

Chapter 13

Evaluation of Stabilizers for Synthetic Vesicles and Milk Fat Globules under Drying Stress

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Dipalmitoylphosphatidylcholine (DPPC) vesicles in aqueous buffer, prepared by sonication and controlled fusion, were characterized by differential scanning calorimetry (DSC) analysis. Size variations in vesicle preparations during fusion were analyzed by spectroturbidity measurements and laser scattering analysis. Inasmuch as the measured turbidity of a vesicle preparation was shown to be directly proportional to the concentration of the phospholipid in the vesicled form, the relative quantity of vesicles remaining after applying a drying and rehydration procedure was estimated from turbidity measurements taken before and after drying. A parameter, η_R , the rehydration efficiency, was defined in terms of turbidity changes to express the resilience of vesicles to the drying-induced stress. Values of η_R were determined for vesicles dried and rehydrated in the presence of candidate preservatives, including carbohydrates and metal ions. Disaccharides stabilized dried phospholipid vesicles better than monosaccharides; polyols were destabilizing at all studied concentrations, and metal ions were mildly stabilizing only at low concentrations. Extracts of milk fat globules (MFG) from whole milk were analyzed for their resilience toward drying-induced rupture in the absence and presence of candidate preservatives by the spectroturbidimetric method. Of the studied additives, maltodextrin M-100 (a hydrolyzed corn starch) was found to be the most effective stabilizer. Analogous studies of dried diluted whole milk samples, in which the major contributor to sample turbidity was due to the component MFGs, indicated that the best single stabilizing additive toward drying-induced rupture of MFGs is maltodextrin M-100.

A fundamental problem with milk and milk-based foods and beverages is that they have limited shelf-lives outside a refrigerator. Although spray-dried milk products having extended storage stability

are commercially available, they usually derive from skim milk and can have an unacceptable flavor or mouth feel to many consumers after they are reconstituted with water. Skim milk is used predominantly to produce dried milk because the prior removal of the milk fat minimizes the subsequent nonenzymatic oxidative rancidity in the dried residue.

The fat content in milk exists as a distribution of tiny fat globules or microspheres, bounded by membranes made up of complex phospholipids and proteins, dispersed throughout the aqueous medium (1). During the drying of milk, the removal of water from contact with the surface membrane of the milk fat globules can lead to their rupture, which can then lead to the dispersion of the fat component throughout the residual dried protein, carbohydrate, and mineral components (2). The blending of the fat with the other milk components facilitates the potential for oxidative decomposition of the dried milk, especially in the more reactive membrane phospholipids, and leads to the production of off-flavors and odors (3).

From published studies of synthetic and natural membrane vesicles, it is known that additives can protect the membranes of component microspheres against drying-induced rupture and fusion (4-6); the additives include mostly sugars and some common salts. Trehalose, a disaccharide, has shown particularly promising potential for maintaining the structural integrity of both natural and synthetic membrane vesicles under drying stress (6-8). This protection has been correlated with the ability of the hydroxyl groups on sugar molecules to replace the stabilizing influence of water molecules on the surface of the dried membrane microspheres (4,5,8,9).

Changes in the surface structure of component vesicles in an emulsion induced by drying or freezing can be accompanied by clustering and fusion phenomena, and by the leakage of a pre-encapsulated solute (4,10). Relatively sophisticated experimental techniques have been used to monitor the effects of such stresses on vesicle preparations, including fluorimetry (7,11), gamma radiation counting (6), electron microscopy (6,7), differential scanning calorimetry (DSC) (5,7,8), dynamic light scattering (5), and gel filtration (5,6,7).

In order to provide a basis for developing a better dried whole milk or nonskimmed filled milk product, we have adapted a spectroturbidimetric technique (12) to assess the effects of additives on maintaining the stability of freeze-dried aqueous preparations of synthetic vesicles and milk fat globules (MFGs). In addition to the potential benefit for improving the stability of dried whole milk, this study may provide insights into protecting liposomes used for active-agent delivery systems in pharmaceutical applications.

Materials and Methods

Additives. All additives used to increase the rehydration efficiencies of dried emulsions were reagent grade from a variety of commercial resources. PEG is polyethylene glycol. Maltodextrin M-100 is a commercial hydrolyzed starch product supplied by Grain Processing Corp., Muscatine, IA; M-100 consists of 89% tetrasaccharides and higher, 6% trisaccharides, 4% disaccharides, and 1% dextrose.

Preparation of FUV. This preparation is based on those of Barenholz et al. (13) and Wong et al. (14). About 125 mg of dipalmitoylphosphatidylcholine (DPPC; Sigma, 99% purity) was suspended in 2.5 mL of 25 mM HEPES buffer previously adjusted to pH 7.0 with tetramethylammonium hydroxide in a 15 mL Pyrex centrifuge tube. The suspension was warmed to 50 °C, vigorously vortexed several times, and then sonicated at 50 °C with a Branson Sonifier 350. The suspension was sonicated for 24 @ 2 min blasts with 1 min breaks; translucent opalescence in the sonicated emulsion was obtained between blasts #15-20.

The sonicated sample was then centrifuged at 15,000 rpm for 50 min at 5-10 °C in a Sorval RC2-B (23,000 x g). The top 3/4 of the supernatant was carefully removed with a Pasteur pipet. At this stage, the emulsion is in the form of small unilamellar vesicles (SUV); this form could be stored at 45 °C for several days. For further processing and curing, the SUVs were stored at 20 °C for two days, and then at 4 °C for 3 days. This storage procedure facilitated the fusion of the SUVs into fused unilamellar vesicles (FUV). The sample was then centrifuged at 15,000 rpm for 45 min at 5-10 °C, from which the top 3/4 of the supernatant was carefully removed and then stored at 20 °C.

A stock preparation of FUVs was essentially stable to further fusion for up to 3 weeks. Greater uniformity of the FUV samples taken from an aging preparation was obtained by taking aliquots from the top of the supernatant after the stock was centrifuged at 6,000 rpm for 20 min. Absorbance scans of the diluted stocks were highly reproducible for batch variations. Samples of the FUV were analyzed for phosphorus by the method of Morrison (15). Yields of the phospholipid in the FUV form of 60-70% were obtained by this preparative method.

Preparation of Milk Fat Globules. This preparation is based on the method of Horisberger et al. (16). Two 5 mL portions of fresh unprocessed whole bovine milk purchased from a local dairy, were centrifuged at 5,000 rpm (8,000 x g) for 5 min. The infranatant skim portion of the milk was then removed and discarded. The remaining cream layer was resuspended without vortexing in 5 mL of a 50 mM TRIS (Sigma) buffer at pH 7.4 containing 0.15M NaCl (Fisher). The resuspension was then centrifuged at 5,000 rpm for 5 min. This procedure of removing the infranatant and resuspending the cream in TRIS buffer was then repeated twice. The final resuspended cream sample was chilled for two hours in an ice bath. After centrifugation at 5,000 rpm for 5 min, the infranatant was carefully removed from the tilted centrifuge tube with a Pasteur pipet, and then centrifuged again at 5,000 rpm for 5 min. This infranatant was carefully decanted into a clean tube, and then filtered through a Whatman No.1 filter paper. This preparative procedure removes the largest portion of the original MFG population. Absorbance scans of the resulting filtrate gave values of $A_{350} : A_{650}$ typically 2.1 - 2.3. Unlike the DPPC vesicles, the MFGs were quite stable to chilling-induced fusion in an ice bath for studied durations of up to two weeks; preparations of MFG emulsions were stored in a refrigerated ice bath.

Spectroturbidimetric Analyses. All the emulsions used in this study were scanned for absorbance, actually turbidity, over the UV-visible range in 10 mm semimicro quartz cuvettes with a Gilford Response™

Spectrophotometer, having the capability of scanning a reference and up to 5 samples in rapid sequence. In all cases, the turbidity maximum was kept to <1.0 to minimize multiple scattering effects.

For the quantitative analyses of DPPC FUV samples, small corrections were made to the measured turbidity values for cuvette mismatch, and for any marginal absorbance due to an additive reagent in the studied wavelength range. All buffers were filtered through BioRad 0.45 μm Prep Discs, and all drying bottles were rinsed free of any detergent residue. New polypropylene microcentrifuge tubes were washed thoroughly in hot water before being used. The cuvettes were kept thoroughly cleaned.

Laser Particle Size Analyses. DPPC microemulsions were analyzed for the size distribution of their component vesicles on a Nicomp Model 200 Laser Particle Sizer (Nicomp Instrument, Inc., Santa Barbara, CA) operated in the distribution mode for vesicular microspheres at 632.8 nm and 5 mW. The output of this instrument provides the mean diameter of the vesicle size distribution, the standard deviation, and the closeness of the fit of the experimental distribution to the Gaussian ideal.

DSC Analyses. Differential scanning calorimetry of the aqueous emulsions was performed on a Microcal MC-2 solution calorimeter (Microcal Inc., Northampton, MA) in the range 20-90 °C at a scan rate of 90 °C/h. The calorimeter is operated by dedicated data acquisition and analysis software that includes an option to estimate ΔH change for a phase transition.

Freeze-Drying. Samples of microemulsions were freeze-dried in an FTS Dura Stop™ Dryer, operated at a shelf-temperature of 20 °C at a pressure of 100-300 mT. Sample volumes of 0.5-1.0 mL were dried in 45x25 mm glass bottles for 80-120 min. Once evacuation begins, the samples freeze within 2-3 mins after supercooling to ca. -5 °C at a pressure < 1T. The frozen samples cool to -25 °C after 6-7 min at a pressure of ca. 100 mT, and then gradually warm to ca. 0 °C after 20 min; the major portion of the drying occurs at ca. 0 °C.

Results

Morphology and Stability of DPPC Vesicles. Preparations of DPPC vesicles in the concentration range 0.1-0.2% in aqueous HEPES buffer at pH 7.0 were analyzed by DSC in the 20-90 °C range. The DSC profiles differ significantly according to the morphology of the vesicle preparations, as shown in Table I. T_c values are the maximum transition temperatures for the gel \Rightarrow liquid crystalline phase change, and ΔH values are the associated enthalpy changes, expressed in the table as the ratio of ΔH at the higher transition

Table I. Solution DSC Characterization of DPPC Vesicles

Treatment	Structural Form	T_c (°C)	$\frac{\Delta H_{hi}}{\Delta H_{lo}}$
Vortexed emulsion	MLV	35,41	6:1
Sonicated microemulsion	SUV	38,41	1:1
Post-sonicated fusion	FUV	41	

temperature to that at the lower temperature. Repeated scan cycles gradually changed the DSC profile of the SUVs into that more characteristic of FUVs. The MLVs were marginally affected by repeated scanning. The profile for the FUVs was entirely unchanged by three heat-cool cycles over the studied temperature range.

Quantitative Spectroturbidimetric Analyses of Vesicle Fusion.

Figure 1 shows the turbidity profile in the wavelength range 250-350 nm of a 1:40 dilution of a freshly prepared 3% DPPC SUV microemulsion; the [DPPC] in the SUV preparations was determined by the Morrison analysis for phosphorus (15). The smooth, featureless profile of decreasing turbidity with increasing wavelength is characteristic of light scattering by the SUV.

In order to accelerate vesicle fusion, freshly prepared SUV microemulsions were placed in an ice bath. Over a duration of at least 8 h, aliquots of the SUV were periodically diluted with HEPES buffer and scanned in the 250-350 nm range on the spectrophotometer. Figure 2 shows a profile of the turbidity at 250 nm normalized for the mM concentration of DPPC against the ratio of the turbidities at 250 and 350 nm, τ_{250}/τ_{350} , for three separate SUV preparations. As vesicle fusion proceeds, turbidities increase nonuniformly across the wavelength range, with relatively greater increases at higher wavelengths. Consequently, vesicle fusion is characterized by a concomitant growth of turbidity at 250 nm and a decrease in τ_{250}/τ_{350} .

In a parallel study, 1:10 dilutions of a fresh SUV preparation, 60 mM in DPPC, were analyzed by a Laser Particle Sizer during fusion at 0 °C. In addition to the expected increase in the mean diameter of the vesicles, the size analysis also indicated that the standard deviation increases uniformly, while the degree of Gaussian fit for the size distribution remains unchanged. The mean diameters of the fusing SUV are profiled in Figure 3 against the τ_{250}/τ_{350} ratio obtained from the simple spectrophotometric analysis.

The turbidity of an emulsion also depends on the concentration of particles in the sample. In Figure 4, the measured turbidities of FUV samples at 250 and 350 nm are plotted against [DPPC]. Turbidity is directly proportional to [DPPC] up to the studied limit of 5.2 mM at both wavelengths, indicating that multiple scattering does not occur in this concentration range.

Rehydration Efficiency of Dried DPPC Vesicles. Because of their inherent stability, FUVs were chosen for the drying studies. FUV samples containing ca. 50 mM DPPC were centrifuged at 6,000 rpm (9,000 x g) for 20 min at 15-20 °C. The top of the supernatant was removed and used to prepare samples containing ca. 2 mM DPPC FUV in HEPES buffer at pH 7, which were UV-scanned. After scanning, 1.00 mL portions were freeze-dried for 80 min in glass bottles. The dried samples were rehydrated with 0.98 mL of distilled water by gentle swirling for ca. 5 min; suspended particles of (presumably) unvesicled DPPC, produced by the drying-induced rupture and coagulation of the original FUVs, were visible.

The rehydrated samples were then centrifuged at 10,000 rpm (15,000 x g) for 30 min at 15-20 °C to spin down the large unvesicled particles; 0.65 mL of the supernatant was then carefully removed from the microcentrifuge tube. The supernatant was UV-scanned on the spectrophotometer. The effect of the centrifugation alone on the UV-profile of undried samples of the FUV was also measured.

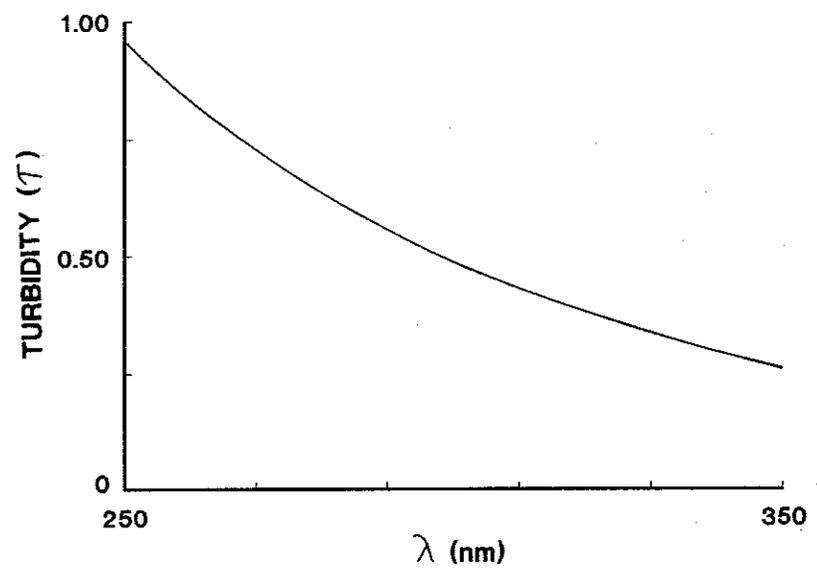


Figure 1. Turbidity-wavelength profile of 0.075% DPPC SUV microemulsion.

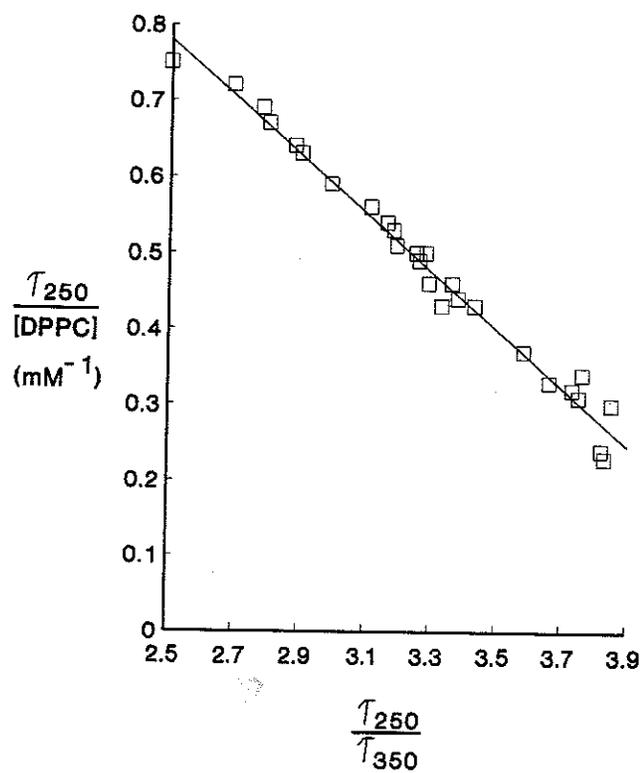


Figure 2. Turbidimetric characteristics of fusing DPPC vesicles; DPPC concentration normalized to 1.0 mM. τ_{250} and τ_{350} are the measured turbidities at 250 and 350 nm, respectively.

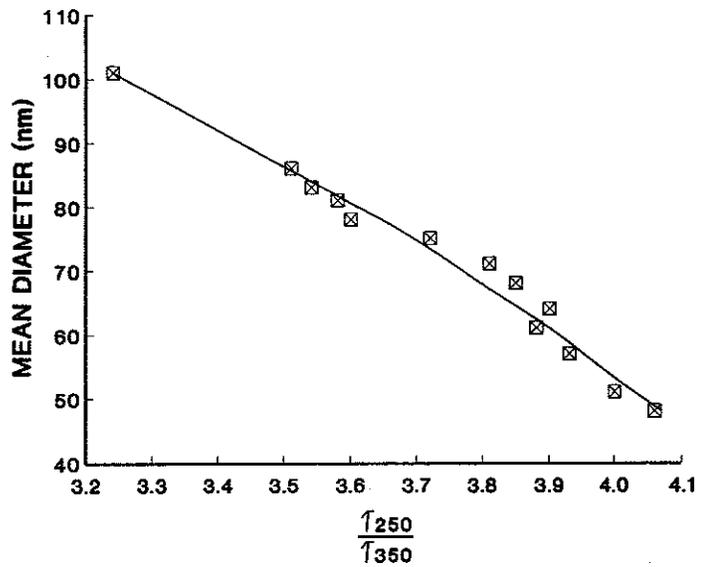


Figure 3. Correlation between the mean diameters and the τ_{250}/τ_{350} ratio of fusing DPPC vesicles.

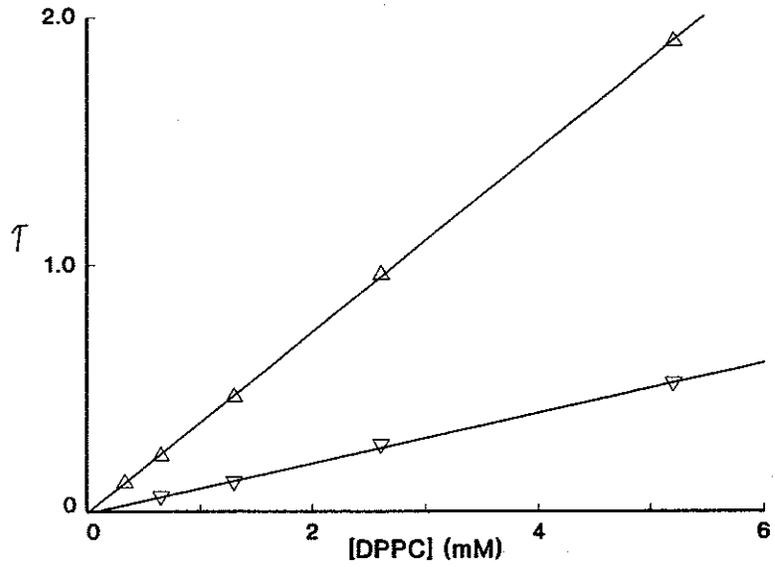


Figure 4. Measured turbidity as a function of the concentration of DPPC FUVs at 250 (Δ) and 350 (∇) nm.

As a result of the drying and rehydration procedure, the turbidity profile in the 250-350 nm range changed relative to that obtained before drying. Both τ_{250} and τ_{250}/τ_{350} were diminished significantly by the processing. As indicated in Figure 2, vesicle fusion alone, characterized by a reduction in the turbidity ratio can change τ_{250} . By measuring the turbidity ratio of the undried and dried DPPC vesicles, after centrifugation, reference to Figure 2 can provide the factor by which τ_{250} increases due to drying-induced fusion alone. In addition, the extent of vesicle fusion can be quantified from Figure 3; for DPPC FUVs having τ_{250}/τ_{350} ca. 3.7 initially, that of the reconstituted dried analogs decreases to ca. 3.3, corresponding to an increase in the mean diameter of the vesicle distribution from ca. 75 nm to ca. 95 nm.

Once τ_{250} measured after rehydration is corrected by the fusion factor, its value correlates with [DPPC] (Figure 4) in a slightly larger reconstituted form of FUVs. The ratio of τ_{250} of a centrifuged rehydrated sample to that of a centrifuged undried sample provides a measure of the rehydration efficiency, η_R , of the dried DPPC FUV. That is,

$$\eta_R = \frac{[\text{DPPC FUV(dried)}]}{[\text{DPPC FUV(undried)}]} = \frac{\tau_{250}(\text{dried})}{\tau_{250}(\text{undried})} \quad (1)$$

where $\tau_{250}(\text{dried})$ is corrected for the increase due to drying-induced fusion, and $\tau_{250}(\text{undried})$ is the value for the corresponding centrifuged undried sample. The value of η_R provides the extent of maintaining the original form of the vesicle population during the stress of drying and rehydration. A value of $\eta_R = 0.18(\pm 0.03)$ was obtained for the dried and rehydrated DPPC FUV, independent of batch-to-batch variations of the stock used. With 10 mM sucrose present in the vesicle sample, the corresponding value of η_R increased significantly to 0.32 (± 0.04).

In parallel studies on dried and undried samples of FUV in the absence and presence of 10 mM sucrose, the [DPPC] values in expression (1) were determined both by the Morrison analysis for phosphorus (15) and by DSC analysis, before and after the processing procedure. The Morrison analysis provides direct input into expression (1), while the DSC approach depends on the direct relationship between the value of ΔH for the FUV phase transition at 41 °C and the amount of DPPC in the sample. Table II shows the comparison of the values of η_R determined by the turbidimetric, phosphorus-content, and DSC methods. The acceptable correlation between these methods justified further application of the spectroturbidimetric method.

Table II. Comparison of Rehydration Efficiencies by Three Methods

Method	η_R	
	no sucrose	10 mM sucrose
Turbidimetry	0.18 (± 0.03)	0.32 (± 0.04)
P-analysis	0.16 (± 0.03)	0.38 (± 0.05)
DSC	0.19 (± 0.02)	0.30 (± 0.02)

Effects of Additives on Resuspension of Dried DPPC Vesicles.

Samples of 2-3 mM DPPC FUV in HEPES buffer at pH 7.0 were dried and rehydrated as described above in the presence of additives offering potential protection against drying-induced vesicle rupture. The values of η_R were determined spectroturbidimetrically using expression (1) for the broad range of additives listed in Tables III and IV. A profile of η_R as a function of [sucrose] is shown in Figure 5; η_R increases uniformly up to a plateau value of ca. 0.6 for [sucrose] > 40 mM, i.e. up to 12 mole sucrose/mole DPPC.

Profiles of η_R against [additive] were also obtained for the additives in Table III, which shows the plateau (or peak) values of η_R and the [additive] at which η_R is > 95% of its plateau value. The effectiveness of an additive in protecting the FUV against drying-induced fusion can be assessed by the tendency for η_R to approach its limiting value of 1.0 at a minimal value of [additive].

In addition, several other additives were compared for their ability to increase η_R of dried DPPC FUV at a single concentration value, rather than over a range of concentrations. Table IV shows the values determined at 15 mM of each additive.

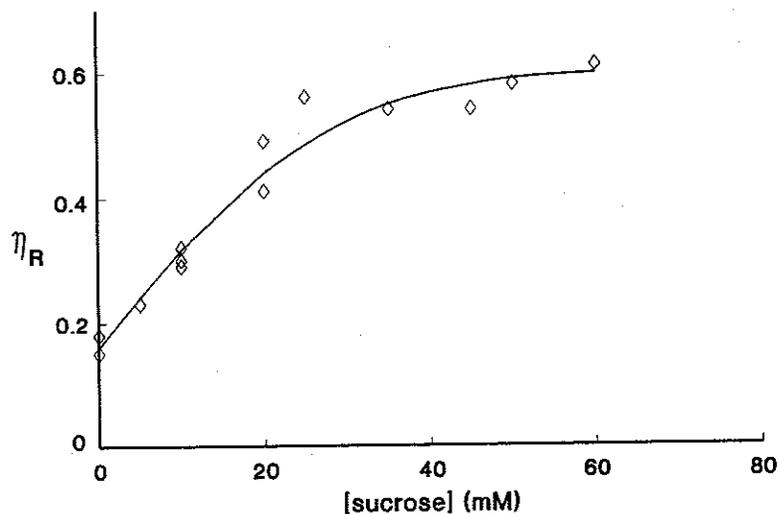


Figure 5. The rehydration efficiency, η_R , of freeze-dried DPPC FUVs (2-3 mM) in the presence of increasing sucrose concentration.

Table III. Plateau Values of η_R and [Additive] at which η_R is >95% of the Plateau Value for Dried DPPC FUV. ([DPPC FUV] is 2-3 mM).

Additive	η_R plateau	[additive] (mM)
none	0.2	-
sucrose	0.6	40
trehalose	0.8	60
lactose	0.7	40
maltotriose	0.8	60
raffinose	0.7	>65
glucose	0.6	>65
xylose	0.6	>65
xylitol	0.2	a
glycerol	<0.1	b
maltodextrin M-100	0.5	5, c
Ca ²⁺	0.5	10, c
Mg ²⁺	0.6	10, c

- η_R is independent of [xylitol] up to 60mM
- no plateau is reached; glycerol is strongly fusogenic
- further increases in [additive] reduce η_R from its peak value.

Table IV. Values of η_R for Freeze-Dried 2-3 mM DPPC FUV in the Presence of 15 mM Additive

Additive	η_R
none	0.18 (± 0.04)
sucrose	0.38*
trehalose	0.43*
lactose	0.45*
maltose	0.40 (± 0.04)
maltotriose	0.44*
raffinose	0.41*
glucose	0.28*
xylose	0.28*
fructose	0.38 (± 0.05)
arabinose	0.36 (± 0.06)
PEG 200, 400, 1000	<0.1
mannitol	0.25 (± 0.03)
sorbitol	0.26 (± 0.01)
Zn ²⁺	0.51 (± 0.05)
betaine	0.25 (± 0.05)
hydroxyproline	0.40 (± 0.03)
alanine	0.23 (± 0.06)
β -alanine	0.17 (± 0.03)
serine	0.23 (± 0.01)
hydroxylysine	0.26 (± 0.01)
sarcosine	0.31 (± 0.06)

* from plots analogous to Figure 5; see Table III.

Effects of Additives on Resuspension of Dried MFG. Emulsions of MFG in TRIS buffer at pH 7.4 were prepared from fresh, unprocessed whole bovine milk. In Figure 6, the turbidity profiles of an MFG sample over the 350-650 nm wavelength range show characteristic light scattering by the fat globules, analogous to the scattering profile in Figure 1 for DPPC FUV. The MFG are considerably larger than the synthetic vesicles (1), however, and show a different scattering profile. Photometric measurements were not taken below 350 nm because protein components absorb in this region. The correlation between τ_{350} and %MFG w/v in a sample was determined by a plot analogous to Figure 4 for DPPC FUV. A direct proportionality between τ_{350} and %MFG up to $\tau_{350} \sim 0.8$ was obtained, beyond which progressive deviation from linearity due to multiple scattering was observed; all subsequent quantitative studies of the MFG emulsions were conducted with $\tau_{350} < 0.8$.

Figure 6 shows the turbidity profile of a 0.4 mg/mL emulsion of MFG taken both before and after the sample was freeze-dried and rehydrated. Unlike the rehydration procedure for DPPC FUV, that of dried MFG involved only the readdition of the original volume of water, followed by gentle swirling before the spectrophotometric scan was taken. Because no fat particles were visible in the resuspension, no centrifugation step was needed. In addition to a 33% decrease in τ_{350} , there is a marginal 2% decrease in τ_{350}/τ_{650} induced by the drying procedure. When the MFG emulsion is dried in the presence of 25 mM sucrose, τ_{350} decreases only 18%, and the turbidity ratio increases 4%. Thus, not only does drying and rehydration induce measurable quantitative changes in the spectrophotometric profile of the MFG, but also the presence of a carbohydrate additive affects the induced changes. These

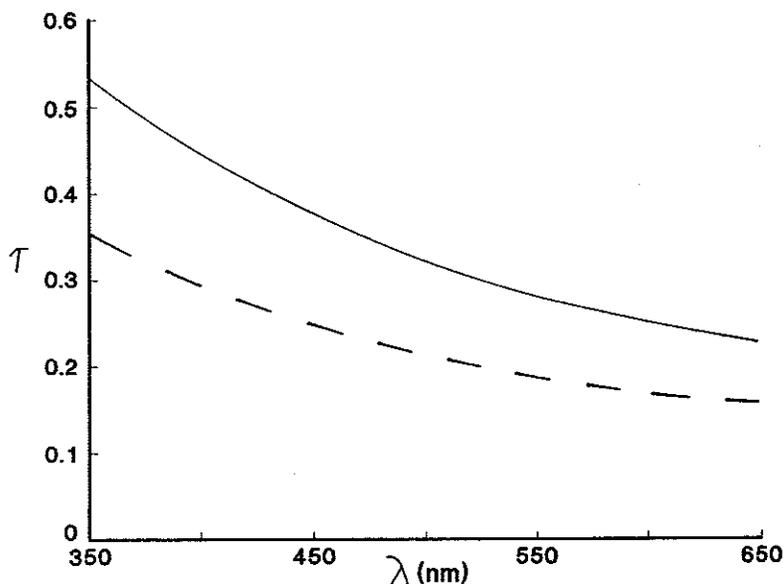


Figure 6. Turbidity-wavelength profiles of a 0.4 mg/mL emulsion of MFGs taken before (—) and after (----) freeze-drying and rehydration.

qualitative features of dried rehydrated MFG provide a basis for assessing the effectiveness of protective additives in an approach analogous to that used for the DPPC FUV, in terms of η_R values.

Figure 7 shows a profile of $\eta_R = \tau_{350}(\text{dried})/\tau_{350}(\text{undried})$ as a function of added [sucrose], analogous to Figure 5 for DPPC FUV. Such profiles were also obtained for the additives listed in Table V, where the plateau values of η_R and the [additive] at which η_R is > 95% of its plateau value are presented.

Table V. Plateau Values of η_R and [Additive] at which η_R is >95% of the Plateau Value for Dried MFG. (*MFG ~0.04%)

Additive	η_R plateau	[additive] (mM)
none	0.6	-
sucrose	0.9	40
trehalose	0.9	40
lactose	0.9	20
glucose	0.9	>100
maltodextrin M-100	0.9	15
Ca ²⁺	a	N/A

a. increased [Ca²⁺] \geq 5 mM decreases η_R

Effects of Additives on Resuspension of Diluted Milk. Samples of commercially available homogenized, pasteurized whole bovine milk, fortified with vitamin D, were diluted 1:250 with deionized water, and scanned in the spectrophotometer over the 350-650 nm wavelength range. As shown in Figure 8, a turbidity profile was obtained similar to that observed for MFG emulsions in Figure 6. When aliquots of the diluted milk were freeze-dried, simply rehydrated, and rescanned over the same wavelength range, a dramatic 75% decrease in τ_{350} was observed, in addition to a marginal 4% decrease in τ_{350}/τ_{650} . For the analogous experiment in the presence of 6 mM lactose, τ_{350} decreased by only 55%, and the turbidity ratio increased by 15%. As for the studies of MFG emulsions, these results suggest that the spectroturbidimetric method can be used to assess the effectiveness of added stabilizers in dried whole milk samples, again in terms of η_R values.

A 1:20 diluted whole milk sample in water was further diluted over a range of 1:10 to 1:67 either with water or 0.5N NaOH solution and left for 30 min with occasional vortexing. Each series of diluted milk samples was then scanned over the 350-650 nm range on the spectrophotometer. Inasmuch as 0.01N NaOH solubilizes casein micelles (17), any difference between the turbidities of the two series can be attributed to scatter by casein micelles. As shown in Figure 9, which profiles τ_{350} against the dilution factor, τ_{350} in the presence of NaOH is ca. 80% of that in its absence. This result indicates that most of the observed scatter at 350 nm is independent of casein and is due to the MFG component of the milk. Furthermore, the values of τ_{350} are linear with decreasing dilution up to $\tau_{350} = 0.8$.

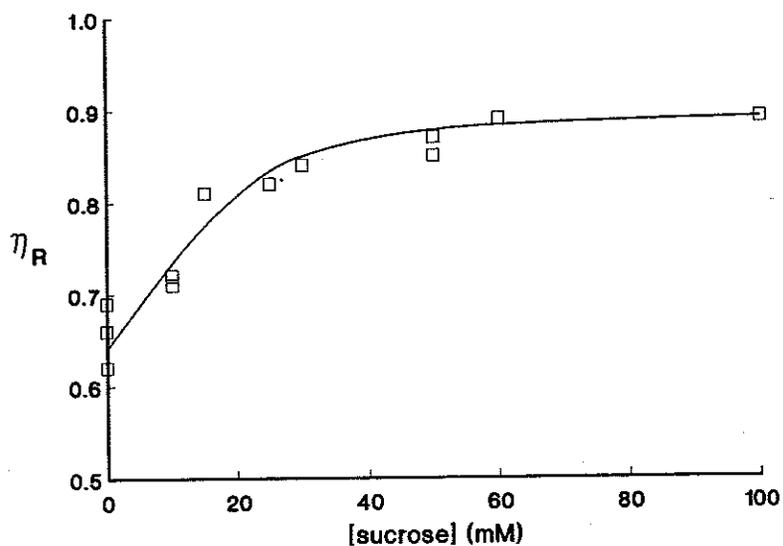


Figure 7. The rehydration efficiency, η_R , of freeze-dried MFGs (0.4 mg/mL) in the presence of increasing sucrose concentration.

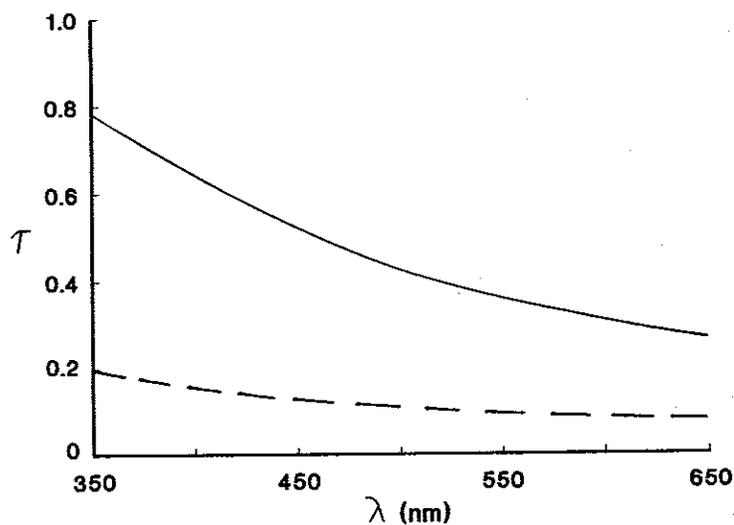


Figure 8. Turbidity-wavelength profiles of a 0.2% v/v diluted sample of whole milk taken before (—) and after (----) freeze-drying and rehydration.

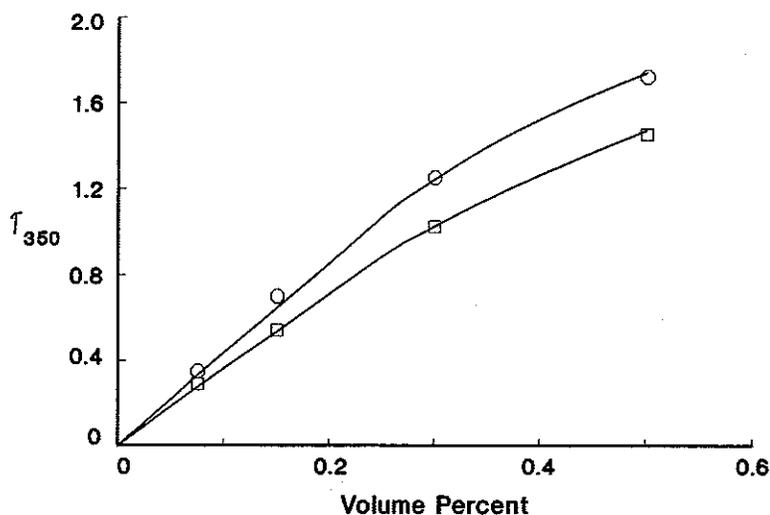


Figure 9. Measured turbidity at 350 nm as a function of the volume percentage of milk in the absence (O) and presence (□) of NaOH solution.

A simple determination of η_R for dried, undiluted milk over a range of [additive] is automatically limited by the solubility of the additive at the levels needed, and by the inherent presence of a background [lactose] in milk of ca. 5% or 0.14M. Figure 10 shows plots of $\eta_R = \tau_{350}(\text{dried})/\tau_{350}(\text{undried})$ against $\log D$, where D is the dilution factor or volume fraction of the milk samples, in the absence and presence of ca. 0.1M lactose. No lactose was added to the undiluted milk sample, and progressively more lactose was added to the more diluted milk samples to maintain the stated constancy of [lactose]. Measurement of τ_{350} values before drying required further dilution of the milk samples; less secondary dilution was required for the turbidity analysis of samples dried at higher primary dilution. Turbidity scans were determined on milk solutions diluted 400-fold overall before and after drying/rehydration. All dilutions were performed with deionized water, and allowance was made for the 12% solids content of the whole milk. Figure 11 clearly shows that η_R increases with increasing [lactose], and that the same effectiveness ($\eta_R \approx 1.0$) is obtained at ca. 0.1M lactose, regardless of the extent of dilution of the dried milk sample.

The values of η_R against [lactose] were then determined at each of $D=0.0032, 0.010, 0.032,$ and 0.10 ; due to the inherent lactose content of the milk, the minimum "default" value of [lactose] decreased with increasing dilution. From Figure 11, in which the profiles of η_R vs [lactose] for $D=0.0032 - 0.10$ overlay each other, it is not only evident that the same plateau value of η_R is reached at the highest [lactose], but also that the same η_R values are obtained at [lactose] values before the plateau is reached, regard-

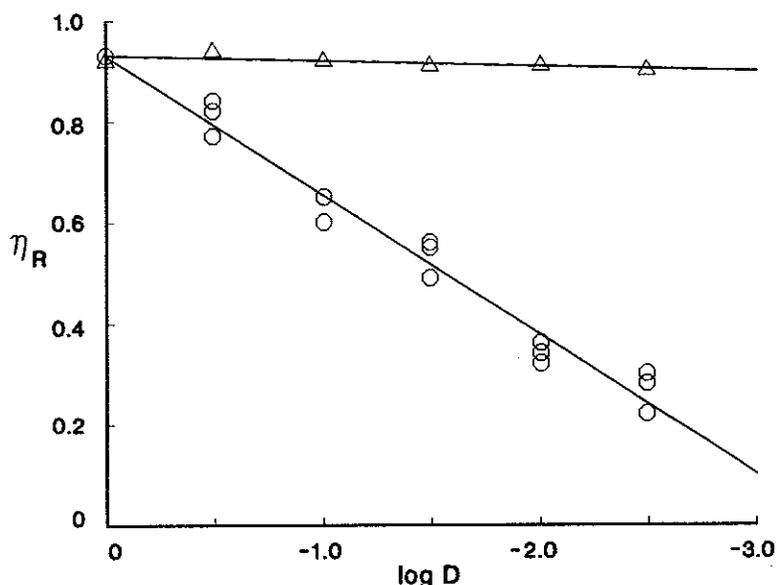


Figure 10. The rehydration efficiency, η_R , of freeze-dried diluted milk samples against the log of the volume fraction, $\log D$, of milk in the sample, without and with added lactose; no added lactose (O), 0.1 M lactose (Δ).

less of the dilution factor. This overlay feature was observed further for undiluted milk samples dried in the presence of 0.14 - 0.49M lactose; $\eta_R \sim 0.97$ was obtained at 0.49M lactose.

That η_R of the dried milk samples depends on [lactose], and not on the dilution factor (i.e., on the % MFG), suggests the same degree of stabilization of the MFGs is offered by a given [lactose], regardless of the concentration of the protected MFGs. That is, the relative concentrations of lactose to MFG are unimportant over the ranges of each component studied here. Consequently, the relative protection of other stabilizers was studied at a nominal dilution factor of 0.010, where the background [lactose] is a marginal 1.4 mM, and a large range of η_R from 0.3 - 1.0 is available for an additive to show its effectiveness.

Values of η_R as functions of [additive] were determined for dried whole milk at a dilution factor of 0.010 for the additives listed in Table VI. The profiles obtained for these additives were characterized by increasing η_R to a plateau value with increasing [additive], analogous to the profile in Figure 11. Table VI contains the plateau (or peak) values of η_R , and [additive] at which $\eta_R > 95\%$ of its plateau value for the dried diluted whole milk samples.

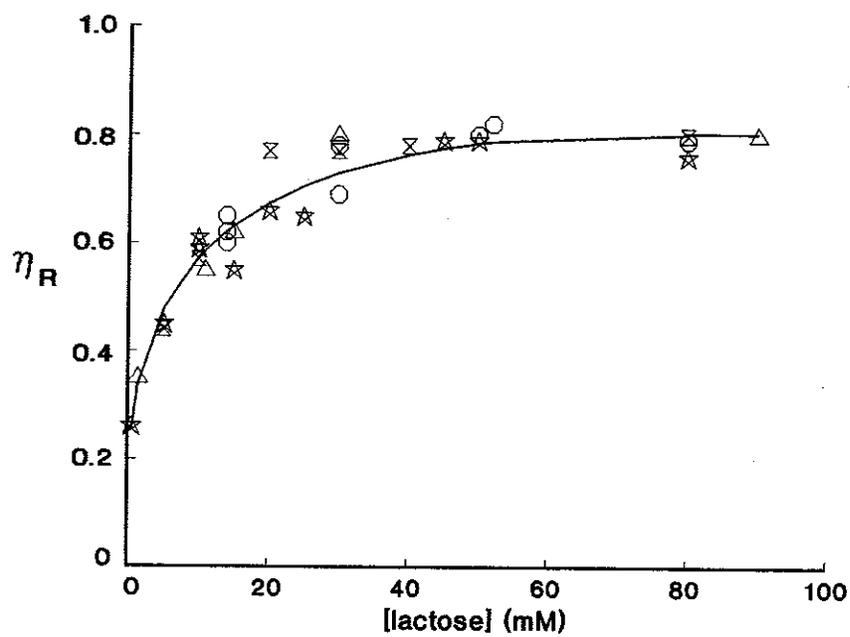


Figure 11. The rehydration efficiency, η_R , of freeze-dried diluted milk in the presence of increasing lactose concentration at different dilution factors, D; D = 0.0032 (★); D = 0.010 (△); D = 0.032 (⊗); D = 0.10 (○).

Table VI. Plateau Values of η_R and [Additive] at which η_R is >95% of its Plateau Value for Dried MFG in Diluted (0.010) Whole Milk.

Additive	η_R plateau	[additive] (mM)
none	0.3	-
maltodextrin M-100	0.9	15
lactose	0.9	35
trehalose	0.9	50
sucrose	0.9	25
glucose	0.9	30
glycerol	0.9	20
PEG 200	1.0	20
PEG 400	1.0	15, a
PEG 1000	0.8	20, a
sorbitol	1.0	20
Ca ²⁺	0.4	20, a

a. increased [additive] decreases η_R .

Discussion

DPPC Vesicles. Fused unilamellar vesicles of DPPC in aqueous buffer can be prepared with a highly reproducible yield and size distribution by the technique described in this study. Although a preparation of FUV requires about a week to complete, the simplicity of the technique allows at least two batches, chronologically displaced by a few days, to be concurrently prepared without excessive consumption of lab time. Although faster methods of preparing stable, unilamellar phospholipid vesicles are available, their technical complexity is generally greater (18,19). Once prepared, the FUV stocks are storage-stable for several weeks at 20 °C; the storage duration can be extended by diluting the stock preparation.

Different precursor forms of the DPPC vesicles can be easily extracted at earlier stages of the overall preparation and can be characterized for their morphology and thermal stability by DSC analysis, as shown in Table I. Although turbidimetric analyses of the DPPC vesicles were performed in the wavelength range 250-350 nm in this study, it is possible similarly to analyze vesicles prepared from unsaturated synthetic phospholipids, or natural lecithins over a wavelength range displaced towards the visible region where the chromophore of the unsaturated lipid does not absorb. Chromophores produced by peroxidation of the lipid may require an extension of the analytical range to still longer wavelengths. The measured values of τ_{250}/τ_{350} for the DPPC vesicles suggest that turbidity varies inversely with wavelength to the power of 3.6 - 3.9, which approaches the ideal $1/\lambda^4$ dependence associated with Rayleigh scattering (20).

The instability of DPPC vesicles in concentrated emulsions towards chilling-induced fusion (14) has enabled the changes in the size distribution of a vesicle population to be quantified by the simple spectroturbidimetric method. From a single spectral scan of diluted FUV sample, reference to Figures 2 and 3 enables an estimation to be made of both the [DPPC] in the microemulsion and the mean diameter of the vesicle population, respectively. Although the technique of spectroturbidimetry has been used previously to provide

relative sizings of fat globules in homogenized milk processing (21), this technique has not been used to assess vesicle stabilizers as it is applied in this study.

Resuspension of Dried DPPC Vesicles. The results in Table II indicate that DPPC FUVs undergo substantial rupture and fusion as a consequence of drying and rehydration stresses. In the absence of an added stabilizer, only ca. 20% of the original FUVs are resuspended in a size distribution comparable to that of the pre-dried emulsion; the remaining DPPC has agglomerated into visible particles, probably unvesicled, that are removed by centrifugation. The extent of drying-induced rupture is diminished by the presence of sucrose, a known stabilizer of dried vesicles (4). The quantitation of these drying effects is independent of the method used to determine the relative amounts of DPPC in the emulsion before and after the stress is applied. Since the turbidimetric technique is substantially faster in providing this quantitation than the other methods used, it provides a better means of determining the values of η_R in expression (1). Furthermore, Figure 3 provides an estimation of the actual increase in the size of the resuspended FUVs, which cannot be provided by the other methods. However, the turbidimetric method does not indicate whether the residual FUVs present after the processing have escaped rupture, or have reformed after prior rupture.

The form of expression (1) indicates that $\eta_R \rightarrow 1.0$ as the extent of drying-induced agglomeration of the vesicles decreases. With the exception of glycerol and xylitol, all of the additives listed in Table III increase η_R for the DPPC FUVs, indicating a stabilizing influence against drying-induced damage. Of the additives that protect the FUVs, trehalose and malto triose increase η_R to the highest observed plateau value of ca. 0.8; however, as much as 20 mole sugar/mole DPPC is required for this level of protection. Lactose provides ca. 10% less protection for ca. 13 moles sugar/mole DPPC. Maltodextrin M-100 stabilizes strongly at < 2 mole dextrin/mole DPPC, but destabilizes at higher relative concentrations. Similar reversals of stabilizing influence were observed for the metal ions Ca^{2+} and Mg^{2+} .

In the broader perspective, it appears that disaccharides offer better protection than monosaccharides, and that simple polyols are destabilizing. Metal ions protect only at low relative concentrations. This general trend has been previously reported (4). The results in Table IV integrate with these trends for the most part; the studied amino acids offer some protection against vesicle rupture, but are not as effective in this regard as the disaccharides.

Resuspension of Dried MFG. MFG emulsions exhibit different spectroturbidimetric profiles from those of DPPC FUV samples. Although the dependence of vesicle scattering on wavelength differs substantially because the MFGs are at least 10 times larger than the FUVs (1), both vesicle types exhibit a similar behavior in response to an applied drying/rehydration stress; the turbidity of a rehydrated sample is diminished significantly relative to an undried sample, and this effect is progressively ameliorated by the addition of known vesicle stabilizers at increasing concentration.

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Of the additives listed in Table V, the maltodextrin is the most effective stabilizer, followed by lactose; Ca^{2+} is moderately destabilizing. Lactose is significantly more protective than the other disaccharides, suggesting that some specificity of interaction exists between the milk sugar and the milk fat globule membrane. As for the DPPC FUVs, the disaccharides are more protective than the monosaccharide.

Resuspension of Dried Whole Milk. The drying of dilute whole milk leads to diminished τ_{350} in the observed spectroturbidimetric profile of the rehydrated samples. The profile is again characteristic of scattering by the MFGs; scattering by casein micelles was shown to be of marginal importance over the studied concentration range (Figure 9). Figure 11 shows that the stabilization of the MFGs in diluted whole milk by lactose depends only on [lactose] and not on the dilution factor of the milk. That the degree of protection offered by this stabilizer is not dependent on the relative concentration of these components, i.e., on the [lactose]:[MFG] ratio, is difficult to interpret and requires further investigation.

The results in Table VI indicate that maltodextrin M-100 is the most protective of the listed additives towards drying-induced modification of the MFG morphology in 1:100 diluted whole milk. Glycerol, PEG 200, and sorbitol are marginally less effective; the higher MW PEGs are destabilizing at concentrations $>$ ca. 20 mM. Lactose is more protective than trehalose, but less so than sucrose. Ca^{2+} is destabilizing beyond 20 mM. These trends are broadly similar to those obtained for dried MFG samples, in Table V, particularly for the best and worst stabilizers. Glycerol and sorbitol exhibit opposite influences of stabilization and destabilization on dried MFG and DPPC FUV, respectively. This contrast probably reflects a favorable structural interaction of these additives with the membrane proteins of the natural emulsion.

It is notable that maltodextrin M-100 shows optimal stabilization for the MFGs in Tables V and VI. Inasmuch as the comparisons of effectiveness of the stabilizers are made on a per mole basis, part of the advantage of the dextrin over the other carbohydrates may be in its large MW; dextrans provide more OH-groups per mole than the other carbohydrates. Not only does this dextrin offer optimal protection, but also it has very low sweetness, high solubility, and is not active in Maillard browning reactions with protein components (22). All these features consolidate the choice of maltodextrin M-100 as the best single stabilizer of dried whole milk. As a secondary protectant, the background concentration of lactose in milk offers some stabilization, but, as long commercial experience with drying whole milk has demonstrated, the extent of this self-protection is adequate only for short-term storage stability of the dried product.

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