Impact of emulsion-based drug delivery systems on intestinal permeability and drug release kinetics

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ABSTRACT

Lipid based drug delivery systems, and in particular self-emulsifying drug delivery systems (SEDDS), show great potential for enhancing oral bioavailability but have not been broadly applied, largely due to lack of general formulation guidance. To help understand how formulation design influences physicochemical emulsion properties and associated function in the gastrointestinal environment, a range of twenty-seven representative self-emulsifying formulations were investigated. Two key functions of emulsion-based drug delivery systems, permeability enhancement and drug release, were studied and statistically related to three formulation properties - oil structure, surfactant hydrophilic lipophilic balance (HLB) values, and surfactant-to-oil ratio. Three surfactants with HLB values ranging from 10 to 15 and three structurally different oils (long chain triglyceride, medium chain triglyceride, and propylene glycol dicaprylate/dicaprate) were combined at three different weight ratios (1:1, 5:1, 9:1). Unstable formulations of low HLB surfactant (HLB = 10) had a toxic effect on cells at high (1:1) surfactant concentrations, indicating the importance of formulation stability for minimizing toxicity. Results also indicate that high HLB surfactant (Tween 80) loosens tight junction at high (1:1) surfactant concentrations. Release coefficients for each emulsion system were calculated. Incorporation of a long chain triglyceride (Soybean oil) as the oil phase increased the drug release rate constant. These results help establish an initial foundation for relating emulsion function to formulation design and enabling bioavailability optimization across a broad, representative range of SEDDS formulations.

Keywords: Oil-in-water emulsions, effect of formulation parameters, self-emulsifying drug delivery systems, SEDDS, experimental design
INTRODUCTION

Although oral delivery is the most preferred method of drug administration, this is not possible for roughly 50% of currently marketed drug compounds due to their low solubility in water and low oral bioavailability. Recent approaches to address this problem include administration of drug components with lipid vehicles such as oils, liposomes, and self-emulsifying formulations [1, 2]. Self-emulsifying drug delivery systems (SEDDS), which are oil in water emulsions, often are studied for the enhancement of bioavailability of hydrophobic drugs. These systems are not commonly used commercially, however, largely due to lack of formulation guidance and knowledge of how these systems function to enhance bioavailability.

SEDDS typically have droplet sizes ranging from a few nanometers to hundreds of nanometers and consist minimally of oil, surfactant, and the drug to be delivered. SEDDS spontaneously emulsify in the gently mixed aqueous gastrointestinal (GI) environment [3], have shown great promise for enhancing oral bioavailability of low-solubility compounds, and offer the advantages of minimal processing and inherent stability. By example, the bioavailability of Tipranavir (TPV), a nonpeptidic protease inhibitor anti-HIV drug, was doubled when dosed to rats in a SEDDS formulation versus delivered as solid powder in a hard filled capsule [1]. Kommuru et al [4] developed a self-emulsifying drug delivery system for Coenzyme Q10 using polyglycolyzed glycerides as emulsifiers with a resultant 150% increase in bioavailability. Numerous other examples exist in the literature demonstrating enhanced bioavailability with self-emulsifying drug delivery systems [2, 5-8], but most focus on a single formulation or small set of formulations with little explanation of how this formulation was developed. Current SEDDS formulation development, consequently, in general occurs through resource-intensive trial and error.

Some mechanistic studies have been conducted exploring a range of SEDDS formulations and a specific aspect of their function, most commonly ability to emulsify [6, 8]. Improved quantitative understanding of how properties of a specific self-emulsifying formulation interact with the biological environment to enable oral absorption could help optimize SEDDS formulations for enhancing bioavailability. Although a significant amount of literature exists from other fields concerning the function of emulsions [9-12], pharmaceutical formulations have not been broadly studied with respect to fundamental aspects of emulsion function, with few studies statistically developing predictive models that relate emulsion properties with formulation parameters [13-16]. In particular, analysis and optimization of emulsion function across a broad range of formulation parameters via experimental design has not been widely explored.

To gain a greater understanding of the dependence of emulsion function in the GI tract on formulation design, we conducted a quantitative study of properties central to emulsion function. A broad range of formulations was studied as follows. Oils from three different structural classes (long chain triglyceride (Soybean oil), medium chain triglycerides (Neobee M5), propylene glycol dicaprylate/dicaprate (Captex 200)) and surfactants with hydrophilic-lipophilic balance (HLB) values ranging from 10-15 (Cremophor EL, Tween 80, a mixture of Capmul MCM and Labrasol) were combined at three different oil-to-surfactant weight ratios (9:1, 5:1, 1:1) utilizing a 3^3 factorial experimental design. Naproxen was included in formulations to test the influence of drug on emulsion properties. Each formulation was characterized for its ability to emulsify, resulting particle size, and surface charge. Two mechanisms of emulsion function in the GI environment, intestinal permeability enhancement and drug release, also were studied for each formulation. The statistical relationships of these
emulsion characteristics and functional parameters to the above emulsion design parameters were determined via analysis of variance and regression modeling.

MATERIALS AND METHODS

Reagents

The following reagents were used in this study, sourced as indicated. Soybean oil, Tween 80, Sodium dodecyl sulfate (SDS), Cremophor EL, Phosphate buffered saline (PBS), Hank’s balanced salt solution (HBSS), antibiotic-antimycotic solution, trypsin solution, MTT toxicity assay, Transwell® inserts, and Lucifer Yellow were purchased from Sigma-Aldrich Incorporated (St. Louis, MO). Captex 200 and Capmul MCM were generously donated by Abitec Corporation (Janesville, WI). Neobee M-5 was received as a gift from Stepan Company (Northfield, IL). Labrasol was purchased from Gattefosse (St-Priest, France). Caco-2 cells, Eagle’s minimum essential medium, and fetal bovine serum were purchased from American Type Culture Collection (Manassas, VA). Naproxen was obtained from MP Biomedicals, Incorporated (Solon, OH).

Preparation and Characterization of Emulsions

In order to study a wide variety of formulation components and SEDDS characteristics, a $3^3$ factorial experimental design was used to combine oils of three different structural classes and surfactants with three HLB values (ranging from 10 to 15) at three different oil-to-surfactant weight ratios: 9:1, 5:1, 1:1 (Table 1). Naproxen was incorporated into formulations as a model drug at 3 mg/ml for characterization studies, and at 7 mg/ml for drug release studies. Formulations without drug were prepared for characterization, permeability, and cytotoxicity studies. Each formulation was prepared in polycarbonate 2 ml centrifuge tubes, vortexed, further mixed using a rotating shaker for three hours at 37° C, and equilibrated at room temperature for 24 hours before use.

<table>
<thead>
<tr>
<th>EXCIPIENTS</th>
<th>OIL</th>
<th>Chemical Structure</th>
<th>surfactant</th>
<th>Name</th>
<th>HLB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean Oil</td>
<td>Long chain triglycerides</td>
<td>C16-C22</td>
<td>Tween 80 (C18)</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Captex 200</td>
<td>Propylene glycol dicaprylate/dicaprate</td>
<td>C8-C10</td>
<td>Cremophor EL (C18)</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Neobee M5</td>
<td>Medium chain triglycerides</td>
<td>C8-C10</td>
<td>56% Labrasol</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>44% Capmul MCM (C8-C10)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For testing of ability to emulsify and emulsion characterization, 10 ml of deionized (DI) water was warmed to 37° C in a 15 ml centrifuge tube. 1:100 dilution and 1:1000 dilution emulsions were prepared by introducing 10 or 100 µl of formulation to the aqueous phase and vortexing for 30 seconds. All formulations were prepared three times and assessed visually for spontaneous emulsification and uniform fine emulsion formation. Droplet size and zeta potential of freshly prepared emulsions were measured using laser light scattering (Brookhaven 90 Plus). Droplet size was measured on three independently prepared formulations, with each measurement averaged from three readings per formulation, whereas zeta potential was ana-
Emulsion Cytotoxicity Measurement

Toxicity of formulations on Caco-2 cell monolayers was tested with the MTT toxicity assay. Cells were seeded on 96 well cell culture plates at a seeding density of 2.5 x 10^5 cells/ml in 200 µl medium 7 days prior to the cytotoxicity assay. Medium was changed every 3 days. On the eighth day, the cell culture medium was removed, and cells were rinsed with HBSS twice and incubated with each of the 27 emulsion formulations for three hours at 37°C. Three wells were used to test each formulation. Emulsion systems were prepared at a 1:100 formulation-to-buffer ratio without the model drug (Naproxen) in HBSS. After three hours, wells were rinsed twice with HBSS and then incubated with 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT substrate) solution at 10µl/100µl HBSS for three hours. MTT solubilization solution then was added to solubilize any formazan crystals formed. After a 12 hour incubation, UV absorbance of wells was measured at 570 nm. Wells incubated only with HBSS were used as a negative control, assumed to have 100% cell viability. 0.1% Sodium dodecyl sulfate (SDS) solution was used as a positive control. Percent viabilities were determined relative to the negative control.

Transport across Caco-2 Cell Monolayers

Transport across intestinal epithelial cells was tested using model drug compounds Naproxen and Lucifer Yellow. Cells were seeded at 2 x 10^6 cells/ml and cultured on 24 well plate Transwell® permeable supports (0.4 um pore size) for 27 days. Cell culture medium was changed every other day. On day 27, cell culture medium was removed from both the apical and basolateral compartments, and cells were rinsed once with HBSS. Apical compartment solution was replaced with emulsion in HBSS (with no drug), and basolateral compartment solution was replaced with HBSS. Emulsion systems were prepared at 1:100 dilution ratio without the model drug, Naproxen, in HBSS. Blank HBSS and 0.1% SDS served as the negative and positive controls, respectively. Cells were exposed to emulsions for 3 hours inside the incubator. To test transport, a solution containing 0.3 mg/ml Naproxen was prepared in PBS. Apical compartment solution was removed and replaced with drug solution after rinsing cells once with HBSS, basolateral compartment solution was replaced with blank PBS, and cells were placed in the incubator. After three hours, samples were taken from each compartment and analyzed for Naproxen content using a UV spectrophotometer at 330 nm wavelength. Paracellular transport was tested following the same procedure except instead of Naproxen, 1 mg/ml Lucifer Yellow was used, and drug content was measured using a fluorescence plate reader at 485 nm excitation and 528 nm emission wavelengths. Transport studies were combined with Trans Epithelial Electrical Resistance (TEER) measurements before and after exposure to formulations (EVOM, World Precision Instruments, Sarasota, FL) in order to assess effect of formulations on cell monolayer integrity. Apparent permeability coefficient, P_{app}, is calculated as [17]

\[ P_{app} = \frac{dQ/dt}{A C_0}, \]

where \( dQ/dt \) is the steady-state flux, \( A \) is the surface area of the membrane, and \( C_0 \) is the initial drug concentration in the donor compartment.
Measurement of Kinetics of Drug Release from Emulsions

Drug release experiments were conducted using a modified dialysis method[18]. Emulsion formulations containing 7 mg/ml Naproxen were introduced inside the tubular regenerated cellulose dialysis membrane (Fisherbrand, MWCO 3500) of 3 ml at a formulation to PBS ratio of 1:50 and gently shaken to enable mixing. The dialysis membrane then was immersed in 35 ml PBS solution in a beaker that served as the receiver fluid maintaining sink conditions. A stir bar was used at a mixing speed of 330 rpm to enable mixing inside the beaker, and a hot plate was used to maintain the temperature inside the beaker at 37°C. Samples (200 μl) were taken every 15 minutes for three hours from the receiver compartment and replaced with fresh PBS. A sample was also taken at 24 hours to assess the amount of drug released at equilibrium. Samples were analyzed using UV spectrophotometry at 271 nm.

The rate of drug release from emulsions, characterized by the release coefficient \( k_{eff} \), was calculated using the mass transfer equations

\[
\ln(1 - \frac{M_T}{M_0}) = 3 \times k_{eff} \times t / r^2
\]

and

\[
\frac{dM_R}{dt} = \rho \times (C_w - C_{wOUT})
\]

where \( M_T/M_0 \) is the fraction of the released drug at time \( t \), \( M_R \) is the drug amount inside the receiver fluid outside the dialysis membrane, and \( C_{wOUT} \) is the concentration of drug in the receiver fluid measured throughout the release experiment as described above. It was assumed that initially drug was only incorporated inside oil droplets. In order to calculate the concentration of free drug in solution inside the membrane \( (C_w) \), the permeability of the membrane to free drug, \( \rho \), was measured using a drug release experiment (with no emulsion) together with Equation [3]. Briefly, 3 ml of 0.14 mg/ml Naproxen solution in PBS was placed inside a dialysis membrane immersed in 35 ml PBS, as described above, and samples were taken from the receiver compartment every 15 minutes for 3 hours.

In order to calculate the fraction of drug released from oil droplets at time \( t \), drug concentration in the aqueous phase inside the dialysis membrane, meaning both micelles and water phase, was calculated using \( C_w \) and an experimentally determined partition coefficient \( K_m \) [19],

\[
K_m = \frac{C_m}{C_w}
\]

where \( C_m \) is the drug concentration in the micellar phase and \( C_w \) is the drug concentration in water. Briefly, 7 mg/ml drug was dissolved in surfactant and 60 ul of surfactant containing drug was mixed with 3 ml PBS inside of a dialysis membrane. This membrane then was immersed in a PBS receiver compartment as described above for release experiments. A sample was taken after 24 hours from the receiver compartment and used to calculate \( C_w, C_m \) then was calculated by mass balance and used to determine the partition coefficient. Drug partitioning into micelles was assumed to be instantaneous.

Micelle volume in each emulsion system, required to calculate the partition coefficient, was measured by a previously established method [20]. Emulsions with Tween 80 were centrifuged for 20 minutes through nanoporous regenerated cellulose centrifuge filters (Amicon Ultra, Millipore Corp, Billerica, MA) of molecular weight cut off 100,000. For emulsions of Cremophor EL, an ultracentrifugation unit (Amicon 8400, Millipore Corporation) and a mem-
brane of 500,000 molecular weight cut off were used. Micelle molecular weight, calculated from the aggregation number and the monomer molecular weight, was used to select the membrane molecular weight cut off that allows micelles but not oil droplets to pass through the membranes[21]. The filtrates from both centrifugation and ultracentrifugation processes containing the micelles and surfactant monomers were analyzed using a methylene blue complexation method [22]. The percentage of surfactant introduced that formed micelles and monomers (as opposed to that associated with emulsion droplets) was calculated.

The parameter \( r \) in Equation [2] is the oil droplet radius, calculated from the average particle size of the emulsions formed at 1:50 ratio in PBS, assuming spherical shape.

**Statistical Analysis and Regression Model Development**

Effect of formulation design parameters on emulsion characteristics was statistically investigated by performing Analysis of Variance (ANOVA) as well as Tukey’s post-hoc pairwise comparison tests using SPSS® software (SPSS Inc., Chicago, IL). Least squared linear regression models of emulsion characteristics were established as a function of formulation design variables using the same software.

**RESULTS AND DISCUSSION**

**Characterization of SEDDS**

The visual observation upon gentle mixing of formulations in aqueous media indicated that the HLB value of surfactant is highly important for formation of a fine, uniform emulsion. Formulations containing surfactant with the lowest HLB value tested (10) did not result in uniform emulsifications. Surfactants that have been used for formulating SEDDS generally range in HLB value from 4 to 15, but it has been previously suggested that surfactants should have a relatively high HLB for spontaneous oil in water emulsion formation [2]. In a study utilizing HLB 10 surfactant, Labrafac CM 10, good emulsification was possible only with a surfactant ratio higher than 50% (1:1) [23]. Our findings imply that for a reasonable oil-to-surfactant ratio (≥ 1:1), a surfactant with HLB value greater than 10 would be necessary for fine and uniform self emulsification. It should be noted that head group structure, in addition to HLB, may play a role in the emulsification process [2]. The formulations that did not spontaneously form emulsions upon gentle mixing were not further analyzed in the permeability enhancement and drug release studies.

All studied systems that did form visibly uniform emulsions, including different formulations and different dilutions in aqueous media, contained droplet sizes in the 100 to 500 nm range (Table 2). Formulations that did not form uniform fine emulsions had larger droplet sizes, with measured averages in the range of hundreds of nanometers to several microns. However, data pertaining to these systems are not reported, since samples taken from these solutions are not representative of the entire system due to lack of uniformity, and the light scattering instrument used accurately measures particle sizes only up to 6 microns.

All three formulation design parameters (oil type, HLB value, oil-to-surfactant ratio) had strong direct or interaction influences on emulsion mean droplet size. Regression analysis yielded good predictive models (Table 3) for mean droplet size for both formulations with drug \( (p < 0.0005, \text{adjusted } R^2 = 0.444) \) and without drug \( (p < 0.0005, \text{adjusted } R^2 = 0.709) \). For 1:1000 dilution emulsions in DI water with drug, an analysis of variance (Table 4) indicated that the primary factors affecting mean droplet size were oil-to-surfactant ratio \( (p = 0.007) \), oil type \( (p = 0.005) \), and surfactant-ratio interaction \( (p < 0.001) \), but interestingly not surfactant
alone ($p = 0.251$). A higher oil-to-surfactant ratio resulted in a larger mean droplet size, with post-hoc pairwise comparisons indicating that mean droplet sizes from the 9:1 oil-to-surfactant ratio systems are significantly larger than those from the 1:1 ratio systems ($p = 0.005$). This relationship agrees with previous findings where a medium chain triglyceride oil and Tagat TO, an HLB 11.5 surfactant, were combined at 9 different surfactant concentrations between 30% and 70% [24]. However, an increase in mean droplet diameter once the surfactant concentration exceeded 60% (wt.) was reported. The effect of surfactant concentration on droplet size has been attributed to viscous gel formation above a certain surfactant level and a consequent retarding of self emulsification [25]. The impact of oil to surfactant ratio on emulsion performance was recently studied by Cuinè et al. [26]. They utilized Soybean oil: Maisine 35-1 (1:1 w/w) as the oil phase, Cremophor EL as the surfactant and ethanol as the co-surfactant and formulated excipients at the following oil to surfactant ratios 2:1, 1.6:1, 1.5:1, 1.2:1, 1.1:1, 0.7:1, 0.3:1. A notable effect of oil to surfactant ratio on emulsion particle size and drug release rate was not observed with this relatively narrow range of ratios; however, drug solubilization into the aqueous phase during in vitro digestion was highly affected by oil to surfactant ratio, which was also shown to affect overall oral absorption.

### Table 2. Droplet sizes of emulsion systems

<table>
<thead>
<tr>
<th>Oil</th>
<th>Surfactant</th>
<th>Weight ratio</th>
<th>1:1000 dilution with 3 mg/ml drug in DI water</th>
<th>1:1000 dilution without drug in DI water</th>
<th>1:100 dilution without drug in HBSS</th>
<th>1:50 dilution with 7 mg/ml drug in PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>S.D.</td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>Cremophor EL</td>
<td>9:1</td>
<td>259.8</td>
<td>16.8</td>
<td>443.6</td>
<td>141.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5:1</td>
<td>285.6</td>
<td>20.9</td>
<td>386.8</td>
<td>40.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1</td>
<td>300.6</td>
<td>59.3</td>
<td>338.1</td>
<td>24.3</td>
</tr>
<tr>
<td></td>
<td>Tween 80</td>
<td>9:1</td>
<td>340</td>
<td>49.9</td>
<td>320.2</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5:1</td>
<td>290.2</td>
<td>34.8</td>
<td>298.5</td>
<td>20.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1</td>
<td>396.6</td>
<td>41.6</td>
<td>313.5</td>
<td>65.1</td>
</tr>
<tr>
<td>Captex 200</td>
<td>Cremophor EL</td>
<td>9:1</td>
<td>398.8</td>
<td>94.1</td>
<td>362.8</td>
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<tr>
<td></td>
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<td>5:1</td>
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<td></td>
<td>Tween 80</td>
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<td>209.1</td>
<td>22.7</td>
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<tr>
<td></td>
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<td>5:1</td>
<td>209.8</td>
<td>8.4</td>
<td>230.4</td>
<td>13.8</td>
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<tr>
<td></td>
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<td>1:1</td>
<td>115</td>
<td>65.5</td>
<td>197</td>
<td>6.8</td>
</tr>
<tr>
<td>Neobee M5</td>
<td>Cremophor EL</td>
<td>9:1</td>
<td>373.6</td>
<td>51.3</td>
<td>436.6</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5:1</td>
<td>338.6</td>
<td>48.7</td>
<td>388.1</td>
<td>30.7</td>
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<td></td>
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<td>1:1</td>
<td>106.1</td>
<td>12.3</td>
<td>493.3</td>
<td>50.2</td>
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<tr>
<td></td>
<td>Tween 80</td>
<td>9:1</td>
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<td>21.3</td>
<td>285.5</td>
<td>22.1</td>
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<td></td>
<td></td>
<td>5:1</td>
<td>223</td>
<td>19.5</td>
<td>257.8</td>
<td>58.9</td>
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<td></td>
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<td>1:1</td>
<td>240.1</td>
<td>6.8</td>
<td>273.1</td>
<td>57</td>
</tr>
</tbody>
</table>

*a Each measurement was repeated with three independent samples. M.D. indicates mean diameter and S.D. indicates standard deviation. Droplet size measurements of formulations with HLB 10 surfactant, which did not spontaneously emulsify, are not reported here.

The oil studied with the longest carbon chain length, Soybean oil (C16-C22), had a significantly larger mean particle diameter ($p = 0.012$) than Captex 200 and Neobee M5 as indicated by Tukey’s multiple comparison post-hoc tests (Figure 1). Conversely, medium chain...
triglyceride, Neobee M5, and Captex 200, both of which have alkyl chain length of C8-C10 showed similar effects on droplet size. The phenomenon occurring might be low levels of partitioning of the surfactant at the oil water interface as a result of the low driving force, which would be the difference in carbon chain lengths between surfactant and oil. It has been proposed that oil with carbon chain length similar to that of surfactant penetrates more into the interfacial film, decreasing surfactant partitioning and droplet stabilization [27]. This relationship agrees with the results of James-Smith et al [20], who correlated carbon chain length with mean emulsion diameter. Their studies, conducted with SDS as the surfactant and alkane oils ranging in carbon chain length from 8 to 16, also showed that droplet size is largest for systems containing oil with chain length closest to that of the surfactant. Surfactants utilized in our formulations, Tween 80 and Cremophor EL, have alkyl carbon chain lengths of 18, which are in the range of Soybean oil chain lengths [28].

Our results also graphically suggest that for each oil type, particle size tends to be smaller for formulations with HLB 13 surfactant (Cremophor EL) compared to HLB 15 surfactant (Tween 80) (Figure 1). It was somewhat surprising, however, that there was not significant dependence of particle size on surfactant HLB over the 13 to 15 range ($p = 0.251$), given the inability of low HLB surfactant to spontaneously form emulsions, although this may be due to the narrow remaining test range (13 to 15 HLB) or the low statistical power of 3 measurements.

For systems without drug formed with 1:1000 dilution in water, factors demonstrating statistically significant influences (ANOVA) on droplet size were oil-to-surfactant ratio ($p = 0.034$), oil type ($p < 0.001$), surfactant HLB ($p < 0.001$), oil-surfactant interaction ($p = 0.018$), and oil-ratio interaction ($p = 0.037$) (Table 4). Tukey’s post-hoc tests again indicated that mean diameters of 1:1 oil to surfactant ratio systems were significantly smaller compared to 9:1 ratio emulsions ($p = 0.032$), agreeing with the above results for 1:1000 dilution emulsions in DI water with drug. Mean droplet size of formulations containing Captex 200 was smaller than that of systems containing other oils ($p < 0.0005$), again indicating that a shorter oil carbon chain length relative to surfactant may contribute to a smaller droplet size.

Overall, formulations with drug had slightly smaller mean droplet size compared to those without drug at 1:1000 dilutions in DI water. This can be attributed to the drug partitioning at the oil water interface. Naproxen has a logP of 3.29 and is thus relatively hydrophobic, but may still partition between the oil and aqueous layer along with the surfactant due to limited solubility in oil. Therefore a decrease in interfacial tension might occur that leads to an overall decrease in droplet size. Another potential reason for the change in droplet size may be the change in surfactant critical micelle concentration (CMC) due to addition of drug, which can increase surfactant aggregation number and decrease CMC [29]. At the same surfactant concentrations, systems with lower CMC values tend to have smaller droplet size [30]. The opposite effect of drug, however, has been observed for some systems. When Simvastatin concentration was increased from 2.4% to 18.3% in formulations containing Capryol 90, Laurog-

**Figure 1.** Droplet size of 50% surfactant concentration formulations with drug in DI water at 1:1000 dilution. For all oils, particle size is larger for formulations with Tween 80 (HLB 15) and smaller for formulations with Cremophor EL (HLB 13). Droplet size of the emulsion system with the most lipophilic triglyceride (soybean oil) was the largest for both surfactants ($p = 0.012$, Tukey post-hoc test). Error bars indicate standard deviation of three independent measurements. ‘*’ shows significance compared to other values (without ‘*’).
lycol 90, and Cremophor EL, an increase in droplet size from 33 nm to 150 nm was observed [8]. These phenomena are likely drug dependent, and drugs with a wider range of properties need to be studied to better understand the influence of drug on emulsion characteristics.

Particle sizes of systems prepared with 1:100 dilution in HBSS without drug and 1:50 in PBS with 7 mg/ml Naproxen are also summarized in Table 2. The 1:100 dilution emulsions in HBSS without drug were used to test the influence of emulsions on intestinal monolayer permeability, and 1:50 dilution formulations in PBS with 7 mg/ml Naproxen were used to test drug release kinetics. Droplet sizes of 1:1000 dilution systems with no drug in DI water appear similar to those of 1:100 dilution systems with no drug in HBSS, indicating a lack of impact on particle size of a range of dilution factors typically observed in the literature. The 1:50 formulations have larger mean emulsion particle size than emulsions prepared at greater dilution levels, possibly related to the increase in drug concentration or the decrease in dilution factor.

Zeta potential measurements of emulsions confirmed the stability of the systems and ranged from approximately -18 mV to -41 mV for all systems except for 1:50 dilution formulations in PBS with 7 mg/ml drug concentration (Table 5). For these systems, zeta potential ranged from -6 to -15 mV and was generally of smaller magnitude compared to those of other systems. This decrease in zeta potential magnitude can be correlated with the comparatively larger droplet sizes as well as the increased drug concentration of these same systems. Surface charge of the droplets was not statistically related to any of the formulation parameters (p = 0.424) (Table 4); the regression model therefore is not shown in Table 3.

Table 3. Linear regression analysis of formulation design variables and emulsion characteristics

<table>
<thead>
<tr>
<th>Response</th>
<th>Y&lt;sub&gt;i&lt;/sub&gt;</th>
<th>Model</th>
<th>p</th>
<th>R&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Droplet size</td>
<td>Droplet size of 1:1000 dilution emulsions in DI water with drug in nm</td>
<td>Y&lt;sub&gt;1&lt;/sub&gt; = 797.2 - 138.5<em>A&lt;sub&gt;1&lt;/sub&gt; + 43.9</em>A&lt;sub&gt;2&lt;/sub&gt; - 279.6<em>B - 245.5</em>C + 62.2<em>A&lt;sub&gt;1&lt;/sub&gt;<em>C + 62.9</em>A&lt;sub&gt;1&lt;/sub&gt;<em>B - 24.8</em>A&lt;sub&gt;2&lt;/sub&gt;<em>B - 3</em>A&lt;sub&gt;2&lt;/sub&gt;<em>C + 121</em>C</em>B</td>
<td>&lt;0.0005</td>
<td>0.444</td>
</tr>
<tr>
<td></td>
<td>Droplet size of 1:1000 dilution emulsions in DI water without drug in nm</td>
<td>Y&lt;sub&gt;2&lt;/sub&gt; = 677.3 - 59.9<em>A&lt;sub&gt;1&lt;/sub&gt; - 137</em>A&lt;sub&gt;2&lt;/sub&gt; - 229.2<em>B - 35.4</em>C - 39.2<em>A&lt;sub&gt;1&lt;/sub&gt;<em>C + 88.4</em>A&lt;sub&gt;1&lt;/sub&gt;<em>B - 76.3</em>A&lt;sub&gt;2&lt;/sub&gt;<em>C + 121.4</em>A&lt;sub&gt;2&lt;/sub&gt;<em>B + 30.9</em>C</em>B</td>
<td>&lt;0.0005</td>
<td>0.709</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>Percentage cell viability</td>
<td>Y&lt;sub&gt;3&lt;/sub&gt; = 80.7 - 11.8<em>A&lt;sub&gt;1&lt;/sub&gt; + 1.7</em>A&lt;sub&gt;2&lt;/sub&gt; + 17.5<em>B + 30.6</em>C - 0.4<em>A&lt;sub&gt;1&lt;/sub&gt;<em>B + 4.3</em>A&lt;sub&gt;1&lt;/sub&gt;<em>C + 9.8</em>A&lt;sub&gt;2&lt;/sub&gt;<em>B - 7.6</em>A&lt;sub&gt;2&lt;/sub&gt;<em>C - 21.2</em>B</em>C</td>
<td>&lt;0.0005</td>
<td>0.466</td>
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<tr>
<td>Permeability</td>
<td>Percentage Naproxen permeation across cell monolayer</td>
<td>Y&lt;sub&gt;4&lt;/sub&gt; = 35.8 + 3.7<em>A&lt;sub&gt;1&lt;/sub&gt; - 0.9</em>A&lt;sub&gt;2&lt;/sub&gt; - 4.6<em>B - 3.4</em>C - 1.6<em>A&lt;sub&gt;1&lt;/sub&gt;<em>B + 3</em>A&lt;sub&gt;1&lt;/sub&gt;<em>C + 3.5</em>A&lt;sub&gt;2&lt;/sub&gt;<em>B - 0.9</em>A&lt;sub&gt;2&lt;/sub&gt;<em>C + 2.2</em>C</em>B</td>
<td>0.028</td>
<td>0.289</td>
</tr>
<tr>
<td>Release Coefficient</td>
<td>Release constant of Naproxen in nm²/min</td>
<td>Y&lt;sub&gt;5&lt;/sub&gt; = 2300.1 - 1793.6<em>A&lt;sub&gt;1&lt;/sub&gt; - 799.5</em>A&lt;sub&gt;2&lt;/sub&gt; + 542.7<em>B - 28.6</em>C + 1267.3<em>A&lt;sub&gt;1&lt;/sub&gt;<em>B + 52.9</em>A&lt;sub&gt;1&lt;/sub&gt;<em>C - 691.3</em>A&lt;sub&gt;2&lt;/sub&gt;<em>B + 498.3</em>A&lt;sub&gt;2&lt;/sub&gt;<em>C - 193.8</em>C</em>B</td>
<td>&lt;0.0005</td>
<td>0.594</td>
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</table>

<sup>b</sup> Independent variables are: A<sub>1</sub>: Soybean Oil, A<sub>2</sub>: Captex 200; B: Surfactant HLB; C: Oil to surfactant ratio. Oil type is coded as (0,1) indicator variables, with Soybean oil included by setting A<sub>1</sub>=1 and A<sub>2</sub>=0, Captex 200 included by setting A<sub>1</sub>=0 and A<sub>2</sub>=1, and Neobee M5 included by setting A<sub>1</sub>=0 and A<sub>2</sub>=0 (note only one oil type can be included in any given model). Zeta potential and integrity were statistically insignificant and are not included in the table.
Table 4. Analysis of variance of formulation design parameters affecting emulsion performance.

<table>
<thead>
<tr>
<th>Response</th>
<th>Main effects</th>
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<th>Interactions</th>
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<tr>
<td></td>
<td></td>
<td>Surf. HLB</td>
<td>Oil type</td>
<td>Ratio</td>
</tr>
<tr>
<td>Droplet size with drug</td>
<td>$F$</td>
<td>1.363</td>
<td>6.151</td>
<td>5.735</td>
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<tr>
<td></td>
<td>$p$</td>
<td>0.251</td>
<td>0.005</td>
<td>0.007</td>
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<tr>
<td>Droplet size without drug</td>
<td>$F$</td>
<td>36.194</td>
<td>18.626</td>
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<tr>
<td></td>
<td>$p$</td>
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<td>0.000</td>
<td>0.034</td>
</tr>
<tr>
<td>Zeta potential</td>
<td>$F$</td>
<td>0.879</td>
<td>1.115</td>
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<tr>
<td></td>
<td>$p$</td>
<td>0.361</td>
<td>0.349</td>
<td>0.126</td>
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<td>1.917</td>
<td>14.546</td>
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<tr>
<td></td>
<td>$p$</td>
<td>0.000</td>
<td>0.157</td>
<td>0.000</td>
</tr>
<tr>
<td>Cell integrity</td>
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<tr>
<td></td>
<td>$p$</td>
<td>0.299</td>
<td>0.586</td>
<td>0.606</td>
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<tr>
<td>Permeability</td>
<td>$F$</td>
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<td>8.304</td>
<td>0.502</td>
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<td></td>
<td>$p$</td>
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<td>Release</td>
<td>$F$</td>
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<tr>
<td></td>
<td>$p$</td>
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<td>0.000</td>
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Table 5. Surface charges of emulsion droplets<sup>c</sup>

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<th>Oil</th>
<th>Surfactant</th>
<th>Weight ratio</th>
<th>1:1000 with 3 mg/ml drug in DI water</th>
<th>1:1000 without drug in DI water</th>
<th>1:100 without drug in HBSS</th>
<th>1:50 with 7 mg/ml drug in PBS</th>
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</thead>
<tbody>
<tr>
<td>Soybean oil</td>
<td>Cremophor EL</td>
<td>9:1</td>
<td>-31.5</td>
<td>-35</td>
<td>4.9</td>
<td>-7.3</td>
</tr>
<tr>
<td></td>
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<td>5:1</td>
<td>-31.8</td>
<td>-40.4</td>
<td>2.2</td>
<td>-10.3</td>
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<td></td>
<td></td>
<td>1:1</td>
<td>-19.7</td>
<td>-25.3</td>
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<td>-9.7</td>
</tr>
<tr>
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<td></td>
<td>-39.6</td>
<td>-39.8</td>
<td>6.6</td>
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<td>-35</td>
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<td>5:1</td>
<td>-27.6</td>
<td>-35</td>
<td>11.1</td>
<td>-14.7</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>-33.3</td>
<td>-36.3</td>
<td>3.1</td>
<td>-11.7</td>
</tr>
<tr>
<td>Tween 80</td>
<td>Cremophor EL</td>
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<td>-29.1</td>
<td>-42</td>
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<td>-8</td>
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<tr>
<td></td>
<td></td>
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<td>-17.9</td>
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<td>-9.3</td>
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<tr>
<td>Captex 200</td>
<td>Cremophor EL</td>
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<td>-27.1</td>
<td>-36.1</td>
<td>1.6</td>
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</tr>
<tr>
<td></td>
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<td>5:1</td>
<td>-39.7</td>
<td>-6.4</td>
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</tr>
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<td></td>
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</tr>
<tr>
<td>Tween 80</td>
<td>Cremophor EL</td>
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<td>-24.4</td>
<td>-27.3</td>
<td>9.7</td>
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<tr>
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<td>-36.4</td>
<td>12.9</td>
<td>-6.3</td>
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<tr>
<td></td>
<td></td>
<td>1:1</td>
<td>-20.3</td>
<td>-31</td>
<td>16.5</td>
<td>-9.5</td>
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<tr>
<td>Neobee M5</td>
<td>Cremophor EL</td>
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<td>-24.4</td>
<td>-27.3</td>
<td>9.7</td>
<td>-5</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>-36.4</td>
<td>12.9</td>
<td>-6.3</td>
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<tr>
<td></td>
<td></td>
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<td>-20.3</td>
<td>-31</td>
<td>16.5</td>
<td>-9.5</td>
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</table>

<sup>c</sup> Each measurement is repeated with two independently prepared samples. M.Z. indicates mean zeta potential value and S.D. indicates standard deviation. Zeta potential measurements of formulations with HLB 10 surfactant, which did not spontaneously emulsify, are not reported here.
Effect of emulsions on Caco-2 cell monolayer cytotoxicity and permeability

Since intestinal permeability enhancement sometimes can be attributed to damage to the intestinal epithelial barrier, Caco-2 cell monolayers were used to test the cytotoxicity of the formulations. The surfactant seems to be the major factor in cytotoxicity either as a main or interaction effect, with cytotoxicity in the MTT assays affected by surfactant type \((p < 0.001)\), oil-to-surfactant ratio \((p < 0.001)\), and surfactant-ratio interaction \((p < 0.001)\). Cells exposed to formulations composed of 1:1 ratio mixture surfactant (56% Labrasol, 44% Capmul MCM), regardless of the oil type, showed significant cytotoxicity \((p < 0.001)\) after three hours of exposure compared to the negative control (Figure 2). The significant toxicity of the unstable formulations (HLB 10 surfactant at 1:1 oil-to-surfactant ratio (but not the higher ratios)) implies that emulsion stability might be an important factor in maintaining cell viability. The cytotoxicity also might be due, however, to the toxic nature of the specific surfactant mixture, as suggested by the statistically significant surfactant-ratio interaction above. This was investigated by exposing Caco-2 monolayers to single surfactant solutions in buffer (data not shown). Surfactant mixtures of Labrasol and Capmul MCM showed higher cytotoxicity levels compared to other surfactants; however, even though the concentrations of surfactant were higher in solution in these experiments than in tests of emulsion cytotoxicity, surfactant alone did not decrease cell viability as much as emulsion formulations with surfactant at 1:1 ratio. Testing all formulation components individually on Caco2 was not possible since oils without surfactant do not form a uniform mixture in the aqueous cell culture medium. In a recent study by Sha et al. [31] performed on Caco2 it was shown that Labrasol alone induced cytotoxicity dramatically at dilutions higher than 1:100 (w/v), in agreement with our findings. However, an increase in paracellular drug transport with no toxicity was reported with a formulation consisting of 30% Maisine 35-1, 38% Cremophor EL, 10% Labrasol, 23% Transcutol P; enhancement in paracellular permeability was suggested to be due to Labrasol at 1:1000 overall dilution.

Changes in intestinal permeability were assessed by measuring drug transport across epithelial cell layers, and epithelial tight junction integrity was assessed by measuring transepithelial electrical resistance (TEER). Two model drugs were used in permeability assessment: Naproxen and Lucifer Yellow. Lucifer Yellow is known to be transported by the paracellular route while Naproxen is transported mostly by the transcellular route. Formulations tested did not appear to influence the transcellular transport route. No increase in permeability to Naproxen was observed with exposure to emulsion systems (Figure 3); permeability values were in the range of 2.9 to 3.5 \(\times 10^{-5}\) cm/s.

In contrast, the integrity of tight junctions as assessed by TEER values appeared to be influenced by the emulsion formulations. TEER values were measured before and after exposure to emulsion systems during transport studies. Cell monolayer resistance was between 503 and 630 ohms for all wells before exposure to emulsion systems. Figure 4 shows the decrease in cell monolayer resistance after exposure to emulsion systems.
monolayer integrity due to exposure to SEDDS. Formulations with Tween 80 at 1:1 oil-to-surfactant ratio resulted in the most notable decrease in cell monolayer integrity; although only decreases after exposure to formulations with Soybean oil (at a 1:1 ratio) were found to be statistically significant ($p = 0.01$) compared to negative control HBSS. Since the formulations that decreased monolayer integrity do not result in transcellular permeability enhancement or a toxic affect on cells as indicated by the MTT assay results, the decrease in cell monolayer integrity can be attributed to the loosening of tight junctions due to Tween 80. Decrease in cell-cell junction integrity due to the surfactant Tween 80 was also indicated in a study by Levy et al [32]. They studied transport of barbituates across goldfish membrane and showed that the nonionic surfactant Polysorbate 80 (Tween 80) with a concentration below the CMC increased drug absorption, while it decreased absorption at concentrations above the CMC [32]. The increase in absorption was interpreted as being due to the enhanced permeability of the biological membrane. The decrease at higher concentrations, conversely, might have been due to the increased solubilizing effect of the micellar surfactant structures slowing the rate of absorption or to the layering of free surfactant monomers on the cell culture monolayer and related blocking of drug transport.

Lucifer Yellow transport, another indicator of paracellular transport and thus tight junction integrity, was influenced by exposure to different emulsion systems (Figure 5). Due to large variances in experimental measurements, it was not possible to distinguish any significant enhancement in permeability due to any specific formulation, although additional experiments might increase statistical power. To test the specific influence of Tween 80, the effect of surfactants alone on Lucifer Yellow transport also was studied. Exposure to Tween 80 solution for three hours tended to improve paracellular drug transport (Figure 5).

**Rate of drug release from emulsions**

Drug release from emulsions involves interfacial transport across the layer of surfactant coating the emulsion droplet followed by diffusive and convective transport through the surrounding aqueous medium. The appropriate kinetic expression depends upon (1) whether the drug enters the aqueous phase as a free molecule which then “reacts” with micelles or enters the aqueous phase by partitioning directly into a micelle and (2) the rate-limiting step in transport: interfacial transport, bulk transport, or reaction with micelles. In order to explore drug release kinetics and dependence on emulsion formulation characteristics, we used a mass transfer model that assumes interfacial transport limited kinetics (Equation [2]) [33] to calculate the release coefficient from emulsions. The magnitude of this coefficient can be compared to that expected if different mechanisms were dominating drug release for insight into drug release mechanism [34].

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**Figure 4.** Effect of formulations on cell monolayer integrity. Exposure to SEDDS formulations with high levels of high HLB surfactant (Tween 80) resulted in decreased cell layer integrity potentially indicating loosening of tight junctions. Soybean oil-Tween 80 formulation at 1:1 oil to surfactant weight.

**Figure 5.** Effect of emulsion systems and surfactants on Lucifer Yellow transport across Caco-2 cell monolayers. Tween 80 solution in HBSS at 1:100 dilution tends to improve Lucifer Yellow permeability. Error bars indicate standard deviation of two independent measurements.
<table>
<thead>
<tr>
<th>Oil</th>
<th>Surfactant</th>
<th>Weight Ratio</th>
<th>$k_{\text{eff}}$ (nm$^2$/min)</th>
<th>% drug released at 15 min</th>
<th>% drug released at 24 hr</th>
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<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Std Dev</td>
<td></td>
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<tr>
<td>Soybean Oil</td>
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<td>904.2</td>
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<td></td>
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<td></td>
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<td>1561.1</td>
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</table>

For most of the formulations, release of Naproxen from oil droplets was mostly completed in the first fifteen minutes of the experiment. However, it was also observed that for some formulations release was not complete even after 24 hours. Release coefficients reported as $k_{\text{eff}}$ represent effective release coefficients for formulations calculated for the first fifteen minutes of the experiment. Based on the analysis performed with release rate constants ($k_{\text{eff}}$) all of the formulation parameters (oil type $p < 0.0005$, surfactant HLB $p < 0.0005$, oil-to-surfactant ratio $p < 0.0005$) had significant influence on rate of release constant. It was statistically observed that release rate constants were higher for formulations with the least surfactant ratio (oil to surfactant ratio 9:1) compared to other ratios ($p < 0.0005$, Tukey’s post-hoc test) which might be due to the low level of partitioning of the surfactant at the interface resulting in a decrease in the interfacial barrier. It was previously suggested that decreasing the total surfactant amount in a system may cause a decrease in the amount of surfactant located at the interface [20]. Release coefficients were also overall highest for formulations containing Soybean oil and lowest for those containing Captex 200 ($p < 0.0005$, post-hoc tests) (Table 6). Soybean oil has a carbon chain length closest to that of the surfactants used. As proposed above, penetration of soybean oil into the interfacial film [27] might decrease the interfacial barrier at the oil water interface, which is proposed to be the rate-limiting factor for release from emulsion systems [35].

**CONCLUSIONS**

The results of this study represent initial efforts to develop guidance for emulsion-based formulation design based on experimental-mechanistic understandings of how formulation parameters relate to function. Representative formulations of emulsion based drug delivery systems based on different oil structural classes, surfactant HLB, and oil-to-surfactant ratios were...
prepared and tested for their ability to spontaneously emulsify and several emulsion characteristics. The impact of these formulation parameters on mean droplet size, cytotoxicity, intestinal permeability, release kinetics, and droplet surface charge were studied via analysis of variance and regression models.

Results identify the strong influences of certain formulation parameters and interactions on emulsion characteristics and function. The effect of surfactant HLB on emulsion formation was underscored by formulations with HLB 10 not spontaneously emulsifying. Exposure to high concentrations of high HLB surfactant (Tween 80), however, resulted in decreased cell monolayer integrity, possibly due to loosening of tight junctions. Release rates were related to oil-surfactant pair carbon chain length and surfactant concentration.

Additional analysis of fundamental mechanisms of function across broader ranges of oils, surfactants, and drugs will help further generalize these results and form the basis for an overall mechanistic model to optimize drug absorption using emulsion-based drug delivery. Regression models such as those presented here can be used to design emulsions with specific desired characteristics or estimate key parameters reflective of performance of specific formulations (e.g., drug release kinetics, intestinal permeability enhancement). Such regression models eventually could be incorporated into an overall systems-based model predicting drug absorption upon dosing with SEDDS, based on mass transfer expressions for drug release and drug permeation across the intestinal membrane; these mass transfer expressions would contain parameters predicted by regression models. In parallel, experimental design methods could be used to determine optimal formulations that balance multiple desirable properties (e.g. multivariate optimization, desirability and loss functions, goal programming, stochastic optimization, et cetera). This may be especially helpful for optimizing inherent tradeoffs between different combinations of formulation values that separately optimize mean particle size, toxicity, permeability, and release.

While not the focus of this paper, another set of important results is the significant amount of experimental variability (standard deviation) in specific characteristics within some formulations, and differences between formulations in the size of these within-formulation variabilities, which typically would not be known via physical models. These results have implications on formulation design optimization, as it may be desirable to identify formulations that both give desirable mean results but also high consistency (i.e. low variability) in performance. Future potential useful analyses from the experimental design field therefore might include variability minimizing and robust designs that perform desirably well with maximum consistency across uncontrollable (e.g. within and between patient) in vivo environments.

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