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Emulsion-Based Delivery Systems for Tributyrin, a Potential Colon Cancer Preventative Agent

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Tributyrin, a short-chain triglyceride oil used as a food additive, has been reported to be a potential preventive agent against colon cancer. The purpose of this study was to develop tributyrin delivery systems based on food-grade oil-in-water emulsions that could potentially be incorporated into foods. Emulsions containing only tributyrin as the lipid phase were highly unstable to droplet growth due to Ostwald ripening (OR) because of the relatively high water solubility of this low molecular weight triacylglycerol. The stability of the emulsions to OR could be greatly improved by incorporating ≥15−25% corn oil (a food-grade oil with a low water solubility) into the lipid phase. In addition, the tendency for droplet sedimentation to occur was reduced because the density contrast between the lipid and water phases was reduced in the mixed tributyrin/corn oil systems. The potential anticarcinogenic ability of the tributyrin emulsions was demonstrated using a cell culture model. Treatments with emulsions containing tributyrin significantly inhibited the viability of HT29 colon carcinoma cells. These results have important implications for the development and testing of nutraceuticals encapsulated in food-grade delivery systems as anticancer agents.

KEYWORDS: Tributyrin; colon cancer; emulsions; cell culture; Ostwald ripening

INTRODUCTION

Colon cancer is a major public health problem in most economically developed countries (*1*, *2*). It has been suggested that a significant fraction of colon cancers could be prevented by moderate changes in diet and lifestyle (*3*, *4*). Epidemiological and experimental studies suggest that dietary fiber is protective against the development of colon cancer, with these effects being mainly attributed to the production of short-chain fatty acids (*5*−*8*). For over 20 years, butyric acid, an important short-chain fatty acid produced by dietary fiber fermentation in the colon, has been investigated for its potential preventive effects. Butyrate has been reported to inhibit cell proliferation and to stimulate differentiation of cancer cells (*5*, *9*). As well as being produced by microbial fermentation of dietary fiber, butyric acid is also found naturally in vegetable oils and animal fats. Although the potential of butyrate as an antitumor agent has been recognized, its application as a therapeutic agent has been hampered because of difficulties in consistently delivering physiologically efficacious concentrations to the site of action, that is, the colon (*5*, *9*). The poor clinical response is believed to be related to butyrate's rapid metabolism and very short half-life in human plasma (<6 min), which causes inability to reach efficacious serum concentrations (*9*−*11*). In addition, butyrate produces a strong odor that is unacceptable for direct oral consumption by the majority of humans (*5*).

On the basis of encouraging preclinical data, there has been an interest in developing derivatives of butyrate (pro-drugs) that are more efficacious (*9*). Tributyrin is a triacylglycerol, consisting of three butyric acids esterified to a glycerol, which is less volatile and has less of an off-odor than butyrate. In addition, tributyrin is well tolerated orally and is approved as an additive in the United States and Canada. Studies have shown that the effectiveness of tributyrin as an anticarcinogenic agent was higher than that of butyrate (*5*, *9*, *11*). It has also been shown that the effectiveness of tributyrin on cell cultures is higher when used in an emulsified rather than nonemulsified form (*10*).

A practical problem that currently limits the incorporation of emulsified tributyrin into food products is the fact that tributyrin emulsions are highly unstable to Ostwald ripening (OR) (*12*). OR is a process whereby larger droplets grow at the expense of smaller droplets due to molecular diffusion of oil molecules through the continuous aqueous phase separating the droplets (*13*). The driving force for OR is the fact that the solubility of the oil contained within an emulsion droplet increases as the curvature of the droplet increases, so that there is a larger oil concentration around small droplets than around large droplets. The relatively low molar volume of tributyrin means that it has a relatively high water solubility and is therefore prone to OR. Previous studies have shown that OR can be greatly retarded by mixing water-soluble oils with water-insoluble oils through an entropy of mixing effect that counteracts the imbalance of droplet size effect (*12*, *14*). In this study, we examine the possibility of mixing tributyrin with long-chain triglycerides (corn oil) to reduce the tendency for OR to occur and, thereby, form food-grade delivery systems for tributyrin. In addition, we test these delivery systems using a cell culture model (HT29) to demonstrate their potential efficacy at preventing colon cancer.

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MATERIALS AND METHODS

Materials. Tributyrin (98%), sodium dodecyl sulfate SDS (99%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin, and Tween 20 were purchased from Sigma-Aldrich Co. (St. Louis, MO). Corn oil was purchased from a local supermarket and used without further purification. Food-grade lyophilized β-lactoglobulin was donated by Davisco Foods International, Inc. (Le Sueur, MN). Sodium caseinate was obtained from NZMP (ALANATE180, lot 0034-w5166). Powdered lecithin was obtained from ADM-Lecithin (Decatur, IL). Trypsin EDTA 1× was obtained from Cellgro (Media Tech Inc., Manassas, VA). Fetal bovine serum was purchased from Hyclone (Thermo Fisher Scientific Inc.). McCoy's 5A medium and HT29 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA).

Emulsion Preparation. *Emulsion Preparation for Ostwald Ripening Studies*. Lipid phases were prepared by mixing different tributyrin-to-corn oil mass ratios (from 0 to 100 wt % corn oil). Aqueous phases were prepared by dispersing 0.5 wt % emulsifier in buffer solution (5 mM phosphate, pH 7.0). Oil-in-water emulsions were then prepared that consisted of 5 wt % lipid phase and 95 wt % aqueous phase.

Coarse emulsions were prepared by homogenizing 5 wt % lipid phase and 95 wt % aqueous phase in a high-speed blender (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland) for 2 min at room temperature. Fine emulsions were then prepared by passing the coarse emulsions through a high-pressure homogenizer (M-110Y Microfluidizer with a F20Y 75 μm interaction chamber, Microfluidics, Newton, MA). The emulsions were passed through the homogenizer five times at 612 bar at ambient temperature. After preparation, the emulsions were stored at 25 °C.

Emulsion Preparation for Cancer Cell Studies. Oil-in-water emulsions were prepared with 20 wt % lipid phase and 80 wt % aqueous phase. The lipid phase consisted of pure corn oil (control) or 50% corn oil and 50% tributyrin (a composition found to inhibit Ostwald ripening). The aqueous phase consisted of emulsifier (2 wt % SDS, lecithin, β-lactoglobulin, caseinate, or Tween 20) dissolved in deionized water. The remainder of the emulsion preparation protocol was the same as that used for the Ostwald ripening experiments described above.

Emulsion Characterization. *Particle Size Measurement*. The mean particle sizes (z-average) of the emulsions were measured using dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, U.K.), with each individual measurement being the average of 13 runs. The emulsions were diluted prior to analysis using either distilled deionized water (cell culture experiments) or buffer solution (5 mM phosphate, pH 7.0; OR experiments) to avoid the effects of multiple scattering. The aqueous phases used to dilute the emulsions were saturated with tributyrin to avoid migration of tributyrin from the droplets to the surrounding water upon emulsion dilution. Results are reported as the average of three measurements on freshly prepared samples.

Cell Culture Experiments. *Cell Cultures*. The HT29 human colon carcinoma cells were maintained in McCoy 5A medium supplemented with 5% heat-inactivated FSB, 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin at 37 °C with 5% CO_2 and 95% air. Cells were kept subconfluent, and media were changed twice a week. All cells used were between 3 and 30 passages.

Treatment Procedures. HT29 (7500 cells/well) cells were seeded in 96-well plates. After 24 h, cells were treated with serial concentrations of different emulsions in 200 μL of serum complete media. After 48 h, cells were subjected to MTT assay.

MTT Assay. Cell proliferation was determined by MTT assay. Media were replaced by 100 μL of fresh media containing 0.5 mg/mL of MTT. After 2 h of incubation at 37 °C, MTT-containing media were removed and the reduced formazan dye in the cells was solubilized by adding 100 μL of DMSO to each well. After gentle mixing, the absorbance was monitored at 550 nm using a 96-well plate reader (Biotek).

It should be noted that the MTT assay measures only whole cell viability, with the results being indicative of relative cell numbers and their viability in terms of mitochondria functions. An observed inhibition of cell number and viability could be due to enhanced apoptosis and/or other types of cell death (e.g., necrosis) and/or decreased cell proliferation. The MTT assay alone does not provide information on apoptosis and differentiation, and further analyses would be needed to determine the precise mechanism of inhibition.

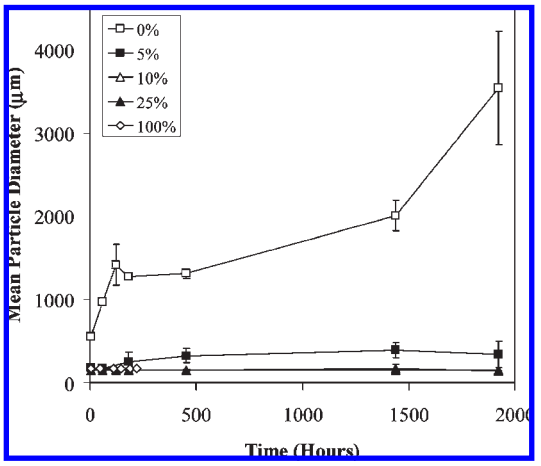

Figure 1. Dependence of mean droplet diameter on time for oil-in-water emulsions containing different ratios of tributyrin and corn oil in the lipid phase. The emulsions were all stabilized by Tween 20.

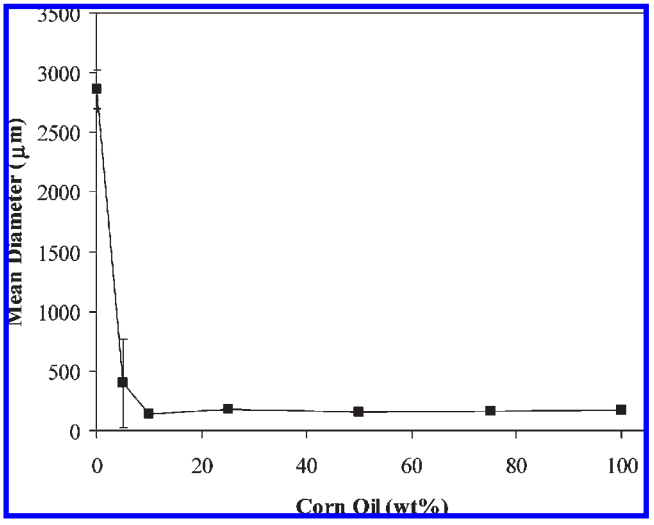

Figure 2. Dependence of mean droplet diameter after 2 days of storage on oil composition for oil-in-water emulsions containing different ratios of tributyrin and corn oil in the lipid phase. The emulsions were also stabilized by Tween 20.

Statistical Analysis. Experiments were performed at least two or three times using freshly prepared samples. Averages and standard deviations were calculated from these measurements (Excel, Microsoft).

RESULTS AND DISCUSSION

Stability to Ostwald Ripening. Initially, we examined the effect of oil composition (tributyrin-to-corn oil mass ratio) and surfactant type (SDS, Tween 20, β-lactoglobulin, and caseinate) on the stability of tributyrin oil-in-water emulsions to droplet growth during storage. The evolution in mean droplet diameter for Tween 20 stabilized emulsions with different initial oil compositions is shown in **Figure 1**. The impact of droplet composition on emulsion stability to droplet growth is shown in **Figure 2**, which gives the mean particle diameter after 2 days of storage. Changes in the particle size distribution of the emulsions over storage are shown in **Figure 3**. Emulsions prepared using a lipid phase consisting of 100% tributyrin were highly unstable to droplet growth, with a rapid increase in droplet diameter occurring immediately after preparation (**Figure 1**). In addition, the droplets

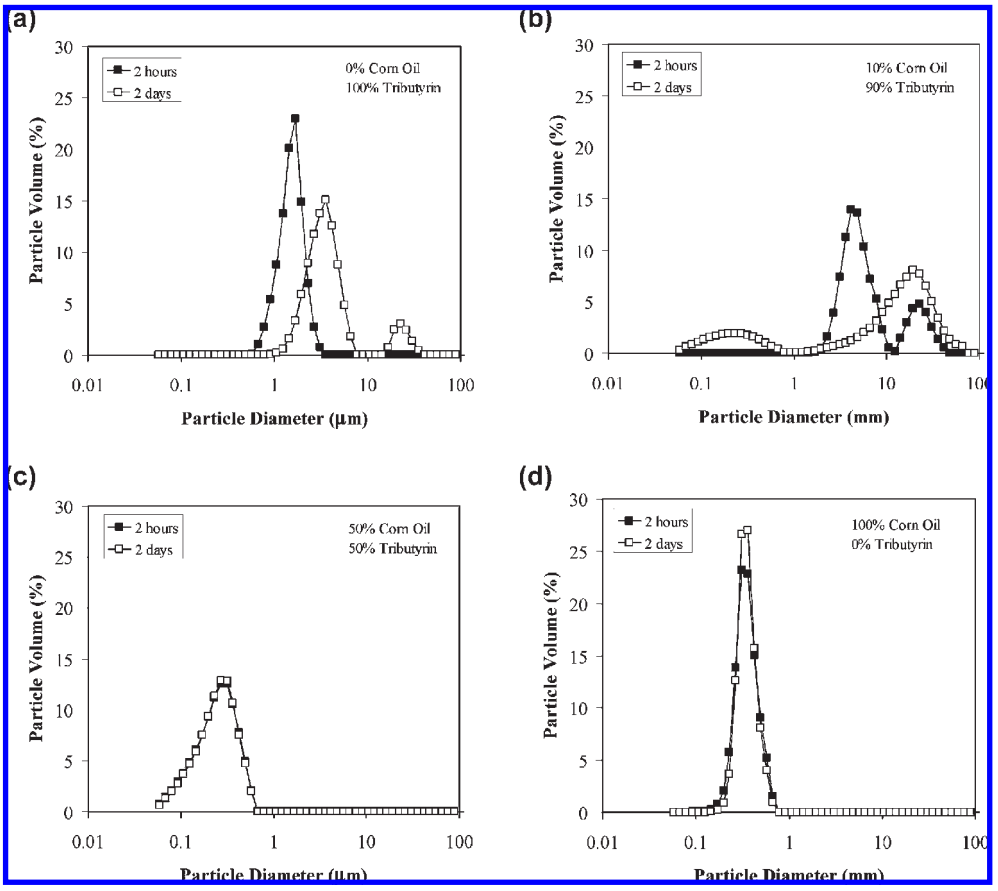

Figure 3. Dependence of particle size distributions during storage on oil composition for oil-in-water emulsions containing different ratios of tributyrin and corn oil. The emulsions were stabilized by Tween 20.

in these emulsions sedimented to the bottom of the test tubes during storage (**Figure 4**), which can be attributed to the large droplet size and the relatively high density of pure tributyrin (ρ = 1032 kg m^{-3}) compared to water (ρ = 1000 kg m^{-3}). As the concentration of corn oil in the lipid phase increased, the rate of droplet growth decreased (**Figure 1**). When the corn oil concentration in the lipid phase exceeded about 15−25%, the rate of droplet growth decreased dramatically (**Figure 2**). This effect can be attributed to the fact that compositional ripening effects oppose Ostwald ripening (*12*, *14*).

The increase in mean droplet size (r) with time (t) due to Ostwald ripening of a one-component emulsified lipid phase in the steady state regime is given by (*15*)

$$r^3 - r_0^3 = \omega t = \frac{4}{9}\alpha c D t \qquad (1)$$

Here, ω is the Ostwald ripening rate, α ($= 2\gamma V_m/RT$, where γ is the interfacial tension, V_m is the molar volume of the lipid, R is the gas constant, and T is the absolute temperature), c is the solubility of the lipid in the continuous phase, and D is the translational diffusion coefficient of the solute through the continuous phase. Equation 1 accounts for the large difference in the OR stability of the pure tributyrin and pure corn oil emulsions. Tributyrin is a relatively small nonpolar molecule that has a relatively high water solubility and is therefore highly prone to Ostwald ripening (*12*). On the other hand, corn oil contains relatively large nonpolar triacylglycerol molecules with a relatively low water solubility, and therefore emulsions prepared from this oil are highly stable to OR (*16*). Recently, it has been shown that the stability of emulsified tributyrin to OR can be greatly improved by mixing

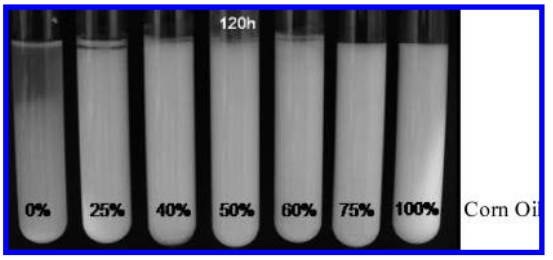

Figure 4. Photographs of 5 wt % oil-in-water emulsions (stabilized by β-lactoglobulin) with different ratios of corn oil and tributyrin after storage at ambient temperature for 120 h. The percentages indicated on the tubes are the percentage of corn oil in the lipid phase.

it with high molecular weight hydrocarbons or triacylglycerols (*16*). In the current study, we examined the possibility of improving the OR stability of our emulsions by mixing tributyrin with corn oil, because this is a food-grade lipid that could be used as part of an edible delivery system.

The ability of low water solubility oils to retard OR in emulsions containing high water solubility oils can be attributed to an entropy of mixing effect that opposes droplet growth due to differences in curvature (*14*). Consider an oil-in-water emulsion that contains droplets composed of two different lipid components: a water-insoluble component (such as corn oil) and a water-soluble component (such as tributyrin). The water-soluble molecules will diffuse from the small to the large droplets due to OR. Consequently, there will be a greater percentage of water-soluble molecules in the larger droplets than in the smaller droplets after OR occurs. Differences in the composition of

Table 1. Parameters Required To Predict OR Stability Ratio[a]

	MW (g/mol)	V_m (cm^3/mol)	γ (mN/m)
corn oil	933	330	30
tributyrin	302	959	30

[a] We also used a d_0 = 100 nm and assumed the interfacial tension was the same as for corn oil−water (*12*).

emulsion droplets are thermodynamically unfavorable because of entropy of mixing: it is more favorable to have the two lipids distributed evenly throughout all of the droplets rather than concentrated in particular droplets. Consequently, there is a thermodynamic driving force that operates in opposition to the OR effect. The change in droplet size distribution with time then depends on the concentration and solubility of the two components within the oil droplets.

The dependence of the OR stability of O/W emulsions on droplet composition can be divided into three regimes for systems containing a water-soluble and a water-insoluble component (*14*, *15*):

$$\text{unstable regime} : X_2 < \frac{2\alpha_1}{3d_0} \quad (2.\text{i})$$

$$\text{kinetically stable regime} : \frac{2\alpha_1}{3d_0} < X_2 < \frac{2\alpha_1}{d_0} \quad (2.\text{ii})$$

$$\text{thermodynamically stable regime} : X_2 > \frac{2\alpha_1}{d_0} \quad (2.\text{iii})$$

Here, X_2 is the initial mole fraction of the water-insoluble component present within the overall disperse phase, α_1 ($= 2\gamma V_m/RT$) is the characteristic length scale of the high-solubility component, and d_0 is the initial mean droplet diameter. In the unstable regime, the droplet curvature effect dominates the entropy of mixing effect because the initial concentration of water-insoluble component within the droplets is insufficient. Hence, the droplets continue to grow at a rapid rate through OR. Usually, a bimodal distribution is observed after a certain time with a population of large droplets that are enriched with the water-soluble component (*14*, *15*). In the thermodynamically stable regime, the entropy of mixing effect dominates the droplet curvature effect and the droplets are stable to OR. Under these conditions, the size and composition of the lipid droplets in the emulsion remain constant over time (*13*, *14*). In the kinetically stable regime, droplet growth is thermodynamically favorable, but there is a kinetic energy barrier that retards growth. If the initial particle size distribution (PSD) is fairly narrow, then the emulsion may remain relatively stable to OR for some time, but if the PSD is broad, then OR occurs but at rate appreciably lower than that of the pure water-soluble component.

The particle size distributions of emulsions containing low and high concentrations of corn oil are compared in **Figure 3**, where it can be seen that the initial monomodal distribution changes to a bimodal distribution over time at low corn oil concentrations (< 10−25%), but remains monomodal at high corn oil concentrations.

We calculated the critical concentrations of water-insoluble component (corn oil) required to provide stability against OR using equation 2.ii and the parameters in **Table 1**: $0.063 < X_2 < 0.188$ for kinetic stability and $X_2 > 0.188$ for thermodynamic stability. These mole fractions are equivalent to mass fractions of $\phi_m = 0.17$ and 0.42 (i.e., 17 and 42% corn oil, respectively). In our study, we found that the OR rate was relatively slow at corn oil ratios of between about 10 and 25% (**Figure 2**), which is in reasonable agreement with the predicted value.

The impact of emulsifier type on the rate of OR in the tributyrin emulsions was also studied, because different types of emulsifiers may be used to formulate emulsion-based delivery systems in practice. In this study, we examined an anionic surfactant (SDS), a nonionic surfactant (Tween 20), a globular protein (β-lactoglobulin), and a flexible protein (caseinate). We found similar dependences of the OR stability of the emulsions on lipid composition for the different emulsifiers (data not shown), indicating that emulsifier type did not have a major impact on OR stability.

A lipid composition of 50% tributyrin and 50% corn oil was selected for the lipid phase composition used in the cell culture experiments to ensure that the emulsions were stable to OR.

Stability to Gravitational Separation. Another major factor that affects the stability of emulsions is the tendency for the droplets to either cream (move upward) or sediment (move downward) due to gravitational separation. Ideally, it would be advantageous to have emulsion-based delivery systems that were stable to gravitational separation during storage. The stability of an emulsion to gravitational separation depends on the density contrast between the oil and water phases, the droplet size, and the continuous phase viscosity. To retard creaming or sedimentation, it is necessary to decrease the density contrast, reduce the droplet size, or increase the continuous phase viscosity. At ambient temperature (25 °C), tributyrin (ρ = 1032 kg m^{-3}) has a higher density than water (ρ = 998 kg m^{-3}), whereas corn oil (ρ = 920 kg m^{-3}) has a lower density. In principle, it may therefore be possible to retard gravitational separation by matching the density of the lipid phase (ρ_O) to that of the aqueous phase (ρ_W). The density of the lipid phase is given by the equation

$$\rho_O = \phi\rho_2 + (1-\phi)\rho_1 = \rho_1\rho_2/[\phi_m\rho_1 + (1-\phi_m)\rho_2] \quad (3)$$

where ρ_1 and ρ_2 are the densities of the water-soluble and water-insoluble components present, ϕ is the volume fraction of water-insoluble component within the lipid phase, and ϕ_m is the mass fraction of the water-insoluble component in the lipid phase. The critical mass fraction (ϕ_m') of the water-insoluble component in the lipid phase required to give density matching with the surrounding aqueous phase can be predicted from the following equation:

$$\phi_m' = \frac{\rho_2(\rho_1 - \rho_w)}{\rho_w(\rho_1 - \rho_2)} \quad (4)$$

We calculated a critical mass fraction of corn oil of 26% of the lipid phase to give density matching between the lipid and aqueous phases. In practical applications, it may be useful to use this lipid composition within the droplets to prevent creaming instability, but one would also have to ensure that the emulsions were thermodynamically stable to OR (not just kinetically stable). The emulsions prepared in this study appeared to be stable to gravitational separation when the corn oil concentration exceeded 25% (**Figure 4**).

Impact of Emulsified Lipids on Cell Culture Viability. *Impact of Emulsifier Type*. Emulsified lipids may be stabilized by a variety of different kinds of surface-active materials, which may have their own impact on cell viability in addition to that of the lipid phase. Consequently, we examined the impact of emulsifier type (Tween 20, sodium caseinate, β-lactoglobulin, and lecithin) on cell viability. Potentially, an emulsifier may affect cell viability due to the direct effects of the free emulsifier molecules on the cells or due to their ability to alter the interactions between the lipid droplets and the cells. Initially, we therefore examined the effects of different emulsifiers on cell viability, in the absence of any lipids (**Figure 5**).

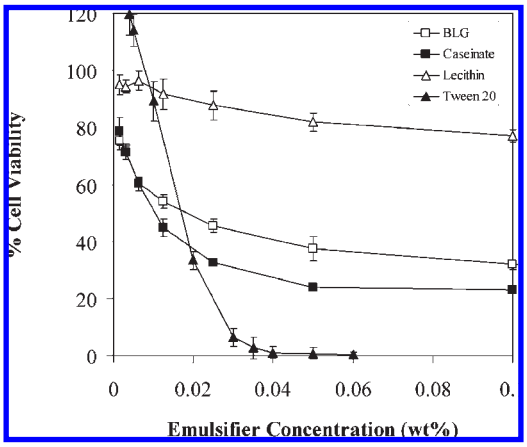

Figure 5. Impact of emulsifier type on the viability of HT29 cells 48 h after treatment. In these experiments emulsifiers were dispersed in aqueous solutions.

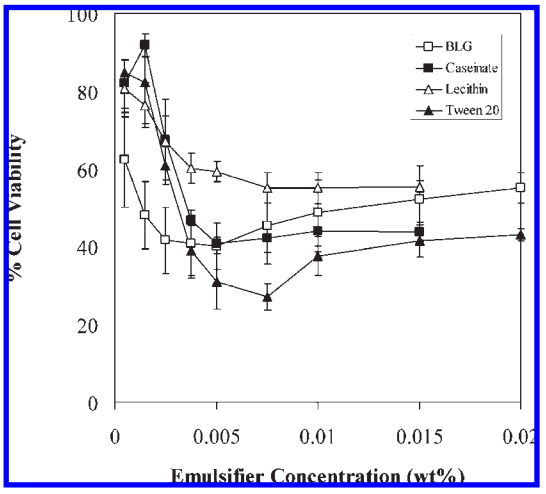

Figure 6. Impact of emulsifier type on the viability of HT29 cells 48 h after treatment. In these experiments emulsifiers were dispersed in oil-in-water emulsions.

Lecithin had the least impact on cell viability of the four emulsifiers studied, causing a small decrease in cell viability with increasing emulsifier concentration from 0 to 0.1 wt % (**Figure 5**). In contrast, Tween 20 had the greatest impact on cell viability, causing a steep decline in cell viability with increasing surfactant concentration and effectively eliminating any cell viability above 0.035 wt %. The two proteins, β-lactoglobulin and caseinate, had an intermediate effect, causing an appreciable decrease in cell viability when their concentrations increased from 0 to 0.1 wt % (**Figure 5**). The differences in the impact of the emulsifiers on cell viability can be highlighted by examining the cell viability at the highest emulsifier concentration used (0.1 wt %): 0, 23, 32, and 77% for Tween 20, caseinate, β-lactoglobulin, and lecithin, respectively. These results show that emulsifiers themselves may promote loss of cell viability in the in vitro cell culture model used. This finding has important consequences in the design and testing of delivery systems for anticarcinogenic components via surfactant-based or emulsion-based delivery systems. Previous studies have also found that various kinds of surface active molecules may decrease the viability of model colon cancer cells, including bile salts, phospholipids, anionic surfactants, cationic surfactants, and nonionic surfactants (*17*−*19*). The reduction in cell viability observed in the cell culture models may have occurred for a number of reasons: (i) the emulsifiers may have physically disrupted the cell membranes; (ii) the emulsifiers may have altered active transporters in the cell membranes; or (iii) the emulsifiers may have competed with the cells for the surface of the cell culture plates, thereby promoting cell detachment (*17*). Further studies would be required to identify the relative importance of these and other potential mechanisms of reducing cell viability.

The impact of the same emulsifiers on cell viability was also tested when cancer cells were exposed to corn oil-in-water emulsions. The mass ratio of emulsifier-to-oil in these emulsions was 1:10. Corn oil was used as a neutral lipid for these studies because it is not believed to have a major impact on cell viability itself under the conditions used in our study. In all of the emulsions studied there was an appreciable decrease in cell viability with increasing emulsifier concentration (**Figure 6**). As was observed for the emulsifiers in solution, the decrease in cell viability was lowest for the systems containing lecithin and greatest for the systems containing Tween 20. The decrease in cell viability observed in these emulsions can therefore largely be attributed to the presence of the emulsifiers. Having said this, the decrease in cell viability observed in the emulsions (**Figure 6**) was somewhat less than that observed in the solutions at the same Tween 20 concentrations (**Figure 5**). For example, at 0.02 wt % emulsifier, the solution containing Tween 20 gave a cell viability of $33 \pm 3\%$, whereas the emulsion containing Tween 20 gave a cell viability of $43 \pm 2\%$. This difference can be accounted for by the fact that in emulsions the emulsifier molecules are distributed between the lipid droplet surfaces and the continuous aqueous phase; hence, the total free emulsifier concentration in the continuous phase of the emulsions is less than the total amount present. These experiments clearly indicate that emulsifiers may cause a decrease in cell viability, which should be taken into account when the results from cell culture models are interpreted.

Impact of Oil Type. In this series of experiments we compared the impact of oil type (corn oil vs tributyrin oil) on cell viability using two emulsions stabilized by the same type of emulsifier. Lecithin was selected as an emulsion stabilizer for these studies because it was the emulsifier with the least impact on cell viability (**Figures 5** and **6**). Oil-in-water emulsions were prepared that contained either 100% corn oil as the dispersed phase or 50% corn oil/50% tributyrin. HT29 cells were incubated with the emulsions for 48 h, and then cell viability was determined (**Figure 7**). These experiments clearly show that the emulsions containing tributyrin are considerably more effective at decreasing cell viability than those containing only corn oil. In the emulsions containing 50% corn oil/50% tributyrin the cell viability was almost completely lost at total oil concentrations of 0.2 wt % (tributyrin = 0.1 wt %). Our results support those of earlier in vitro studies, which also found that emulsified tributyrin has considerable potential as an effective anticancer agent (*10*,*11*). Indeed, it was reported that 0.12% emulsified tributyrin (4 mM) stabilized by phospholipids decreased the percentage of viable cells by >80% in both Caco-2 and HepG2 cells(*10*), which is in close agreement with our data (**Figure 7**). At present the molecular mechanisms by which tributyrin inhibits cell growth are still poorly understood, although recent studies suggest that induction of apoptosis in cancer cells is an important mechanism (*10*). Further work would be required using more detailed cell culture models to identify the precise mechanism.

Impact of Emulsifier Type in Emulsions Containing Tributyrin. Finally, we examined the impact of emulsifier type on the effectiveness of emulsified tributyrin at decreasing cell viability (**Figure 8**). Oil-in-water emulsions stabilized by different kinds of emulsifiers were prepared that all had 50% corn oil/50% tributyrin as the lipid phase. These emulsions were then incubated on

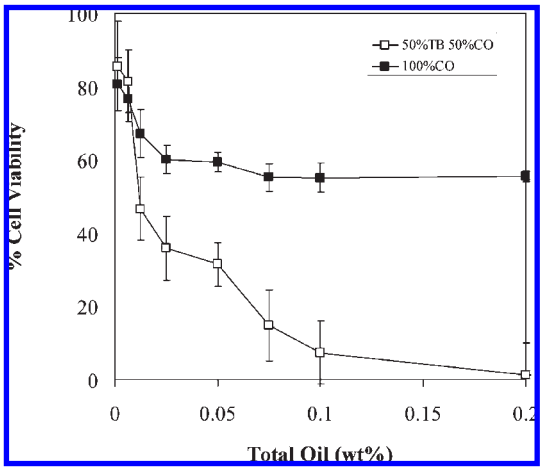

Figure 7. Impact of oil type on the viability of HT29 cells 48 h after treatment. In these experiments lecithin stabilized oil-in-water emulsions contained either 100% corn oil or 50% tributyrin/50% corn oil.

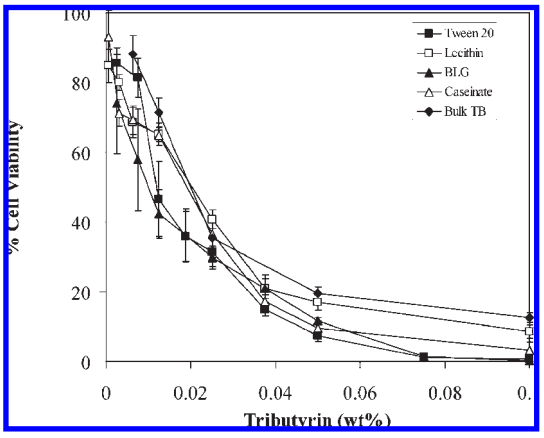

Figure 8. Impact of emulsifier type (shown in caption) on the viability of HT29 cells 48 h after treatment. In these experiments oil-in-water emulsions contained 50% tributyrin/50% corn oil. In addition, the data are compared with samples containing nonemulsified (bulk) tributryin, which was dispersed in DMSO.

the cell culture plates, and cell viability was measured after 48 h. We also carried out a parallel experiment in which we incubated cells with different concentrations of tributyrin directly dissolved in culture media using DMSO as a carrier solvent (**Figure 8**). Overall, the general trend in the dependence of cell viability on total tributyrin concentration was similar for all of the samples studied, regardless of whether the tributyrin was in solution (DMSO) or in lipid droplets stabilized by different kinds of emulsifier. These results suggest that it is the tributyrin, rather than the other components in the system, that dominates the effects on cell viability in these emulsions. Having said this, emulsifier type does appear to have some effect. For example, at 0.1 wt % tributyrin, the cell viability decreased in the following order for the different systems: bulk tributyrin (12.5%) > lecithin (8.5%) > caseinate (3.3%) > Tween 20 (0.8%) > β-lactoglobulin (0.2%). This trend is fairly similar to that observed in the corn oil emulsions, with the exception of the β-lactoglobulin sample.

In practice, the nature of the emulsifier layer that coats any lipid droplets reaching the colon will be highly dependent on the various physicochemical and physiological processes that occur when the droplets pass through the gastrointestinal (GI) tract, that is, the mouth, stomach, and small intestine. There may be appreciable changes in the interfacial composition from the values of the ingested emulsion due to competitive absorption processes between the original emulsifiers and exogenous and endogenous surface-active components present in the GI tract, for example, phospholipids, bile salts, fatty acids, proteins, and digestion products (*20*). It may be possible to control the interfacial properties of emulsified lipids in the digestive tract using structural design principles, for example, by structuring the interface or by careful selection of carrier oils and surfactants (*20*−*22*).

This study has shown that pure tributyrin oil-in-water emulsions are highly unstable to droplet growth and sedimentation because of Ostwald ripening, which is caused by the relatively high water solubility of this low molecular weight triacylglycerol. OR could be effectively inhibited by incorporating a highly water-insoluble oil (such as corn oil) into the lipid droplets to generate an entropy of mixing effect ("compositional ripening") that opposes the OR effect. We found that mixing tributyrin oil with > 10−25% corn oil prior to homogenization inhibited OR in the emulsions.

We also found that both emulsifier type and oil type affect cell viability in colon cancer cell culture models. The efficacy of the emulsifiers in inhibiting cell viability decreased in the following order: Tween 20 (a nonionic surfactant) > β-lactoglobulin and caseinate (two milk proteins) > lecithin (a phospholipid). The possible reduction of cell viability by emulsifiers therefore needs to be taken into account when the results of cell culture models are interpreted. Tributyrin was found to appreciably inhibit cell viability (> 50% reduction) at levels exceeding about 0.02 wt % (i.e., > 0.7 mM). These experiments suggest that emulsified tributyrin (or triglyceride fractions rich in butyrate) may be effective food-grade components to target colon cancer. If sufficient levels of tributyrin could be regularly delivered to the colon through food, then it may be possible to develop dietary intervention strategies to reduce the incidence of colon cancer using tributyrin-enriched functional foods. Nevertheless, there are a number of technical challenges that need to be overcome. In particular, tributyrin is normally digested and absorbed within the small intestine and so does not reach the colon at sufficiently high levels to be efficacious. Hence, the conventional emulsions used in this study are currently unsuitable as colonic delivery systems for tributyrin. It may be possible to overcome this problem by developing emulsion-based delivery systems that resist digestion in the mouth, stomach, and small intestine, but then release the tributyrin in the colon. Our laboratory is currently working on the development of various delivery systems to achieve this goal, such as trapping lipid droplets within nanolaminated coatings or hydrogel beads fabricated from dietary fibers that are resistant to digestion in the stomach and small intestine (*20*, *23*). Indeed, preliminary studies in our laboratory suggest that dietary fiber coatings may be able to retard the digestion of emulsified lipids in the small intestine, for example, coating lipid droplets with chitosan (*24*).

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