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Encapsulation of Curcumin in O/w Nanoemulsions and Its Bioaccessibility After In Vitro Digestion

Kashif Ahmed
University of Massachusetts Amherst

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ENCAPSULATION OF CURCUMIN IN O/W NANOEMULSIONS AND ITS BIOACCESSIBILITY AFTER IN VITRO DIGESTION

A Thesis Presented

by

KASHIF AHMED

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Food Science
ENCAPSULATION OF CURCUMIN IN O/W NANOEMULSIONS AND ITS BIOACCESSIBILITY AFTER IN VITRO DIGESTION

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KASHIF AHMED

Approved as to style and content by:

_____________________________________
H. Xiao, Chair

_____________________________________
D.J. McClements, Member

_____________________________________
E.A. Decker, Member

E.A. Decker, Department Head
Department of Food Science
DEDICATION

I dedicate this work to my parents. I am very appreciative of their love and support. Without their guidance, this would not be possible.
ACKNOWLEDGMENTS

I would like to acknowledge all of those associated with the Food Science Department at the University of Massachusetts Amherst. Their collective efforts have made my time here an enriching experience.
ABSTRACT

ENCAPSULATION OF CURCUMIN IN O/W NANOEMULSIONS AND ITS BIOACCESSIBILITY AFTER IN VITRO DIGESTION

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M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor H. Xiao

The functional ingredient curcumin has a variety of biological and pharmacological actions, such as anti-tumor, anti-inflammatory, anti-virus, anti-oxidant, and anti-HIV properties coupled with low toxicity. However, curcumin possesses low bioavailability due to its poor solubility in water. The purpose of this study was to investigate the impact of different lipid-based formulations of curcumin on in vitro solubilization and bioaccessibility. Oils representing LCT, MCT, LCT:SCT mix and SCT were used to prepare O/W (nano)emulsions with droplet sizes as low as 174 nm. An in vitro digestion model simulating the small intestine milieu in the fasted and fed state was used to characterize rate, extent, and particle size associated with emulsion digestion. Rate and extent were oil dependent, but not particle size. SCT emulsions digested at the fastest initial rate, but MCT emulsions were digested to the largest extent. Bioaccessibility, a precursor to eventual bioavailability, was determined after digestion using a curcumin:lipid content dependent and independent method. MCT produced the highest bioaccessibility of curcumin for each method. Nanoemulsion digestion and bioaccessibility results were compared to conventional emulsions because an appropriate comparison was needed to determine the merits of the nanoemulsion delivery system. There was no significant difference in particle size and bioaccessibility between the conventional and nanoemulsions.

Key words: Curcumin; Bioaccessibility; Nanoemulsion; In vitro digestion
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CHAPTER 1
LITERATURE REVIEW

1.1 General Information of Curcumin

Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, is a natural polyphenolic phytochemical extracted from the powdered rhizomes of the spice turmeric (Curcuma longa) (Khanna 1999). It constitutes approximately 3-4% of the composition of turmeric. Along with the other curcuminoids, curcumin is responsible for the yellow color of turmeric. Turmeric is prominently used in South and East Asian countries for culinary, medicinal, and cultural uses. As an additive, turmeric can improve the palatability, aesthetic appeal, and shelf life of perishable food items. In the Ayurvedic system of medicine, turmeric is used as a tonic, blood purifier, and topical ointment (Joe, Vijaykumar et al. 2004).

Curcumin can exist in at least two tautomeric forms, diketo and keto-enol. The structures are seen in Figure 1 (Kolev, Velcheva et al. 2005):

![Figure 1: Diketo and keto-enol forms of curcumin.](image-url)
The keto-enol form is strongly favored by intramolecular H-bonding and is more energetically stable in the solid phase and in solution (Roughley and Whiting 1973; Pedersen, Rasmussen et al. 1985; Tonnesen 1991; Litwinienko and Ingold 2004). The central β-diketone moiety is suggested to be likely responsible for the high beneficial activities of curcumin mentioned above (Osawa and Namiki 1985; Ruby, Kuttan et al. 1995).

1.2 Potential Biological Properties of Curcumin

The structure of curcumin is important to its proposed biological properties and beneficial effects. The central β-diketone moiety is suggested to be likely responsible for the high beneficial activities of curcumin (Osawa and Namiki 1985; Ruby, Kuttan et al. 1995; Sreejayan and Rao 1996; Sreejayan and Rao 1997). The enol form is more energetically stable in the solid phase and in solution. The dried rhizome of the *Curcuma longa* plant is very rich in phenolics, with curcumin included. Curcumin has attracted considerable attention in recent years due to its great variety of beneficial biological and pharmacological activities (Duvoix, Blasius et al. 2005).

1.3 Bioavailability of Curcumin and Current Approaches for its Delivery

The term bioavailability is defined as the fraction of an ingested component that eventually ends up in the systemic circulation (Versantvoort, Oomen et al. 2005). The overall bioavailability ($F$) of poorly water-soluble lipophilic components depends on a number of physicochemical and physiological factors, as signified in Equation 1:

$$F = F_B \times F_A \times F_M$$

(1)

Here, $F_B$ is the fraction of lipophilic component released from the food matrix into the juices of the gastrointestinal tract to become bioaccessible, $F_A$ is the fraction of the released component that is absorbed across the intestinal epithelia, and $F_M$ is the fraction of the absorbed component that reaches the systemic circulation without being metabolized. The major physicochemical and physiological processes that determine these parameters for lipophilic components have been identified (Bermudez, Pacheco et al. 2004; Fave, Coste et al. 2004; Bauer, Jakob et al. 2005):

- **Bioaccessibility:** A poorly water-soluble lipophilic component must be released from the food matrix and solubilized within mixed micelles present in the small intestine before it can be absorbed. These mixed micelles consist of bile salts and phospholipids secreted by the body, as well as lipid digestion products (monoacylglycerides and free fatty acids). The solubilization step therefore determines the fraction of lipophilic component that is bioaccessible ($F_B$).

- **Absorption:** The mixed micelles transport the solubilized lipophilic components across the intestinal lumen, through the mucous layer (“unstirred layer”), and to the surface of the intestinal enterocyte cells. The lipophilic components are then incorporated into the enterocyte cells through either a passive or an active
mechanism (Singh, Ye et al. 2008). This step determines the fraction of the solubilized lipophilic component that is absorbed ($F_A$) by the body.

- **Metabolism:** Once the lipophilic component has been incorporated into the enterocyte cells it is transported to the systemic circulation by a route that depends on the chemical nature of the lipophilic component. High molecular weight lipophilic components tend to be transported *via* the lymphatic route, whereas short and medium molecular weight lipophilic components tend to be transported *via* the portal vein and liver. Lipophilic components are often metabolized when they pass through the liver before reaching the systemic circulation. The pathway that the lipophilic component is transported to the system circulation therefore determines the fraction of the lipophilic component that survives metabolism ($F_M$).

A graphic representation is seen in Figure 2 (Porter, Trevaskis et al. 2007):
Lipids can affect drug absorption in three ways: by enhancing drug (D) solubilization in the intestinal milieu through alterations to the composition and character of the colloidal environment — for example, vesicles, mixed micelles and micelles (a); by interacting with enterocyte-based transport and metabolic processes, thereby potentially changing drug uptake, efflux, disposition and the formation of metabolites (M) within the enterocyte (b); or by altering the pathway (portal vein versus intestinal lymphatic system) of drug transport to the systemic circulation — which in turn can reduce first-pass drug metabolism as intestinal lymph travels directly to the systemic circulation without first passing through the liver (c). Cellular junctions are represented by green ovals and a representative transport protein is depicted by a blue oval.

In Figure 2, parts a, b, and c are equivalent to bioaccessibility, absorption, and metabolism, respectively.

A lack of information regarding the mechanism of action of curcumin and its low bioavailability have resulted in low amounts of clinical use in western countries (Joe, Vijaykumar et al. 2004). A prominent reason for its low bioavailability is that curcumin is virtually insoluble in water at acidic or neutral pH (Maiti, Mukherjee et al. 2007). The maximum solubility reported was 11 ng/mL in plain aqueous buffer (Tonnesen, Masson et al. 2002). Another problem is that curcumin, after oral dosing, undergoes rapid
degradation and molecular fragmentation to several reduced products (Wang, Pan et al. 1997; Pan, Huang et al. 1999; Lin, Pan et al. 2000; Ireson, Orr et al. 2001). Thus, curcumin’s use as a therapeutic agent suffers from many shortfalls.

Several approaches have been investigated to improve the bioavailability of curcumin. Specific approaches have included loading curcumin into liposomes or nanoparticles (Anand, Kunnumakkara et al. 2007; Marczylo, Verschoyle et al. 2007; Mukerjee and Vishwanatha 2009; Anand, Nair et al. 2010) and interacting curcumin with polysaccharides (Tonnesen, Masson et al. 2002), and phospholipids (Liu, Lou et al. 2006; Maiti, Mukherjee et al. 2007).

Anand et al., (2010) employed a polymer-based nanoparticle approach to improve the bioavailability of curcumin. The focus of their study was to prepare and characterize the curcumin nanoparticles using various methods, with a goal to enhance its bioavailability without loss of biological activity. Curcumin was encapsulated in biodegradable nanoparticulate formulation based on poly (lactide-co-glycolide) (PLGA) and a stabilizer polyethylene glycol (PEG)-5000. Dynamic laser light scattering and transmission electron microscopy indicated a particle diameter of 80.9 nm. These nanoparticles were examined for cellular uptake, the ability to induce apoptosis and suppress proliferation of tumor cells, suppression of NF-kB and NF-kB-regulated gene products, and in vivo bioavailability. For most studies, human leukemia KBM-5 cells were used, as the effects of curcumin on these cells have been well described. The nanoparticle curcumin treatment was compared to native curcumin for this study and the treatment showed a greater efficacy than native curcumin for the attributes listed.
Mukerjee and Vishwantha (2009) formed poly (lactic-coglycolic acid) (PLGA) nanospheres using a solid/oil/water emulsion solvent evaporation method to encapsulate curcumin. In the nanosphere formulation process, the polyvinyl (PVA) solution acted as the stabilizer and the ethanol acted as the nonsolvent. Briefly, 30 mg of the polymer PLGA, was dissolved in chloroform. Free curcumin was added to the PLGA/chloroform solution and sonicated at 55 W for 1 minute to produce the s/o primary emulsion. This emulsion was then added to a solution of 2% PVA and ethanol (1:1) and again sonicated at 55 W for 2 minutes to form the final s/o/w emulsion. The particle size distribution showed a range of 35 nm to 100 nm, with the mean particle size being 45 nm. Characterization of the nanospheres was done by particle size analysis, encapsulation efficiency, percent yield, and various microscopy methods. They investigated the ability of these nanospheres to be endocytosed by cells, specifically the cancer cell lines, DU145, PC3 and LNCaP. These were incubated with curcumin-loaded Nile red-labeled PLGA nanospheres for three hours and results depicted robust uptake of the nanospheres in all three prostate cancer cell lines.

Marczylo et al., (2007) produced a formulation of curcumin with soy phosphatidylcholine-phospholipid complex called “Meriva” in Europe to enhance systemic bioavailability of curcumin in male Wistar rats following oral gavage. The preparation of Meriva used EpiKuron130 P, a de-oiled, powdered soybean lecithin enriched with 30% phosphatidylcholine. Peak plasma levels and area under the plasma concentration time curve (AUC) values for parent curcumin after administration of Meriva were 5-fold higher than those seen after administration of unformulated curcumin. Similarly, liver levels of curcumin were higher after administration of Meriva.
as compared to unformulated curcumin. In contrast, curcumin concentrations in the gastrointestinal mucosa after ingestion of Meriva were somewhat lower than those observed after administration of unformulated curcumin. Similar observations were made for curcumin metabolites as for parent compound. Extracts of bio-matrices were subjected to HPLC mass spectrometric analysis and the analysis furnished incontrovertible proof of presence of curcumin based on specific MRM transitions in the gut mucosa, plasma, and liver. The results suggested that curcumin formulated with phosphatidylcholine furnished higher systemic levels of parent agent than unformulated curcumin.

Tonnesen et al., (2002) complexed curcumin with a variety of cyclodextrins in order to improve its stability and retard the degradation process. Cyclodextrins were used as pharmaceutical excipients, mainly as solubilizing and stabilizing agents for lipophilic substances in aqueous preparations. A number of molecules were solubilized in cyclodextrin solutions through formation of an inclusion complex. The cyclodextrins used to encapsulate curcumin were hydroxypropyl-α-cyclodextrin, hydroxypropyl-β-cyclodextrin, hydroxypropyl-γ-cyclodextrin, randomly methylated β-cyclodextrin, sulfobutylether-β-cyclodextrin, and hydroxytrimethylammoniumpropyl-β-cyclodextrin. The water and lytic stability of curcumin in the cyclodextrin complexes was determined and found to be superior to curcumin in organic solvent systems. However, the photostability was greatly diminished in these complexes.

Maiti et al., (2007) produced a formulation of curcumin with hydrogenated soy phosphatidyl choline (HSPC) to improve its therapeutic efficacy, overcome the limitation of absorption, and to investigate the protective effect of curcumin–phospholipid complex
on carbon tetrachloride induced acute liver damage in rats. The complex was prepared with curcumin and HSPC at a molar ratio of 1:1. The mixture was refluxed at a temperature not exceeding 60 °C for 2 h. The curcumin–phospholipid complex was precipitated and the precipitate was filtered and dried under vacuum to remove traces of solvents. The complex’s yield was 88% weight/weight basis. The complex’s antioxidant activity in carbon tetrachloride intoxicated rats was found to be greater than that of pure curcumin using the thiobarbituric acid reactive substance (TBARS), superoxide dismutase (SOD), and catalase (CAT) tests. Free curcumin at the dose of 200 mg/kg prevented the adverse conditions in rats created by CCl₄ intoxication. The complex also restored the normal condition of rat liver enzymes.

1.4 Nanoemulsion Approach to Curcumin Encapsulation

Emulsions are mixtures of two or more immiscible liquids. Many bioactive components or ingredients are not soluble in water. In an emulsion-based delivery system, the component, in my case a lipophilic component, can be first dissolved into an oil phase and then emulsified with a water phase to make a homogenous emulsion. These systems have been widely used in the food industry to protect active ingredients against extreme conditions, to enhance their stability, to maintain their effectiveness, and to mask bad odors and bitter taste (Madene, Jacquot et al. 2006).

However, emulsions are subject to various destabilization mechanisms such as gravitational separation, coalescence, flocculation, sedimentation, creaming, and Ostwald ripening. The small droplet size of nanoemulsions makes them resistant to physical destabilization via gravitational separation, flocculation and/or coalescence due to effective steric stabilization (Capek 2004; Tadros, Vandamme et al. 2004; Solans,
Izquierdo et al. 2005). Several phytochemicals have already been encapsulated using nanoemulsions (Garti 2005).

Nanoemulsions, as a lipid-based solubilized drug formulation, can enhance drug solubilization within the GI tract by avoiding solid-state limitations of the drug and increasing solubilization capacity via enhanced secretion of biliary-derived solubilizing compounds (Porter, Trevaskis et al. 2007). Micelles, and other digestion products such as vesicles and liposomes, are formed from free fatty acids that have evolved from the digestion of triglycerides. Nanoemulsions, by increasing the solubility of a hydrophobic compound like curcumin, can enhance the absorption in the gastrointestinal tract (Patel and Sawant 2007).

Recently, different variations of emulsion-based delivery systems have been used (Bisht, Feldmann et al. 2007; Wang, Jiang et al. 2008; Cui, Yu et al. 2009; Lin, Lin et al. 2009) with curcumin. These systems can produce nanoemulsions with droplet sizes from 50-200 nm and microemulsions with droplet sizes less than 20 nm. Conventional emulsions have a size range from 1-100 µm. Nanoemulsions possess high kinetic stability due to their extremely small emulsion droplet sizes in the range of 50-200 nm (Sonneville-Aubrun, Simonnet et al. 2004; Solans, Izquierdo et al. 2005).

Cui et al., (2009) utilized a self-microemulsifying drug delivery system (SMEDDS) to improve the solubility and oral absorption of curcumin. Variable proportions of oil, surfactant and co-surfactant were added into a 10ml screw-capped glass tube, and the components were mixed by gentle stirring. After complete dissolution, SMEDDS, a clear and transparent solution, was obtained. The optimal microemulsion formulation was determined by pseudoternary phase diagrams and consisted of oil (ethyl
oleate), surfactant (emulsifier OP: Cremorphor EL = 1:1 w/w), co-surfactant (PEG 400) and water. The average particle size of the curcumin-loaded SMEDDS was about 21 nm. Size and curcumin content in the SMEDDS showed no significant variation when stored for three months at 4 °C. In order to investigate the transport mechanism of curcumin in SMEDDS, the method of perfusion in rat intestine was used because it can give a reliable prediction of oral absorption of drug in humans (Zakeri-Milani, Valizadeh et al. 2007). Curcumin-loaded SMEDDS demonstrated concentration-independent absorption in the rat’s intestine, which might contribute to intestinal absorption of curcumin in SMEDDS via passive transfer by diffusion across the lipid membranes.

Lin et al., (2009) also used a ternary phase diagram of a curcumin-encapsulated O/W microemulsion system. Specifically, their formulation used food-acceptable components lecithin and Tween 80 as the surfactants and ethyl oleate as the oil phase. A microemulsion was prepared when the weight ratio of aqueous phase (deionized water): surfactants (Tween 80 and lecithin): oil phase ranged from 10:5:1 to 10:1:0.3 at the fixed lecithin to Tween 80 molar ratio of 0.3. Homogeneous and yellow transparent curcumin encapsulated microemulsions were obtained using isopropyl myristate (IPM) and ethyl oleate (EO) as the oil phase. The composition of the curcumin microemulsion (DI water: surfactants (the mole ratio of lecithin/Tween 80 was 0.3): EO = 10:1.7:0.4 in wt ratio) was stable for 2 months with an average diameter of 71.8 ± 2.45 nm. Microemulsion formulations prepared from loading curcumin of different doses were tested for their permeation into BALB/c mouse skin. In the in vitro skin permeation study, both the dose–response and time-dependent studies of the encapsulated curcumin equations showed that MEC2 (30 mg of curcuminoids loaded into 10 mL of microemulsion
solution) was the most suitable formulation with reduced particle diameter and maximum permeation capability.

Wang et al., (2008) produced and characterized O/W nanoemulsions consisting of medium chain triacylglycerols (MCT) as the oil phase and Tween 20 as the emulsifier. An amount of 1% curcumin was able to be loaded in MCT. The mixtures of MCT, Tween 20, and water were mixed under magnetic stirring for 30 min at a ratio of 10/10/80 to fabricate O/W emulsions. Some emulsion premixes were subjected to high-speed homogenization for 10 min. Some high-speed homogenized emulsions were further homogenized by using a high-pressure homogenizer for 6 cycles. Mean droplet sizes ranged from 618.6-79.5 nm. The oral administration of 1% curcumin in Tween 20 water solution showed little or no inhibition effect of TPA-induced edema of mouse ear. On the contrary, it was found that curcumin O/W emulsions could significantly improve the inhibition effect of TPA-induced edema of mouse ear from 43% to 85% for curcumin emulsion with droplet size of 618.6 nm and 79.5 nm, respectively. The 1% curcumin O/W emulsions showed improved inhibition on the edema of mouse ear, and such anti-inflammation activity was further enhanced when the emulsion droplet sizes were reduced to below 100 nm.

Bisht et al., (2007) synthesized a polymeric nanoparticle encapsulated formulation of curcumin – nanocurcumin – utilizing the micellar aggregates of cross-linked and random copolymers of Nisopropylacrylamide (NIPAAm), with N-vinyl-2-pyrrolidone (VP) and poly(ethylene glycol) monoacrylate (PEG-A). Curcumin loading in the polymeric nanoparticles was done by using a post-polymerization method. In this process of loading, the drug was dissolved after the co-polymer formation has taken place. The
physical entrapment of curcumin in NIPAAM/VP/PEG-A polymeric nanoparticles was carried out as follows: 100 mg of the lyophilized powder was dispersed in 10 ml distilled water and was stirred to re-constitute the micelles. Free curcumin was dissolved in chloroform (CHCl₃; 10 mg/ml) and the drug solution in CHCl₃ was added to the polymeric solution slowly with constant vortexing and mild sonication. Curcumin was directly loaded into the hydrophobic core of nanoparticles by physical entrapment. The drug-loaded nanoparticles were then lyophilized to dry powder for subsequent use. Characterization of the polymeric nanoparticles by dynamic laser light scattering and transmission electron microscopy confirmed a narrow size distribution in the 50 nm range. The nanoparticles were also characterized via ¹H NMR and FT-IR studies. Cell viability (MTT) assays were performed using equivalent dosages of free curcumin and nanocurcumin in a panel of human pancreatic cancer cell lines. The assay was terminated at 72 hours, and colorimetric determination of cell viability performed. Four of six cell lines demonstrate response to nanocurcumin (defined as an IC₅₀ in the 10–15 µM range) – BxPC3, ASPC-1, PL-11 and XPA-1, while two lines were curcumin resistant – PL-18 and PK-9. Nanocurcumin inhibited NFκB function in pancreatic cancer cell lines and downregulated multiple pro-inflammatory cytokines.

### 1.5 In Vitro Digestion

Digestion within humans is a complicated process because of the compounds involved in aiding the process and the different environments encountered by the ingested food. These environments include the mouth, stomach, and small intestine. Lipid-based oral delivery systems have been utilized as a strategy to enhance bioavailability of poorly water soluble, lipophilic drugs (Humberstone and Charman 1997). *In vitro* lipid digestion
models are used to find \textit{in vitro-in vivo} correlations, which are a key element for drug development. A marked advantage of using \textit{in vitro} models is the ability to stimulate gastric or intestinal fluids to assess their \textit{in vivo} performance.

Recently, there has been an effort to develop lipid-based formulations of poorly soluble compounds for oral administration. The use of \textit{in vitro} lipolysis models can attempt to probe solubilization in the aqueous phase during the progress of enzymatic degradation of lipid-based formulations (Fatouros and Mullertz 2008). With this information, \textit{in vitro} lipid digestion models can serve as a screening tool for formulating and optimizing lipid-based formulations of drugs. Figure 3 shows an example of a dynamic \textit{in vitro} digestion model (Porter, Trevaskis et al. 2007):
Figure 3: Lipid digestion models for *in vitro* assessment of lipidic formulations. (A) The models are built around a temperature-controlled (37°C) vessel that contains digestion buffer, bile salt (BS), and phospholipid (PL) (to represent a model intestinal fluid) into which lipid-based formulations are introduced. Digestion is initiated by the addition of pancreatic lipase and co-lipase. The onset of lipid digestion results in the liberation of fatty acid (FA), which in turn causes a transient drop in pH. (B) The drop in pH is quantified by a pH electrode that is coupled to a pH-stat meter controller and autoburette, which together automatically titrate the liberated FA by the addition of an equimolar quantity of NaOH. This maintains the pH at a set point (thereby allowing the pH-sensitive process of digestion to continue) and facilitates indirect quantification of the extent of digestion (by quantification of the rate of NaOH addition and assumption of a stoichiometric reaction between FA and NaOH).
The digestion model used in my study incorporated bile, lipase, and calcium chloride, while having a starting condition similar to a fasted state. Digestion products of triglyceride lipids may enhance drug solubilization and dissolution by stimulating bile salt and phospholipid secretion into the GI lumen (Hernell, Staggers et al. 1990) and enhance the solubilization capacity of endogenous bile salts/phospholipid mixed micelles by intercalation into the micellar structure (Laher and Barrowman 1983; Vetter, Carey et al. 1985). Thus, the formed micellar structure is very important to lipolysis digestion models.

An ideal outcome of the digestion model is for the lipase to effectively digest the triglycerides and have the free monoglycerides form micelles encapsulating the curcumin. First, the triglyceride is hydrolyzed to form a single fatty acid and diglyceride. Next, the diglyceride is hydrolyzed to produce a second fatty acid and a corresponding 2-monoglyceride. The 2-monoglyceride may isomerize into 1-monoglyceride and be further hydrolyzed into a third fatty acid and glycerol, but this process is generally believed to be limited in vivo (Mattson and Volpenhein 1964).

The progress of triglyceride lipolysis is generally monitored in vitro using an automated pH-stat titration apparatus (Alvarez and Stella 1989; MacGregor, Embleton et al. 1997). The limitation of this methodology is that it quantifies the rate and extent of digestion indirectly via titration of fatty acids produced. However, direct detection and quantification of lipolytic products such as triglycerides, diglycerides, monoglycerides, and fatty acids is difficult because of the lack of chromopohoric groups on these molecules (Sek, Porter et al. 2001). The accuracy of the pH-stat titration technique in
quantifying the fatty acid produced during lipid digestion is highly dependent on the ionization of fatty acid and the availability of titratable fatty acid (Sek, Porter et al. 2002).

1.6 Caco-2 Cell Line

Recently, new insights about the mechanisms underlying intestinal transport of carotenoids have been provided by the use of human intestinal cell lines. Several attempts to obtain differentiated intestinal cell lines from normal tissues have not been successful, but established differential intestinal lines have been developed from human colon adenocarcinoma cells (Ranaldi, Bellovino et al. 2007). The majority of in vitro transport studies reported have used Caco-2 cells because these cells show, in vitro, most of the characteristics of enterocyte cells in the small intestine (Pinto, Robineleon et al. 1983).

Caco-2 cells have been extensively used to study intestinal transport of nutrients, drugs, and xenobiotics (Ranaldi, Islam et al. 1992; Ferruzza, Ranaldi et al. 1995; Shah, Jogani et al. 2006). The differentiative process of Caco-2 cells is a spontaneous event dependent on the time that cells are maintained in culture. It has been reported that differentiation occurs 2 – 3 weeks after initial confluency (Hidalgo, Raub et al. 1989; Chantret, Rodolosse et al. 1994; Lind, Jacobsen et al. 2007). Upon differentiation, cells give rise to a monolayer of polarized epithelial cells joined by a functional tight junction system and display several biochemical and functional characteristics typical of a mature enterocyte cell. Figure 4 depicts a differentiated Caco-2 cell (Ranaldi, Bellovino et al. 2007):
Figure 4: Schematic representation of Caco-2 cell grown on filter. Monolayers on the apical side of the cell are easily accessible, making it an ideal model to study both absorption and excretion.

For transport experiments, cells are plated in the Caco-2 culture system, which represents the intestinal mucosa environment in which the lumen is separated from the bloodstream by the intestinal epithelial monolayer. Trans-epithelial passage of molecules from the apical to the basolateral side of the monolayer can be easily measured in different experimental conditions, thus allowing to discriminate factors involved in transport mechanisms (Ranaldi, Bellovino et al. 2007).
CHAPTER 2

FORMULATE AND CHARACTERIZE O/W NANOEMULSIONS USING
DIFFERENT CHAIN LENGTH OILS WITH ENCAPSULATED CURCUMIN

2.1 Introduction

Curcumin has attracted considerable attention in recent years due to its great variety of beneficial and biological and pharmacological activities (Duvoix, Blasius et al. 2005). However, curcumin suffers from low bioavailability and is essentially insoluble in water at acidic or neutral pH (Maiti, Mukherjee et al. 2007). Many attempts have been made to improve water solubility and bioavailability of curcumin. Previous delivery systems have used complexation of curcumin with macromolecules including polysaccharides and phospholipids (Tonnesen, Masson et al. 2002; Liu, Lou et al. 2006). These delivery systems have the disadvantage of using non-food grade components and organic solvents.

Emulsion-based delivery systems are being increasingly used for encapsulating lipophilic bioactive components (Porter, Trevaskis et al. 2007). The curcumin can be solubilized in the oil, dispersed, phase and then homogenized with the water, continuous, phase. Nanoemulsions can be prepared using a dual high-speed and high-pressure homogenization technique to create droplet sizes in the 20 – 200 nm range (Solans, Izquierdo et al. 2005).

The objective of this chapter is to formulate and characterize curcumin-loaded O/W nanoemulsions. Specific conditions involving nanoemulsion stability are also noted.
2.2 Materials and Methods

2.2.1 Materials

Curcumin (~95.2% pure, from *Curcuma longa*) was purchased from Bepharm Ltd. (Shanghai, China) and used without further purification. Powdered BioPURE β-Lactoglobulin (β-Lg; emulsifier) was provided by Davisco Foods International, Inc. (Le Sueur, MN). Corn oil (oil, LCT) was purchased from a local market and was used without further purification. Miglyol® 812 (oil; MCT) was purchased from SASOL (Houston, TX). Tributyrin (98%) (oil; SCT) was purchased from Sigma Aldrich (St. Louis, MO). Double deionized water was used in all experiments. A 5 mM pH 7 phosphate buffer solution (PBS) was produced by dispersing 0.011% of anhydrous NaH$_2$PO$_4$ and 0.058% of anhydrous Na$_2$HPO$_4$. The chemicals for the phosphate buffer preparation were purchased from Sigma Aldrich and Fisher, respectively.

2.2.2 Maximum Solubility of Curcumin

The maximum solubility of curcumin in each oil phase that had been heated to 60 °C was determined. To ensure maximum curcumin dissolution, the heated oil phase containing excess curcumin was magnetically stirred for 10 min and then sonicated for 20 min at 60 °C. The resulting solution was centrifuged (1750 rpm, Fisher Scientific 225A, Fisher) at room temperature for 10 min and the supernatant was saved. The supernatant was then diluted to an appropriate concentration to be analyzed by UV-VIS (wavelength dependent on oil type for emulsion, Ultrospec 3000 Pro, GE) with the pure oil used as the blank.
2.2.3 Curcumin Photostability

Curcumin stability in oil was measured by UV-VIS spectroscopy. Curcumin-loaded oil was incubated at 37 °C with shaking of 150 rpm for 48 hours. Aliquots were measured for absorbance at 1-12, 24, 36, and 48 hours. The oil without curcumin was used as a blank.

2.2.4 O/W Nanoemulsion Preparation

Curcumin-loaded O/W nanoemulsions were prepared. To maximize the curcumin dissolution into the oil phase, the oil and aqueous phases were prepared separately. The aqueous emulsifier solution containing 1 wt% protein was prepared by dispersing β-LG into PBS and stirring for at least two hours to ensure complete hydration. The oil phase was prepared by adding 0.15% curcumin by weight into the heated 60 °C oil. To ensure maximum curcumin dissolution, the heated oil phase was magnetically stirred for 10 min and sonicated for 20 min at 60 °C. O/W nanoemulsions were prepared by homogenizing 10 wt% oil phase with 90 wt% aqueous emulsifier solution in a M133/1281 high-speed blender (Biospec Products, Inc., ESGC, Switzerland) for 2 min at room temperature, followed by five passes at 9000 psi (~620 bars) through a Microfluidics M-110Y Microfluidizer™ (MFIC Corporation, Newton, MA, USA) with a F20 Y 75 µm interaction chamber. After preparation, emulsions were stored at 4 °C.

2.2.5 Emulsion Characterization

The particle size distribution (PSD) of the O/W nanoemulsions was measured using dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, U.K.), with each individual measurement being the average of 12 runs. Results were
reported as the average size (Z-average) and width (polydispersity index-PDI). Emulsions were diluted prior to analysis using buffer solution (5mM phosphate, pH 7.0) to avoid the effects of multiple scattering.

2.3 Results and Discussion

2.3.1 Curcumin Solubility and Photostability in Oil

From now on, LCT refers to long chain triglyceride (corn oil), MCT refers to medium chain triglyceride (Miglyol® 812), and SCT refers to short chain triglyceride (tributyrin). Before the nanoemulsion could be formulated, the maximum curcumin-loading capacity in each oil phase was determined. The maximum solubility of curcumin in different oils at 60 °C is seen below:

<table>
<thead>
<tr>
<th>Oil Type</th>
<th>Maximum Solubility of Curcumin (% by weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCT</td>
<td>0.30 ± 0.10</td>
</tr>
<tr>
<td>MCT</td>
<td>0.79 ± 0.25</td>
</tr>
<tr>
<td>LCT:SCT Mix</td>
<td>1.94 ± 0.44</td>
</tr>
<tr>
<td>SCT</td>
<td>2.98 ± 0.18</td>
</tr>
</tbody>
</table>

Table 1: Effect of oil chain-length on maximum solubility of curcumin. Each measurement has been correlated to a previously made standard curve of 0.08% curcumin in the oil phase. Results are reported as the average of three measurements on freshly prepared samples.

As seen in Table 1, as oil chain length decreased the ability to solubilize curcumin increased. This phenomenon could be due to entropy of mixing, which states that entropy change is not from the intermingling of the particles, but rather the change in the available volume per particle (Huyskens and Haulaitpirson 1985). Since SCT’s are
smaller molecules than MCT and LCT’s, adding curcumin to a given area does not change the molecular configuration as it would for MCT and LCT’s. Another explanation is that, at a given mass of oil, there are more SCT molecules than higher chain length triglycerides because of the lower molecular weight. The amount of oxygen atoms on each triglyceride molecule are the same, regardless of fatty acid chain length. Thus, at a given mass, there are more available oxygen molecules to hydrogen bond to curcumin in SCT as compared to MCT and LCT. Interesting results were seen for the LCT:SCT mix because the average solubility was approximately halfway between the average solubility of only LCT and SCT. There was no synergistic or inhibitory effect seen with mixing the two oils. With these results, I selected 0.15% by weight curcumin in oil phase for my nanoemulsions so as to make sure that 100% solubilization of curcumin in the oil was possible in the experimental conditions.

A temperature of 60 °C was selected for initial dissolution because it was a temperature that could be replicated in industrial conditions and curcumin-loaded emulsions have been previously prepared with curcumin being dissolved into hot oil (Wang, Jiang et al. 2008). Subsequent use of the curcumin-loaded oil at temperatures ranging from 25 - 60 °C (doing dilutions, making emulsions, etc., would use oil whose temperature would fall to between 25 - 60 °C) showed that the curcumin stayed solubilized in the oil. Noticeable recrystallization of curcumin only occurred after 24 hour storage in refrigeration (4 °C) for MCT and LCT. SCT and LCT:SCT mix showed no noticeable recrystallization in refrigeration after 24 hours.
Curcumin stability in oil was tested at 37 °C over a 48 hour time span to see if there was any decrease in absorbance, which correlated to curcumin falling out of oil. The results are seen in Figure 5:

![Figure 5: Effect of storage time on curcumin stability in oil. Results are reported from one experiment on freshly prepared samples. Absorbance did not decrease an appreciable amount over a 48 hour time period. Thus, photo-stability was not heavily influenced when the curcumin was in oil. With these results, I hypothesized the curcumin that was incorporated into the emulsion stayed encapsulated in the oil phase during the duration of the emulsion’s use and its photo-stability would not wane. Further experiments could be done to test this by dissolving aliquots of the emulsion in organic solvent (such as chloroform) and test the absorbance as a function of storage time.

2.3.2 O/W Nanoemulsion Characterization

Curcumin-containing O/W nanoemulsions were formulated using a microfluidizer. A general diagram of the microfluidizer is seen below with a targeted view of the interaction chamber (Lagoueyte and Paquin 1998; Vladisavljevic, Lambrich et al. 2004):
Figure 6: Diagram of microfluidizer used for O/W nanoemulsion formulation.

Figure 6 showed how nanoemulsions were produced with a high-pressure apparatus following initial homogenization by a high-speed blending process (pre-emulsion). All nanoemulsions produced by microfluidization were 10% O/W (by weight) with 0.15% curcumin (by weight) in the oil phase. The particle size distributions (PSD) of the nanoemulsions are presented in Table 2 as the mean (Z-average) and width (polydispersity index-PDI):
Table 2: PSD of curcumin-loaded nanoemulsions. PSD analysis was performed by dynamic light scattering. Results shown are from the “Intensity (%)) parameter. Results are reported from two measurements on freshly prepared samples.

Figure 7 shows graphical representations of results used to obtain values in Table 2:

(A) LCT with curcumin (intensity %)

(B) LCT with curcumin (volume %)

(Continued on next page)
Figure 7: Particle size distribution (PSD) of 10% oil with 0.15% curcumin O/W emulsion. Results are reported as one sample used in PSD analysis.

In Figure 7, for LCT (A, B) and SCT (C, D) with curcumin, PSD was shown using intensity and volume percentage as two parameters for how PSD data can be collected. These are two common representations used for PSD data and it is important to note the differences that result when selecting parameters to show PSD data. The Z-average and PDI data could change to a significant extent based on which parameter the data is being reported (as seen in the graphs). From now on, I will present PSD data using the “Intensity (%))” parameter.
The curcumin-containing O/W nanoemulsions were prepared using a dual high-speed and high-pressure homogenization technique. Emulsions are considered nanoemulsions if they have a droplet size range between 20 – 200 nm. Microemulsion droplet sizes range from 1 – 20 nm and conventional emulsion droplet sizes range from 1 – 100 µm (Solans, Izquierdo et al. 2005; Wooster, Golding et al. 2008).

As seen in Table 2, the homogenization technique used produced nanoemulsions in a limited droplet size range independent of oil type, except for SCT. In Figure 7, the PSD showed a system that was monomodal for LCT. Sometimes there was a slight peak at the 4 – 6 µm range, making the distribution bimodal. This could be due to aggregated emulsion droplets. These trends were also seen for MCT and LCT:SCT mix. However, the emulsions made with SCT showed droplet growth and/or aggregation, as seen by the multimodal PSD. Emulsions containing SCT in the lipid phase are highly unstable and have been prone to droplet growth. This is due to Ostwald ripening because of the relatively high water solubility of the low molecular weight triacylglycerol (Li, Le Maux et al. 2009).

LCT and MCT nanoemulsions were kept in refrigeration for up to 10 days (duration of emulsion use) without any noticeable phase separation or curcumin fallout observed. LCT:SCT mix nanoemulsions were kept in refrigeration for up to 3 days (duration of emulsion use) without any noticeable phase separation or curcumin fallout observed. SCT nanoemulsions showed separation within hours and need to be made fresh everyday. As stated before, further experiments could be done to test the instability mechanism and determine the maximum storage life of these emulsions at 4 ºC.
2.4 Conclusions

Curcumin-loaded O/W emulsions were prepared. For LCT, MCT, and LCT:SCT mix, these were considered nanoemulsions because their particle size was under 200 nm. SCT emulsions were not considered nanoemulsions because their size distribution was most closely linked to conventional emulsions. The theoretical maximum amount of curcumin able to be loaded at 60 °C was determined. As chain-length decreased, maximum curcumin solubility increased and this was most likely due to the entropy of mixing effect. With this knowledge, nanoemulsions with 0.15% curcumin were selected for further comparative studies involving the different chain-length oils.

Curcumin photo-stability in the pre-emulsion oil phase (up to 48 hours) was confirmed and it was hypothesized that the curcumin stayed encapsulated in the oil phase during the duration of the nanoemulsion’s use. Minimum storage time limits for nanoemulsions at 4 °C were identified and were oil dependent. Future work can be performed to determine the maximum storage limit of these nanoemulsions and see which mechanism, gravitational separation, curcumin fallout, or a combination of both, is responsible for the destabilization.
CHAPTER 3

CHARACTERIZE THE DIGESTION OF CURCUMIN O/W NANOEMULSIONS USING AN IN VITRO DIGESTION MODEL

3.1 Introduction

The use of lipid and surfactant-based formulations is one of several approaches that have been applied in order to improve the oral bioavailability of poorly aqueous soluble compounds intended for oral administration (Fatouros and Mullertz 2008). Lipids may enhance the absorption of poorly water-soluble, lipophilic drugs by their actions on gastric transit, intestinal permeability, and drug metabolism. However, lipids are most widely used to enhance drug solubilization and dissolution in the gastrointestinal tract (Constantinides 1995; Charman, Porter et al. 1997).

Gastrointestinal lipid digestion is a complex process and the impact of digestion on the drug-solubilization process needs to be elucidated. In vitro digestion models have recently gained much attention as a tool to understanding the digestion process. The use of an in vitro lipolysis model has been proposed as an approach to probe solubilization in the aqueous phase during the progress of enzyme degradation of lipid-based formulations (Zangenberg, Mullertz et al. 2001; Zangenberg, Mullertz et al. 2001; Pouton 2006) and for understanding the partitioning of the drug substance between the formed colloid phases during the digestion of lipids (Porter, Trevaskis et al. 2007). Ultimately, a successful in vitro digestion model will be able to predict in vitro-in vivo correlations because this is a prerequisite for rational development for drug delivery systems (Fricker, Kromp et al. 2010).
The objective of this chapter is to use an *in vitro* digestion model to characterize initial rate and extent of digestion of the previously formulated (nano)emulsions.

### 3.2 Materials and Methods

#### 3.2.1 In Vitro Digestion Model

The dynamic *in vitro* digestion model used was a modification of those described by Mun et al. and Zanenberg et al. (Zangenberg, Mullertz et al. 2001; Mun, Decker et al. 2006). This model was meant to mimic the environment of the GI tract in a fasted state. The procedure was as follows: Emulsion and PBS (30.0 mL in total) was placed in a clean beaker in a water bath at 37.0 °C for 10 min and adjusted to pH 7 with NaOH solution (range from 0.05 – 1M). Then, 4.0 mL of bile extract solution containing 187.5 mg of bile extract dissolved in PBS (pH 7.0) and 1.0 mL of CaCl$_2$ solution containing 110 mg of CaCl$_2$ dissolved in PBS (pH 7.0) was added into the 30 mL emulsion under stirring. The resultant mixture was then adjusted to pH 7. Finally, 2.5 mL of freshly prepared pancreatin suspension containing 60 mg of lipase dissolved in phosphate buffer (pH 7.0) was added to the mixture. The bile solution and lipase solution concentrations were 5.0 mg/mL and 1.6 mg/mL respectively. At this point, the pH-stat was started. The pH-stat (Metrohm, USA Inc.) was used to monitor and control the pH (at pH 7) of the digestion solution. The volume of added NaOH solution (500 mM) reflected the amount of free fatty acids generated by lipolysis of the initial triacylglycerols. Experiments ran for 2 hours.
3.2.2 Post-Digestion Characterization

After in vitro digestion, emulsions were centrifuged (12500 rpm, Sorvall RC 6C Plus, DuPont) at 25 °C for 30 min. Aliquots were taken from the supernatant and were either filtered using a 0.45 µm filter or left unfiltered. The PSD of the post-digested supernatant was measured using dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, U.K.), with each individual measurement being the average of 12 runs. The emulsions were diluted prior to analysis using buffer solution (5mM phosphate, pH 7.0) to avoid the effects of multiple scattering.

3.3 Results and Discussion

3.3.1 In Vitro Digestion Titration Profiles

Conditions mimicking the fasted state are used with the caveat that a higher calcium concentration (20 mmoles) was used. This was done to ensure that complete digestion could be achieved. At much lower calcium concentrations (5 mmoles), complete digestion was never able to be achieved. The dynamic in vitro digestion model used a pH-stat method that indirectly quantified digestion extent. I characterized and compared digestion for 1, 1.5, and 2% (nano)emulsions using the four different oils mentioned in Chapter 2.

Digestion extent was calculated from Equations 2 and 3:

\[
NaOH_{\text{Theo}} = \frac{2 \left( \frac{\text{mass}_{\text{oil}}}{\text{MW}_{\text{oil}}} \right) (1000) \left( \text{conc}_{\text{NaOH}} \right)}{\text{conc}_{\text{NaOH}}}
\]  

\[
\% \text{ FFA Released} = \frac{\text{NaOH}_{\text{Exp}}}{\text{NaOH}_{\text{Theo}}} \times 100\%
\]
Equation 2 gave the theoretical volume, in mL, of sodium hydroxide (NaOH) titrant needed to neutralize the free fatty acids released from the triacylglycerols and was referred to as “NaOH\textsubscript{Theo}”. The mass\textsubscript{oil} was the mass of the oil phase in each titration run (300 g), MW\textsubscript{oil} was the molecular weight of the oil used, and conc\textsubscript{NaOH} was the concentration of titrant used in the titration run (0.50M). I hypothesized that each triacylglycerol would release two free fatty acids from its structure from the sn-1 and sn-3 positions upon digestion because pancreatic lipase has an affinity for primary ester bonds on the outer portion of the molecule (Carey, Small et al. 1983). Pancreatic lipase hydrolyzes primary ester bonds of tri- and diglycerides to generate 2-monoglycerides and fatty acids, which are adsorbed through the intestinal barrier in the form of mixed micelles with bile salts (Schmid and Verger 1998). Equation 3 gave indirect digestion extent, percent free fatty acids released (% FFA released), with NaOH\textsubscript{Exp} being the experimental volume of NaOH titrated. Titration profiles were generated from experiments with the \textit{in vitro} digestion model and measured digestion extent (% FFA released; Equation 3) as a function of time. The titration profiles for nanoemulsions are seen in Figure 8:
(A) LCT

(B) MCT

(C) LCT:SCT Mix

(Continued on next page)
(D) SCT

**Figure 8: Effect of droplet concentration on digestion extent for different oils.**
Prepared nanoemulsions containing 0.15% curcumin in LCT (A), MCT (B), LCT:SCT mix (C), and SCT (D) were titrated for two hours. Results with “*” were statistically different from 1% droplet concentration. Results are reported as the average of three measurements on freshly prepared samples.

As can be seen in Figure 8, there were differences in the profiles depending on the oil type used in the nanoemulsion. One common outcome for all oils was an initial period during which the rate of FFA released was relatively fast (from 0 to 1200 s), followed by another period when the rate decreased appreciably.

LCT (Fig. 8A) showed that digestion extent was dependent on droplet concentration because as droplet concentration increased, digestion extent decreased. The amount of FFA released increased rapidly with time almost immediately after digestion starts and then leveled off as the reaction continued. The reduction in the rate of lipolysis may be due to the saturation of phospholipid/bile salt mixed micelles with digestion products at the oil:water interface as digestion progressed. Long-chain lipids generate water-insoluble products, which can interfere with lipase activity at the interfacial layer.
The LCT within the droplets had not been fully digested because the slope was still increasing after 2 hours (7200 sec).

MCT (Fig. 8B) showed a statistical correlation of digestion extent being dependent on droplet concentration because as droplet concentration increased, digestion extent decreased. Values for 1.5 and 2% were similar, but the difference between 1 and 2% was statistically significant. An interesting note about the results for MCT was that each droplet concentration produced a digestion extent greater than 100%. This suggests that MCT could release >2 FFA’s because Equation 3 used a maximum of 2 FFA’s released in determining digestion extent. MCT has been previously shown to go through interesterification and release 2 FFA’s from the sn-1 and sn-3 position. The products of triacylglycerol hydrolysis by gastric and pancreatic colipase-dependent lipases are FFA’s released from the sn-1,3 positions and sn-2 monoacylglycerols. This may be important because approximately 70% of the fatty acids absorbed as sn-2 monoacylglycerols are conserved in the original position during re-esterification to triacylglycerols in the intestinal cells (Small 1991). The 2-monglyceride is then able to be absorbed through the intestinal epithelial barrier. Thus, the ability to release >2 FFA’s seems unlikely. An alternative explanation for MCT’s results was that digestion products evolved during the digestion process are via breakdown of phospholipids and/or proteins. This can cause a drop in pH and results in increased volume of titrant added to the reaction vessel. However, more work needs to be done to verify if the high digestion extent is due to the broken down components or some other cause.

LCT:SCT mix (Fig. 8C) showed no statistical correlation that digestion extent was dependent on droplet concentration. The values for 1.5 and 2% were similar. This
trend was similar to MCT’s titration profile, which suggests that the lipase and/or bile concentrations used may be limiting factors for digestion. Of note is that the titration profile followed a similar slope and shape to tributyrin (Fig. 8D) in that digestion extent was almost wholly completed within 1 hour (3600 sec).

SCT (Fig. 8D) showed a dissimilar trend to LCT in that as droplet concentration increased, digestion extent was similar. At all three droplet concentrations tested, the titration profile was similar in slope and position. The SCT within the droplets cannot be said to be fully digested, but the digestion had come to near completion because the digestion extent did not increase appreciably after approximately 1 hour (3600 sec). This suggests that lipase had equal access to the oil droplets at all three droplet concentrations and that the lipase and/or bile concentration would have a limiting effect on the ability to hydrolyze oil droplets.

This *in vitro* digestion model was designed to simulate lipid digestion within the small intestine, where the majority of lipid digestion normally occurs, and is based on measurements of the amount of FFA released from lipids (usually triacylglycerols) after lipase addition at pH 7. The pH-stat reaction vessel contained components (lipase, bile, calcium) of digestive fluids that interact with the nanoemulsions and influence digestion rate and extent. In reality, ingested lipid droplets have to pass through the mouth and stomach before they reach the small intestine (McClements, Decker et al. 2009; Singh, Ye et al. 2009). Consequently, the physicochemical properties of the originally ingested lipid droplets may have been altered appreciably due to their interactions with other molecules in the mouth and stomach such as salts, acids, mucin, gastric lipase, and proteases. Gastric lipase initiates lipid digestion in the stomach, which results in the
partial digestion of triglyceride to diglyceride and fatty acid (Hamosh, Scanlon et al. 1981; Abrams, Hamosh et al. 1988). Although gastric lipolysis is a minor contributor to overall lipid digestion, it has been suggested to be responsible for up to 25% of acyl chain hydrolysis (Carriere, Barrowman et al. 1993; Renou, Carriere et al. 2001). The potential changes to emulsion structure during digestion can be seen in Figure 9 (Singh, Ye et al. 2009):

Figure 9: Diagram of possible changes in emulsions as they pass through the gastrointestinal tract.

These interactions were ignored in my study because I wished to focus on the physicochemical processes that occurred within the small intestine, where the majority of lipid digestion normally occurs.
Lipase is a surface-active protein that can compete for the oil-water interface with other surface-active components (Reis, Holmberg et al. 2008), such as the \(\beta\)-lactoglobulin initially coating the lipid droplets or the bile salts added to the reaction vessel. Two important factors which determine lipase actions are the affinity of the enzymes for the substrate droplets and the ability of the enzymes to hydrolyze the substrate lipid of the emulsion particle (Verger and Dehaas 1976). At low lipase concentrations, there may be insufficient lipase present to displace \(\beta\)-lactoglobulin from the oil-water interface. Thus, the enzyme cannot come into close contact with the lipid substrate within the lipid droplets. My model simulated the fasted state, so this could explain not achieving 100% digestion within the 2 hour timeframe.

The emulsion surfactant, \(\beta\)-lactoglobulin was used, is important to digestion because it reduces the free energy and interfacial tension of the system by adsorbing at the interface. The main thermodynamic driving force is the removal of hydrophobic groups from the aqueous phase by displacing water molecules from the interface (Singh, Ye et al. 2009). \(\beta\)-lactoglobulin, a whey protein, can adsorb to the interface and undergo unfolding and rearrangement to form a compact, stabilizing adsorbed layer (Dalgleish 1990).

Digestion is also dependent on the presence of bile salts because they can either inhibit or stimulate digestion based on certain environmental parameters. Bile has been shown to displace surface-active materials from the droplet interface, bind lipase to the interfacial layer, and accelerate lipid digestion (Gargouri, Julien et al. 1983; Ivanova, Panaiotov et al. 1990; Mun, Decker et al. 2005). In the absence of co-lipase, bile salts have been shown to inhibit the ability of pancreatic lipase to digest LCT and the
suggested mechanism of inhibition is bile-salt-mediated desorption of lipase from the oil-water interface (Vandermeers, Vandermeerspiret et al. 1976). In the presence of co-lipase, bile salts increase the initial rate of FFA release by most likely reducing emulsion droplet size, thereby increasing the surface area available for pancreatic lipase binding (Borgstro.B and Erlanson 1973).

Activity at the oil-water interface of emulsion droplets is a crucial factor in the digestion process. My *in vitro* digestion model incorporated bile, calcium, and lipase to reproduce conditions in the small intestine when a lipid-based food is consumed. With these various components, many conflicting activities may influence digestion. Calcium can activate lipase and removes FFA’s from the oil-water interface so lipase can attach. In this sense, it is similar to co-lipase in that can inhibit pancreatic lipase desorption from the oil-water interface by removing FFA’s and other digestion products. However, co-lipase, as a small protein, maintains the ability to anchor the lipase to the oil-water interface via binding to the interface itself (Lowe 1997). Bile can change the surface of the interface, thus allowing lipase to attach and it also removes poorly water-soluble lipolytic products from the oil-water interface by solubilization (Carey, Small et al. 1983; MacGregor, Embleton et al. 1997). In contrast, phospholipids have been shown to inhibit lipase activity at the interface (Larsson and Erlanson albertsson 1986). Some theorize that phospholipids can mix with bile salt to form mixed micelles that may act as an alternate binding site for co-lipase during digestion. This leads to reduced interfacial co-lipase concentrations and diminished pancreatic lipase binding at the oil-water interface (Patton and Carey 1981).
The rate and extent of lipid digestion was clearly higher when MCT was used as the lipid phase than when LCT was used. It has been previously shown that lipolytic digestion rates were substantially higher on MCT as compared to LCT emulsions (Deckelbaum, Hamilton et al. 1990). This effect can be attributed to the fact that the medium chain FFA digestion products arising from MCT have a higher dispersibility in aqueous media than the long chain FFA digestion products arising from LCT. The medium chain FFA’s produced during digestion of MCT are able to migrate rapidly into the surrounding aqueous phase and therefore do not inhibit the lipase reaction at the oil-water interface. The long chain fatty acids produced by LCT tend to accumulate at the oil-water interface and inhibit lipase activity until they are removed by being solubilized in mixed micelles or precipitated by calcium ions into calcium soaps. Nanoemulsions containing SCT can be digested quickly because tributyrin is water-soluble, which allows lipase to access to the oil-water interface easily. Also, the short-chain FFA digestion products can be solubilized or complexed with calcium away from the interface much easier than long-chain FFA digestion products because of their greater amphiphilic nature.

3.3.2 Lipase Effect on Digestion Extent

The proposed in vitro digestion model simulated digestion using a fasted state. Digestion components of the model included lipase, bile, and calcium. One would expect the rate of lipid digestion to increase as the lipase concentration increased because there would be more total enzyme present in the system to catalyze the conversion of triacylglycerols to free fatty acids (Reis, Holmberg et al. 2009). In addition, the amount of lipase present at the oil-water interface, where the lipolysis reaction occurs, would
increase as the lipase concentration increased. Figure 10 showed experiments that had lipase concentration as a function of droplet concentration and also at 1.6 mg/mL:

![Graph showing lipase effect on droplet concentration for LCT.](image)

**Figure 10: Lipase effect on droplet concentration for LCT.** Percentages without additional numbers have a lipase concentration of 1.6 mg/mL. Percentages with additional numbers have the additional number’s lipase concentration. Results with “*” were statistically different from samples with a lipase concentration of 1.6 mg/mL at the same droplet concentration. Results are reported as the average of at least two measurements on freshly prepared samples.

As can be seen in Figure 10, increasing lipase concentration resulted in increased digestion extent for LCT. All other factors (bile = 5 mg/mL; calcium = 20 mmoles) were held constant as lipase concentration was increased as a function of droplet concentration. Titration profiles for 1% droplet concentration were not included because their lipase concentration always stayed at 1.6 mg/mL. At higher lipase concentrations the adsorption and displacement processes occur rapidly, so that digestion can begin almost immediately after the enzyme was added to the reaction vessel. MCT (not pictured) had a similar trend to LCT in that initial digestion rate and digestion extent were increased as lipase concentration increased, but the differences were not statistically significant. These results suggest that at a higher lipase concentration, as would be seen in the fed state, digestion extent would increase. Thus, more work would need to be done with conditions...
mimicking the fed state. SCT and LCT:SCT mix did not have enough samples to warrant graphical representation.

### 3.3.3 Effect of Oil Type on Digestion

A comparison of oil types used for nanoemulsion digestion was needed to see similarities and/or differences in the titration profiles. Figure 11 shows the different oil types for 2% droplet concentration samples:

![Graph showing effect of oil type on digestion at set droplet concentrations.](image)

**Figure 11: Effect of oil type on digestion at set droplet concentrations.** All samples titrated with 5 mg/mL bile, 1.6 mg/mL lipase, and 20 mmoles calcium. Results with “*” were statistically different from LCT. Results are reported as the average of three measurements on freshly prepared samples.

Results in Figure 11 for 2% samples were similar at 1 and 1.5% samples (not pictured). However, for 1 and 1.5% droplet concentration only MCT was statistically different from LCT. SCT and LCT:SCT mix had greater digestion extent, but not in a statistically significant manner. First, MCT produced the highest digestion extent at >100% digestion for each droplet concentration. This suggests that MCT can be hydrolyzed to produce >2 FFA’s per triacylglycerol, or, more likely, assembled digestion products are formed from broken phospholipid and protein breakdown that resulted in a pH drop independent of the drop resulting from FFA release. Second, those nanoemulsions containing SCT in the droplet, either wholly or combined with LCT, had the fastest initial digestion rates. SCT,
a water-soluble oil, could also have a faster rate because the oil and digestion products were better solubilized in the continuous water phase. Lipase would have greater access to the oil-water interface because digestion products would not compete for the oil-water interface as greatly when all components are solubilized in the continuous phase. In all three droplet concentrations, LCT was digested to the least extent. This can be due to the released FFA’s and other digestion products not being able to be removed effectively from the oil-water interface, not being solubilized by the bile, or not being complexed with calcium ions into calcium soaps. It has been shown that long chain lipolytic products are limited to bile salt concentration and the solubilization capacity of the media, while medium chain lipolytic products were able to rapidly dissociate from the oil-water interface to form colloidal dispersions or precipitate as soaps (Sek, Porter et al. 2002).

It is important to note the initial digestion rate, in addition to final digestion extent. The initial digestion rate of the different oil nanoemulsions is seen in Figure 12:

![Figure 12: Effect of oil type on initial digestion rate. These curves are from the first 800 seconds of Figure 11. Results with “*” were statistically different from LCT samples.](image)

A more comprehensive breakdown of FFA release can be seen in Table 3:
<table>
<thead>
<tr>
<th>Oil Type</th>
<th>100% FFA Released (mmoles)</th>
<th>Initial FFA Released (mmoles)</th>
<th>Total FFA Released (mmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCT</td>
<td>1.99</td>
<td>1.49 ± 0.03</td>
<td>1.85 ± 0.08</td>
</tr>
<tr>
<td>MCT</td>
<td>1.15</td>
<td>0.65 ± 0.20</td>
<td>1.56 ± 0.11</td>
</tr>
<tr>
<td>LCT:SCT Mix</td>
<td>1.34</td>
<td>1.06 ± 0.08</td>
<td>1.33 ± 0.10</td>
</tr>
<tr>
<td>LCT</td>
<td>0.69</td>
<td>0.40 ± 0.02</td>
<td>0.63 ± 0.06</td>
</tr>
</tbody>
</table>

(A) 1% Droplet Concentration

<table>
<thead>
<tr>
<th>Oil Type</th>
<th>100% FFA Released (mmoles)</th>
<th>Initial FFA Released (mmoles)</th>
<th>Total FFA Released (mmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCT</td>
<td>2.98</td>
<td>2.26 ± 0.26</td>
<td>2.90 ± 0.44</td>
</tr>
<tr>
<td>MCT</td>
<td>1.73</td>
<td>0.93 ± 0.14</td>
<td>2.08 ± 0.10</td>
</tr>
<tr>
<td>LCT:SCT Mix</td>
<td>2.00</td>
<td>1.38 ± 0.01</td>
<td>1.82 ± 0.05</td>
</tr>
<tr>
<td>LCT</td>
<td>1.03</td>
<td>0.52 ± 0.04</td>
<td>0.83 ± 0.10</td>
</tr>
</tbody>
</table>

(B) 1.5% Droplet Concentration

<table>
<thead>
<tr>
<th>Oil Type</th>
<th>100% FFA Released (mmoles)</th>
<th>Initial FFA Released (mmoles)</th>
<th>Total FFA Released (mmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCT</td>
<td>3.97</td>
<td>2.71 ± 0.11</td>
<td>3.64 ± 0.33</td>
</tr>
<tr>
<td>MCT</td>
<td>2.31</td>
<td>1.13 ± 0.22</td>
<td>2.62 ± 0.13</td>
</tr>
<tr>
<td>LCT:SCT Mix</td>
<td>2.67</td>
<td>1.70 ± 0.08</td>
<td>2.38 ± 0.07</td>
</tr>
<tr>
<td>LCT</td>
<td>1.38</td>
<td>0.61 ± 0.05</td>
<td>1.83 ± 0.17</td>
</tr>
</tbody>
</table>

(C) 2% Droplet Concentration

Table 3: Effect of oil type on digestion rate and extent at set droplet concentrations (for microfluidized emulsions).

As can be seen in Figure 12, those nanoemulsions containing SCT, wholly or mixed with LCT, released FFA’s at a much faster rate (in a statistically significant manner) than nanoemulsions containing only LCT or MCT. These results were similar at all three droplet concentrations. The interfacial layer of SCT emulsions would be accessed by lipase at a faster rate because of the oil’s high amphiphilic nature. LCT had the slowest digestion rate suggesting that released, insoluble long chain FFA’s interfere with the oil:water interface for a longer extent than other oils, which inhibits the ability of lipase to hydrolyze the triacylglycerols.
In Table 3, column “100% FFA Released” is the theoretical amount of FFA’s that would be released (Equation 4) if 100% digestion was achieved. The “initial FFA released” column is the mmole value after 800 sec of titration time, while “total FFA released” is the mmole value after 7200 sec of titration time. The value of 800 sec was selected for calculating the initial digestion rate because the curve was still comparatively linear at that time point. Interesting results were seen for MCT because that oil was the only one whose initial FFA release was a value that was <50% of the total FFA released at each droplet concentration. This could be due to the type of MCT used, rather than an intrinsic property of MCT because medium chain lipolytic products have been previously shown to dissociate rapidly from the digestion interface to form colloidal dispersion or precipitated soaps as compared to LCT (Sek, Porter et al. 2002).

After in vitro digestion, the PSD of the produced digestion products was determined. This was done because, in vivo, it is the colloidal structures formed during digestion that become the actual carriers of lipophilic curcumin in the aqueous phase. These results can be seen in Table 4:
<table>
<thead>
<tr>
<th>Oil Type</th>
<th>Z-average (nm)-Filtered</th>
<th>PDI (nm)-Filtered</th>
<th>Z-average (nm)-Unfiltered</th>
<th>PDI (nm)-Unfiltered</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCT</td>
<td>155 ± 19</td>
<td>0.16 ± 0.07</td>
<td>99 ± 14</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>MCT</td>
<td>95 ± 16</td>
<td>0.36 ± 0</td>
<td>161 ± 39</td>
<td>0.51 ± 0.08</td>
</tr>
<tr>
<td>LCT:SCT Mix</td>
<td>42 ± 7</td>
<td>0.55 ± 0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>SCT</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

(A) 1% Droplet Concentration

<table>
<thead>
<tr>
<th>Oil Type</th>
<th>Z-average (nm)-Filtered</th>
<th>PDI (nm)-Filtered</th>
<th>Z-average (nm)-Unfiltered</th>
<th>PDI (nm)-Unfiltered</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCT</td>
<td>81 ± 15</td>
<td>0.17 ± 0.01</td>
<td>113 ± 5</td>
<td>0.37 ± 0.18</td>
</tr>
<tr>
<td>MCT</td>
<td>122 ± 18</td>
<td>0.22 ± 0.04</td>
<td>121 ± 13</td>
<td>0.20 ± 0.01</td>
</tr>
<tr>
<td>LCT:SCT Mix</td>
<td>56 ± 33</td>
<td>0.34 ± 0.19</td>
<td>131 ± 19</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>SCT</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

(B) 1.5% Droplet Concentration

<table>
<thead>
<tr>
<th>Oil Type</th>
<th>Z-average (nm)-Filtered</th>
<th>PDI (nm)-Filtered</th>
<th>Z-average (nm)-Unfiltered</th>
<th>PDI (nm)-Unfiltered</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCT</td>
<td>88 ± 19</td>
<td>0.17 ± 0.01</td>
<td>161 ± 10</td>
<td>0.42 ± 0.09</td>
</tr>
<tr>
<td>MCT</td>
<td>62 ± 39</td>
<td>0.21 ± 0.02</td>
<td>68 ± 1</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>LCT:SCT Mix</td>
<td>82 ± 19</td>
<td>0.28 ± 0.04</td>
<td>120 ± 13</td>
<td>0.29 ± 0</td>
</tr>
<tr>
<td>SCT</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

(C) 2% Droplet Concentration

Table 4: Effect of filtering post-digested aqueous phase on PSD (for microfluidized emulsions). Results are reported as the average of at least two measurements on freshly prepared samples.

Statistical analysis could not be performed on Table 4 because “n” values differed between 2 – 4 for filtered and unfiltered samples at the different oils. These differences were due to some samples not being categorized as “quality results” by the DLS computer program. Those samples were not recorded for use in Table 4 and new samples had to be prepared for analysis to make sure each sample (oil type; droplet concentration; filtered vs. unfiltered) had at least two values. As seen, LCT and LCT:SCT mix filtered samples showed a trend of particle size decreasing as droplet concentration increased. For
unfiltered samples, there was a trend between increasing droplet concentration and
decreasing particle size for MCT and the opposite trend for LCT. I hypothesize that most
of the digestion products were mixed bile salt micelles because the released FFA’s could
self-assemble into these structures during digestion. These colloidal structures formed
during lipid digestion prevent precipitation and enhance absorption of the drug. Other
structures include vesicles and liposomes because these products have previously been
shown to have a PSD in the 100 – 200 nm range (Olson, Hunt et al. 1979; Szoka, Olson
et al. 1980). Of note is that products with only SCT did not consistently have a size in the
low nm range. All attempts produced polydisperse readings that the instrument did not
quantify as “quality results” by the computer program. Explanations given were: particle
size was too high for proper measurement, sample was aggregating or sedimenting,
and/or sample size was too low. Thus, they were omitted from Table 4. This observation
could be due to SCT being prone to Ostwald ripening, which results in droplet instability
and growth (Li, Le Maux et al. 2009).

Some post-digested samples were filtered before measurement because there were
larger digestion products present that distorted the measurement due to the polydispersity
(case of all SCT samples). This was dependent on the samples themselves and the need
for filtering could only be identified after an initial run on the Zetasizer. Even then, some
filtered samples did not register as “quality results”. The products, due to their large µm
size, could be larger vesicles and/or liposomes that caused the isolated aqueous phase to
not be transparent. Only filtered and unfiltered samples that were considered “quality
results” by the computer program were included in Table 4. These samples ranged form
monomodal to trimodal distributions. As PDI increased, peak number increased, but there
was no definitive cut-off value seen for differentiating between monomodal, bimodal, and trimodal distributions. A general trend followed was that PDI values under 0.20 frequently generated monomodal distributions, PDI values between 0.20 – 0.60 were either bimodal or trimodal, and PDI values above 0.60 resulted in polymodal distributions that did not fit “quality results” for the instrument.

Attempts were made to obtain the zeta potential, surface charge distributions of particle droplets, of the post-digested aqueous phase. With surface active components such as bile, insights into how the charge distributions could be related to particle size or what factors affect digestion extent would be useful for modeling processes associated with digestion. However, no results were ever deemed “quality results” by the DLS computer program.

3.4 Conclusions

Nanoemulsions were subject to *in vitro* digestion and their digestion profiles were examined. Nanoemulsions containing MCT showed >100% digestion. This suggested that that MCT can release >2 FFA’s per triglyceride or, more likely, additional digestion products were formed by breakdown of phospholipids and/or proteins, which caused a drop in pH not associated with FFA release. This caused additional titrant to be added during digestion. More studies are needed to characterize formed digestion products and examine their role during *in vitro* digestion. A useful method is HPTLC, which can differentiate between mono-, di-, and tri-glycerides. Previous work has used this method to determine relative distributions of lipid digestion products throughout the digestion process (Sek, Porter et al. 2001; Sek, Porter et al. 2002).
When lipase was added as a droplet concentration dependent value to LCT and MCT, the digestion extent increased significantly. These results suggest the need for a study using the fed state. These could produce different titration profiles for LCT and MCT because more lipase, as well as more bile and calcium, is present in the fed state. This could also affect the bioavailability of curcumin after *in vitro* digestion because of the greater possibility of curcumin being encapsulated in mixed micelles and other digestion products after digestion. More studies need to be performed to see the effect of digestion parameters, fasted vs. fed state, affecting digestion rate, digestion extent, and bioavailability.

In a comparison study between oil types, corn oil was digested the least after 2 hours and this suggested that the long chain FFA’s released cannot be effectively removed from the oil-water interface by the concentrations of bile and calcium present. Thus, lipase faced more interference at the oil-water interface. Another explanation is that the long chain FFA’s were not solubilized as quickly as short and medium chain FFA’s, which affected lipase access to the oil-water interface of the emulsions.

On the effect of oil type on post-digestion particle size, statistical analysis could not be used due to varying “n” values. Some trends showed a decrease in particle size as droplet concentration increased. SCT did not have any values for a post-digestion micelle size that was not too polydisperse for proper measurements. This implied that short chain FFA’s were unable to effectively self-assemble into mixed bile salt micelles after *in vitro* digestion.
CHAPTER 4
DETERMINE BIOACCESSIBILITY OF CURCUMIN AFTER IN VITRO DIGESTION

4.1 Introduction

The term bioavailability is defined as the fraction of an ingested component that eventually ends up in the systemic circulation (Versantvoort, Oomen et al. 2005). The overall bioavailability ($F$) of poorly water-soluble lipophilic components depends on a number of physicochemical and physiological factors such as bioaccessibility, absorption, and metabolism. A more complete description of those processes was seen in Chapter 1.3.

There is growing interest in understanding and controlling the digestibility of emulsified lipids as they pass through the human gastrointestinal (GI) tract (Porter and Charman 2001; Pafumi, Lairon et al. 2002; Porter, Trevaskis et al. 2007; McClements, Decker et al. 2009; Singh, Ye et al. 2009). This knowledge is being used by the pharmaceutical industry to rationally design emulsion-based delivery systems to carry biologically active agents to specific locations within the GI tract and release them at a controlled rate (Pouton 2006; Porter and Wasan 2008). These delivery systems have been proposed as a means of increasing the bioavailability of highly lipophilic nutraceuticals or as a means of delivering functional food ingredients to specific locations within the GI tract.

Curcumin possesses low bioavailability, as only a small fraction of the administered dose reaches systemic circulation. Curcumin is only slightly absorbed into the gastrointestinal tract due to its poor solubility in water (Tonnesen, Masson et al.)
Curcumin also suffers from rapid degradation into several reduced products after oral dosing, which limits its bioavailability (Ireson, Orr et al. 2001). Emulsion-based delivery systems represent a promising avenue to increase bioavailability of curcumin because curcumin can be solubilized in the lipid phase before being homogenized with another liquid, usually water-based.

The objective of this chapter was to determine the percent bioaccessibility of curcumin from O/W nanoemulsions that had been subject to in vitro digestion.

4.2 Materials and Methods

4.2.1 Determination of Percent Bioaccessibility

After in vitro digestion, emulsions were centrifuged (12500 rpm, Sorvall RC 6C Plus, DuPont) at 25 °C for 30 min. Aliquots of 5 mL were taken from the aqueous phase, washed with 5 mL of chloroform, and centrifuged (1750 rpm, Fisher Scientific 225A, Fisher) at room temperature for 10 min. The bottom layer was collected, top layer was washed with 5 mL of chloroform, and centrifuged (1750 rpm) at room temperature for 10 min. The bottom layer was added to the previously set aside bottom layer, lightly mixed, and analyzed by UV-VIS spectrophotometry (wavelength dependent on oil type used in emulsion, Ultrospec 3000 Pro, GE) with chloroform used as the blank.

4.3 Results and Discussion

4.3.1 Steps leading to Bioaccessibility Determination

Equations 4 and 5 were used for determining percent bioaccessibility (%BIO) of the isolated aqueous phase:
\[
\text{Mass}_{\text{CurcFinal}} = \text{Mass}_{\text{CurcInitial}} \times \frac{\text{Mass}_{\text{OilEmulsion}}}{\text{Mass}_{\text{OilTotal}}} \times \frac{\text{Mass}_{\text{EmulsionTitrator}}}{\text{Volume}_{\text{Titrator}}} \times \frac{\text{Volume}_{\text{Aliquot}}}{\text{Volume}_{\text{Emulsion}}} \times 1000 \quad (4)
\]

\[
\% \text{Bioaccessibility} = \frac{\text{Mass}_{\text{CurcAliquot}}}{\text{Mass}_{\text{CurcFinal}}} \times 100\% \quad (5)
\]

Equation 4 determined the theoretical maximum mass, in mg, of curcumin present in the entire aqueous phase after \textit{in vitro} digestion (\text{Mass}_{\text{CurcFinal}}). The right side of the equation made up the dilutions that were done to get the final mass amount of curcumin theoretically present in the aqueous phase. The \text{Mass}_{\text{CurcInitial}} was the mass of curcumin initially solubilized in the oil, \text{Mass}_{\text{OilTotal}} was the total mass of oil containing solubilized curcumin, \text{Mass}_{\text{OilEmulsion}} was the mass of oil used to produce the 10\% O/W nanoemulsion from the initial oil containing curcumin, \text{Mass}_{\text{Emulsion}} was the total mass of the generated nanoemulsion, \text{Mass}_{\text{EmulsionTitrator}} was the mass of the sample used in an experiment in the \textit{in vitro} digestion model, \text{Volume}_{\text{Titrator}} was the initial volume of all components used in the \textit{in vitro} digestion model (37.5 mL), and \text{Volume}_{\text{Aliquot}} was the volume of the aliquot of post-digested solution taken out and used for further studies (35 mL). Equation 5 determined the mass of curcumin present in the isolated aqueous phase (\text{Mass}_{\text{CurcAliquot}}), which was then calculated into \%\text{BIO}.

The \text{Mass}_{\text{CurcAliquot}} and subsequent \%\text{BIO}, was determined from Figure 13:
Figure 13: Standard curve of total curcumin in aqueous phase. This is a composite of three standard curves of curcumin dissolved in chloroform and formatted to correlate UV-VIS absorption to mass of curcumin in the isolated aqueous phase. The equation is $y = 1.9218x - 0.0023$. The $R^2$ value = 0.9926.

The aqueous phase was isolated after centrifugation of the post-digested solution. Figure 14 shows the separated layers and theoretical make-up of these layers:

(A) LCT  
(B) MCT

(Continued on next page)
Figure 14: Super-speed centrifugation of post-digestion solution. At left is the post-digested sample of LCT (A). At right is the post-digested sample of MCT (B). At bottom is the theoretical composition of post-digested solution after ultra-centrifugation (C), as taken from (Porter, Trevaskis et al. 2007).

As suggested by Fig. 14C, the pellet at the bottom of the tube consisted of precipitated curcumin and calcium complexed with oil (calcium soaps). The lower liquid phase (overall middle phase) was the aqueous phase I isolated to determine %BIO of curcumin. It most likely consisted of mixed micelles with curcumin as well as larger digestion products, vesicles and/or liposomes, which caused the phase to look translucent instead of transparent. Based on DLS measurements, these other products could be liposomes or vesicles because these products have previously been shown to have a PSD in the 100 – 200 nm range (Olson, Hunt et al. 1979; Szoka, Olson et al. 1980). It should be noted that the upper liquid (overall upper) phase was only seen for LCT (Fig. 14A). Previous work showed that LCT was not able to be digested fully, so the undigested oil that still contained curcumin could be seen at the top. The MCT sample (Fig. 14B) did not have an undigested oil layer, as its titration profile suggested complete digestion of the oil phase.
It must be noted that the color of the aqueous phases for the four oil formulations was different. Thus, oil type affected the composition of micelles in the aqueous phase and may be related to each oil producing different extents of digestion. For example, the SCT aqueous phase was colored, but the %BIO was close to zero percent. Even though SCT was hydrolyzed to a large extent, there seemed to be little curcumin in micelles or other colloidal structures. LCT, on the other hand, had less digestion extent and an appreciable amount of curcumin was solubilized in micelles. These results suggested that colloidal structure compositions were dependent of fatty acid chain length. More work needs to be done to elucidate these findings.

Figure 15 shows the emulsion at various stages of the experiment to illustrate how %BIO was calculated:

**Figure 15: Different stages of experiment to calculate %BIO (for microfluidized emulsions).** Far left is the stock emulsion (1.5% MCT) with 0.15% curcumin, middle left is the post-digested solution after *in vitro* digestion, middle right is the isolated aqueous phase after super-speed centrifugation, and far right is the chloroform with curcumin measured by UV-VIS (bottom layer).

The far right picture in Fig. 15 was the first wash of chloroform (5 mL) on the isolated aqueous phase (middle right of Fig. 15). The bottom layer was collected and a second wash of 5 mL of chloroform was done to obtain residual curcumin in the aqueous phase.
that was not obtained from the first wash. Rough estimates (data not shown) showed that approximately 85 - 90% of the final amount of obtained curcumin was in the first wash, while the other approximate 10 - 15% was obtained in the second wash. It must be noted that these percentages relate to the amount of curcumin that was actually collected (%BIO) and not the total amount of curcumin that was in the system (MassCurcFinal). Thus, two washes of chloroform were necessary for getting an accurate assessment of curcumin present in the aqueous phase. An interesting difference between the centrifuged samples is seen in Figure 10:

Figure 16: Post-centrifugation of first wash of 1.5% LCT with chloroform.
The sample in Figure 16 was LCT and no other oil used produced the three layers seen when centrifuging after chloroform addition. The precipitate layer was found at all three droplet concentrations. Other oils produced a thin, delicate layer of precipitate between the bottom chloroform and upper aqueous layer. The bottom layer contained dissolved curcumin in chloroform, while the upper layer contained other solubilized digestion products. I hypothesized that the middle layer in LCT samples consisted of undigested oil and precipitated digestion products such as mono- and di-glycerides that did not form larger micellar structures. Further analysis is needed to determine the identity and ratio of
contents within this precipitate layer. A useful method is HPTLC, which can differentiate between mono-, di-, and tri-glycerides. Previous work has used this method to determine relative distributions of lipid digestion products throughout the digestion process (Sek, Porter et al. 2001; Sek, Porter et al. 2002).

4.3.2 Bioaccessibility Determination

The %BIO of curcumin after in vitro digestion is seen in Table 5:
Table 5: Effect of oil type on %BIO (for microfluidized emulsions). Results with “*” were statistically different from 1% samples. Results are reported as the average of three measurements on freshly prepared samples.

As can be seen in Table 5 (A – C), LCT, MCT, and LCT:SCT mix all had increasing %BIO as droplet concentration increased. This suggests that increasing available oil for lipophilic compounds increases the likelihood of forming mixed phospholipid/bile salt micelles that incorporate curcumin during digestion. SCT (Table 4D) did not show the above trend. An explanation could be that short chain FFA’s are water soluble and cannot
be expected to interact with, or form, mixed bile salt micelles (Fatouros and Mullertz 2008). This would result in digestion products that could not be considered mixed micelles, vesicles, and/or liposomes. They would be continually being aggregated or broken up. The PSD data results for SCT (Table 4) may be due to this phenomenon. During super-speed centrifugation, almost all free curcumin in solution would then be in the pellet phase with the calcium soaps and undigested tributyrin because free curcumin’s solubility in water is low. LCT:SCT mix (Table 4C) showed better results for bioaccessibility compared to SCT because LCT was incorporated. The long chain FFA’s would be better able to incorporate curcumin into stable mixed micelles or micellar structures because of the larger solubilization capacity of long chain FFA’s.

More work needs to be done to identify curcumin concentration in the two – three layers (dependent on oil type; Fig. 14C) immediately following super-speed centrifugation because then one would be able to see the distribution of curcumin within the sample. Initial attempts proved difficult because the top, undigested oil layer in LCT was also stuck to the sides of the centrifuge tube and totally isolating the pellet phase was not feasible because the solid would stick to the bottom and sides of the centrifuge tube.

4.3.3 Theoretical Bioaccessibility Maximum

MCT (Table 5B) had reached a maximum bioaccessibility of curcumin because the lipid has been fully digested, according to Equation 3. Comparing MCT to LCT (Table 5A) yielded interesting results because the bioaccessibility was higher for LCT at 1 and 1.5% droplet concentration, but higher for MCT at 2%. Previous work has shown that, at low lipid concentrations, effective drug solubilization is possible with LCT, but the use of MCT can lead to drug precipitation (Porter, Kaukonen et al. 2004). However, a
further look was needed because not all of the LCT had been digested. If one assumes that the %BIO of curcumin is proportional to the amount of lipid digested, one can estimate the theoretical maximum %BIO of the system. Taking the average percent bioaccessibility of LCT currently and multiplying it by a factor that accounted for complete digestion results in Equation 6:

\[
\% B_A = \% B_i \times \frac{100\%}{\% D_i}
\]  

(6)

Here, \(\% B_A\) is the adjusted %BIO, \(\% B_i\) is the initial %BIO, and \(\% D_i\) is the initial percent lipid digested in the 2 hour timeframe (right hand column of Table 5A). MCT was not subject to this equation, i.e. average values did not change, because >100% lipid had been digested. Calculations are seen in Table 6:

<table>
<thead>
<tr>
<th>Droplet concentration (%)</th>
<th>LCT (%)</th>
<th>MCT (%)</th>
<th>LCT + SCT mix (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>1.5</td>
<td>50</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>58</td>
<td>22</td>
</tr>
</tbody>
</table>

Table 6: Theoretical %BIO if total digestion is achieved (for microfluidized emulsions). These do not take standard deviation into account, just average values.

As can be seen in Table 6, there seemed to be a limit to the bioaccessibility of curcumin and it was independent of oil type (results for LCT and MCT). Titrations were run simulating fasted conditions, so there may not be enough bile, lipase, and/or calcium to increase the number of mixed micelles and other digestion products available that contain curcumin. Thus, more work needs to be done simulating fed conditions to see if an increase in available bile salts overcomes this apparent %BIO threshold of my system.
4.4 Conclusions

The %BIO of nanoemulsions subject to *in vitro* digestion was determined. For LCT, MCT, and LCT:SCT mix, an increase in droplet concentration resulted in an increase of %BIO. For SCT, there was low %BIO at each droplet concentration and this can be attributed to short chain FFA’s not being able to assemble into stable micellar structures during digestion. MCT produced the highest overall %BIO at 2% droplet concentration. Under fasted conditions, there theoretically appears to be a maximum of bioaccessibility at approximately 60%, as seen in Table 6. More studies need to be done that simulate fed conditions to see if more than approximately 60% of the curcumin is bioaccessible. Also, further analysis is needed to determine the identity and ratio of contents within the isolated aqueous phase after *in vitro* digestion. This information can then be used to predict lipid behavior during digestion and its effectiveness at creating carrier digestion products for curcumin.
CHAPTER 5

COMPARE CURCUMIN ENCAPSULATED O/W NANOEMULSIONS TO CONVENTIONAL EMULSIONS

5.1 Introduction

Curcumin is a highly lipophilic, poorly water-soluble bioactive component. Emulsion-based delivery systems have been proposed as a means of increasing the bioavailability of highly lipophilic nutraceuticals or as a means of delivering functional food ingredients to specific locations within the GI. To investigate their potential benefits, the emulsion-based delivery systems should be compared to other delivery systems of curcumin.

Previous studies have compared curcumin-encapsulated delivery systems to free curcumin in water in order to show potential benefits of the proposed curcumin-encapsulated delivery system (Bisht, Feldmann et al. 2007; Wang, Jiang et al. 2008; Mukerjee and Vishwanatha 2009; Yu and Huang 2010). However, the maximum solubility of curcumin in water is 11 ng/mL (Tonnesen, Masson et al. 2002). Also, there is no surfactant in the system, so free curcumin in water is not a comparable delivery system to use.

A more appropriate delivery system for comparison is a conventional emulsion because lipophilic curcumin can be solubilized greater in the dispersed oil phase and the presence of surfactant allows the dispersed phase to be maintained in the continuous water phase. Main differences between conventional emulsions and nanoemulsions include droplet size and creaming stability. Nanoemulsions have been produced with droplet sizes from 50-200 nm and conventional emulsions have a size range from 1-100
The objective of this chapter is to compare nanoemulsions to conventional emulsions in the parameters of digestion rate, digestion extent, PSD of aqueous phase, and %BIO to see if one delivery system was superior to the other.

5.2 Materials and Methods

5.2.1 Formulation and Characterization of Conventional Emulsions

Curcumin-loaded O/W conventional emulsions were prepared in a similar fashion to the O/W nanoemulsions, except that they were not subject to the high-pressure homogenization technique after initial blending.

The O/W conventional emulsions were measured using static light scattering (SLS) (Mastersizer S, Malvern Instruments, Worcestershire, U.K.). The volume moment mean ($D_{4,3}$) and surface area moment mean ($D_{3,2}$) were obtained. The emulsions were diluted prior to analysis using buffer solution (5mM phosphate, pH 7.0) as per instrument specifications.
5.2.2 In Vitro Digestion of Conventional Emulsions

The O/W conventional emulsions were subject to the in vitro digestion model and the post-digested solution was analyzed by UV-VIS spectrophotometry for %BIO as prescribed in the methods for Chapters 3 and 4.

5.3 Results and Discussion

5.3.1 Conventional Emulsion Characterization

The droplet sizes for the conventional emulsions are shown in Table 7:

<table>
<thead>
<tr>
<th>Oil Type</th>
<th>Volume Moment Mean (µm)</th>
<th>Surface Area Moment Mean (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCT</td>
<td>20 ± 11</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>MCT</td>
<td>22 ± 6</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>LCT:SCT Mix</td>
<td>19 ± 5</td>
<td>10 ± 6</td>
</tr>
<tr>
<td>SCT</td>
<td>14 ± 2</td>
<td>12 ± 2</td>
</tr>
</tbody>
</table>

Table 7: Conventional emulsion SLS mean values. Results are reported as the average of three measurements on freshly prepared samples.

Graphical representations of results used to produce values in Table 7 are shown in Figure 17:
Figure 17: SLS graph of 0.15% curcumin conventional emulsion.

The droplet sizes for the conventional emulsions were in the µm range, while droplet sizes for the nanoemulsions were in the nm range. This was a result of nanoemulsions being subject to a dual high-speed and high-pressure homogenization technique, while conventional emulsions were subject only to high-speed homogenization. Two means were presented because the volume moment mean (De Brouckere Mean Diameter) represented the size distribution of the droplet population, while the surface area moment mean (Sauter Mean Diameter) represented the mean diameter of the particles. In literature, these are two common means to present. These means are similar to moments of inertia and indicate the central point of the frequency around which the surface area or
volume/mass distribution would rotate. In effect, they are centers of gravity of the respective distributions (Berchane, Carson et al. 2007). A large standard deviation of the volume and surface area moment means would signify a larger distribution of particles at differing sizes.

As seen in Table 7, the trend between values for volume moment and surface area moment mean were only similar for SCT. This suggested that the blending homogenization process produced many particles with a mean diameter of approximately 12 µm and a relatively small peak width (Fig. 17A). The disparate relationship between values for the other oils (much smaller surface area moment means than volume moment means) suggested that while the majority of particles were smaller than the volume moment mean, there were also particles with larger sizes than the volume moment means or the width of the peak(s) would be sizeable as compared to the average size value (Fig. 17B). Thus, blending homogenization would not have been an appropriate homogenization process if a small distribution of evenly sized particles was wanted.

5.3.2 Conventional vs. Nanoemulsion-Digestion

It would be prudent to note differences in titration profiles between the two types of emulsions to see if digestion extent is proportional to initial droplet size. Titration profiles for the conventional emulsions and their comparisons to the nanoemulsions are seen in Figure 12:
Figure 18: Effect of emulsion preparation method on digestion extent. The top graph is that of the conventional emulsion, while the middle and bottom graphs are both the conventional and nanoemulsion. For 18B and 18C, droplet concentrations with “-N” are those made by microfluidization (nanoemulsion) and those with “-C” are made via blending only (conventional). Results with “*” were statistically different from the 1% sample in 18A and corresponding nanoemulsion in 18C. Results are reported as the average of three measurements on freshly prepared samples.
For MCT (Fig. 18A), digestion at 2% droplet concentration of the conventional emulsion was significantly greater than 1% digestion. This difference was also seen in LCT (not shown). For MCT (Fig. 18B), digestion extent for conventional emulsions was greater than the nanoemulsion, but not in a statistically significant manner. For SCT (Fig. 18C), digestion at 1% droplet concentration of the conventional emulsion was significantly greater than the nanoemulsion. For LCT:SCT mix (not shown), digestion at 2% droplet concentration of the conventional emulsion was significantly greater than the nanoemulsion. No results were seen that had significantly greater digestion with nanoemulsions as compared to conventional emulsions.

These results were interesting because the nanoemulsions’ smaller droplet sizes have a higher surface area to volume ratio and lipase would then have greater access to the oil:water interface for the nanoemulsions. Also, with smaller particle size, nanoemulsions would have a higher kinetic activity that would allow the oil:water interfaces to be less prone to being shielded by released FFA’s. Thus, digestion extent should be more complete for the nanoemulsions as compared to conventional emulsions. However, this was not seen. An alternative explanation is that pancreatic lipase would have the same affinity for the conventional emulsion droplets because, component-wise, they were the same as nanoemulsions. Thus, differences in digestion extent would be minimal. Previous work has suggested that product performance is poorly related to initial particle size of a dispersed formulation and instead is correlated more with the patterns of solubilization obtained after dispersion and digestion of the formulation in simulated intestinal fluid (Porter, Kaukonen et al. 2004; Sek, Boyd et al. 2006).
It is important to note the initial digestion rate, in addition to final digestion extent. The initial digestion rate of the different oil conventional emulsions is seen in Figure 19:

(A) Conventional Emulsion

(B) Conventional Emulsion vs. Nanoemulsion

**Figure 19: Effect of emulsion preparation method on digestion rate at 1% droplet concentration.** For 19B, droplet concentrations with “-N” are those made by microfluidization (nanoemulsion) and those with “-C” are made via blending only (conventional). Results with “*” were statistically different from LCT (Fig. 19A) and corresponding nanoemulsion (Fig. 19B). Results are reported as the average of three measurements on freshly prepared samples.

As seen in Figure 19, initial digestion rates of the conventional emulsions mirrored those of the nanoemulsions (Fig. 12) in that emulsions containing SCT, either wholly or mixed with LCT, had a significantly higher initial digestion rate than emulsions containing only
LCT or MCT. The lower graph in Figure 19 compared titration profiles of nanoemulsions (Fig. 8) to conventional emulsions. In all three droplet concentrations, conventional emulsions initially digested at a faster rate than nanoemulsions for every different oil type. However, statistical significance was only achieved for SCT at 1%, MCT at 2%, and LCT:SCT mix at 2% droplet concentrations. One explanation is that nanoemulsions, being of such a small size and having a higher kinetic activity than conventional emulsions, are more dispersed throughout the titration sample. Conventional emulsions are more prone to gravitational separation than nanoemulsions because the homogenization technique used to create conventional emulsions is not as rigorous. Within a short period of time, the oil phase can partially separate from the water phase and collect near the top of the reaction vessel. Also, conventional emulsions were prepared by blending in a vessel where the oil phase was wholly on top of the water phase before blending. Some of the oil droplets may have stayed towards the top of the reaction vessel and not be as dispersed in the continuous phase due to blending limitations of the handheld homogenizer. In contrast, nanoemulsions prepared with a microfluidizer were more completely mixed because the initially blended oil and water phase traveled a path through a series of small tubes where homogenization occurred. Upon addition of lipase, to the top of the reaction vessel, more conventional emulsion droplets may be hydrolyzed as opposed to the nanoemulsions dispersed throughout the sample. However, this seems unlikely as the reaction vessel had a stirrer to keep the solution in motion, which would not have been a deterrent to lipase being able to access emulsion droplets of conventional or nanoemulsions. A difference in titrant concentration
(0.50M NaOH) also seems unlikely because titrant solution was made every few weeks and showed no precipitation during use.

A more comprehensive breakdown of FFA release of conventional emulsions can be seen in Table 8:

<table>
<thead>
<tr>
<th>Oil Type</th>
<th>100% FFA Released (mmoles)</th>
<th>Initial FFA Released (mmoles)</th>
<th>Total FFA Released (mmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCT</td>
<td>1.99</td>
<td>1.79 ± 0.07</td>
<td>2.18 ± 0.12</td>
</tr>
<tr>
<td>MCT</td>
<td>1.15</td>
<td>0.83 ± 0.23</td>
<td>1.60 ± 0.11</td>
</tr>
<tr>
<td>LCT:SCT Mix</td>
<td>1.34</td>
<td>1.18 ± 0.12</td>
<td>1.39 ± 0.05</td>
</tr>
<tr>
<td>LCT</td>
<td>0.69</td>
<td>0.42 ± 0.04</td>
<td>0.68 ± 0.11</td>
</tr>
</tbody>
</table>

(A) 1% Droplet Concentration

<table>
<thead>
<tr>
<th>Oil Type</th>
<th>100% FFA Released (mmoles)</th>
<th>Initial FFA Released (mmoles)</th>
<th>Total FFA Released (mmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCT</td>
<td>2.98</td>
<td>2.50 ± 0.02</td>
<td>3.26 ± 0.09</td>
</tr>
<tr>
<td>MCT</td>
<td>1.73</td>
<td>1.39 ± 0.15</td>
<td>2.28 ± 0.22</td>
</tr>
<tr>
<td>LCT:SCT Mix</td>
<td>2.00</td>
<td>1.69 ± 0.12</td>
<td>2.07 ± 0.10</td>
</tr>
<tr>
<td>LCT</td>
<td>1.03</td>
<td>0.55 ± 0.06</td>
<td>0.90 ± 0.08</td>
</tr>
</tbody>
</table>

(B) 1.5% Droplet Concentration

<table>
<thead>
<tr>
<th>Oil Type</th>
<th>100% FFA Released (mmoles)</th>
<th>Initial FFA Released (mmoles)</th>
<th>Total FFA Released (mmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCT</td>
<td>3.97</td>
<td>3.04 ± 0.02</td>
<td>4.24 ± 0.15</td>
</tr>
<tr>
<td>MCT</td>
<td>2.31</td>
<td>1.73 ± 0.34</td>
<td>2.85 ± 0.19</td>
</tr>
<tr>
<td>LCT:SCT Mix</td>
<td>2.67</td>
<td>2.07 ± 0.13</td>
<td>2.75 ± 0.10</td>
</tr>
<tr>
<td>LCT</td>
<td>1.38</td>
<td>0.67 ± 0.09</td>
<td>1.00 ± 0.14</td>
</tr>
</tbody>
</table>

(C) 2% Droplet Concentration

Table 8: Effect of oil type on digestion rate and extent at set droplet concentration for conventional emulsions.

In Table 8, column “100% FFA Released” is the theoretical amount of FFA’s that would be released (Equation 3) if 100% digestion was achieved. The “initial FFA released” column is the mmole value after 800 sec of titration time, while “total FFA released” is the mmole value after 7200 sec of titration time. The value of 800 sec was selected for
calculating the initial digestion rate because the curve was still comparatively linear at that time point. For all oils at each tested droplet concentration, at least 50% of digestion was achieved during the first 800 sec. These results were similar to microfluidized emulsions (Table 3).

### 5.3.3 Conventional vs. Nanoemulsion-Bioaccessibility

The %BIO of curcumin after *in vitro* digestion is seen in Table 9:

<table>
<thead>
<tr>
<th>Droplet concentration (%)</th>
<th>LCT (%)</th>
<th>Lipid Digested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38 ± 22</td>
<td>98 ± 16</td>
</tr>
<tr>
<td>1.5</td>
<td>56 ± 9</td>
<td>88 ± 8</td>
</tr>
<tr>
<td>2</td>
<td>50 ± 10</td>
<td>73 ± 10</td>
</tr>
</tbody>
</table>

(A) LCT

<table>
<thead>
<tr>
<th>Droplet concentration (%)</th>
<th>MCT (%)</th>
<th>Lipid Digested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3 ± 0</td>
<td>139 ± 9</td>
</tr>
<tr>
<td>1.5</td>
<td>17 ± 6</td>
<td>132 ± 13</td>
</tr>
<tr>
<td>2</td>
<td>59 ± 5*</td>
<td>123 ± 8</td>
</tr>
</tbody>
</table>

(B) MCT

<table>
<thead>
<tr>
<th>Droplet concentration (%)</th>
<th>Corn Oil and Tributyrin (LCT &amp; SCT) (%)</th>
<th>Lipid Digested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 ± 3</td>
<td>104 ± 4</td>
</tr>
<tr>
<td>1.5</td>
<td>20 ± 6*</td>
<td>103 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>45 ± 7*</td>
<td>103 ± 4</td>
</tr>
</tbody>
</table>

(C) LCT:SCT mix

<table>
<thead>
<tr>
<th>Droplet concentration (%)</th>
<th>SCT (%)</th>
<th>Lipid Digested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0 ± 0</td>
<td>110 ± 6</td>
</tr>
<tr>
<td>1.5</td>
<td>0 ± 0</td>
<td>110 ± 3</td>
</tr>
<tr>
<td>2</td>
<td>0 ± 0</td>
<td>107 ± 4</td>
</tr>
</tbody>
</table>

(D) SCT

**Table 9: Effect of oil type on %BIO for conventional emulsions.** Results with “*” were statistically different from 1% samples. Results are reported as the average of three measurements on freshly prepared samples.
For MCT and LCT:SCT mix, an increase in droplet concentration corresponded to an
significant increase in %BIO. For LCT, the 1.5% droplet concentration produced the
highest %BIO, but the 2% droplet concentration value was similar. However, it must be
noted that digestion extent for LCT decreased as droplet concentration increased, which
would explain the similar %BIO values for LCT. If one assumes that the %BIO of
curcumin is proportional to the amount of lipid digested, one can estimate the theoretical
maximum %BIO of the system. If Equation 6 was applied to the LCT results to account
for 100% digestion, those results are seen in Table 10:

<table>
<thead>
<tr>
<th>Droplet concentration (%)</th>
<th>MCT (%)</th>
<th>LCT (%)</th>
<th>LCT + SCT mix (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>39</td>
<td>5</td>
</tr>
<tr>
<td>1.5</td>
<td>17</td>
<td>64</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>59</td>
<td>68</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 10: Theoretical %BIO if total digestion is achieved. These do not take standard
deviation into account, just average values.

As seen in Table 10, there was a maximum theoretical %BIO of LCT at 68%. For the
nanoemulsions (Table 6), there was a maximum value of 60%. Titrations were run
simulating fasted conditions, so there may not be enough bile, lipase, and/or calcium to
increase the number of carrier digestion products containing curcumin. With these
conditions there could be a maximum amount of mixed bile salt micelles that could form,
thus, limiting the amount of curcumin solubilized in the aqueous phase. Thus, more work
needs to be done simulating fed conditions to see if there is a higher %BIO attainable.

The results for Table 9 need to be compared to those of the nanoemulsions (Table
5) to see which emulsion produced a greater percent bioaccessibility. The percent
bioaccessibility of the conventional vs. nanoemulsions is seen in Table 11:
<table>
<thead>
<tr>
<th>Droplet concentration (%)</th>
<th>Nanoemulsion (%)</th>
<th>Conventional Emulsion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20 ± 10</td>
<td>38 ± 22</td>
</tr>
<tr>
<td>1.5</td>
<td>40 ± 6</td>
<td>56 ± 9</td>
</tr>
<tr>
<td>2</td>
<td>41 ± 4</td>
<td>50 ± 10</td>
</tr>
</tbody>
</table>

(A) LCT

<table>
<thead>
<tr>
<th>Droplet concentration (%)</th>
<th>Nanoemulsion (%)</th>
<th>Conventional Emulsion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8 ± 1*</td>
<td>3 ± 0</td>
</tr>
<tr>
<td>1.5</td>
<td>20 ± 7</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>2</td>
<td>58 ± 6</td>
<td>59 ± 5</td>
</tr>
</tbody>
</table>

(B) MCT

<table>
<thead>
<tr>
<th>Droplet concentration (%)</th>
<th>Nanoemulsion (%)</th>
<th>Conventional Emulsion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3 ± 1</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>1.5</td>
<td>9 ± 3</td>
<td>20 ± 6*</td>
</tr>
<tr>
<td>2</td>
<td>20 ± 3</td>
<td>45 ± 7*</td>
</tr>
</tbody>
</table>

(C) LCT:SCT mix

<table>
<thead>
<tr>
<th>Droplet concentration (%)</th>
<th>Nanoemulsion (%)</th>
<th>Conventional Emulsion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>1.5</td>
<td>1 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>2</td>
<td>1± 1</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

(D) SCT

Table 11: Effect of emulsion preparation method on %BIO at set droplet concentrations. Results with “*” were statistically different from their corresponding emulsion preparation at set droplet concentration. Results are reported as the average of three measurements on freshly prepared samples.

With statistical significance, LCT:SCT mix showed greater %BIO for the conventional emulsion at droplet concentrations of 1.5 and 2%. MCT showed significantly greater %BIO for the nanoemulsion at 1%. For SCT, all three droplet concentrations were similarly low and suggest that short chain FFA’s cannot self-assemble into stable micelles.

These results implied that nanoemulsions were not significantly better delivery systems for curcumin than conventional emulsions. However, emulsion stability is an
issue that favors the use of nanoemulsions. Conventional emulsions creamed within hours, while nanoemulsions were much more stable to creaming. Conventional emulsions needed to be made fresh everyday. The stability difference is seen in Figure 20:

![Figure 20: Stability difference between nanoemulsions vs. conventional emulsions. At left is a conventional emulsion after 4 hours. At right is a nanoemulsion after 4 hours.](image)

LCT and MCT nanoemulsions were kept in refrigeration for up to 10 days (duration of emulsion use) without any noticeable phase separation or curcumin fallout observed. LCT:SCT mix nanoemulsions were kept in refrigeration for up to 3 days (duration of emulsion use) without any noticeable phase separation or curcumin fallout observed. SCT nanoemulsions showed separation within hours and need to be made fresh everyday. As stated before, further experiments could be done to test the instability mechanism and determine the maximum storage life of these emulsions at 4 °C.

5.4 Conclusions

As compared to conventional emulsions, nanoemulsions were not significantly better delivery systems for curcumin. However, nanoemulsions are more stable than conventional emulsions in storage and may present a better alternative in industry to improve curcumin bioaccessibility and eventual bioavailability.
CHAPTER 6
ONGOING WORK

6.1 Introduction

As seen in my results, there seemed to be a maximum theoretical %BIO of approximately 60% for nanoemulsions and 68% for conventional emulsions. This could be due to starting conditions that mimicked the fasted state instead of the fed state. Thus, more work needs to be done with conditions resembling the fed state as a means of comparison to my previous work.

My previous work with emulsions shows, often, an increase in droplet concentration correlating to an increase in %BIO of curcumin. However, some may argue that a different approach is required to investigate %BIO because the amount (mass) of curcumin in each droplet concentration was not the same. My previous stock emulsions used for titration samples were 10% O/W, so diluting to a sample at 2% droplet concentration would have twice as much curcumin as a 1% sample. Thus, new experiments have been performed that keep the amount of curcumin the same and the additional droplet concentration is supplemented by an O/W emulsion of the same oil without curcumin.

By its definition in Equation 1, bioavailability is comprised of bioaccessibility, metabolism, and absorption. My work has shown the bioaccessibility of curcumin in emulsions that are subject to in vitro digestion. To be able to effectively predict eventual bioavailability, a common tool used is the Caco-2 cell line. Caco-2 cell models have been used for predicting eventual bioavailability because mature, differentiated cells have the same characteristics as enterocyte cells in the small intestine. Uptake experiments with
Caco-2 cells are commonly used for determining eventual concentration of lipophilic drugs.

6.2 Materials and Methods

6.2.1 Fed State Conditions

To simulate the fed state, a similar procedure to the \textit{in vitro} digestion model of Chapter 3 was used with different concentrations of bile and lipase. In the simulated fed state, 4.0 mL of bile extract solution containing 750 mg of bile extract dissolved in PBS (pH 7.0) and 1.0 mL of CaCl$_2$ solution containing 110 mg of CaCl$_2$ dissolved in PBS (pH 7.0) was added into the 30 mL emulsion under stirring. The resultant mixture was then adjusted to pH 7. Finally, 2.5 mL of freshly prepared pancreatin suspension containing 90 mg of lipase dissolved in phosphate buffer (pH 7.0) was added to the mixture. The bile solution and lipase solution concentrations were 20 mg/mL and 2.4 mg/mL respectively. At this point, the pH-stat was started. The pH-stat (Metrohm, USA Inc.) was used to monitor and control the pH (at pH 7) of the digestion solution. The volume of added NaOH solution (0.50M) reflected the amount of free fatty acids generated by lipolysis of the initial triacylglycerols. Experiments ran for 2 hours.

6.2.2 Alternative Method to Determine Bioaccessibility

The different approach to determining %BIO of curcumin was only slightly different than my procedure outlined in Chapter 4. Emulsions were created by the Sonic Dismembrator 500 (Fisher Scientific) by pulsing for 12 min and amplitude of 70%. The pulse rate alternated between on and off every 0.5 sec. For the \textit{in vitro} digestion model, curcumin-containing stock emulsion (10% O/W) was added up to 1%. For samples at 1.5
and 2%, the remaining lipid was supplemented with diluted 10% O/W emulsion without curcumin diluted to the extra droplet concentration. After \textit{in vitro} digestion, the method described in Chapter 4 was used to determine \%BIO.

Aliquots were taken from the supernatant and were left unfiltered. The mean particle size (z-average) and width (PDI) of the post-digested aqueous phase was measured using dynamic light scattering (Zetasizer Nano-ZS, Malvern Instruments, Worcestershire, U.K.), with each individual measurement being the average of 12 runs. The emulsions were diluted prior to analysis using buffer solution (5mM phosphate, pH 7.0) to avoid the effects of multiple scattering.

6.2.3 Caco-2 Cell Culture

The human colon adenocarcinoma cell line Caco-2 was obtained from American Type Cell Collection (ATCC, Manassas, VA, USA). They were cultured in Dulbecco’s modified eagle medium (DMEM) containing glucose, L-glutamine, and sodium pyruvate, supplemented with 10% fetal bovine serum, 0.1% MEM non-essential amino acid, 6mM HEPES, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in an atmosphere of 5% CO\textsubscript{2} and 95% air. The cells were kept subcultured at 70-90% confluency and media was changed every 3 days. For initial cytotoxicity experiments, cells utilized were between 15 and 50. Subcultured cells were placed in 6-well plates with the medium to obtain 250,000 cells/mL. The total volume was 2 mL in each well. Cells were grown for 21 – 24 days before use. Before the experiment, cells were cultured with pH 6.5 Hanks’ Blank Balanced Solution (HBSS) (9.8 g/L Hanks’ Blank Salt Solution powder, 0.37 g/L sodium bicarbonate, and 2.86 g/L HEPES) for 1 hour. The filtered, post-digested aqueous phase of nanoemulsion samples were put on the wells after the HBSS was removed.
Samples were diluted from 100 – 12.5% with pH 6.5 HBSS. Photos were taken at times specified (0.5 – 8 h) to examine cell viability.

6.3 Results and Discussion

6.3.1 Fasted vs. Fed-Digestion and Bioaccessibility

Statistical analysis of the fasted vs. fed state could not be performed because results for the fed state were from duplicate samples, while fasted state results were from triplicate samples. Instead, I detailed trends between the fasted and fed state. The %BIO of nanoemulsions subjected to the fed state, with comparison to the fasted state, are seen in Table 12. Percent lipid digested is also noted:
### Table 12: Effect of digestion state on %BIO

Results for the fed state are reported as the average of two measurements on freshly prepared samples, while results for the fasted state are reported as the average of three measurements.

The results for 2% LCT were not able to be produced because the middle layer after the first wash with chloroform (see Figure 16) was too thick to accurately collect the bottom chloroform layer containing curcumin.

As seen in Table 12, *in vitro* digestion of curcumin-containing emulsions in the simulated fed state showed a trend of increasing %BIO as droplet concentration increased. This could be due to several factors. First, the increased bile concentration (20 mg/mL vs. 5 mg/mL for fasted) could increase the solubilization capacity of digestion products and increase the potential number of mixed carrier products able to encapsulate...
curcumin. Second, the increased amount of lipase (2.4 mg/mL vs. 1.6 mg/mL for fasted) could hydrolyze fatty acid chains at the oil:water interface at an increased extent. Third, there could be an additive combination between bile and lipase. Increased lipase concentration can hydrolyze triacylglycerols at a faster rate in the fed state and the increased bile concentration can solubilize and remove the released FFA’s from the oil:water interface at a faster rate than the fed state. This allows greater access to the interface for lipase. To discover which explanation is more likely, it is necessary to see the titration profiles to examine initial digestion rate and eventual digestion extent. These are seen in Figure 21:
**Figure 21: Effect of feeding state on digestion extent.** Lipid concentrations (percentages) with “FA” were titrated with fasted state conditions, while concentrations with “FE” were titrated with fed state conditions. Results for the fed state are reported as the average of two measurements on freshly prepared samples, while results for the fasted state are reported as the average of three measurements.

As seen in Figure 21, LCT had a higher digestion extent in the fed state vs. the fasted state at each lipid concentration, while MCT showed an opposite trend. In both cases, initial digestion rate was faster for fasted conditions. The most likely cause for LCT’s results (Fig. 21A) was the increased concentration of bile in the fed state solubilizing and removing digestion products from the oil:water interface, which allowed lipase greater access to the interface to hydrolyze emulsion droplets. The presence of released long chain FFA’s in solution can have an impact on digestion extent as results from titrations...
performed with fasted conditions (Fig. 11) showed that LCT had the lowest digestion extent. The higher extent of long chain lipid digestion with greater bile salt concentrations is in agreement with previous studies (Borgstro.B and Erlanson 1973; Alvarez and Stella 1989; MacGregor, Embleton et al. 1997). For initial digestion rate in the fed state (Fig. 21A), there is an early period of some hydrolysis (0 – 800 s) followed by a period of rapid hydrolysis (800 – 2000 s). This effect is most likely due to droplet flocculation, which minimizes the surface area of droplets that lipase can act upon. Indeed, the sample looked turbid and precipitates were seen as the experiment initially started. As time progressed, the solution appeared less turbid as lipase was able to hydrolyze the nanoemulsion droplets and many of those products were solubilized in solution. So, initially, the released long chain FFA’s would limit access of lipase to the oil:water interface. As digestion continued, the higher concentration of bile and lipase would result in a higher efficiency at solubilizing digestion products and greater enzyme activity to hydrolyze emulsion droplets.

Results for MCT (Fig. 21B) illustrated another, different effect that bile can have on digestion. Fasted conditions produced a slightly higher digestion extent than fed conditions and this can be due to having a high concentration of bile in solution that interfered with lipase access to the oil:water interface. It has been previously shown that increased bile concentrations can inhibit lipase activity by mixed micelles forming alternate binding sites for co-lipase, which can diminish binding of pancreatic lipase to the oil:water interface (Patton and Carey 1981). This interference can also explain the higher initial digestion rates for nanoemulsions at the fasted state.

The PSD of fasted vs. fed (unfiltered) samples is seen in Table 10:
Table 13: Effect of digestion state on PSD. Samples of post-digested aqueous phase were analyzed unfiltered. Results are reported as the average of at least two measurements on freshly prepared samples.

As seen in Table 13, there are some trends between the fasted and fed state. For LCT, the PDI for the fed state was smaller at each droplet concentration than the fasted state. For MCT, Z-average and PDI values in the fed state were similar at each droplet concentration. However, these trends do not seem to explain differences in %BIO results seen in Table 12.

If Equation 6 was applied to the LCT results to account for 100% digestion (assuming curcumin % BIO is proportional to digestion extent), those results are seen in Table 14:
Table 14: Theoretical %BIO if total digestion is achieved. These do not take standard deviation into account, just average values.

There was no data for 2% droplet concentration because the middle oil layer produced after centrifugation on the first wash with chloroform (Fig. 16) was too thick to be removed effectively to be able to isolate the bottom chloroform layer. Multiple attempts produced curcumin-containing chloroform layers interspersed with solids that made UV-VIS readings unreliable. The unattained data was crucial because it would have shed insight on the limitations of my nanoemulsion delivery system in terms of %BIO of curcumin. A %BIO value significantly higher then 60% would have shown that bioaccessibility was measurably enhanced by fed conditions. If the value would have been similar to 60%, then we would know the limitations of the nanoemulsion delivery system and then be able to formulate ways to increase %BIO of curcumin. Even in the fed state for MCT (2% droplet concentration; Table 12B), the maximum theoretical %BIO (assuming curcumin %BIO is dependent on digestion extent) was approximately 58%, a value similar to maximum %BIO values seen in Tables 6 and 10.

Results for the fasted vs. fed state need to be standardized because some may say my fasted state was not accurate due to the high concentration of calcium (20 mmoles) used to make 100% digestion possible. The following values I propose to use as differentiating between the fasted and fed state were obtained from combining various conditions selected from literature (Zangenberg, Mullertz et al. 2001; Sek, Porter et al.)

<table>
<thead>
<tr>
<th>Droplet Concentration (%)</th>
<th>%BIO Fasted (%)</th>
<th>%BIO Fed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>1.5</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>---</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Droplet Concentration (%)</th>
<th>%BIO Fasted (%)</th>
<th>%BIO Fed (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>1.5</td>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>---</td>
</tr>
</tbody>
</table>
The proposed values are seen in Table 15:

<table>
<thead>
<tr>
<th>Experimental Parameter</th>
<th>Fasted</th>
<th>Fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Reaction Cell Volume</td>
<td>37.5 mL</td>
<td>37.5 mL</td>
</tr>
<tr>
<td>Temperature</td>
<td>37 ºC</td>
<td>37 ºC</td>
</tr>
<tr>
<td>Stirring Speed</td>
<td>4 s⁻¹</td>
<td>4 s⁻¹</td>
</tr>
<tr>
<td>[NaOH] in Titration Unit</td>
<td>0.50 M</td>
<td>0.50 M</td>
</tr>
<tr>
<td>Lipid content in Reaction Cell</td>
<td>300 mg</td>
<td>300 mg</td>
</tr>
<tr>
<td>CaCl₂ in Reaction Cell</td>
<td>5 mmoles</td>
<td>20 mmoles</td>
</tr>
<tr>
<td>Bile Extract in Reaction Cell</td>
<td>5 mg/mL</td>
<td>20 mg/mL</td>
</tr>
<tr>
<td>Pancreatin Lipase</td>
<td>0.4 mg/mL</td>
<td>2.4 mg/mL</td>
</tr>
</tbody>
</table>

Table 15: Proposed values for studies involving fasted vs. fed state.

Going forward, I believe these parameters would be best for using in studies that compare results of the fasted vs. fed state. My formulation (calcium = 20 mmoles; bile = 5 mg/mL; lipase = 1.6 mg/mL) was used because a set, lab-wide protocol with those concentrations for the *in vitro* digestion model was only determined as my initial work began. Of importance in the protocol was the ability to achieve 100% digestion of a sample.

However, there is some variability in the conditions for the fasted and fed state. Even in the literature listed for Table 15, ranges were used for component concentrations. There would not be one set value for these states, so I selected the smallest (fasted) and largest (fed) concentrations to be the proposed values.

### 6.3.2 Sonicated Emulsions-Digestion and Bioaccessibility

The titration profile of sonicated emulsions, in the simulated fasted state, is seen in Figure 22:
Figure 22: Effect of oil type on digestion extent for sonicated emulsions. Results are reported as the average of three measurements on freshly prepared samples.

Figure 22 followed similar results in that increasing droplet concentration resulted in lower digestion extent. Thus, this emulsion preparation method should not be noticeably different than the other methods (microfluidizer and blending only) used to prepare emulsions. The relationship between emulsion preparation method and digestion extent are seen in Table 16:

<table>
<thead>
<tr>
<th>Preparation Type</th>
<th>Lipid Digested-1% (%)</th>
<th>Lipid Digested-1.5% (%)</th>
<th>Lipid Digested-2% (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano</td>
<td>91 ± 9</td>
<td>80 ± 9</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>Conventional</td>
<td>98 ± 16</td>
<td>88 ± 8</td>
<td>73 ± 10</td>
</tr>
<tr>
<td>Sonicated</td>
<td>121 ± 6</td>
<td>102 ± 7</td>
<td>82 ± 7</td>
</tr>
</tbody>
</table>

(A) LCT

<table>
<thead>
<tr>
<th>Preparation Type</th>
<th>Lipid Digested-1% (%)</th>
<th>Lipid Digested-1.5% (%)</th>
<th>Lipid Digested-2% (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nano</td>
<td>135 ± 9</td>
<td>120 ± 6</td>
<td>113 ± 6</td>
</tr>
<tr>
<td>Conventional</td>
<td>139 ± 9</td>
<td>132 ± 13</td>
<td>123 ± 8</td>
</tr>
<tr>
<td>Sonicated</td>
<td>155 ± 19</td>
<td>158 ± 18</td>
<td>148 ± 8</td>
</tr>
</tbody>
</table>

(B) MCT

Table 16: Effect of emulsion preparation method on digestion extent. Results are reported as the average of three measurements on freshly prepared samples.
For LCT, each preparation method showed a trend of increasing droplet concentration resulting in a decrease in digestion extent. Sonicated emulsions had the highest digestion extent at all droplet concentrations, but this could be due to differences in pancreatic lipase activity on an experiment-by-experiment basis. Pancreatic lipase is a mixture of at least three distinct lipolytic enzymes (Reis, Holmberg et al. 2009), so activity could change slightly. Droplet size should not be the reason for the results seen in Table 16 because sonicated emulsions produced droplet sizes (200 – 600 nm) in between nanoemulsions and conventional emulsions. If digestion was dependent emulsion droplet size, with the trend seen between nanoemulsions and conventional emulsions, sonicated emulsions would digest to an extent between the other two preparation methods and not to a much higher extent. For MCT, sonicated emulsions deviated from other preparation methods because all three droplet concentrations produced similar extents of digestion.

The PSD of sonicated emulsions are seen in Table 17:
<table>
<thead>
<tr>
<th>Droplet Concentration (%)</th>
<th>Z-average (nm)</th>
<th>PDI (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>114 ± 7</td>
<td>0.30 ± 0.04</td>
</tr>
<tr>
<td>1.5</td>
<td>151 ± 11</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>2</td>
<td>146 ± 5</td>
<td>0.21 ± 0.01</td>
</tr>
</tbody>
</table>

(A) LCT

<table>
<thead>
<tr>
<th>Droplet Concentration (%)</th>
<th>Z-average (nm)</th>
<th>PDI (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>130 ± 33</td>
<td>0.29 ± 0.06</td>
</tr>
<tr>
<td>1.5</td>
<td>113 ± 15</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>87 ± 24</td>
<td>0.29 ± 0.09</td>
</tr>
</tbody>
</table>

(B) MCT

Table 17: PSD of sonicated emulsions. Results are reported as the average of three measurements on freshly prepared samples.

There was no statistical significance between particle size and droplet concentration for LCT and MCT.

The %BIO of the sonicated nanoemulsions is seen in Table 18:

<table>
<thead>
<tr>
<th>Oil Type</th>
<th>1% Droplet Concentration</th>
<th>1.5% Droplet Concentration</th>
<th>2% Droplet Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCT</td>
<td>50 ± 6</td>
<td>59 ± 3</td>
<td>53 ± 11</td>
</tr>
<tr>
<td>MCT</td>
<td>5 ± 1</td>
<td>28 ± 8*</td>
<td>68 ± 5*</td>
</tr>
</tbody>
</table>

Table 18: Effect of increased droplet concentration on %BIO of sonicated emulsions. Results with “*” were statistically different from the 1% sample. Results are reported as the average of three measurements on freshly prepared samples.

If Equation 6 was applied to the results to account for 100% digestion (assuming curcumin % BIO is proportional to digestion extent), those results are seen in Table 19:
As seen in Table 18, the addition of available phospholipids increased %BIO of curcumin in MCT in a statistically significant manner, but not for LCT. MCT produced the highest %BIO between the oils. For LCT, addition of lipid produced only slightly higher %BIO that was not proportional to the greater amount of lipid present. With long chain FFA’s, there may be conflicting processes that result when high lipid concentrations are present that inhibit the ability to increase the amount of mixed bile salt micelles to incorporate curcumin in the aqueous phase. These experiments simulated fasted conditions, but the relationship of additional lipid only slightly increasing %BIO was not seen for the other emulsion preparations, nanoemulsion vs. conventional emulsion, that simulated fasted conditions (Table 11A). For those samples, an increase from 1 to 1.5% droplet concentration produced a sizable increase in %BIO and an increase from 1.5 to 2% showed %BIO values that were similar to each other. The latter trend remained for the sonicated emulsions, but the former trend (large %BIO increase from 1 to 1.5%) was not seen.

As seen in Table 19, there seems to be a theoretical maximum %BIO for curcumin in the 60th percentile range. Sonicated emulsions were made in a similar fashion to nanoemulsions with a two-step homogenization procedure. During this time the microfluidizer was not operational, so this new method of making nanoemulsions was

<table>
<thead>
<tr>
<th>Droplet Concentration (%)</th>
<th>%BIO MCT (%)</th>
<th>%BIO LCT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>1.5</td>
<td>28</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>65</td>
</tr>
</tbody>
</table>

Table 19: Theoretical maximum %BIO for sonicated emulsions. These do not take standard deviation into account, just average values.
adopted. While slightly different from nanoemulsions produced with the microfluidizer, the emulsions produced were in the 200 – 600 nm range and there was no correlation between oil type and differing droplet size for sonicated emulsions. The trend of increased lipid leading to increased %BIO for MCT was previously seen for the other emulsion preparations, nanoemulsion vs. conventional emulsion, also simulating fasted conditions (Table 11B). The results from Table 17 suggested that more hydrolyzed medium chain FFA’s, as compared to long chain, are needed to self-assemble into mixed bile salt micelles that contain curcumin because MCT always produced >100% digestion at each droplet concentration. LCT rarely produced 100% digestion and, yet, the theoretical %BIO for LCT was often found to be similar to MCT (Tables 6, 14, and 19) in that %BIO was in the 60th percentile range. It must also be noted the results from Figure 16 where centrifuging after the first wash of chloroform produced an oil layer that I hypothesize consisted of FFA’s, monoglycerides, diglycerides, and calcium precipitated digestion products. MCT samples with the first wash of chloroform produced a thin, delicate layer between the aqueous and chloroform layers, which would be expected if complete digestion of the emulsion droplets was achieved. If LCT can have a theoretical maximum %BIO similar to MCT, with many more digestion products not formed into curcumin-containing micelles, then it can be reasoned that these digestion products have the potential to form micelles, vesicles, and/or liposomes under more favorable conditions. With those available products being able to form micelles, vesicles, and/or liposomes, LCT seems to have the potential to be the superior oil capable of producing the highest %BIO of curcumin. However, more work needs to be done to examine the
precipitated layer (Fig. 16) and to formulate conditions that would allow these digestion 
products to become potential carriers for curcumin after digestion.

6.3.3 Caco-2 Cell Viability

I calculated %BIO for curcumin, but that value was not the same as the eventual 
bioavailability for curcumin, i.e. the amount of curcumin that ends up in systemic 
circulation. The amount that ends up in the bloodstream is most likely less than the 
amount that is bioaccessible in the small intestine because there are metabolic and 
absorption processes that the mixed micelles are subject to before the curcumin can get to 
the bloodstream (Fig. 2). Specific processes can include metabolism via the liver and 
bacteria in the small intestine. Caco-2 cell models have been used for predicting eventual 
bioavailability because mature, differentiated cells have the same characteristics as 
enterocyte cells in the small intestine.

Uptake experiments with Caco-2 cells are commonly used for determining 
eventual concentration of lipophilic drugs. The drug would absorb from the apical side to 
the basolateral side. Unfortunately, I have had much trouble finding a suitable medium 
for the curcumin because the filtered post-digestion solution killed the Caco-2 cells in a 
matter of hours as seen in Table 20:
<table>
<thead>
<tr>
<th></th>
<th>0.5 Hr</th>
<th>1 Hr</th>
<th>2 Hr</th>
<th>4 Hr</th>
<th>8 Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
</tr>
<tr>
<td>100%</td>
<td>Dead</td>
<td>Dead</td>
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</tr>
<tr>
<td>75%</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>50%</td>
<td>Dead</td>
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<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>25%</td>
<td>Alive</td>
<td>Mix</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>12.5%</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Mix</td>
<td>Mix</td>
</tr>
</tbody>
</table>

(A) LCT

<table>
<thead>
<tr>
<th></th>
<th>0.5 Hr</th>
<th>1 Hr</th>
<th>2 Hr</th>
<th>4 Hr</th>
<th>8 Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
</tr>
<tr>
<td>100%</td>
<td>Dead</td>
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</tr>
<tr>
<td>75%</td>
<td>Dead</td>
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<td>Dead</td>
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<tr>
<td>50%</td>
<td>Dead</td>
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<td>Dead</td>
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<td>Dead</td>
</tr>
<tr>
<td>25%</td>
<td>Dead</td>
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</tr>
<tr>
<td>12.5%</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Mix</td>
</tr>
</tbody>
</table>

(B) MCT

**Table 20: Caco-2 cell viability with post-digested nanoemulsion.** The percentages on the left column are the dilutions of the filtered post-digestion solution.

I hypothesized that the bile concentration was too large and that is what caused the cells to die. Bile acts as a detergent and this property caused the cells to be transplanted from their positions on the cell plate, which is a result of cell death. Previous studies have 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 (Boulenc, Breul et al. 1995;
Dimitrijevic, Shaw et al. 2000; Ingels and Augustijns 2003). Viable (alive) vs. dead cells are seen in Figure 23:

![ALIVE](image1)

![DEAD](image2)

**Figure 23: Photographs of Caco-2 cell viability.**

As seen in Figure 23, dead cells did not show the differentiation and compartmentalization of viable cells. More studies were done to see the effect of bile concentration on Caco-2 cell viability and these results are seen in Table 21:
<table>
<thead>
<tr>
<th></th>
<th>0.5 Hr</th>
<th>1 Hr</th>
<th>2 Hr</th>
<th>4 Hr</th>
<th>8 Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
</tr>
<tr>
<td>100%</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
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<tr>
<td>75%</td>
<td>Mix</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>50%</td>
<td>Alive</td>
<td>Mix</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>25%</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Mix</td>
<td></td>
</tr>
<tr>
<td>12.5%</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Mix</td>
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</tbody>
</table>

(A) 5 mg/mL Bile

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<th>4 Hr</th>
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</tr>
</thead>
<tbody>
<tr>
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<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
</tr>
<tr>
<td>100%</td>
<td>Alive</td>
<td>Mix</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>75%</td>
<td>Alive</td>
<td>Alive</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>50%</td>
<td>Alive</td>
<td>Alive</td>
<td>Mix</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>25%</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Mix</td>
<td>Dead</td>
</tr>
<tr>
<td>12.5%</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
</tr>
</tbody>
</table>

(B) 2.4 mg/mL Bile

<table>
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<th>2 Hr</th>
<th>4 Hr</th>
<th>8 Hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
</tr>
<tr>
<td>100%</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>75%</td>
<td>Alive</td>
<td>Alive</td>
<td>Dead</td>
<td>Dead</td>
<td>Dead</td>
</tr>
<tr>
<td>50%</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Mix</td>
<td>Dead</td>
</tr>
<tr>
<td>25%</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Mix</td>
</tr>
<tr>
<td>12.5%</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
<td>Alive</td>
</tr>
</tbody>
</table>

(C) 0.4 mg/mL Bile

**Table 21: Effect of bile on Caco-2 cell viability.** Each sample has 2% MCT with 0.15% curcumin post-digested solution as the beginning nanoemulsion. Lipase (1.6 mg/mL) and calcium (20 mmoles) concentration do not change.
As seen in Table 20, as bile concentration increased, the amount of dead cells increased at a faster time period. Previous studies have used bile concentrations from 0.4 – 12 mg/mL (Garrett, Failla et al. 1999; Liu, Glahn et al. 2004) with similar growing conditions and applications to Caco-2 cells. More studies need to be performed to obtain viable Caco-2 cells to determine uptake, which is similar to determining eventual bioavailability. This will show a more complete picture of the behavior of nanoemulsion delivery systems of lipophilic drugs during digestion.

6.4 Conclusions

Digestion rate, extent, particle size, and %BIO were determined for the fed state and were compared to the fasted state. Particle size showed no trend between the fasted and fed state. %BIO of LCT increased in the fasted state, but MCT produced no such trend. LCT had a higher digestion extent in the fed state vs. the fasted state at each lipid concentration, while MCT showed an opposite trend. In both cases, initial digestion rate was faster for fasted conditions.

Sonicated emulsions were produced to mimic nanoemulsions, while the microfluidizer was unavailable. These emulsions were used for an alternative method to obtaining %BIO, as the amount of curcumin did not change for the samples, but amount of lipid changed. No definitive trends for particle size were seen and digestion extent was greater than prepared nanoemulsions and conventional emulsions. However, this could be due more to the lipase activity during the time these experiments were performed rather than digestion affinity based on droplet size.

%BIO of MCT increased as lipid concentration increased, but LCT showed no such trend for sonicated emulsions. The theoretical %BIO of MCT and LCT were similar
and this suggested that there could be a limit to %BIO that an emulsion-based delivery system could provide. However, the aqueous phase of LCT needs to be examined further to see if there are available digestion products such as FFA’s, monoglycerides, and diglycerides that have the potential to form micelles, vesicles, and/or liposomes to become carriers for curcumin. This potential could increase %BIO and suggest that LCT has a higher potential %BIO than MCT.

Caco-2 cells are used for predicting eventual bioavailability. I have had much trouble with bile acting as a detergent and killing the cells. More work must be performed to obtain a model that withstands the detergent abilities of bile. This model would also need appropriate concentrations of curcumin to be available, so proper determination of curcumin bioavailability after uptake is possible.
BIBLIOGRAPHY


Szoka, F., F. Olson, et al. (1980). "Preparation of Unilamellar Liposomes of Intermediate Size (0.1-0.2-Mumm) by a Combination of Reverse Phase Evaporation and Extrusion through Polycarbonate Membranes." Biochimica Et Biophysica Acta 601(3): 559-571.


