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Size-Dependent Lipid Content in Human Milk Fat Globules

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Human milk fat globules (HMFGs) are considered to constitute a triglyceride-rich source of fat and energy. However, milk contains lipid particles at different sizes ranging from tens of micrometers to less than 1 μm. In particular, the physical, chemical, and biological properties of submicron sized particles are poorly described. Individual HMFGs were analyzed using laser trapping confocal Raman spectroscopy, and their chemical signature was obtained and compared to 1, 5, and 10 μm globules. Significant differences in both lipid composition and relative lipid content were found between the classes of particles with different diameters. A strong Raman peak at 1742 cm^{-1} corresponding to the triacylglycerol core was detected in the 5 and 10 μm diameter globules, whereas in the smaller HMFGs no detectable peak was found. In addition, the submicron particles produced Raman signals consistent with large quantities of unsaturated fatty acids. Moreover, cis and trans isomers of unsaturated fatty acids were found to be unequally distributed between large and small milk fat globules. Interestingly, trans unsaturated fatty acids were found only in 1 and 5 μm globules although more prominent in the 5 μm diameter range. This is the first evidence for size related differential lipid composition of various diameter classes of HMFGs. The results suggest that the milk fat globule size distribution determines milk lipid composition. In addition, large portions of the HMFGs are secreted into milk conspicuously not for fat delivery. Thus, small HMFGs may offer novel metabolic and nutritional functions.

KEYWORDS: Milk fat globules; lipids; laser trap; Raman spectroscopy

INTRODUCTION

The milk fat globule, although acknowledged to consist of a diverse range of size classes, is considered to be exclusively a fat delivery vehicle. This perspective neglects the profound and very basic differences between various size classes of the actual milk fat globules found in human and other mammalian milks. The dominant view of milk fat overlooks the fact that large globules are indeed dominated by their triacylglycerol (TAG) core whereas the small milk fat globule features are dominated by the complex structural lipids including the phospholipids and glycolipids that make up the surface of the globule membrane.

Research to date has focused on understanding, even altering, the concentration of specific fatty acid within the milk fat fraction, mainly of bovine origin (*1*–*3*) but also in humans (*4*). Molecules such as unsaturated fatty acids and conjugated glycolipids (*5, 6*) have been the focus of an array of studies. However, these alterations of specific compounds in the milk lipid fraction were never assessed as a function of different diameters and structures of the milk fat globules.

The technologies proposed up to date for milk fat globule size separation are mainly based on gravity separation either spontaneously (*7*) or enhanced by centrifugation (*8*) or microfiltration based protocols (*8*). From a colloidal point of view, both methods generate a rather broad range of size subclasses and enable rough separation based on diameter exclusion. These techniques generate two main fractions: cream (i.e., large milk fat globules) and skim (i.e., small milk fat globules). Indeed, the "cream" fraction enriched in larger fat globules is not devoid of the smallest globules that are present in large numbers; the semiskimmed fraction containing small globules contains much more than simply the smallest globules and can include globules with diameters as large as several microns. Since all current chemical analyses of milk lipid particles are constrained by outcome of these separation methods, the chemical contribution

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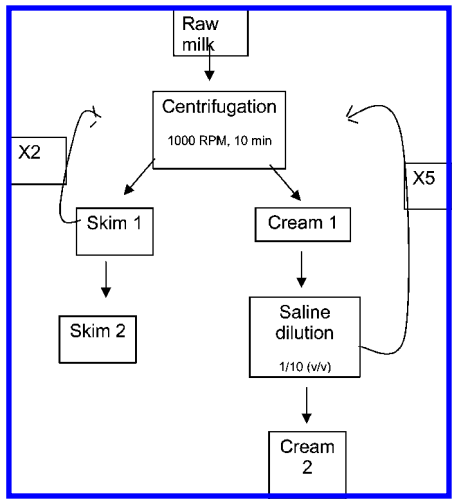

Figure 1. Procedure for milk fat globule size separation.

of the smallest milk fat globules will always be contaminated by the larger milk lipid particles' composition. Moreover, the data generated will constantly be disproportionately biased toward the chemical composition of larger milk fat globules due to their overwhelming volume compared to the smallest milk fat globules.

If there are heterogeneous pathways of milk fat globule assembly, these pathways would potentially result in different globular compositions, structures, and functions ascribable to the various milk fat globules. Thus, understanding their structures both nutritionally and metabolically will require establishing methods that enable chemical as well as nutritional characterization of specific size classes of milk fat globules.

We present first evidence of the unique chemical and physical properties of human milk fat globules (HMFGs) of different size classes within extremely narrow size distributions using laser tweezers and Raman spectroscopy. This approach successfully identified bacterial spores in heterogeneous samples (*9*) and more recently was used to characterize TAG rich lipoproteins (i.e., nanometer sized particles) (*10*). This method was found to be well-suited for the analysis of complex mixtures of individual nano- to microscale particles because of the immobilizing of the particle in the tightly focused laser beam. As a result, the acquisition of the Raman signal from a single particle becomes possible. Single colloidal particles can be trapped and analyzed while in their natural suspension, without disrupting them.

This method was used to distinguish between milk's large and small lipid assemblies and produce chemical fingerprints of particles while they are sized. The application of laser trapping confocal Raman spectroscopy opens up an area of biology and food functionality that was never appreciated due to inadequate methods for lipid particle analysis at the nanometer scale length. This is the first evidence for chemical analysis of such milk lipid globules.

MATERIALS AND METHODS

Human milk samples were provided by Prolacta Bioscience Inc. (Monrovia, CA). Milk samples that were tested and found unsuitable for infant consumption were chosen for these studies as they provided a suitable material for research purposes. Milk samples were frozen at −20 until their use. Human breast milk of four volunteers was collected at different lactation stages and separated into skim and cream fractions (**Figure 1**). Subsequently, the fractions were further separated to obtain maximum segregation of milk fat globules according to their size.

Milk fat globule sizes were measured in fresh cream or skim milk samples from human mature breast milk in triplicate using a particle size analyzer with a single modular light-scattering system (Microtrac UPA; Leeds and Northrup, North Wales, PA). For each milk sample, fat globule size was calculated as the mean diameter (in microns) of the area distribution of the particles in solution obtained from two 180 s scans. Mean diameter $= \sum V_i/\sum(V_i/d_i)$, where V is the volume percentage in channel size and d is the channel diameter (width of path of light). To prevent artifacts regarding submicron-sized globules, the casein micelles were dissociated by diluting the sample in 35 mM EDTA (pH 7) prior to measurement.

Raman spectroscopy was applied to samples from both fractions, and the data were divided into four different size classes of milk fat globules, as determined from the images obtained with the optical microscope: submicron, 1, 5, and 10 μm. Raman spectra of three globules from the submicron class and four globules from the other classes from each milk donor were plotted to generate a representative average spectrum for each globule diameter class. To validate that the separation process did not add any artifact, globules from whole milk samples were also analyzed by Raman spectroscopy, and the spectra were added to the averaged spectra.

Raman spectra of HMFGs were collected using a laser tweezers Raman spectroscopy setup. The 647 nm laser line of an ArKr multiline laser (Coherent Innova C90) was focused on a diffraction limited spot (approximately 400 nm diameter) by a 60×, 1.45NA Olympus TIRF objective mounted on a fully automated optical microscope (iMic,TILL Photonics, Munich, Germany). Individual HMFGs were trapped by the laser light, and their Raman spectra measured in epidetection through the same objective via a grating spectrometer (Acton Research model no. 2300i, 600 L/mm, blazed at 1 μm) and a thermoelectrically cooled CCD camera (Pixis100, 1340 × 100 pixels, cooled to −70 °C). The combination of a narrow spectrometer entrance slit (50 microns) and narrow strip on the CCD camera (10 pixels) ensures good rejection of out-of-focus light and confocal detection of the Raman signal.

Approximately 5 mW laser power was used to excite Raman vibrations, and spectra were collected in 30 s each. Spectra were calibrated to the known spectrum of small (2 μm diameter) polystyrene beads trapped by the laser in the same way as the HMFGs. Spectra of each size class were averaged, and then a background correction was performed by subtraction of a third-order polynomial baseline fit. Normalization of the intensities of Raman peaks was calculated by measuring the area for a Gaussian fit of each peak. Relative lipid composition in each HMFG diameter ($n = 7$ for each diameter class) was calculated as follows and expressed in arbitrary units: relative intensities of the Raman peaks were obtained by normalizing to the averaged Raman spectra of the antisymmetric CH_2 stretching mode at 2885 cm^{-1}, which would be the closest indicative of total lipid content, as was previously described (*11*). Differences between means were tested by Tukey's test, and the significance was $p < 0.05$ unless otherwise stated. Values are represented as mean ± SEM. JMP version 5.1 was used for all analyses (*12*).

RESULTS

Human milk fat globules were separated by size using the common centrifugation techniques (**Figure 1**), and there was a reasonable separation achieved between the large and small diameter globules (**Figure 2** inserts **1** and **2**). The "cream 2" fraction consist of average milk fat globules diameter of 7.02 (±1.3) μm, whereas the "skim 2" fraction consist of milk fat globules in the average diameter of 3.8 (±1.9) μm.

The major Raman peaks and their assignments are listed in **Table 1**. The diameter classes of the milk fat globules were found to differ in both their lipid composition (**Figure 3**) as well as in the relative concentration of the various lipid components (**Table 2**, **Figure 4**).

The peaks observed in the Raman spectra of milk fat particles can be readily assigned to well-known major bands of lipid vibrations by comparing them to previously published results or native lipid spectra. We have summarized the most significant

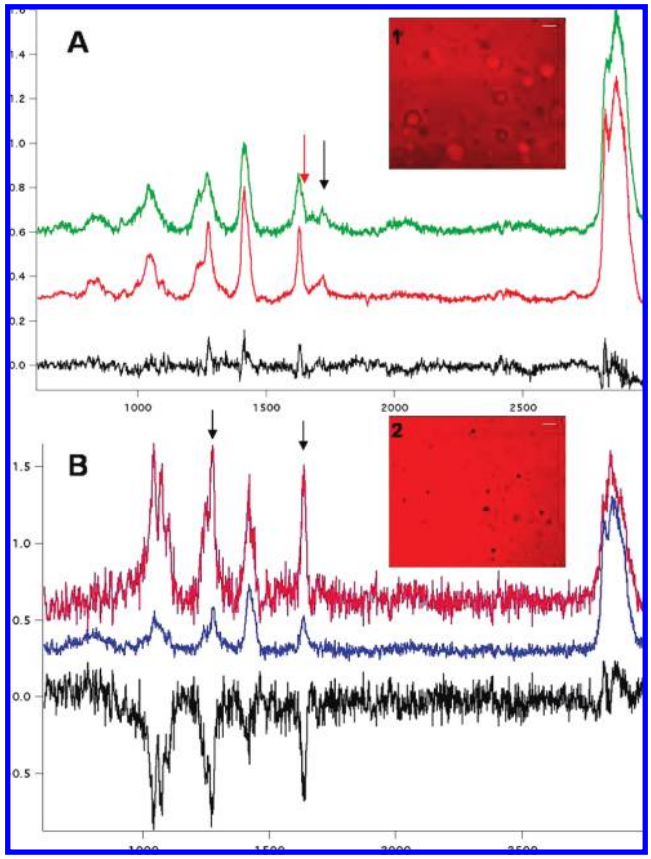

Figure 2. (**A**) Raman spectra of 5 μm (green) and 10 μm (red) HMFGs. Arrows represent the peaks assigned to triacylglycerol and unsaturated C═C double bonds, trans conformation (red and black, respectively). The differences between the two human milk fat globules size groups are indicated in the bottom (black). Inset represents the isolated fraction "cream 2", which is largely composed of large milk fat globules but also smaller globules. (**B**) Raman spectra of submicron (green) and 1 μm (blue) HMFGs. Arrows represent the peaks assigned to cis conformation of C═C double bonds. The differences between the two human milk fat globules size groups are indicated in the bottom (black) Inset: isolated fraction of "skim 2" composed of nanoscale length as well as microscale length milk fat globules (bar indicates 5 μm).

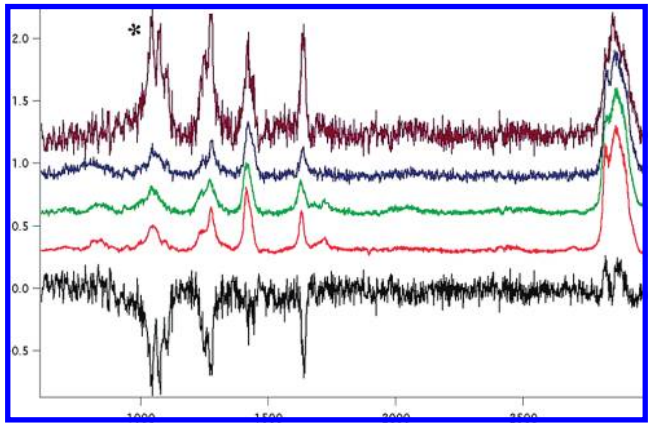

Figure 3. Raman spectra of milk fat globules of different sizes: submicron (purple), 1 μm (blue), 5 μm (green), and >10 μm (red). The differences between the submicron and the largest milk fat globules size group are indicated in the bottom (black). An asterisk marks the three distinct peaks that have been assigned to different saturated fatty acids. The separation of these three peaks only occurs in the submicron HMFG and provides a unique fingerprint to this diameter class.

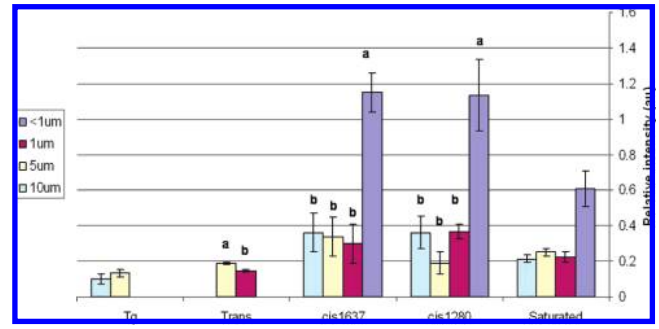

Figure 4. Dependence of lipid content of HMFGs on particle size. The vertical bars indicate five major Raman peak intensities relative to the main C−H asymmetric vibration at 2885 cm^{-1} (dark horizontal line).Values significantly different ($p < 0.05$) between lipid particles diameter classes are designated by different letters. Saturated, Raman peak at 1076 cm^{-1} assigned for saturated fatty acids; cis 1266, Raman peak at 1266 cm^{-1} assigned for unsaturated carbon double bond, cis conformation; cis 1650, Raman peak at 1650 cm^{-1} assigned for unsaturated carbon double bond, cis conformation; trans, Raman peak at 1668 cm^{-1} assigned for unsaturated carbon double bond, trans conformation; TAG, Raman peak at 1742 cm^{-1} assigned for triacylglycerol.

Table 1. Raman Shift Assignments for Raman Bands (cm^{-1})

group	vibration	chemical bond	molecule type
1000	976	δ(═C—H)	
	1066	φ(C—C)	saturated
	1076	φ(C—C)	
	1129	φ(C—C)	saturated
1266	1265	δ(═C—H) cis	unsaturated
	1302	δ(CH2) twisting	
1427.5	1439	δ(CH2)	
	1455	δ(CH2)	
1650	1654	φ(C═C) cis lipid	unsaturated
		φ(OH) water	
	1668	φ(C═C) trans lipid	unsaturated
1724	1742	φ(C═O) in CH_2—COOR	triacylglycerol
3000	2870		cholesterol
	2885		CH_2 antisymmetric stretch

Table 2. Relative Lipid Composition of Raman Peaks of HMFGs in the Fingerprint Region Relative to the Symmetric C−H Vibration at 2845 cm^{-1} ($n = 6$ for Each Diameter Class)[a]

	SAT 1066	SAT 1066	SAT 1129	cis 1266	cis 1650	trans	TAG
submicron	1.103	0.609	0.559	1.135	1.148	UD	UD
1 μm	UD	0.225	UD	0.364	0.298	0.155	UD
5 μm	UD	0.252	UD	0.191	0.337	0.193	0.133
10 μm	UD	0.214	UD	0.362	0.362	UD	0.101
SEM		0.02		0.09	0.11	0.006	0.02

[a] UD, under detectable levels; SAT1044, Raman peak at 1044 cm^{-1} assigned for saturated fatty acids; SAT1076, Raman peak at 1076 cm^{-1} assigned for saturated fatty acids; SAT1129, Raman peak at 1129 cm^{-1} assigned for saturated fatty acids; cis 1266, Raman peak at 1266 cm^{-1} assigned for unsaturated carbon double bond, cis conformation; cis 1650, Raman peak at 1650 cm^{-1} assigned for unsaturated carbon double bond, cis conformation; trans, Raman peak at 1668 cm^{-1} assigned for unsaturated carbon double bond, trans conformation; TAG, Raman peak at 1742 cm^{-1} assigned for triacylglycerol.

Raman peaks and their assignments in **Table 2**. Most peaks in the Raman fingerprint range of a milk fat particle are due to carbon bond vibrations, and similar peaks have been discussed in detail in ref (*10*). Here, we briefly summarize some of the more important peaks. Raman peaks of acyl chains in the 1000−1200 cm^{-1} range are typically due to C—C stretching vibration. Specifically, the peaks at 1066, 1076, and 1129 cm^{-1}

are known to be very sensitive indicators of acyl chain order in lipids. These peaks usually indicate close chain packing typical of the structure of saturated fatty acids at room temperature. The 1266 cm^{-1} peak is due to an in-plane C—H bending mode in a double bond and thus a marker for unsaturation. The same is true for the 1654 cm^{-1} C═C stretch mode. It is further known (*13*) that spectra of triglycerides exhibit the presence of a peak at 1742 cm^{-1} (C═O bond in an ester). Also, the location of the 1650 cm^{-1} band corresponds to a cis conformation in a C═C double bond, which would otherwise be located at 1668 cm^{-1} for the trans conformation, and is therefore another important structural marker for unsaturated bonds. Raman bands due to proteins that might be associated with the milk fat globules are typically difficult to identify because of the overwhelming contributions from lipids. A distinguishing mode from phospholipids other than the acyl chain vibrations discussed above is typically observed at ~860 cm^{-1}, but this mode is too weak to provide any useful information at the single particle level. The Raman spectral range in the region around 2700−3200 cm^{-1} is composed of modes mostly due to hydrogen bonds, most prominently methylene and methyl group vibrations. These have been characterized in detail in ref (*11*). The most prominent C—H bond vibrations in CH_2 groups are located at approximately 2845 cm^{-1} (symmetric stretching mode), 2885 cm^{-1} (antisymmetric stretching mode), and a weak mode at 2925 cm^{-1} (symmetric stretching mode). The same bands also show Raman active modes for bonds in a CH_3 group, with the addition of a weak peak at 2960 cm^{-1} due to a symmetric methyl stretching mode. Cholesterol and cholesterol ester exhibit an additional peak at 2870 cm^{-1} (*14*). Lastly, it has been suggested that a C—H stretching mode of a C═C double bond resides at ~3010 cm^{-1}. Thus, by acquiring the entire Raman spectral range from ~400−3200 cm^{-1}, laser tweezers Raman spectroscopy can quickly and nondestructively determine the lipid composition of individual milk fat particles.

The differences between the two subpopulations of the milk fat globules are represented by the subtraction of the largest milk fat globule average Raman spectra from the smallest, submicron milk fat globules average spectra (**Figure 3**, black line). Sensational differences between the submicron- and the micron-sized particles are found in both the relative intensities of the peaks and the presence and absences of specific signals. For example, the submicron MFG spectra showed a triplet peak corresponding to saturated fatty acids in the 1000 cm^{-1} region (**Figure 3**, asterisk) that are not discernible in any larger MFG classes. In addition, the relative intensities of the peaks near 2800 are lower in the submicron particles compared to the larger particles. These peaks contain signals due to CH_2 group and cholesterol that are evident when the peaks are deconvoluted (results not shown).

On the basis of the spectra, quantification of various functional groups was performed and summarized in **Figure 4**. The values were normalized as indicated in the Materials and Methods section. The results indicate that the lipid composition of smallest milk fat globules (submicron) differ significantly from the other MFG larger classes. The 5 and 10 μm size particles contain approximately the same amounts of TAG, which are absent in both the 1 μm and submicron particles. The relative quantity of polyunsaturated fatty acids with cis conformations (1266 and 1654 cm^{-1}, respectively; **Figure 4**) within the submicron group was found to be consistently 3- or 4-fold higher than in the other groups. Interestingly, unsaturated fatty acids with double bonds in trans conformation were found only in the 5 and 1 μm diameter particles and were specifically enriched in the 5 μm particles

Comparisons between the submicron and the larger globules are made further in **Figure 2**.The comparison between the submicron and the 1 μm diameter particle (**Figure 2B**, black spectrum) showed the same major differences described previously, while the same comparison between the large MFG particles (**Figure 2A**, black spectrum) showed only minor differences. The factor of the differences between the two class sizes in **Figure 2** indicates that the variations between the particles cannot be solely accounted for by the TAG core. There appears to be a fundamental variation in composition between the submicron and micron sized particles.

DISCUSSION

Despite the fact that milk fat globules are colloidal lipid assemblies, little research has focused on the striking diversity of sizes, structures, and compositions within human or any other mammalian milks. Moreover, the evolutionary mechanisms responsible for this diversity have not been explored leading to the widespread belief that the biological role of all milk fat globules is solely to deliver energy rich fuel in the form of triglycerides to the infant. In the present study, the milk lipid fraction was analyzed using laser trapping confocal Raman spectroscopy, revealing the power of this distinctive method to analyze specific submicron sized, nanoscale population of milk fat globules. Moreover, this is the first evidence for a subset of milk lipid assemblies secreted into milk not for the sole purpose of energy delivery, as there is no evidence of significant levels of triglycerides in these particles.

By using common techniques based on centrifugation, it is possible to reduce the average size and the size distribution of the skim milk only to a finite extent. It has not been possible to attain solely submicron particles (*7, 8*). Thus, all chemical analyses of such fractions give rise to a bulk average composition of milk lipid particles with a broad distribution around 1 μm. Hence, novel techniques are needed to either separate the submicron globule and study their nutritional and beneficial affects or obtain in situ chemical analysis of explicit particles.

By comparing the Raman spectra that were obtained in this study for milk fat particles of different sizes, the following general observations can be made. The spectra obtained for large milk fat particles (5−10 μm large compared to the wavelength of the probe laser) are relatively similar and are triglyceride-rich as evidenced by the 1742 cm^{-1} peak. These spectra are also similar to those previously obtained for lipoproteins in the fasting state (*10*). Upon closer inspection of the spectra, it also appears that the large diameter particles show peaks at 2870 cm^{-1} indicative of cholesterol contributions, as well as a peak at 860 cm^{-1} indicating phospholipids. Because milk globules consist of a triglyceride core and a phospholipid covering (*15*), these observations indicate therefore that the spectra probe both the contributions of the bulk and membrane. However, smaller particles (1 μm and submicron particles) show distinct characteristics. Because the wavelength of the probe laser is the same size as the particle or larger, it is more likely that the composition of the entire particles is examined and even though their surface-to-volume ratio is significantly higher. For these particles, the contributions from cholesterol and triglyceride peak at 1742 cm^{-1} disappears. We can therefore conclude that, while the nanoscale particles lack a significant triglyceride component, they are enriched with unsaturated cis isomers of fatty acids. These results are surprising given that the sole purpose of milk fat globules has been attributed mainly as deliverers of triglyc-

eride (*16*). These results further suggest a new function for the nanosized milk globules.

The nanoscale milk lipid globules can therefore no longer be termed "milk fat globules", which suggest simply that they deliver triglycerides. We propose a different term "lactosomes" to describe them and distinguish their unique structural compositional and potentially nutritional properties from the large, triglyceride-rich fat globules. The lack of a TAG core implies a new mechanism of formation and a new class of lipid particles perhaps analogous to phospholipids rich particles as lamellar bodies (*17*).

The nanoscale particles produced Raman spectra characterized by narrower peaks than the larger particles. This spectral property is reminiscence of other nanoparticles that undergo a step function in its properties as it transitions from bulk to discreet states. This phenomenon was also present in the spectral characteristics between the 1 μm diameter and submicron particles, indicating that the phenomenon is simply not related to the lack of the TAG core.

Enrichment of the unsaturated fatty acid was also present in the submicron particles. This trend is probably due to the relatively higher phospholipid content that naturally contains more polyunsaturated fatty acids (*18*). The increase in unsaturated fatty acids is in agreement with a previous study that compared fatty acids profiles between large and small MFGs (*16*). The presence of increasing double bonds is consistent with possibly longer unsaturated fatty acids that in turn may contain more double bonds in the phospholipid alkyl chain.

One observation is perplexing and may yet indicate an even further complexity of the milk globule assembly. The Raman spectra show that fatty acid double bonds with the trans conformation were found exclusively in the 1 μm and the 5 μm size milk fat globules. Trans fatty acids in human milk have attracted attention for its relationship to cancer occurrence (*19*). Additionally, its presence in milk has been associated with compromised metabolism of very long chain polyunsaturated fatty acid in infants (*20*). The presence of these compounds in specific milk globule diameter classes and their relationships to health make these lipid globules also of considerable interests and warrant further investigations.

At this point, it is not possible to further discriminate the submicron (nanoparticle) sized particles, the lactosome. However, we foresee the development of more precise analytical tools that can further differentiate these classes of particles. Future studies may even determine the nature of the physiological conditions to their formation allowing precise production of specifically desired distribution. This possibility will further drive more biological studies relating them to nutritional roles and potential therapeutic properties.

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