared with NMR spectra of phosphatidylcholine vesicles with and without added TG. Identification of carbonyl peaks intermediate between those of phosphatidylcholine carbonyls and bulk TG confirmed the presence of surface TG in each emulsion. The surface of emulsions contained 2.2 mol % tripalmitin and 1.4 mol % triolein, but significantly more medium-chain TG, 9.1 mol % trioctanoin, as predicted by measurements of TG in phospholipid vesicles. Thus, medium-chain TGs are more accessible than long-chain TGs to enzymes or pro-oxidants in the continuous phase of phospholipid-stabilized emulsion systems. The quantitative determination of surface-located TG in intact particles will advance the understanding of emulsion colloidal properties, physicochemical stability, and metabolic behavior.

Keywords: *Triacylglycerol; surface; interface; emulsion; ^{13}C NMR; triolein; tripalmitin; trioctanoin*

INTRODUCTION

The basic structure of a triacylglycerol (TG) emulsion particle consists of a hydrophobic core of TG molecules surrounded by a monolayer of surfactant molecules. A small but finite amount of TG is present in the surface monolayer of emulsions (*1*). Determining the presence and quantity of surface TG is critical to understanding both the physicochemical stability and metabolic behavior of emulsion particles. Several studies have shown that the oxidative stability of emulsions is greatly affected by the concentration and specific location of antioxidants (*2*, *3*). Knowing the interfacial substrate concentration, that is the concentration of TG at the emulsion surface, would greatly increase understanding of the oxidation of emulsified TG. Surface-located TG is more accessible to both enzymes and pro-oxidants in the continuous phase than bulk TG, and may directly affect the rates of particle oxidation or lipolysis. For example, although lipoprotein lipase and hepatic lipase both have greater affinity for long-chain TG, emulsions and vesicles containing medium-chain TG are hydrolyzed at a faster rate than those containing long-chain TG because of the greater solubility of medium-chain TG in the particle surface (*4*). To date, the presence and quantity of TG in emulsion surfaces have been based on chemical determination of TG in ultracentrifugally separated surface and core phases (*1*); or estimated from measurements of ^{13}C carbonyl-enriched TG in phospholipid vesicles using ^{13}C NMR spectroscopy (*4*−*6*). Although phospholipid vesicles may provide a reasonable estimate of the amount of surface-located TG in emulsions, they differ significantly in physical structure. Vesicles have a phospholipid bilayer and lack a hydrophobic core, whereas emulsions have a phospholipid monolayer surrounding a bulk hydrophobic core. ^{13}C NMR spectra of various TG-containing emulsion particles have been published (*7*, *8*); however, identification of surface TG in intact emulsion particles has not been reported. Carbonyl signals from surface TG were either of insufficient intensity to be detected or were obscured by the intensity of signal arising from bulk TG (Boyle-Roden, E., unpublished observations). Thus, emulsion particles composed of ^{13}C carbonyl-enriched TG and a non-carbonyl-containing hydrophobic core filler were developed to provide both sufficient signal-to-noise and spectral resolution for the detection of surface TG. The objective of this study was to quantify directly the amount of surface-located tripalmitin, trioctanoin, and triolein in intact emulsion particles and to compare the concentrations of surface TG in emulsions with those in phosphatidylcholine (PC) vesicles.

MATERIALS AND METHODS

Materials. [$^{13}C_3$-1,1,1]-tripalmitin, [$^{13}C_3$-1,1,1]-trioctanoin, [$^{13}C_3$-1,1,1]-triolein, and [$^{13}C_2$-1,1]-dipalmitoylphosphatidylcholine ($^{13}C_2$-DPPC) were purchased from Cambridge Isotope Labs (Cambridge, MA). Egg yolk PC was purchased from Avanti Polar Lipids (Alabaster, AL). Paraffin oil was obtained from Fisher Scientific (Philadelphia, PA). Deuterium oxide (D_2O, 99% purity) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Lipid standards were purchased from Nuchek Prep (Elysian, MN). All other reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Emulsions. The initial lipid weight composition of emulsions is given in Table 1. ^{13}C-enriched TG were dispersed in chloroform and analyzed for purity by ^{13}C NMR prior to emulsion sample preparation. Appropriate volumes of lipids were transferred from stock organic solutions to a 4-mL glass vial and mixed on a vortex mixer for 15 s. Organic solvent was evaporated under N_2, and the dried lipid film was desiccated for 2−4 h at room temperature. Mixtures containing trioctanoin or triolein were dispersed in D_2O that contained

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Table 1. Initial Weight Composition of Emulsions (in mg)

	emulsions			vesicles	
component	tripalmitin	triolein	trioctanoin	DPPC	2% TP
^{13}C tripalmitin	40				1
^{13}C triolein		28			
^{13}C trioctanoin			28		
paraffin oil	35	24.5	24.5		
^{13}C-DPPC	1	2	2	6.2	6.1
PC	24	15.5	15.5	43.8	42.9
total	100	70	70	50	50

0.15 M NaCl and 0.01 M Tris-HCl, pH 7.4, at room temperature and were prepared in triplicate. Mixtures containing tripalmitin were heated to >65 °C, and disposed in D_2O that contained 0.15 M NaCl and 0.01 M Tris-HCl, pH 7.4, warmed to >45 °C, and were prepared in duplicate. Each dispersion was sonicated 30−40 min in pulse mode using a Fisher Scientific model 550 sonic dismembrator (Philadelphia, PA). Samples were dispersed in 2.0 mL of buffer to give a final lipid concentration of 50 mg/mL, and were transferred directly to 10-mm NMR tubes following sonication; or dispersed in 1.0 mL of buffer to yield a final lipid concentration of 70 mg/mL prior to ultracentrifugation.

Preparation of Phospholipid Vesicles. PC vesicles were used to confirm both the absence of vesicles in emulsion preparations and the identification of peak assignments in ^{13}C NMR spectra. A total of 50 mg lipid of PC with 2 wt% tripalmitin, or PC and $^{13}C_2$-DPPC in a 7:1 weight ratio was transferred from stock organic solutions into a glass vial, dried to a thin lipid film under N_2, and dispersed in 1 mL of 20 wt % D_2O that contained 0.15 M NaCl and 0.01 M Tris-HCl, pH 7.4. The mixtures were sonicated for 30 min in pulse mode.

Separation of Emulsions and Phospholipid Vesicles. To remove potentially contaminating PC vesicles, emulsions were ultracentrifuged after sonication using a procedure adapted from that of Walzem et al. (*9*). A 1-mL portion of emulsion was transferred to a Beckman centrifuge tube and overlayed with 8 mL of D_2O that contained 0.196 M NaCl and 0.245 M NaBr, density 1.0255 g/mL. Emulsions were centrifuged at 46,700 rpm (200,000*g*) for 12 h at 22 °C in a Beckman 70.1 Ti rotor and Beckman model L7 Ultracentrifuge (Palo Alto, CA). The top 1.0 mL was aspirated and placed in 5-mm NMR tubes. Removal of PC vesicles by ultracentrifugation was confirmed by centrifuging 1 mL of 20 mg/mL PC vesicles under the conditions given above in duplicate. The top 1 mL of the supernatant fraction was placed in a cuvette and inserted into a PSS Nicomp model 370 submicron particle analyzer (Particle Sizing Systems, Santa Barbara, CA). The absence of intensity output indicated the absence of particles scattering light. Two additional 1-mL aliquots of the supernatant fraction were also analyzed. All intensity readings for aspirated supernatant fraction from ultracentrifuged PC vesicles were ≤10 kHz. For comparison, background buffer intensity readings were 3−8 kHz and 5 μL of 20 mg/mL PC vesicles dispersed in 1 mL of water yielded intensity readings of 130 kHz.

Particle Size Analysis. Aliquots, approximately 5 μL each, of emulsions were transferred to a glass cuvette containing 1 mL of H_2O to yield an intensity output of >200 kHz on the PSS Nicomp model 370 submicron particle analyzer, and data were collected in 5 cycles of 5 min each. Emulsions were sized both prior to and immediately after NMR analysis. Size distribution is presented as mean particle diameter ± one standard deviation on a number-weight basis; for bimodal distributions, the percentage of total particles within each distribution is shown in parentheses.

^{13}C NMR Spectroscopy. ^{13}C NMR spectra were acquired at 100 MHz on a Bruker AM 400 NMR spectrometer (Billerica, MA) using a 10-mm heteronuclear probe or at 75.6 MHz, or a QE 300 NMR spectrometer (Fremont, CA) using a 5-mm ^{1}H/^{13}C probe. Following are the acquisition parameters that were used: sweep width, 20,000 Hz; block size, 16 k points; excitation pulse, 60°; recycle time, 2.0 s; and with broadband decoupling, the number of acquisitions was 8192 unless specified otherwise. All spectra were obtained at 25 °C following ^{1}H NMR analysis of a sucrose standard for magnetic homogeneity. Data were processed with baseline correction, exponential multiplication of 3.0 Hz unless noted otherwise, Fourier transformation and phase correction using MacFID software by Teqmag (Houston, TX). Chemical shift values (δ) were referenced to the terminal methyl carbon at 14.10 ppm (*5*). Peak assignments were made on the basis of published values for TG in PC vesicles and native lipoprotein particles (*5*, *7*). Line width at half-height ($v_{1/2}$), area, and intensity values were measured digitally using MacFID software. Areas were referenced to the sn-1,3 carbonyl resonance of TG in the core phase set to 100.

Analysis of Lipid Composition. The lipid composition of ultracentrifuged emulsions was analyzed by thin-layer chromatography and quantified by gas chromatography. PC and TG were separated via thin-layer chromatography using a mobile phase of benzene/ethyl ether/ethyl acetate/acetic acid, 80:10:10:0.2 (v/v/v/v), and identified by comparison with authentic lipid standards (*10*). Lipids were extracted from silica with 1 mL of benzene and transmethylated with acetyl chloride/methanol (1:15, v/v). The resulting fatty acid methyl esters (FAME) were extracted into hexane and quantified by gas chromatography using a Hewlett-Packard 5890 series gas chromatograph equipped with a DB 23 capillary column (J&W Scientific, Folsom, CA), automatic controller, autosampler, and Hewlett-Packard 3396A integrator. Total FAME were corrected for percentage methylation and extraction using internal standards, converted to milligrams of free fatty acid, and the total TG and PC were calculated from the quantity of free fatty acid according to equations 1 and 2.

$$\mu\text{g } TG = \frac{\mu\text{g } FFA}{\mu\text{L } emulsion} \times \frac{1 \text{ umol } FFA}{MW_{FFA}} \times \frac{1 \text{ umol } TG}{3 \text{ umol } FFA} \times (3 \times MW_{FFA} + 41.1) \quad (1)$$

$$\mu\text{g } PL = \frac{\mu\text{g } FFA}{\mu\text{L } emulsion} \times \frac{1 \text{ umol } FFA}{MW_{FFA}} \times \frac{1 \text{ umol } PL}{2 \text{ umol } FFA} \times (2 \times MW_{FFA} + 221.4) \quad (2)$$

Where MW_{FFA} is the average molecular weight of fatty acyl chains calculated from the fatty acid profile of TG and PL respectively; 41.1 and 221.4 are the molecular weights of the three carbon backbones less the fatty acyl chains, respectively. Additionally, PC content of 10- and 20-μL aliquots of emulsions, as well as PC extracted from silica, was determined using a colorometric reaction between phospholipid and ferrothiocyanate (*11*).

Calculation of Surface Triacylglyceride Content. The surface TG content was estimated by three different sets of calculations. The first calculations were based on comparison of the integrals of TG surface carbonyls to TG core carbonyls and the second and third sets of calculations were based on the comparison of the signal-to-noise intensity or integrals of the PC carbonyls to the TG carbonyls, respectively. Although NMR signal intensities are not directly quantitative, they are presented for comparison with the peak integrals because of the relatively small size of surface TG peaks located on the shoulder of the large core TG peak and thus, the inherent difficulty of integrating the smaller peaks. The percentage of TG in the surface ($\%TG_{SURFACE}$) versus the core of emulsions was calculated from the ratio of areas of ^{13}C carbonyl resonances of TG in the core and surface phases as described by Hamilton and Small (*5*) according to eq 3:

$$\%TG_{SURFACE} = (\text{Area } TG_{SURFACE})/(\text{Area } TG_{SURFACE} + \text{Area } TG_{CORE}) \times 100 \quad (3)$$

The nuclear overhauser enhancement values for TG in core and surface phases were assumed to be equivalent on the basis of published values for triolein in the surface of PC vesicles and triolein in a neat oil phase (*5*). The mass of TG in the surface phase was calculated based on the $\%TG_{SURFACE}$ and

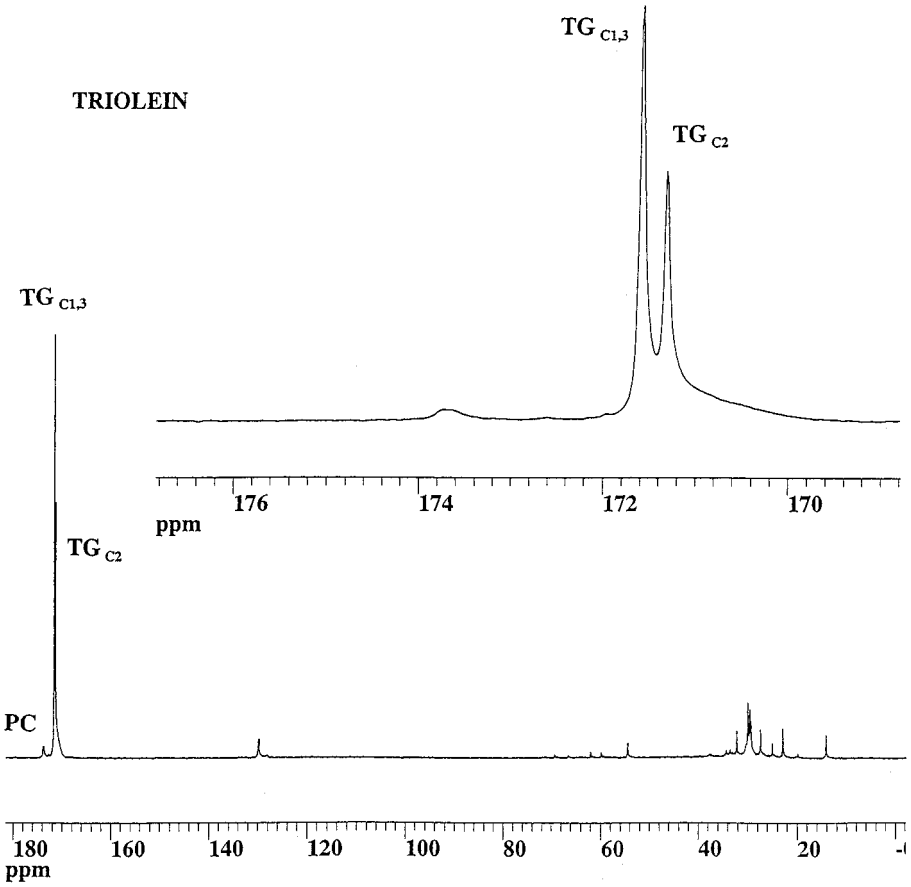

Figure 1. ^{13}C NMR spectrum and enlarged carbonyl region of $^{13}C_3$-triolein emulsions. The spectrum is 8192 acquisitions processed with baseline correction, exponential multiplication of 3.0 Hz, and Fourier transformation. Peaks are the sn-1,3 carbonyls of TO in the core ($TG_{C1,3}$), sn-2 carbonyls of TO in the core (TG_{C2}), and PC carbonyls (PC).

the chemical analysis of TG in each emulsion post-NMR ($TG_{EMULSION}$) according to eq 4:

$$\text{mg } TG_{SURFACE} = \%TG_{SURFACE} \times \text{mg } TG_{EMULSION} \quad (4)$$

The total mass of PC determined via chemical analysis of each emulsion post-NMR (mg $PC_{EMULSION}$) was assumed to be surface located, thus the total lipid mass of the surface was equal to the sum of mg $PC_{EMULSION}$ and mg $TG_{SURFACE}$. The weight and mol percentage of surface TG in emulsions ($SurTG_{WT\%}$ and $SurTG_{MOL\%}$) were calculated according to eqs 5 and 6:

$$SurTG_{WT\%} = \text{mg } TG_{SURFACE} / (\text{mg } TG_{SURFACE} + PC_{EMULSION}) \quad (5)$$

$$SurTG_{MOL\%} = (\text{mg } TG_{SURFACE} / MW_{TG}) / [(\text{mg } TG_{SURFACE} / MW_{TG}) + (PC_{EMULSION} / MW_{PC})] \quad (6)$$

Surface TG content was also estimated using the ratios of either the signal-to-noise intensity or integrals of the PC carbonyls to the $TG_{SURFACE}$ carbonyls, respectively. When the mass and ^{13}C enrichment levels of PC and TG are known, the expected ratio of the intensity of PC carbonyls to TG carbonyls in an NMR spectra can be calculated as described by Hamilton and Small (*5*). The calculated TG_{SIGNAL} is derived from eq 7 which states that the experimental PC:TG carbonyl ratio is equivalent to the calculated PC:TG carbonyl ratio. The calculated PC_{SIGNAL} is the expected signal intensity from the known amount of PC in the emulsion surface. The calculated TG $_{SIGNAL}$ is the expected signal intensity of a finite but unknown amount of TG in the surface of the emulsion. Thus, the theoretical calculated TG_{SIGNAL} can be estimated only by using the experimental PC and TG signals from the NMR spectra. The mass of TG in the surface is then calculated from eq 8 using the known percentage of ^{13}C enrichment in the TG.

$$\frac{\text{Calculated } PC_{SIGNAL}}{\text{Calculated } TG_{SIGNAL}} = \frac{\text{Experimental } PC_{SIGNAL}}{\text{Experimental } TG_{SIGNAL}} \quad (7)$$

$$\text{mg } TG = \text{Calculated } TG_{SIGNAL} \times \frac{MW_{TG}}{1 \text{ mmol } TG} \times \frac{1 \text{ mmol } TG}{3 \text{ carbonyls}} \times \frac{1}{\%^{13}C \text{ enrichment}} \quad (8)$$

Where MW_{TG} is the molecular weight of TG, and mg *TG* is the mass of TG in the surface. The mass of PC and $^{13}C_2$-DPPC were calculated using the assumption that the total mass of PC determined via chemical analysis had the same relative proportions of $^{13}C_2$-DPPC and non-^{13}C-enriched PC. The weight and mol % of TG in the surface of emulsions was then calculated using eqs 5 and 6 above.

The limitations and assumptions used to estimate surface lipid content included: (1) integration accuracy of the small surface TG peaks relative to core TG peaks; (2) the assumption that TG carbonyls in surface and core phases and PC carbonyls experience similar mechanisms and degree of relaxation during acquisition; (3) the assumption that TG carbonyls in surface and core phases of emulsions have the same nuclear Overhauser enhancement (NOE) factor; and (4) the accuracy of TG and PC compositional analysis. Integration of core-located TG resonances yielded values within 5−6% of the true values based on measurements of the ratio of core-located TG sn-1,3 carbonyls to sn-2 carbonyls. Integration of surface-located TG resonances was estimated to have an experimental error of ±10%.

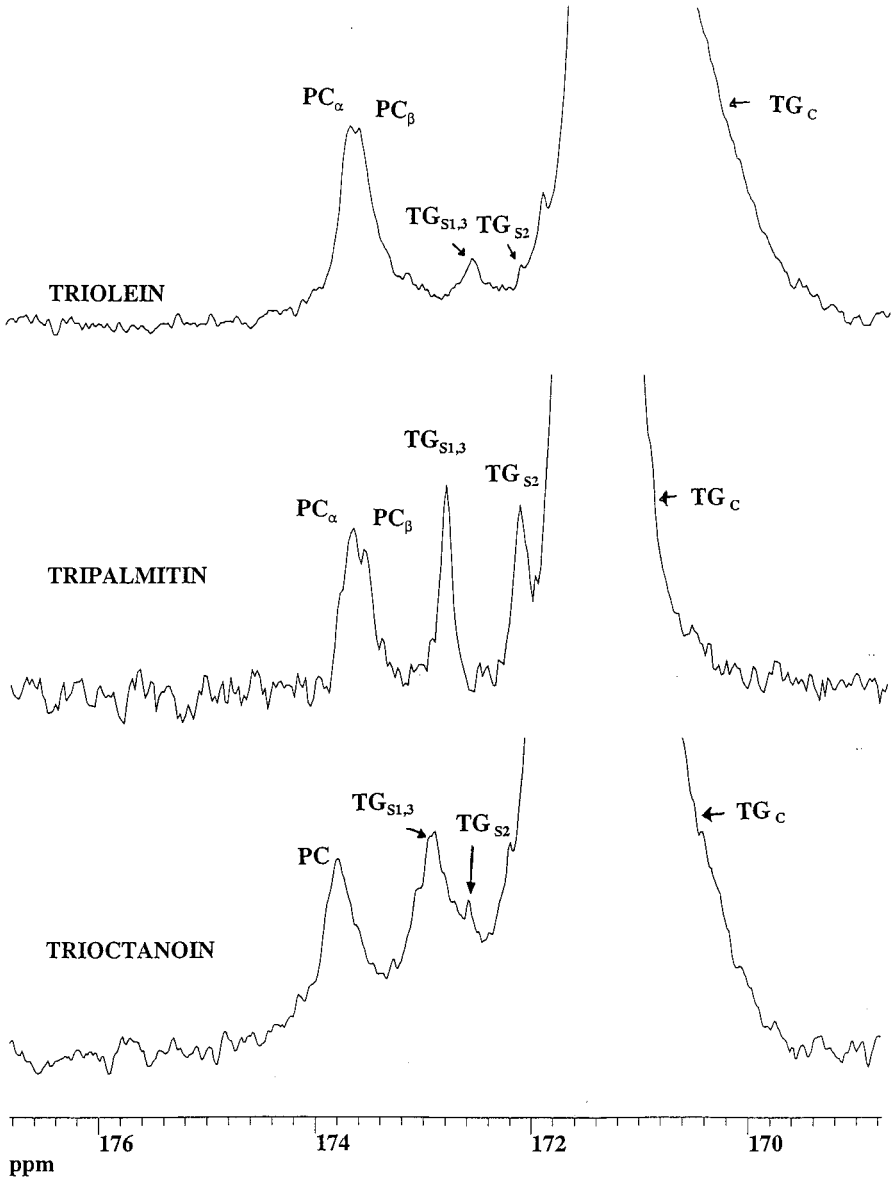

Figure 2. Enlarged carbonyl region of ^{13}C NMR spectra of $^{13}C_3$-triolein, $^{13}C_3$-tripalmitin, and $^{13}C_3$-trioctanoin emulsions. Each spectrum is 8192 acquisitions processed with baseline correction, exponential multiplication of 3.0 Hz, and Fourier transformation. Peaks are the carbonyls of TG in the core (TG_C), sn-1,3 carbonyls of TG in the surface ($TG_{S1,3}$), sn-2 carbonyls of TG in the surface (TG_{S2}), sn-1-$^{13}C_2$-DPPC carbonyls (PC_α), and sn-2-$^{13}C_2$-DPPC carbonyls (PC_β).

Table 2. ^{13}C NMR Chemical Shift Value, Line Width, and Area of Carbonyl Resonances in Emulsions

	tripalmitin			triolein[a]			trioctanoin[a]		
resonance	δ ppm[b]	$v_{1/2}$ Hz	area[c]	δ ppm	$v_{1/2}$ Hz	area[c]	δ ppm	$v_{1/2}$ Hz	area[c]
PC_D	173.72		1.40	173.72		4.67	173.76		3.84
$TG_{S1,3}$	172.87	5.71	1.65	172.61	6.5	1.82	172.61	6.7	5.95
TG_{S2}	172.19	nm[d]	0.63	172.33	nm	0.68	172.18	nm	2.64
unknown				171.97	1.37				
$TG_{C1,3}$	171.56	1.75	100	171.57	0.75	100	171.79	0.64	100
TG_{C2}	171.30	6.2	50.73	171.32	0.28	57.98	171.52	2.8	57.50
terminal -CH_3	14.10	2.77	5.91	14.10	1.37	5.20	14.10	1.20	2.36

[a] All values were measured in triplicate. [b] Chemical shift values were referenced to the terminal methyl carbon at 14.10 ppm. [c] Area values were normalized to the $TG_{C1,3}$ at 100. [d] nm, not measured.

RESULTS

^{13}C NMR Spectroscopy. The full ^{13}C NMR spectrum and the enlarged carbonyl region of the triolein emulsion are shown in Figure 1. ^{13}C signals from the sn-1,3 and sn-2 carbonyls of triolein in the core clearly dominated the spectrum. Surface-located TG was present in each emulsion composition as shown in the enlarged carbonyl region of the ^{13}C NMR spectra (Figure 2). Six distinct carbonyl resonances were detected in emulsions containing tripalmitin and trioctanoin, whereas emulsions containing triolein had seven distinct carbonyl resonances (Table 2). Carbonyl resonances were identified on the basis of published values for TG in PC vesicles and native lipoprotein particles (*5*, *7*) and by direct

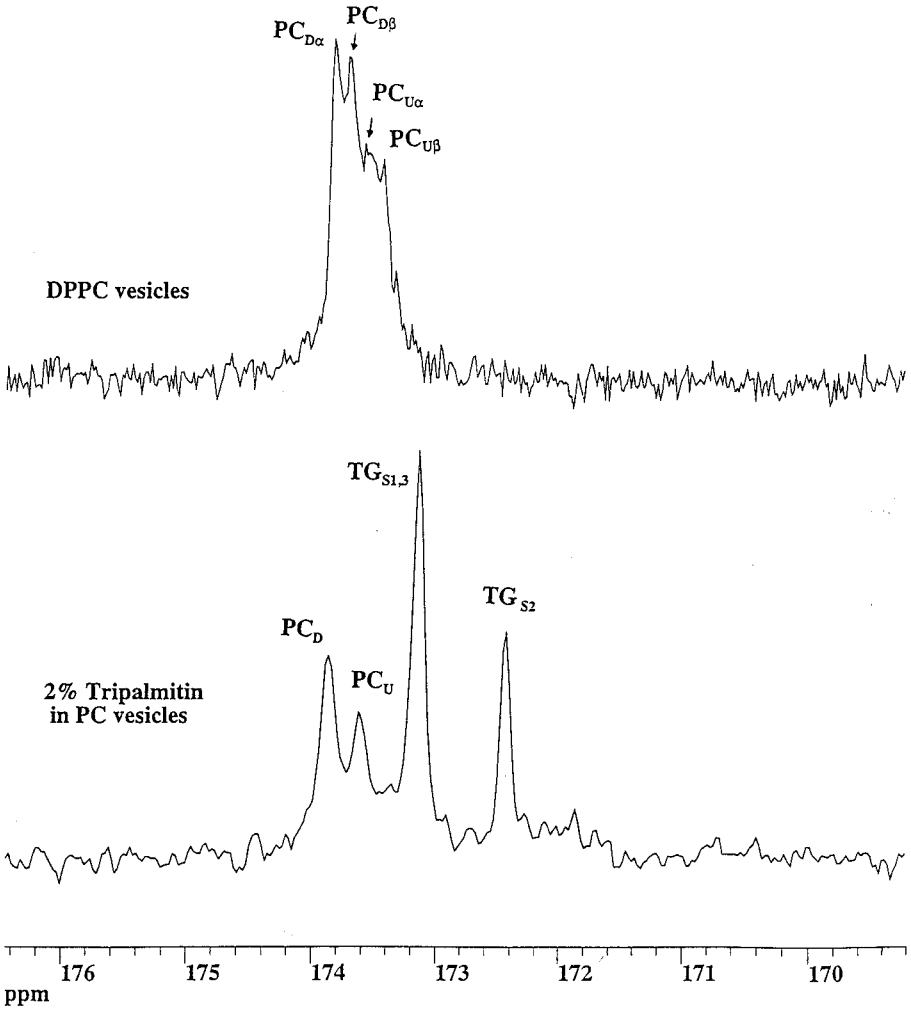

Figure 3. Enlarged carbonyl region of ^{13}C NMR spectra of $^{13}C_2$-DPPC vesicles and PC vesicles with 2 wt % of tripalmitin. Spectrum of $^{13}C_2$-DPPC vesicles is 1024 acquisitions and spectrum of PC vesicles with 2 wt % of tripalmitin is 20,000 acquisitions. Each spectrum is processed with baseline correction, exponential multiplication of 0.5 Hz, and Fourier transformation. Peaks are the sn-1,3 carbonyls of TP in the surface ($TG_{S1,3}$), sn-2 carbonyls of TP in the surface (TG_{S2}), PC carbonyls of the outer leaflet (PC_D), PC carbonyls of the inner leaflet (PC_U), sn-1-$^{13}C_2$-DPPC carbonyls of the outer leaflet ($PC_{D\alpha}$), sn-2-$^{13}C_2$-DPPC carbonyls of the outer leaflet ($PC_{D\beta}$), sn-1-$^{13}C_2$-DPPC carbonyls of the inner leaflet (PC_U), and sn-2-$^{13}C_2$-DPPC carbonyls of the inner leaflet ($PC_{U\beta}$).

comparison with carbonyl resonances in $^{13}C_2$-DPPC vesicles and PC vesicles containing 2% tripalmitin (Figure 3). In the order of decreasing chemical shift value, the carbonyl resonances in the spectrum of tripalmitin emulsions (Figure 2, middle panel) were sn-1 PC carbonyls (PC_α), 173.72 ppm; sn-2 PC carbonyls (PC_β), 173.62 ppm; sn-1,3 carbonyls of tripalmitin in the surface, 172.87 ppm; sn-2 carbonyls of TP in the surface, 172.19 ppm; sn-1,3 carbonyls of TP in the core, 171.56 ppm; and sn-2 carbonyls of tripalmitin in the core, 171.30 ppm. Carbonyl resonances in emulsions containing triolein and trioctanoin varied slightly in chemical shift value but followed the same order as those in emulsions containing tripalmitin. The triolein emulsion contained a seventh unknown carbonyl resonance at 171.97 ppm that was present in each replicate. The spectra of trioctanoin also exhibited a low, broad signal from 172.2 to 173.2 ppm. The chemical shift value, line width at half-height, and integral of carbonyl resonances for each emulsion are presented in Table 2; the chemical shift value and intensity of carbonyl resonances in vesicles and emulsions are presented in Table 3.

$^{13}C_2$-DPPC vesicles had four distinct carbonyl resonances corresponding to sn-1 PC carbonyls of the outer leaflet ($PC_{D\alpha}$), sn-2 PC carbonyls of the outer leaflet ($PC_{D\beta}$), sn-1 PC carbonyls of the inner leaflet ($PC_{U\alpha}$), and sn-2 PC carbonyls of the inner leaflet ($PC_{U\beta}$). The more downfield $^{13}C_2$-DPPC carbonyl resonance of each pair was identified as the sn-1 DPPC carbonyl based on published literature values for $^{13}C_2$-DPPC and $^{13}C_1$-DPPC vesicles (*12*). The difference in chemical shift values between sn-1 and sn-2 $^{13}C_2$-DPPC carbonyls within the same leaflet was 0.12−0.15 ppm, and the difference in chemical shift values between $^{13}C_2$-DPPC carbonyls on the outer and inner leaflets was 0.24−0.27 ppm. The ratio of intensities between the sn-1 and sn-2 $^{13}C_2$-DPPC carbonyls within the same leaflet was 1.04:1, and the ratio of intensities between $^{13}C_2$-DPPC carbonyls on the outer and inner leaflets was 1.41:1. The chemical shift difference and intensity ratio between the PC carbonyls of the outer and inner leaflets of PC vesicles containing 2% tripalmitin were 0.22 and 1.38 ppm, respectively.

Particle Size. The mean particle diameters of emulsions ranged from 64 to 107 nm and varied with the TG composition. Triolein emulsions exhibited a bimodal particle size distribution, and the other emulsion compositions all had a typical Guassian size distribution (Table 4). The mean particle diameters of vesicles were 20 ± 4 nm for $^{13}C_2$-DPPC vesicles and 27 ± 8 nm for

Table 3. Chemical Shift Value and Relative Intensity of Carbonyl Resonances in Phosphatidylcholine (PC) Vesicles and Emulsions

	vesicles					emulsions					
	2% tripalmitin		^{13}C-DPPC			tripalmitin		triolein		trioctanoin	
	δ[a]	I[b]	δ	I		δ	I	δ	I	δ	I
$PC_{D\alpha}$	173.87	9.12	173.87	1.08	PC_{α}	173.72	0.22	173.72	0.51	173.76	0.62
$PC_{D\beta}$			173.75	1.04	PC_{β}	173.62	0.21	173.64	0.51	173.64	0.61
$PC_{U\alpha}$	173.65	6.58	173.63	.77							
$PC_{U\beta}$			173.48	.73							
$TAG_{S1,3}$	173.16	2.88				172.87	0.96	172.61	0.41	172.61	2.4
TAG_{S2}	172.44	1.42				172.19	0.94	172.33	0.39	172.18	2.1
Δ $PC_{\alpha} - PC_{\beta}$[c]			0.12			0.10		0.08		0.12	
Δ $PC_D - PC_U$	0.22		0.25								
PC_{α}:PC_{β}[d]				1.04			1.03		1.01		1.10
PC_D:PC_U		1.38		1.41							

[a] Chemical shift values were referenced to the terminal methyl carbon at 14.10 ppm. [b] Intensity values were measured digitally after processing spectra with exponential line broadening of 0.5 Hz. [c] Difference in chemical shift value and [d] Ratio of intensities of PC carbonyls: PC_{α}, sn-1 PC carbonyls; PC_{β}, sn-2 PC carbonyls; $PC_{D\alpha}$, sn-1 PC carbonyls of outer leaflet; $PC_{U\alpha}$, sn-1 PC carbonyls of inner leaflet; $PC_{D\beta}$, sn-2 PC carbonyls of outer leaflet, and $PC_{U\beta}$, sn-2 PC carbonyls of inner leaflet.

Table 4. Lipid Composition and Particle Size of Triacylglycerol (TG) Emulsions

component	tripalmitin	triolein[a]	trioctanoin[a]
^{13}C TG (mg)	40	22.1 ± 3.4	11.3 ± 1.1
phosphatidylcholine (mg)	25	18.0 ± 6.5	9.4 ± 2.8
diameter (nm)	64 ± 9	91 ± 19 (89%) 275 ±51 (11%)	107 ± 42

[a] Values are the average ± SD of 3 replicates.

Table 5. Calculated and Experimental Ratios of PC and TG Carbonyl Signal Ratios in Emulsions

	calculated PC:TG[a]	experimental PC:TG	
		intensity[b]	area[c]
tripalmitin	0.016	0.007	0.009
triolein	0.053	0.032 ± 0.003	0.051 ± 0.008
trioctanoin	0.029	0.018 ± 0.003	0.038 ± 0.001

[a] Theoretical ^{13}C NMR carbonyl peak area ratio of ($^{13}C_2$-DPPC + PC):TG calculated from eqs. 7 and 8 (ref *5*). [b] Calculated using the ratio of the signal-to-noise intensity of surface triacylglycerol carbonyls to signal-to-noise of phosphatidylcholine carbonyls. [c] Calculated using the ratio of the area of surface triacylglycerol carbonyls to the area of phosphatidylcholine carbonyls.

PC vesicles containing 2% tripalmitin. Both particle diameter and size distribution of all emulsion and vesicle samples remained stable throughout the course of NMR analysis.

Emulsion Surface Composition. Tripalmitin crystallized out during ultracentrifugation destabilizing emulsions, thus, a single emulsion containing tripalmitin was analyzed by NMR directly after sonication, and the TG and PC composition of the tripalmitin emulsion post-NMR analysis was assumed to be equivalent to the initial composition. The TG and PC compositions of triolein and trioctanoin emulsions varied from their initial compositions and are given in Table 4. The calculated and experimental ratios of the PC:TG surface carbonyl signal intensities and integrals are presented in Table 5. The average surface TG concentration in emulsions was 2.2−4.4 mol % of tripalmitin, 1.4−1.8 mol % of triolein, or 9.1−15.5 mol % of trioctanoin. The smaller values of surface TG contents were calculated using the integrals of surface and core TG carbonyls whereas the larger values were calculated using ratios of PC:TG surface carbonyl signal intensities or areas (Table 6).

DISCUSSION

Triacylglycerols of varying chain length and saturation were identified and quantified in the surface of intact emulsion particles. Emulsions contained similar amounts of triolein and tripalmitin, and significantly more medium-chain TG than long-chain TG in their surface, as predicted by measurements of TG in phospholipid vesicles (*4*−*6*, *13*). PC vesicles contain 10−11 mol % of trioctanoin, 2.4 mol % of triolein, or 2.7 mol % of tripalmitin in the surface phase (*4*−*6*, *13*). The values for surface TG reported here also agree with the range of surface TG values reported for ultracentrifugally separated emulsions and lipoproteins that contained 2−5 wt % of TG (*14*). All emulsions used in this study contained amounts of surface-located TG within the range of predicted values using small unilamellar PC vesicles. Thus, monolayer and bilayer PC surfaces accommodate similar amounts of TG. The amount of surface TG calculated using ratios of PC:TG signal

Table 6. Estimation of Surface Triacylglycerol Content in Emulsions

	PC: TG				$TG_{SURFACE}$: TG_{CORE}	
	intensity[a]		area[b]		area[c]	
	wt%	mol %	wt%	mol %	wt%	mol %
tripalmitin	3.8	3.6	4.4	4.4	2.3	2.2
triolein[d]	2.1 ± 0.04	1.8 ± 0.04	2.0 ± 0.4	1.8 ± 0.3	1.6 ± 0.1	1.4 ± 0.1
trioctanoin[d]	8.2 ± 2.5	12.6 ± 3.6	10.2 ± 0.2	15.5 ± 0.3	5.8 ± 0.1	9.1 ± 0.2

[a] Calculated using the ratio of the signal-to-noise intensity of surface TG carbonyls to signal-to-noise of PC carbonyls. [b] Calculated using the ratio of the area of surface TG carbonyls to the area of PC carbonyls. [c] Calculated using the ratio of the area of surface TG carbonyls to the area of core TG carbonyls. [d] $n = 3$ replicates.

intensity or integrals were all greater than corresponding measurements using integrals of surface and core TG. The differences in calculated values from PC:TG ratios versus TG surface and core integrals are attributed to variations in $^{13}C_2$-DPPC content of emulsions and the assumption that the relative proportion of $^{13}C_2$-DPPC to non-^{13}C enriched PC remained the same following ultracentrifugation during emulsion preparation. Variations in the calculated and experimental ratios of PC:TG carbonyl signals and integrals were attributed to variations in lipid composition of emulsions.

The presence of carbonyl peaks located between PC carbonyls and core-located TG carbonyls in ^{13}C NMR spectra of each emulsion clearly indicates the presence of surface-located TG. Each emulsion also contained more than one PC carbonyl resonance, indicating the potential presence of PC vesicles in emulsion preparations. However, analysis of the difference in chemical shift values and the ratio of intensities for PC carbonyl resonances indicated that vesicles were not present in emulsion samples during NMR analysis. In phospholipid vesicles, the more downfield carbonyl resonance arises from PC carbonyls on the outer leaflet, PC_D, while the more upfield resonance arises from PC carbonyls on the inner leaflet, PC_U (*12*). The ratio of intensities of PC carbonyl resonances on the outer and inner leaflets of vesicles is approximately 1.4:1, as the outer leaflet has more PC molecules than the inner leaflet. If both emulsions and PC vesicles were present in emulsion samples, the ratio of intensities of PC carbonyl resonances on the outer and inner leaflets would be ≥ 1.4:1 as the PC carbonyls of the outer leaflet of vesicles and the PC carbonyls on the surface of emulsions would both contribute to the intensity of the more downfield resonance (*7*, *12*). The ratios of intensities of PC carbonyls in TG emulsions were all < 1.03:1. Additionally, the differences in chemical shift values between the PC_D and PC_U carbonyls of emulsions (0.10−0.12 ppm) were consistently less than the differences in chemical shift values between PC_D and PC_U carbonyls published for small unilamellar PC vesicles (0.23−0.27 ppm). $^{13}C_2$-DPPC was used to prepare emulsions, thus, the two PC carbonyls in emulsion spectra could arise from the sn-1 and sn-2 carbonyls of $^{13}C_2$-DPPC. To examine this possibility, PC vesicles containing the same proportion of $^{13}C_2$-DPPC to PC used in emulsions were prepared and analyzed by ^{13}C NMR. In the $^{13}C_2$-DPPC vesicles, both the sn-1 and sn-2 carbonyls of $^{13}C_2$-DPPC were visible on the outer and inner leaflets. The difference in chemical shift values between sn-1 and sn-2 carbonyls of $^{13}C_2$-DPPC was 0.12−0.15 ppm, and the difference in chemical shift values between $^{13}C_2$-DPPC carbonyls of the outer and inner leaflets was 0.24−0.27 ppm. Thus, the two PC carbonyl resonances of emulsions did not arise from the presence of vesicles but from the sn-1 and sn-2 carbonyls of $^{13}C_2$-DPPC.

Detection of discreet surface and core TG signals indicates that the rate of exchange of TG between the surface and core of emulsions must be slow on the NMR time scale. The chemical shift value of the unknown carbonyl resonance in triolein emulsions did not correlate with the known chemical shift values for oleic acid, dioleoylglycerol, or monooleoylglycerol in emulsions or vesicles (*15*−*17*, and Kalambur, S., Li, R., and Boyle-Roden, E. unpublished observations). The contaminating compound(s) must contain a ^{13}C-enriched carbonyl group to be visible in the spectra and could potentially be an oxidized product of hydrolyzed oleic acid. Additionally, chemical analysis of triolein emulsions post-NMR revealed only triolein and PC. As the size distribution of triolein emulsions was bimodal, one speculation is that the carbonyl peak at 171.97 ppm arises from triolein in the surface of emulsion particles of the larger size distribution. The appearance of a low, broad signal in trioctanoin emulsions indicated a decrease in molecular mobility for trioctanoin in the emulsion surface. However, line width values for core TG carbonyls and terminal methyl carbons in each emulsion indicated the bulk of TG experienced similar degrees of molecular mobility.

These results provide a direct measurement of surface-located TG in intact emulsion particles and confirm that PC vesicles are a reasonable model for examining the solubility of TG in the surface of emulsions. Medium-chain TGs have a greater surface concentration in emulsions than long-chain TGs, and thus are more accessible to enzymes or other molecules in the continuous phase. No difference was found in the surface concentration of the long-chain saturated or monounsaturated TG measured; however, a heterogeneous acyl chain composition or increased unsaturation of the acyl chains may greatly affect surface availability of TG in emulsions. The quantitative determination of surface-located TG in intact particles will advance our understanding of the physicochemical stability and colloidal properties of emulsions in food systems, as well as enhance our knowledge of the metabolic behavior of lipoproteins in vivo.

ABBREVIATIONS USED

Triacylglycerol, TG; deuterium oxide, D_2O; phosphatidylcholine, PC; dipalmitoylphosphatidylcholine, DPPC; sn-1 PC carbonyls, PC_α; sn-2 PC carbonyls, PC_β; sn-1 PC carbonyls of outer leaflet, $PC_{D\alpha}$; sn-1 PC carbonyls of inner leaflet, $PC_{U\alpha}$; sn-2 PC carbonyls of outer leaflet, $PC_{D\beta}$; sn-2 PC carbonyls of inner leaflet, $PC_{U\alpha}$.

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