**Controlling the gastrointestinal fate of nutraceutical and
pharmaceutical-enriched lipid nanoparticles: From mixed
micelles to chylomicrons**

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ABSTRACT

The oral bioavailability of lipophilic bioactive compounds such as many pharmaceuticals and nutraceuticals can be enhanced using triacylglycerol-based lipid nanoparticle delivery systems. These digestible lipid nanoparticles are disassembled in the gastrointestinal tract to form mixed micelles that solubilize and transport the lipophilic bioactives to the intestinal epithelium cells where they are absorbed. In these cells, the lipid digestion products and bioactive agents contained within the mixed micelles are then packaged into biological lipoprotein nanoparticles (e.g., chylomicrons) that are secreted into the lymph. In this study, we examined the influence of fatty acid type (i.e., oleic acid, linoleic acid, and linolenic acid) on the properties of mixed micelles, cellular lipid droplets, and lipoprotein nanoparticles, and on the bioavailability of a highly lipophilic nutraceutical: 5-demethylnobiletin (5DN). There were distinct differences in the structural properties of lipoprotein nanoparticles formed depending on fatty acid unsaturation. Oleic acid (C18:1) was most effective in enhancing intestinal uptake of 5DN and led to the formation of the largest chylomicrons. Linoleic acid (C18:2) and linolenic acid (C18:3) also promoted intestinal uptake of 5DN and formation of chylomicrons, but they were less efficient than oleic acid. The metabolism of 5DN within the intestinal epithelium cells was greatly reduced when 5DN was incorporated into chylomicrons, presumably because they were isolated from metabolic enzymes in the cytoplasm. These results have important implications for the rational design of lipid nanoparticle-based delivery systems for lipophilic nutraceuticals and pharmaceuticals by targeting them to the lymphatic circulation.

KEYWORDS: lipid nanoparticles; chylomicrons; mixed micelles; bioavailability; metabolism; nutraceuticals; polymethoxyflavones.

1. Introduction

Oral administration of pharmaceuticals and nutraceuticals has been considered to be a more convenient and desirable delivery route where possible, since other routes such as intravenous delivery are often painful, inconvenient, and burdensome (Ng, Lovell, & Zheng, 2011). However, many of these bioactive compounds are so highly lipophilic that they have relatively low and variable oral bioavailability (Gonnet, Lethuaut, & Boury, 2010; Porter, Trevaskis, & Charman, 2007). Hence their potential beneficial health effects are not fully realized due to their poor absorption and extensive metabolism by the human body. An effective way to enhance the oral bioavailability of lipophilic bioactive compounds is to encapsulate them within delivery systems containing digestible lipids (McClements, Decker, Park, & Weiss, 2009; Porter, Trevaskis, & Charman, 2007; Yeap, Trevaskis, Quach, Tso, Charman, & Porter, 2013).

Triacylglycerols (TGs) are the most common form of digestible lipid used in lipid-based delivery systems, with over 95% of them typically being digested within the human gastrointestinal tract (GIT) (Williams, Trevaskis, Charman, Shanker, Charman, Pouton, et al., 2013). After ingestion, TGs are hydrolyzed by digestive enzymes (lipases) in the stomach and small intestine to form free fatty acids and monoacylglycerols (Williams, et al., 2013). These lipid digestion products interact with biological surfactants secreted by the body (e.g., phospholipids, bile salts, and cholesterol) to form various nanostructured assemblies collectively known as “mixed micelles”. These mixed micelles have hydrophobic regions capable of incorporating lipophilic bioactive agents, *e.g.*, the non-polar interior of micelles or the non-polar bilayers of liposomes. The mixed micelles can then travel through the intestinal lumen, across the mucus layer, and to the apical side of the epithelial cells, where they release their contents for absorption by various passive and active transport mechanisms.

After absorption by the epithelium cells, the biological fate of lipophilic molecules depends on their molecular characteristics as well as that of any co-absorbed lipid digestion products (Porter, Trevaskis, & Charman, 2007; Yanez, Wang, Knemeyer, Wirth, & Alton, 2011). For example, after intracellular trafficking, medium-chain or short-chain fatty acids may directly enter the portal vein, while long-chain fatty acids are likely incorporated into chylomicrons and then transported to the lymph, thus avoiding the first pass metabolism in the liver (Shen, Howles, & Tso, 2001). A chylomicron (CM) is a biological lipid nanoparticle assembled in the epithelium cells that consists of a hydrophobic core containing TGs, cholesterol, and lipophilic bioactives, and a hydrophilic shell consisting of phospholipids and proteins (Bateman, Jackson, Maitin, Yaqoob, & Williams, 2007).

Previous studies have reported that the formation and properties of chylomicrons depend on the nature of the fatty acids entering the epithelium cells. Monounsaturated fatty acids and polyunsaturated fatty acids were found to produce larger CMs than saturated fatty acids (Bateman, Jackson, Maitin, Yaqoob, & Williams, 2007). Oleic (C18:1) and linoleic (C18:2) acids were also reported to promote greater secretion of CMs than steric (C18:0) and palmitic (C16:0) acids (van Greevenbroek & de Bruin, 1998). Studies have also shown that the bioavailability of ingested bioactive components depended on the nature of the fatty acids they are ingested with (Holm, Mullertz, Christensen, Hoy, & Kristensen, 2001; van Greevenbroek & de Bruin, 1998). However, there is not always a close correlation between the bioavailability of lipophilic bioactive agents and the nature of the CMs formed. For example, oleic acid was found to stimulate the formation of larger and more numerous CMs than linoleic acid, but the lymphatic transport of a lipophilic bioactive component (halofantrine) was reported to be higher for linoleic acid than oleic acid (Holm, Mullertz, Christensen, Hoy, & Kristensen, 2001; van

Greevenbroek & de Bruin, 1998). This means that there is not always a simple correlation between the production of CMs and the lymphatic transport of bioactive components.

Our previous research using a Caco-2 cell culture model demonstrated that mixed micelles, consisting of oleic acid and sodium taurocholate, increased the trans-intestinal transport of an encapsulated lipophilic nutraceutical, 5-demethylnobiletin (5DN), by promoting its incorporation into chylomicrons (Mingfei Yao, 2013). The purpose of the current study was to examine the influence of fatty acid type (C18:1, C18:2, C18:3) on the formation and structure of mixed micelles, lipid droplets, and chylomicrons, as well as on the incorporation of a bioactive lipophilic agent, 5DN into the chylomicrons. 5DN is high lipophilic nutraceutical mainly found in the citrus fruits and has been reported to have various beneficial biological effects (Mingfei Yao, 2013). The current study is particularly important as polyunsaturated fatty acids have been increasingly utilized in the human diet due to their potential beneficial health effects in human (Nelson, 2005) (Abeywardena & Head, 2001; Djousse, Arnett, Carr, Eckfeldt, Hopkins, Province, et al., 2005; Mozaffarian & Wu, 2011; Wijendran & Hayes, 2004). The knowledge gained from this study could be used to rationally design lipid-based delivery systems for pharmaceuticals and nutraceuticals with improved efficacy by oral administration.

2. Materials and methods

2.1. Materials. The following products were purchased from Sigma Chemicals (St. Louis, MO): OsO₄, oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), taurocholic acid (TC), Sulfatase from Heli pomatia, phosphatungstic acid (PTA) and OptiprepTM density gradient medium. All other chemicals and solvents were of analytical grade and were obtained from Fisher Scientific (Pittsburgh, PA). Apolipoprotein B (Apo B) human ELISA kit was purchased

from Abcam (Cambridge, MA). 5-Demethylnobiletin (5-hydroxy-6, 7, 8, 3', 4'-pentamethoxyflavone, 5DN) was synthesized as we described previously (J. Zheng, Song, Dong, Qiu, Guo, Zhong, et al., 2013).

2.2. Cell culture. Caco-2 cells (passage 55~65) were cultured in complete Dulbecco's modified essential medium (DMEM) containing high glucose, 10% fetal bovine serum (FBS), 1% antibiotic, and 1% amino acids. Cells were seeded at 3×10^5 cells/mL on transwells (Corning Inc., MA, USA) containing polyester filters (3 μ m pore size and 4.7 cm² surface area) and grown for 21 days. The transepithelial electrical resistance (TEER) was measured at 37 °C using a Millicell® ERS-2 epithelial voltammeter (World Precision Instruments, Sarasota, FL). Data were expressed as $\Omega \times \text{cm}^2$. Before the start of different fatty acid treatments, Caco-2 monolayers were washed and incubated for 4 h with serum-free complete medium as described previously (Mingfei Yao, 2013).

2.3. Preparation of mixed micelle systems. Mixed micelles systems containing different types of fatty acids were prepared using a method described previously (Mingfei Yao, 2013). Three types of mixed micelles were prepared: oleic acid-sodium taurocholate (C18:1-TC); linoleic acid-sodium taurocholate (C18:2-TC) and linolenic acid- sodium taurocholate (C18:3-TC), with the same fatty acid-to-TC molar ratio of 1.6:0.5. A known amount of fatty acids was added to a TC solution and then homogenized with a sonicator at 4 °C. The solutions were then swirled overnight under a nitrogen atmosphere before being stored at -20°C prior to use. The particle diameter (Z-average) and charge (ζ -potential) were measured using a combined dynamic light scattering/particle electrophoresis instrument (NanoZS, Malvern Instruments, Malvern, UK) to make sure the consistency of the property of mixed micelles. Samples were diluted with DMEM prior to their application to cell culture.

2.4. Quantification of 5DN and its metabolites. Caco-2 cell monolayers were cultured in trans-well for 21 days, then were incubated with cell culture media containing C18:1-TC and 5DN, C18:2-TC and 5DN, C18:3-TC and 5DN or 5DN alone at the apical side. Aliquots of basolateral medium (100 μ L) were collected at 1, 2, 4, 8 and 24 h, and replaced with equal volume of complete basolateral medium each time. The apical medium and the cell monolayers were collected at 24 h. Samples were extracted twice with ethyl acetate to obtain 5DN and its metabolites, i.e., 5, 3'-dihydroxy-6, 7, 8, 4'-tetramethoxylflavone (M1), 5, 4'-dihydroxy-6, 7, 8, 3'-tetramethoxylflavone (M2) and their corresponding conjugates M1' and M2'. Selected samples were incubated with sulfatase at 37°C to deconjugate M1' and M2' before being extracted with ethyl acetate. Extracts were dried under vacuum. The resulting residue was then dissolved with 100 μ L of mobile phase for HPLC analysis. The concentrations of 5DN and its metabolites were determined using a HPLC system (CoulArray®, Chelmsford, MA, USA) equipped with a multi-channel electrical conductivity detector (Model 6210, CoulArray®, Chelmsford, MA, USA) as we previously described (Dong, Qiu, Zhu, Li, Ho, McClements, et al., 2010; J. K. Zheng, Bi, Johnson, Sun, Song, Qiu, et al., 2015).

2.5. Isolation characterization of lipoprotein by TEM. Lipoproteins secreted to the basolateral side by Caco-2 cell monolayer were isolated by density gradient ultracentrifugation (Bateman, Jackson, Maitin, Yaqoob, & Williams, 2007). The media collected from the basolateral side were preserved in saline EDTA mixed with optiprep (60% iodixanol) at the ratio of 4:1(v/v) with 9% idoixanol-PBS layered on top. The lipoproteins were separated by centrifuging at 28,000 rpm for 3 hours in an ultracentrifuge (SW40 rotor, Beckman Coulter, Indianapolis, IN) at 20 °C. The top 1 mL ($d < 1.006 \text{ g cm}^{-3}$) was collected for further analysis.

Lipoproteins were stained by 4% OsO₄ and then 1% PTA before transmission electron microscopy (TEM, Philip, tecnai 12) analysis.

2.6. Apolipoprotein B analysis. The medium in the basolateral side was collected and centrifuged at 2,000 g for 10 minutes to remove any debris. Supernatants were collected and concentrated in Vivaspins tubes and then stored at -20 °C before analysis. Cells in the transwells were rinsed with ice-cold PBS containing 0.5M EDTA and then scraped into a tube with PBS containing 0.5M EDTA. The suspension was then centrifuged at 2,000 g for 10 min at 4 °C and the supernatant was collected. The cells were then lysed to release the proteins and the supernatant was collected after centrifugation at 20,000 g for 30 min at 4 °C. Apo B was detected using a specific ELISA kit (ab108807, UK).

2.7. Transmission Electron microscopy. Transmission electron microscopy (TEM) was used to visualize mixed micelles and intracellular changes in lipid structure and distribution within Caco-2 monolayers. Mixed micelles were analyzed using cryo transmission electron microscopy (Cryo-TEM) as described previously (Zhao & Craig, 2003). A 10 µl of appropriate concentrated mixed micelles was applied to both sides of a 400 mesh grid coated with a holey carbon film glow-discharged in n-amylamine vapor for three minutes before use. After allowing the micelles to adsorb to the grid for 30 seconds, the grid was placed in a humidity chamber (~70% relative humidity). The grid was blotted to a thin film using Whatman No. 42 filter paper, then immediately plunged under gravity into liquid ethane cooled by liquid nitrogen. Grids were stored under liquid nitrogen. The cryo EM images were recorded by Gatan Camera in a Philips CM120 electron cryomicroscope at 120kV with a magnification of 66,000X and a defocus of 2.3 µm using a Gatan (Pleasanton, CA) 626DH cryo-holder at ~ -184°C. Cells were exposed to different treatments for 24 h before being fixed and prepared for electron microscopy using a

method described previously (Leonard, Creed, Brayden, & Baird, 2000). Sections were then examined under TEM.

2.8. Statistical analysis. All values are expressed as mean $\pm$ standard deviation (SD) unless stated otherwise. The difference among samples were analyzed by ANOVA with significance level of $p < 0.05$.

3. Results

3.1. Morphology of mixed micelles. In order to determine the impact of fatty acid type on the gastrointestinal fate of nutraceutical-enriched lipid nanoparticles, three types of mixed micelles (i.e., C18:1-TC, C18:2-TC, and C18:3-TC) were prepared with different fatty acids as described in the methods. The mean particle diameter of all three mixed micelle systems was around 60 nm and their electrical charge (ζ -potential) was around -50 mV, independent of initial fatty acid type. To confirm the formation of mixed micelles, three systems were subjected to cryo-TEM analysis. As shown in Figure 1, simple micelles, uni-lamellar vesicles, bi-lamellar vesicles, and multi-lamellar vesicles coexisted in all the mixed micelle systems formed. These structures have previously been shown to increase the solubility of lipophilic compound in intestinal fluids (Almgren, 2000; Mullertz, Fatouros, Smith, Vertzoni, & Reppas, 2012). Interestingly, the degree of unsaturation of the initial fatty acids did not have a major impact on the nature of the mixed micelles formed. Nevertheless, there did appear to be more lamellar layers in multi-lamellar vesicles in the C18:1 system than C18:2 and C18:3 systems (Figure 1). There also appeared to be more multi-vesicular vesicles in the C18:2 and C18:3 systems than C18:1 system. Multi-vesicular vesicles consisted of smaller vesicles trapped inside of larger vesicles. The

approximate thickness of the individual bilayers was estimated from the TEM images to be 10.8 ± 0.4 nm, 14.4 ± 0.4 nm, and 11.3 ± 0.7 nm for the C18:1, C18:2 and C18:3 mixed micelles systems, respectively. These values were approximately equal to the chain lengths of two fatty acid chains (Israelachvili, 2011), which may be present in phospholipids, monoacylglycerols, or free fatty acids in the mixed micelle phase.

3.2. Trans-enterocyte uptake of 5DN. To determine the effects of different mixed micelle systems (containing fatty acids with different degrees of unsaturation) on the trans-enterocyte uptake of lipophilic agents, Caco-2 cell monolayers in transwells were treated with different mixed micelle systems containing same level of 5DN at the apical side of the transwells. None of these systems caused any significant change in the TEER value during the 24 hours of treatment (data not shown), suggesting no damage has been done in the integrity of the cell monolayer during the experimental period. The levels of 5DN and its major metabolites were determined in the basolateral sides of the transwells by the HPLC methods as we reported previously (Dong, et al., 2010; J. Zheng, et al., 2013; J. K. Zheng, et al., 2015). When 5DN cross the intestinal cells, it was exposed to many drug metabolizing enzymes capable of changing its chemical structure. As shown in figure 2, in the Caco-2 cells, 5DN firstly underwent phase I metabolism to yield demethylated metabolites M1 and M2. M1 and M2 underwent further phase II metabolism to yield their corresponding conjugates M1' and M2'. These conjugates could be glucuronides and/or sulfates.

As shown in figure 3A, the levels of 5DN in the basolateral media increased rapidly and reached the maximum around the 4th hour in the presence of the mixed micelle systems, whereas the basolateral 5DN level of the control cells increased at much slower rate and reached the maximum at the 8th hour. After the 8th hour, the basolateral 5DN levels showed decreasing trend

in experimental groups, presumably due to the metabolism of 5DN by Caco-2 cells. Different mixed micelle systems showed different ability in promoting the uptake of 5DN across the intestinal cell monolayer. For example, at the 1st hour, the basolateral 5DN levels of intestinal cell monolayers treated by C18:1, C18:2, and C18:3 mixed micelle systems were 46, 20, and 24-folds higher than that of the control cells, respectively. At the 2nd hour, the basolateral 5DN levels of Caco-2 cell monolayers treated by C18:1, C18:2, and C18:3 mixed micelle systems were 24, 14, and 10-fold higher than that of the control cells, respectively. The total amount of 5DN transported across the Caco-2 monolayers was calculated as the area under the curve (AUC) from time zero to the 4th hour (Figure 3B). The results indicated that during the initial 4 hours, the total amount of 5DN transported across intestinal cell monolayer in the presence of C18:1, C18:2, and C18:3 mixed micelle systems was 13.5, 7.8, and 7.0-fold higher than that of the control cells, respectively.

The basolateral levels of 5DN metabolites were quantified in Figure 4. In the first 2 hours, the basolateral levels of 5DN metabolites were within a close range among the control and mixed micelle treated groups (Fig. 4A-D). However, starting from the 4th hour, the basolateral levels of 5DN metabolites in the control cells increased significantly to the levels much higher than those of mixed micelle-treated cells. The relative abundance of 5DN and its metabolites in the basolateral media at the 24th hour was shown in figure 4E. About 50% of basolateral 5DN was in the form of metabolites for the control cells, whereas only about 13-15% of basolateral 5DN was in the metabolite form in the mixed micelle-treated cells. These results demonstrated a much larger extent of 5DN metabolism in the control cells than in the mixed micelle-treated cells. In another word, mixed micelle systems protected 5DN from being metabolized in Caco-2

cells. The relative abundance of basolateral M1 and M1' was much higher than that of M2 and M2' in all cells.

The intracellular levels of 5DN and its metabolites were measured in the Caco-2 cell monolayer at the 24th hour (Figure 5A). The 5DN levels in Caco-2 cells treated by C18:1, C18:2, and C18:3 mixed micelle systems were 8.6, 6.5, and 3.8-folds higher than that of the control cells, respectively. About 50% of intracellular 5DN was in the form of metabolites for the control cells, whereas only about 10-15% of intracellular 5DN was in the metabolite form in the mixed micelle-treated cells (Figure 5B). The relative abundance of intracellular M1 and M1' was much higher than that of M2 and M2' in all cells. These results were consistent with those found in the basolateral media, suggesting that mixed micelle systems promoted cellular uptake of 5DN, and suppressed 5DN metabolism in intestinal cells.

3.3. Biological nanoparticles formation. The impact of the degree of unsaturation of the fatty acids (C18:1, C18:2, and C18:3) on the formation and secretion of lipoprotein particles in the intestinal cells, such as chylomicrons (CM), was examined. Previous studies have demonstrated that unsaturated fatty acids were more potent in stimulating the formation and secretion of lipoprotein particles than saturated fatty acids (van Greevenbroek, van Meer, Erkelens, & de Bruin, 1996). It was also reported that monounsaturated fatty acids (e.g., C18:1) were more effective at promoting chylomicron or very low density lipoprotein (VLDL) formation than polyunsaturated fatty acids (e.g., C18:2 and C18:3) (Field, Albright, & Mathur, 1988). [Herein](#), TEM was used to observe changes in the number, size, and morphology of the lipoprotein particles secreted by the Caco-2 cells after different treatments, as well as to observe any changes in the morphology of the Caco-2 cells (Figure 6). Viable enterocytes with well-differentiated microvilli were observed in all of the test groups (Figure 6A). In addition, cytosolic lipid

droplets were observed in the Caco-2 cells treated with mixed micelles, but not in the control group. Cytosolic lipid droplets, which typically have diameters around 1 to 4 μm , are important contributors to the control of postprandial triglyceridemia as their lipid content is ultimately transported from the epithelium cells as chylomicrons (Demignot, Beilstein, & Morel, 2014). After 24 h, the cells incubated with mixed micelles containing C18:1 and C18:2 formed more numerous lipid droplets than those incubated with mixed micelles containing C18:3. The amount of lipid accumulated in the Caco-2 cells appeared to be higher in cells treated by mixed micelles containing C18:1 and C18:2 than C18:3.

There is no evidence for the secretion of lipoproteins in the control cells (Figure 6A). All mixed micelle systems promoted the formation and secretion of lipoproteins. Interestingly, TEM images of lipoproteins secreted to basolateral media by Caco-2 cells showed that C18:1 mixed micelle system stimulated the formation of larger lipoproteins than mixed micelles containing C18:2 or C18:3 (Figure 6A). To quantify the particle size of the lipoproteins, we analyzed the TEM images and determine the particle size distribution of the lipoproteins in the basolateral media (Figure 6B). Based on previous studies, particles with diameters from 75 to 1200 nm can be considered to be chylomicrons (CMs), whereas those with diameters from 30 to 80 nm can be considered to be VLDLs (Yanez, Wang, Knemeyer, Wirth, & Alton, 2011). The relative abundance of CMs was much higher in the lipoproteins secreted by C18:1 mixed micelle-treated Caco-2 cells than those treated by C18:2 and C18:3 mixed micelle systems. For example, the volume of CMs was more than 80% of the total lipoprotein particles secreted by Caco-2 cells after C18:1 mixed micelle treatment *versus* about 60% for either C18:2 or C18:3 treatment. This result suggests that more chylomicrons were formed for the monounsaturated mixed micelles than for the polyunsaturated ones.

The levels of Apo B protein in Caco-2 cells and in the basolateral media were determined after the cells were exposed to the control medium or to mixed micelles containing different unsaturated fatty acids for 24 hours. Notably, all the mixed micelle-treated cells produced much more Apo B protein compared to the control in the basolateral media. Moreover, the cells treated with C18:3 showed a lower secretion of Apo B protein than the cells exposed to C18:1 or C18:2 (Figure 7A). It was also observed that all three types of fatty acids in mixed micelle systems increased the intracellular level of Apo B protein in Caco-2 cells in comparison to the control cells, with the C18:1 mixed micelle system being the most effective (Figure 7B).

4. Discussion

In this study, we have examined the influence of unsaturated fatty acid type on the formation and structure of mixed micelles, as well as on the absorption, metabolism, and lipoprotein incorporation of 5DN using an intestinal cell model, Caco-2 monolayer model. In addition, we have examined the influence of fatty acid type on the morphologies and levels of lipid droplets and lipoproteins formed by the intestinal cells. Herein, 5DN was used as a representative lipophilic bioactive compound to demonstrate the critical role of lipid particle-based delivery systems on the trans-intestinal uptake and metabolism of lipophilic bioactive agents, e.g., many pharmaceuticals and nutraceuticals. Previous studies have demonstrated that 5DN is a flavonoid found mainly in citrus fruits, and has been associated with many beneficial bioactivities (Qiu, Dong, Guan, Li, Ho, Pan, et al., 2010; Qiu, Guan, Dong, Li, Ho, Pan, et al., 2011).

Our results showed that mixed micelle systems containing both TC and different unsaturated fatty acids (i.e., C18:1, C18:2 or C18:3) greatly enhanced the trans-intestinal uptake of 5DN (Figure 3). For example, C18:1-based mixed micelle system showed the greatest enhancing

effect by elevating the intestinal uptake of 5DN by about 14 to 46 folds compared to the control during the first 4 hours of treatment (Figure 3). In the meantime, C18:2 or C18:3-based systems showed similar enhancing effects by increasing the 5DN uptake by about 7 to 24 folds. Based on our overall experimental results from this study, this enhancing effects of mixed micelle systems can be attributed to the following: (i) fatty acids and other surface active components in the intestinal lumen such as bile acids spontaneously form mixed micelles that can greatly enhance the solubility of lipophilic agents such as many pharmaceuticals and nutraceuticals in the aqueous intestinal fluid (apical fluid); (ii) fatty acids in the mixed micelle systems can promote the formation of biological lipoprotein nanoparticles (e.g., chylomicrons and VLDL) that can transport these lipophilic agents across the intestinal cells; and (iii) the extent of metabolism of lipophilic agents in the intestinal cells can be reduced when they were incorporated into cytosolic lipid droplets or lipoprotein nanoparticles.

As shown in Figure 1, the mixed micelles formed by different fatty acids and taurocholic acid contained a complex mixture of various types of colloidal particles with single layer, multiple layer, or multiple vesicular structures. These particles have hydrophobic regions that can solubilize lipophilic agents such as 5DN, thereby increasing their solubility within gastrointestinal fluids and enhancing their absorption from the small intestine. Our results showed difference in the structures of the mixed micelles formed by fatty acid with different degree of unsaturation. Currently, little is known about the effects of these structural differences in the gastrointestinal fate of these mixed micelles or their impact on the fate of encapsulated lipophilic agents, which warrants future investigations.

Electron microscopy showed that cytosolic lipid droplets were formed within intestinal cells after the treatment with three mixed micelle systems containing different unsaturated fatty acids

(Figure 6A). These lipid droplets have previously been reported to be the result of fusion of the oil droplets formed during triacylglycerol biosynthesis (Olofsson, Bostrom, Andersson, Rutberg, Perman, & Boren, 2009; Schmitz & R, 2009). Fatty acids in the mixed micelles likely were used to synthesize triacylglycerol, therefore the lipid droplets formed in the intestinal cells after the treatment of mixed micelles would have a high content of fatty acids from the mixed micelles. The dimensions and amount of lipid droplets formed depended on the unsaturation of the fatty acids in the mixed micelles: C18:1 promoted the formation of a relatively higher amount of lipid droplets that had diameters around 1 to 4 μm ; C18:2 promoted the formation of a similar amount of lipid droplets but they had smaller dimensions; C18:3 led to the formation of fewer lipid droplets overall but with some relatively large ones (Figure 6A). It was also found that the morphology of the lipoproteins secreted by the intestinal cells depended on fatty acid type in the mixed micelles. The dimensions of the lipoproteins produced in the presence of C18:1 were significantly larger than those produced in the presence of C18:2 and C18:3 (Figure 6A and B). Previous studies have also highlighted the efficacy of C18:1 for stimulating the formation and secretion of chylomicrons (Luchoomun & Hussain, 1999), while C18:3 resulted in lower chylomicron production and faster clearance in the blood based on the *in vivo* experiments (Harris, Hustvedt, Hagen, Green, Lu, & Drevon, 1997).

Apo B protein is associated with chylomicrons and has been used as a marker to determine relative abundance of chylomicrons. The quantification of Apo B protein within the intestinal cells and in the basolateral media indicated the abundance of chylomicrons assembled in the cells and transported out of them, respectively (Figure 7). Our results demonstrated that the amount of Apo B protein decreased as the degree of unsaturation of the fatty acids increased, which is indicative of decreasing amounts of chylomicrons being formed as the number of double bonds

in the fatty acids increases. Previous studies have also reported that unsaturated fatty acids were capable of elevating Apo B protein secretion by Caco-2 cells (van Greevenbroek, van Meer, Erkelens, & de Bruin, 1996), with polyunsaturated fatty acids being less effective than monounsaturated ones (Ranheim, Gedde-Dahl, Rustan, & Drevon, 1994).

As xenobiotics, pharmaceuticals and nutraceuticals generally undergo extensive phase I & II metabolism in the intestinal epithelium to produce various metabolites. Absorbed parent compounds and their metabolites are transported by portal blood to liver where they are subject to further phase I & II metabolism. Consequently, parental compounds are transformed to more water-soluble phase II metabolites (such as glucuronides and sulfates) that can be quickly cleared from the blood via urine. As the result, phase I & II metabolism significantly decreases bioavailability and bioactivity of pharmaceuticals and nutraceuticals, although there are exceptions. Our results showed that mixed micelles stimulated the production of lipid droplets and lipoprotein nanoparticles (e.g., chylomicrons and VLDLs) (Figure 6 and 7). Lipophilic compounds uptaken by intestinal cells are likely to be associated and then incorporated within lipophilic matrix in the cells such as lipid droplets and lipoprotein nanoparticles. This type of incorporation can protect lipophilic compounds from the action of phase I & II enzymes, since these compounds become inaccessible to the enzymes. Most of these metabolizing enzymes are located on the smooth endoplasmic reticulum (Trevaskis, Charman, & Porter, 2008; Van Veld, Vetter, Lee, & Patton, 1987). In agreement with above description, our results demonstrated that the extent of metabolism of 5DN in the intestinal cells and in the basolateral media was reduced by about 3 to 5 folds in the presence of mixed micelles compared to the control (Figure 4E and 5B). In human small intestine, secreted lipoprotein nanoparticles (chylomicrons and VLDLs) together with lipophilic agents (e.g., 5DN) incorporated in these particles directly enter

lymphatic capillaries that lead to the systemic blood circulation without passing through the liver (O'Driscoll, 2002). In this case, lipophilic agents enter the systemic blood circulation bypassing liver metabolism, which increases their bioavailability (Porter, Charman, & Charman, 1996; Sun, Zhai, Xue, Hu, Yang, Li, et al., 2011; Trevaskis, Charman, & Porter, 2008; Yanez, Wang, Knemeyer, Wirth, & Alton, 2011). Furthermore, it takes a few hours of circulation in the blood before the clearance of lipoprotein nanoparticles (Harris, Hustvedt, Hagen, Green, Lu, & Drevon, 1997). This may allow the lipoprotein nanoparticles and incorporated lipophilic agents to interact with certain therapeutic target tissues such as tumors through the “enhanced permeability and retention” effect to produce enhanced efficacy (Kobayashi, Watanabe, & Choyke, 2013).

5. Conclusions

Our study has shown that long chain unsaturated fatty acids, especially oleic acid (C18:1) were effective in enhancing the bioavailability of a lipophilic nutraceutical (5DN). This increase in bioavailability was attributed to two effects: (i) an increase in the amount of 5DN transported across the intestinal epithelium; and (ii) a reduction in the amount of 5DN that underwent metabolism. This study provides important information for the creation of lipid-based delivery systems designed to increase the oral bioavailability of lipophilic compounds in foods and drug formulations. Since the chylomicrons formed by the intestinal epithelial cells are “natural” nanoparticles that may transport lipophilic bioactive compounds around the body and protect them from first-pass metabolism, the lipid-based delivery systems that mediate the production of chylomicrons may provide a new route for therapy and/or diagnosis.

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Figure legend

Figure 1. Morphology of C18: 1-TC-5DN (A), C18: 2-TC-5DN (B) and C18: 3-TC-5DN (C) mixed micelles observed through cryo-TEM.

Figure 2. Metabolic fate of 5DN in Caco-2 cell monolayer.

Figure 3. Trans-intestinal uptake of 5DN by Caco-2 cell monolayer treated with mixed micelle systems containing fatty acids with different degree of unsaturation. (A) Basolateral levels of 5DN. (B) Area under the curve (AUC) of basolateral 5DN during the first 4 hours of treatment. Different notation indicated statistical significance ($p < 0.05$, $n = 3$).

Figure 4. Production of 5DN metabolites in basolateral media by Caco-2 cells. Basolateral levels of M1 (A), M1' (B), M2 (C), and M2' (D). Relative abundance of 5DN and its metabolites in basolateral media (E). Relative abundance of 4 metabolites in basolateral media (F). Different notation indicated statistical significance ($p < 0.05$, $n = 3$). NS: not statistically significant.

Figure 5. Intracellular levels of 5DN in Caco-2 cells after 24 hours of treatment with different mixed micelle systems (A). Relative abundance of 5DN and its metabolites in Caco-2 cells (E). Relative abundance of 4 metabolites in Caco-2 cells (F). Different notation indicated statistical significance ($p < 0.05$, $n = 3$). NS: not statistically significant.

Figure 6. Morphology of lipid droplets in Caco-2 cell monolayers and secreted chylomicrons by Caco-2 cells treated with mixed micelle systems containing fatty acids with different degree of unsaturation for 24 hours (A). Quantification of lipoprotein particles in the basolateral media after 24 hours of treatment (B). Data represent the results of 3 independent experiments.

Figure 7. The levels of Apo B protein in the basolateral media (A) and in the Caco-2 cells (B) after 24 hours of treatment with mixed micelle systems containing fatty acids with different degree of unsaturation. Different notation indicated statistical significance ($p < 0.05$, $n = 3$).

Figure 1.

551

C_{18:1}-TC-5DN

C_{18:2}-TC-5DN

C_{18:3}-TC-5DN

Figure 2.

552
553

Figure 3.

Figure 4.

555

Figure 5.

Figure 6. 557

B

Figure 7.