Real-time Measurements to Characterize Dynamics of Emulsion Interface during Simulated Intestinal Digestion

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ABSTRACT

Efficient delivery of bioactives remains a critical challenge due to their limited bioavailability and solubility. While many encapsulation systems are designed to modulate the digestion and release of bioactives within the human gastrointestinal tract, there is limited understanding of how engineered structures influence the delivery of bioactives. The objective of this study was to develop a real-time quantitative method to measure structural changes in emulsion interface during simulated intestinal digestion and to correlate these changes with the release of free fatty acids (FFAs). Fluorescence Resonant Energy Transfer (FRET) was used for rapid in-situ measurement of the structural changes in emulsion interface during simulated intestinal digestion. By using FRET, changes in the intermolecular spacing between the two different fluorescent probes labeled emulsifier was characterized. Changes in FRET measurements were compared with the release of FFAs. The results showed that bile salts and pancreatic lipase interacted immediately with the emulsion droplets and disrupted the emulsion interface as evidenced by reduction in FRET efficacy compared to the control. Similarly, a significant amount of FFAs was released during digestion. Moreover, addition of a second layer of polymers at emulsion interface decreased the extent of interface disruption by bile salts and pancreatic lipase and impacted the amount or rate of FFA release during digestion. These results were consistent with the lower donor/acceptor ratio of the labeled probes from the FRET result. Overall, this study provides a novel approach to analyze the dynamics of emulsion interface during digestion and their relationship with the release of FFAs.

Key words: Dynamics of emulsion interface; Simulated intestinal digestion; Fluorescence resonance energy transfer (FRET)

INTRODUCTION

Oil-in-water emulsions have been proposed for encapsulation and delivery of hydrophobic bioactives in food, pharmaceutical and cosmetic industries [1]. In emulsions, the hydrophobic bioactive compounds are often dispersed in the oil phase of emulsions. Release of the encapsulated compounds from these emulsions can be significantly influenced by lipid digestion [2, 3]. Lipid digestion occurs primarily in the small intestine of adults [4], where pancreatic lipase acts in concert with various co-factors (co-lipase, bile salts and calcium) to hydrolyze lipids and produce free fatty acids and monoglycerides [5]. Lipid hydrolysis in emulsions is a dynamic and interfacial process [6], and it is controlled by the interactions of pancreatic lipase, bile salts and its co-factors with the emulsion interface [4, 5].

With this motivation, studies have been conducted to modulate the rate of lipid digestion in emulsion formulations using a combination of physical and chemical approaches to engineer the emulsion interface [7-12]. These approaches include (a) layer-by-layer coatings of polymers at the emulsion interface [7, 8, 10]; (b) increasing steric hindrance using inert polymers at the interface [11] to reduce the rate of binding of bile salts and enzymes such as lipase; and (c) biochemical approaches using modified coating at the emulsion interface to inhibit enzyme activity [12]. Despite significant efforts in designing emulsion interface for controlling the rate of lipid digestion, changes in the emulsion interface upon interactions with bile salts and digestive enzymes are not well understood. Most of our current understanding of the interactions of digestive environment with emulsion interface is based on in-vitro digestion studies. In these in-vitro studies, lipid digestion is usually characterized by measuring the amount of free fatty acids produced based on titration using an alkali solution [13]. To characterize the release of encapsulated compounds from emulsions, the most common approach currently used in these

studies is based on centrifugal separation of the micellar phase from the lipid phase [13, 14]. However, this method often requires high speed centrifugation of the digested emulsion samples [13, 15] for an extended period of time that can influence the structure of colloidal particles and the fate of encapsulated compounds. Despite significant efforts in engineering the interface of emulsions and other colloidal particles, the prior studies provide only limited information regarding the dynamics of the interface during digestion.

Interfacial tension measurements are the leading approach commonly used for characterizing dynamics of the oil-water interface during digestion processes [16]. Typically in these measurements, interactions between the suspended oil droplets and the bulk aqueous phase with simulated digestive environments and interfacial active molecules are measured. This approach is useful for understanding the interactions of simulated digestive environment with simple interfacial compositions, but currently this approach cannot be easily translated to measure complex interfacial compositions such as layer-by-layer coatings. In addition, the interfacial tension measurement approaches cannot characterize the role of size of colloidal particles in influencing lipid digestion and release of encapsulated bioactives [5]. Therefore, there are significant gaps in our current knowledge regarding structural changes in the interface of complex emulsion formulations including layer-by-layer coatings during digestion and their relationship with lipid hydrolysis. In order to understand the dynamics of the interface during digestion, there is a need to develop real-time quantitative methods for rapid in-situ analysis and their application to understand dynamics of complex interfacial compositions. It is expected that these measurements provide fundamental understanding of interactions between the interfacial compositions and digestion processes including enzymatic processes and biochemical interactions. Furthermore, these measurements may not only bridge the gap between the

interfacial tension measurements and the current design of in-vitro digestion studies, but also address some of the limitations of both the approaches.

The focus of this study was to understand structural changes in emulsion interface during simulated intestinal digestion and correlate these structural changes with the release of free fatty acids from emulsion formulations. For characterizing changes in emulsion interface during digestion, an optical method based on fluorescence resonance energy transfer (FRET) was developed. FRET is a distance dependent non-radiative energy transfer process between the donor and the acceptor fluorophores [17, 18]. This energy transfer from the donor to the acceptor fluorophore is mediated by intermolecular long-range dipole–dipole coupling between the fluorophores [17, 18]. FRET is highly efficient if the donor and acceptor fluorophores are positioned within the Förster radius (the distance at which half the excitation energy of the donor is transferred to the acceptor, typically 3–6 nm) [18] and with appropriate alignment of the dipoles. To monitor changes in emulsion interface using FRET, phospholipid emulsifier molecules conjugated with two different fluorescent probes were selected. Changes in the relative positioning of these probes can be tracked in real-time by measuring the ratio of fluorescence intensity of the selected donor and acceptor probes using the donor excitation wavelength. The food-grade lecithin and bile salts stabilized emulsion labeled with FRET pair fluorophores at the interface was selected as a model system to evaluate the dynamics of phospholipid interface during simulated intestinal digestion. To evaluate the influence of a biopolymer coating on the dynamics of an emulsion interface during digestion, the model emulsion was coated with food grade polymers ϵ -polylysine (MW around 5000 Da) and medium molecular weight chitosan (MW around 190-310 kDa) respectively based on electrostatic interactions. ϵ -polylysine has been proposed to inhibit lipase activity and thus may

influence the rate of lipid digestion [12, 19]. Chitosan is an indigestible polysaccharides [20] and prior studies have indicated that chitosan could interfere with lipid digestion [10]. This is due to the gel forming ability or enhanced viscosity of chitosan coatings as has been suggested in some of the prior studies [21, 22]. Dynamics of the emulsion interface measured based on FRET measurements during simulated intestinal digestion was correlated with the release of fatty acids from the model emulsions.

In summary, this study (a) develops a real-time quantitative method to measure structural changes in emulsion interface during simulated intestinal digestion and correlate these changes with the release of free fatty acids; and (b) evaluates the role of biopolymer membrane coatings on emulsion interface in influencing interactions between the interface and simulated intestinal fluid and its individual components. The unique approaches developed in this study will enable detailed understanding of real-time dynamics of emulsion interface during simulated intestinal digestion. These measurements will enable rational design of encapsulation systems to control the release of encapsulated compounds.

MATERIALS AND METHODS

Materials

Bile salts (~50% cholic acid sodium salt and ~50% deoxycholic acid sodium salt), calcium chloride, low molecular weight chitosan (MW around 190-310 kDa), and lipase from porcine pancreas (type II) were obtained from Sigma-Aldrich (St Louis, MO, USA). ϵ -polylysine (EPL, MW around 5000 Da) was a gift from Purac America (Lincolnshire, IL, USA). Oregon Green[®] 488 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (OG-ph) and Texas Red[®] 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (TR-ph)

were purchased from Life Technologies (Carlsbad, CA, USA). Low melting lecithin (ALCOLEC®PC 75) was a gift from American Lecithin Inc. (Oxford, CT, USA). Organic canola oil was a gift from Spectrum Naturals, Inc. (Petaluma, CA, USA). Sodium chloride, tris hydrochloride (pH 7.5) with high purity low metal, sodium hydroxide and chloroform were obtained from Fisher Scientific. Ultra-pure water (16MΩ-cm) was obtained from an in-house water filtration system.

Emulsion Preparation

Lecithin-bile primary emulsion

Oregon Green 488 labeled 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (OG-ph) and Texas Red labeled 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (TR-ph) were selected as the donor and the acceptor of FRET pair, respectively. Lecithin and bile salts were dissolved in water and the final concentrations of lecithin and bile salts were 1.5 w/v % and 0.5 w/v %, respectively. Bile salts were selected to stabilize the lecithin emulsion based on their net negative charge. The concentration of bile salts used was below the CMC (critical micelle concentration) of bile salts, thus cannot disrupt the emulsions. A stock OG-ph or TR-ph solution was prepared by dissolving OG-ph or TR-ph in chloroform. The concentration of the dye labeled phospholipids was 0.1 mol % compared to the total amount of lecithin used. The dye labeled phospholipid solution was dried in a glass vial under a stream of nitrogen then placed in a vacuum desiccator for 1 hour. The lecithin and bile salts solution was mixed with the OG-ph or TR-ph after drying. Coarse emulsions were prepared by dispersing canola oil (4 w/v %) in lecithin and bile salt solution using a hand-held disperser (Ultra-Turrax model T25, IKA Works, Wilmington, NC, USA) set at 9500 rpm for 2 min. The

coarse emulsion was then probe-tip sonicated for 30 seconds at 50 % of maximum amplitude (55W power) with a Qsonica Q55 ultrasonic processor (Newton, CT, USA).

Secondary emulsion with EPL or chitosan coating

A layer of EPL or chitosan was added to the lecithin-bile stabilized primary emulsion by mixing the lecithin emulsion with EPL or chitosan solution at 1:1 volume ratio (5 ml each) to form the secondary emulsion with EPL or chitosan coating. The final concentrations of EPL and chitosan in the final emulsion were 2 w/v % and 0.3 w/v %, respectively. The concentration of coated EPL or chitosan was selected based on prior studies [8, 23].

After preparation, the lecithin-bile primary emulsion was diluted with water at 1:1 volume ratio in order to obtain the same concentration of lecithin (0.75 w/v %), bile salt (0.25 w/v %), oil (2 w/v %) and labeled phospholipids in both lecithin-bile primary emulsion and secondary emulsions. The pH of each of the samples was adjusted to 7.5 with 1 M NaOH and then the samples were subjected to a simulated in-vitro intestinal digestion model.

Droplet size and zeta potential measurements

Hydrodynamic diameter and zeta potential of emulsion droplets was measured using a particle size analyzer (Model: Malvern Nano Series, Malvern Instruments, Inc., Westborough, MA, USA). The settings for the analyzer were- material type: oil, particle refractive index = 1.45, dispersant type: water, dispersant refractive index= 1.33, temperature: 25 °C, model for zeta potential measurements: Smoluchowski. Droplet size measurements were analyzed based on the number average droplet size distribution. The size and zeta potential measurements for lecithin-bile primary emulsion and secondary lecithin-bile-EPL emulsion were carried out at pH

7. For secondary lecithin-bile-chitosan emulsion, the size and zeta potential were measured at pH 5 as the increased pH caused significant droplet aggregation due to loss of positive charge at the emulsion interface.

Fluorescence imaging of OG and TR dyes in lecithin-bile emulsion

Fluorescence images were acquired using coarse emulsion droplets to demonstrate that the OG-ph and TR-ph were colocalized at the interface of lecithin-bile primary emulsion. Olympus IX 71 microscope (Olympus Inc., Center Valley, PA, USA) at a 60× magnification was used to acquire fluorescence images of the emulsion droplets. The images were acquired using an excitation filter: 480/30 nm, an emission filter: 570/60 nm for OG-ph and an excitation filter: 540/30 nm and an emission filter: 605/55 nm for TR-ph. The camera exposure time for image acquisition in both channels was 100 ms.

Measurements of fluorescence resonance energy transfer (FRET) spectra

Emulsion samples (200 µL) with both OG and TR labeled phospholipids were placed in a 96-well plate. Fluorescence emission spectra of the emulsion samples were measured using a plate-reader (Spectramax M5, Molecular Devices, Carlsbad, CA, USA). The samples were excited at 501 nm and the emission scan from 515 nm to 700 nm with a step of 2 nm was recorded. Emulsion samples with only OG-ph and emulsion samples with only TR-ph were used as controls and the emission spectra were also measured using the same excitation wavelength and the emission signal was collected from 515 nm to 700 nm.

Monitoring simulated intestinal digestion using FRET

The pH of emulsion samples containing OG-ph and TR-ph was adjusted to 7.5 before incubating with the simulated intestinal digestion fluid or individual components of the simulated intestinal fluid. The simulated intestinal buffer consisted of 50 mM Tris buffer, 150 mM NaCl and 20 mM CaCl₂ (pH 7.5) [24]. Emulsion samples (50 µL) were mixed with 200 µL solution containing buffer, bile salts and pancreatic lipase. The final concentrations of bile salts and pancreatic lipase were 1 mg/mL and 2 mg/mL, respectively. The mixture was then pipetted into a 96-well plate and the plate was placed in a plate-reader (Spectramax M5, Molecular Devices, Carlsbad, CA, USA). The fluorescence intensity of OG and TR labeled emulsions was measured at a regular interval of 2 min for 3 h with shaking between readings. The excitation wavelength was 501 nm and the emission wavelengths were set at 535 nm (OG emission maximum wavelength) and 615 nm (TR emission maximum wavelength). Emulsion samples mixed with only buffer, buffer and bile salt, and buffer and pancreatic lipase were used as controls and the fluorescence intensity was also measured. Samples were prepared in triplicate and maintained at the standard experimental temperature of 37°C. The donor to acceptor ratio was calculated using the following equation:

$$\text{Donor / Acceptor ratio} = I_{\text{donor}} / I_{\text{acceptor}}$$

where I_{donor} is the fluorescence intensity of the sample at emission wavelength of 535 nm, and I_{acceptor} is the fluorescence intensity of the sample at emission wavelength of 615 nm.

Release of free fatty acids

The release of fatty acid was monitored using the automated pH-stat titration approach. In this approach, the % of FFA formation is calculated based on the volume of NaOH required to neutralize the fatty acid released by lipid hydrolysis for maintaining the pH of the solution at 7.5.

218 The release of free fatty acid during simulated intestinal digestion was calculated using the
219 following equation (assuming 2 FFA produced per 1 triacylglycerol molecule) [25]:

$$220 \quad \%FFA = \frac{V_{NaOH} \times c_{NaOH} \times M_{lipid}}{m_{lipid} \times 2} \times 100$$

221 where V_{NaOH} is the volume of NaOH required to titrate the FFA produced (mL), c_{NaOH} is the
222 concentration of NaOH used (M), m_{lipid} is the total weight of canola oil initially present in the
223 reaction cell (g), and M_{lipid} is the molecular weight of canola oil (g/mol).

224 The rate of release of FFA was also analyzed using the mathematical model developed by [26]:

$$225 \quad \phi = \phi_{\max} \left(1 - \left(1 + \frac{3kMt}{2d\rho} \right)^{-2} \right)$$

226 In this equation

227 ϕ = % of FFA released as a function of time (t)

228 k = Kinetic rate constant ($\mu\text{mol min}^{-1} \text{m}^{-2}$)

229 M = Molecular weight of the triglyceride oil (0.883 kg/mol)

230 ρ = Density of the oil (920 kg/m^3)

231 d = Initial mean droplet diameter (micrometer)

232 Using the Matlab curve fit tool, the data was fitted to this equation and the kinetic rate constant
233 was estimated for the primary and the secondary emulsions.

234 For the pH-stat measurements, emulsion samples (8 mL) were placed in a glass beaker
235 that was incubated in a shaker at 37°C and adjusted to pH 7.5. A solution (32 mL) containing
236 buffer, bile salt and pancreatic lipase was also incubated in a shaker at 37°C and adjusted to pH
237 7.5. The final concentrations of buffer, bile salt and pancreatic lipase were the same as

mentioned in the section above. The emulsion sample and the digestion solution were mixed in the reaction cell and a pH-stat automatic titration system (Denver Instrument, Bohemia, NY, USA) was used to automatically monitor the pH and maintain it at pH 7.5 at 37 °C by titrating with 0.1 M NaOH solution. The pH-stat measurements were carried out for 2 h.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel 2007 (Microsoft Corp., Bellevue, WA). Analysis of Variance (ANOVA) to identify the significant differences between the samples ($\alpha=0.05$) and the Student's t-test was used to identify the significant differences between each pair of treatments ($\alpha=0.05$).

RESULTS

Labeling of lecithin-bile stabilized emulsion interface using FRET pair

Co-localization of the selected FRET pair dyes conjugated to phospholipids emulsifier at the emulsion interface is essential for FRET between the dye pairs. To validate co-localization of OG-ph and TR-ph at the interface of lecithin-bile stabilized primary emulsion, coarse lecithin-bile primary emulsion droplets containing OG-ph and TR-ph at the interface were imaged using fluorescence microscopy (**Fig. 1(a)**). Coarse lecithin-bile primary emulsion droplets were prepared with the same proportions of oil and aqueous phases as in the case of homogenized lecithin-bile primary emulsions using a hand-disperser as described in the materials and methods section. Imaging measurements demonstrate that both the dyes are localized at the emulsion interface. Although some background fluorescence from labeled phospholipids that were not incorporated into the emulsion interface was also observed, the fluorescence intensity of the

labeled phospholipids at the emulsion interface was 3.8-fold higher as compared to the background fluorescence. To visualize co-localization of the OG and TR dyes at the emulsion interface, the individual fluorescence images were overlaid. The results from the overlaid images (combination of green and red color, results in yellow) indicate that significant fraction of OR-ph and TR-ph are co-localized at the interface (**Fig. 1(c)**).

To further evaluate that the co-localized OG-ph and TR-ph at the interface of lecithin-bile primary emulsion could transfer energy (FRET) from the donor (OG) fluorophore to the acceptor (TR) fluorophore, the fluorescence spectra were measured. The donor fluorophore was excited at the wavelength of 501 nm and the emission signal was measured from 515 to 700 nm. Lecithin-bile primary emulsions with only OG labeling and only TR labeling respectively were used as controls. Results in **Fig. 1(b)** show that when excited at 501 nm, the lecithin-bile primary emulsion with only OG labeling showed a relatively high fluorescence intensity at 535 nm which is the emission maximum wavelength for the OG dye. To assess contributions from direct excitation of the TR dye using the donor excitation wavelength (501 nm), the fluorescence spectrum was measured using the TR labeled emulsion samples at the excitation wavelength of 501 nm. The results show limited direct excitation of the TR dye with the 501 nm excitation. When OG and TR dual labeled lecithin-bile primary emulsion was excited at 501 nm, the spectrum showed a decrease in OG emission fluorescence intensity and an increase in TR emission fluorescence intensity compared to the fluorescence intensity of only OG labeled and only TR labeled emulsions, respectively. These results indicate the FRET between the OG and TR fluorophores at the emulsion interface. It is important to note that the concentration of dye labeled phospholipids used for labeling the emulsion interface was maintained the same for all emulsions. To account for FRET contributions from the free OG and TR fluorophores, the

emulsion samples were disrupted with methanol. The results show that a very low emission intensity from TR was observed when excited at 501 nm (OG excitation wavelength), indicating no FRET between the free OG and TR (data not shown).

Monitoring simulated intestinal digestion of lecithin-bile emulsions using FRET

Real-time changes in emulsion interface structure during simulated intestinal digestion were monitored using the donor to acceptor ratio of the FRET pair as described in the materials and methods section. **Fig. 2(a)** shows changes in the donor/ acceptor ratio as a function of time during simulated intestinal digestion. In order to evaluate the individual interactions of bile salts and pancreatic lipase with the emulsion interface during intestinal digestion, the emulsion samples were also incubated with only bile salts and only pancreatic lipase. Results in **Fig. 2(a)** show that the donor/acceptor ratios increased (reduction in FRET between the dyes at the interface) immediately upon addition of bile salts or a combination of bile salt and pancreatic lipase to the emulsions. No significant ($p > 0.05$) increase in the donor/acceptor ratio was observed during the 3 h digestion period after the initial changes in the donor/acceptor ratio for the samples incubated with bile salts. There was a marginal but significant ($p < 0.05$) change in the donor/acceptor ratio during the 3 h digestion period for the samples incubated with bile salts and pancreatic lipase. For the emulsion samples incubated with only pancreatic lipase, the donor/acceptor ratio increased slowly during the incubation period of 3 h. **Fig. 2(b)** shows the donor/acceptor ratios after 3 h of incubation of emulsion samples with bile salts, lipase or bile salts + pancreatic lipase. For emulsion samples incubated with only bile salts and a combination of bile salts and pancreatic lipase, the donor/acceptor ratios were approximately 4 times higher than the control (emulsion samples incubated with only buffer, no addition of bile salts and pancreatic lipase), and for emulsion sample incubated with only pancreatic lipase, the final ratio

was approximately 2.4 times higher compared to the control. It is important to note that the concentration of the labeled phospholipids used was 0.1 mol % compared to the amount of lecithin used for stabilization of emulsion, so the chances of both donor and acceptor to partition into the same mixed micelle or other secondary structures were small as illustrated by the results in Fig. S. 1 (Supplementary Section).

Polymer coated secondary emulsion

Secondary emulsion droplets with EPL or chitosan coating based on electrostatic interactions between these positively charged polymers and negatively charged lecithin-bile salt stabilized interface were characterized using size and zeta potential measurements. **Table 1** shows the droplet size and zeta potential of lecithin-bile primary emulsion and secondary emulsions coated with EPL and chitosan. The mean droplet diameter of the primary emulsion and both secondary emulsions ranged between 150-200 nm. Results in **Table 1** show that the zeta potential of lecithin-bile primary emulsion was approximately -78 mV. The zeta potential of emulsion changed from -78 mV to -7 mV and +76 mV when EPL and chitosan were added to the lecithin-bile emulsion, respectively.

Effect of polymer coatings on disruption of emulsion interface during simulated intestinal digestion

The effect of polymer coatings on dynamics of emulsion interface during simulated intestinal digestion was also evaluated using the FRET approach. FRET between the OG and TR conjugated phospholipids in EPL (**Fig. 3(a)**) or chitosan (**Fig. 3(b)**) coated secondary emulsion was evaluated. The results show a decrease in the fluorescence intensity at 535nm (OG emission maximum wavelength) and an increase in the fluorescence intensity at 615 nm (TR emission

maximum wavelength) when the emulsion interface was labeled with both OG-ph and TR-ph compared to emulsions labeled with only OG-ph and only TR-ph, respectively. For these measurements, all the emulsion samples were excited at 501 nm wavelength.

Results in **Fig. 4** show the donor/acceptor fluorescence intensity ratios of dual labeled lecithin-bile primary emulsion, secondary emulsion coated with EPL and secondary emulsion coated with chitosan after 3 h of simulated intestinal digestion. These emulsion samples (both primary and secondary emulsions) were also incubated with individual bio-chemical components of the simulated intestinal fluid including pancreatic lipase and bile salts to evaluate the interaction of individual components in the simulated intestinal digestion fluid with emulsion interface. These individual components were dispersed in the salt buffer solution simulating the pH and ionic strength of intestinal fluid. For EPL coated and chitosan coated secondary emulsions incubated with only pancreatic lipase, only bile salts and a combination of bile salts and pancreatic lipase, the donor/acceptor ratios were significantly ($p < 0.05$) higher than the control (secondary emulsion samples incubated with only buffer). Similar to the observations in **Fig. 2**, for the emulsion samples incubated with only pancreatic lipase, the donor/acceptor ratio increased slowly during the 3 h incubation period, while the rate of change in donor/acceptor ratio for the secondary emulsion samples upon incubation with only bile salts or a combination of bile salts and pancreatic lipase was instantaneous (data not shown). Thus, the rate of change in donor/acceptor ratio for the secondary emulsions incubated with bile salts or bile and pancreatic lipase was not influenced by the coatings.

These results also demonstrate that the magnitude of change in the donor/acceptor ratio induced by bile salts or bile salts and pancreatic lipase was reduced for the secondary emulsions as compared to the primary emulsion. For emulsion samples incubated with only bile salts, the

donor/acceptor ratio of lecithin-bile primary emulsion was approximately 2.3-fold higher than that of the secondary emulsions. Similarly, for emulsion samples incubated with a combination of bile salts and pancreatic lipase, the donor/acceptor ratio of lecithin-bile emulsion was approximately 1.4-fold and 1.7-fold higher than that of the secondary emulsion coated with EPL and secondary emulsion coated with chitosan, respectively.

Measurements of droplet size and zeta potential of lecithin-bile primary emulsion and EPL and chitosan coated secondary emulsions

Changes in emulsion droplet size and zeta potential after simulated intestinal digestion were shown in **Table 1**. The results show that after 3 h of simulated intestinal digestion, the mean droplet size for all three emulsions ranged between 2200 nm to 2500 nm. The results demonstrate that the droplet size increased significantly compared to the original emulsions and the zeta potential was close to neutral for all three emulsions. To account for any differences in the particle size and zeta potential of the lecithin-bile emulsion at pH 7.0 (solution of the emulsion samples) and pH 7.5 (pH of SIF), the particle size of lecithin-bile emulsion samples were compared. The comparison indicates that there is no significant difference ($p > 0.05$) in the particle size and zeta potential of the selected emulsions at these pH conditions. In order to measure the influence of change in the perimeter of the emulsion droplets during intestinal digestion on FRET signal, a control experiment was designed and the results are shown in the Supplementary Section (Table S.1). These results demonstrate that an increase in the diameter of emulsion droplets (comparison between coarse and fine emulsions) without disruption of the interface, does not result in an increase in donor/acceptor ratio. Together, these results indicate that the increase in donor/acceptor ratio results from disruption of the interface during simulated digestion.

Measurements of free fatty acid (FFA) release during simulated intestinal digestion

Release of free fatty acids from the lecithin-bile primary emulsion and the secondary emulsions with EPL or chitosan coatings is shown in **Fig. 5**. Results in **Fig. 5** show that the release of fatty acid from the primary emulsion reached saturation levels within the first 10 min of simulated intestinal digestion. In the case of EPL coated or chitosan coated secondary emulsion, approximately 65% of maximum FFAs were released within the first 10 min of digestion. After the first 10 min of simulated digestion, the release of FFA from the EPL coated or chitosan coated secondary emulsion was at a relatively lower rate compared to the lecithin-bile primary emulsion. After the first 10 min of simulated intestinal digestion, the FFAs were released gradually from the chitosan coated secondary emulsion and the release of FFA eventually reached 100% at 1 h of digestion. For the EPL coated secondary emulsion, approximately 80% release of FFAs was achieved at 1 h of simulated intestinal digestion and no significant increase was observed over the remaining incubation period. Based on analysis of the kinetic measurements as described in the methods section, the kinetic rate constants for the lecithin-bile primary emulsion, chitosan coated secondary emulsion and EPL coated secondary emulsions were: 17.45 ($R^2 = 0.89$, 95% confidence interval: 11.1, 23.2); 8.2 ($R^2 = 0.96$, 95% confidence interval: 7.2, 9.6); and 17.6 ($R^2 = 0.95$, 95% confidence interval: 14.4, 20.7) $\mu\text{mol min}^{-1} \text{m}^{-2}$. The results of this analysis indicated that the chitosan coating reduced the rate of the FFA formation by almost 50% as compared to the control emulsion (lecithin-bile salt) and the EPL coated lecithin-bile emulsion. In addition, the EPL coated lecithin bile emulsion reduced the total FFA by 20% as compared to the control and chitosan coated emulsions.

DISCUSSION

Role of FRET in understanding interfacial changes during intestinal digestion

FRET approach has been used both as a qualitative and quantitative tool in biological systems to analyze macromolecular interactions [27]. FRET measurement developed in this study can provide information on real-time dynamics of the emulsion interface without any extra sample separation steps and can evaluate the role of complex interfacial compositions in influencing interactions of colloidal systems with simulated digestion environment. In this study, emulsion interface was labeled with two fluorescent probes. Changes in the relative spacing of labeled emulsifiers during simulated intestinal digestion were evaluated based on changes in the donor/acceptor ratio of the FRET pair. Disruption of the emulsion interface increases the distance between the donor and acceptor molecules which were initially co-localized at the emulsion interface. This process results in decrease in efficiency of energy transfer from the donor to the acceptor fluorophore. As a result, both quenching of donor fluorophore and the energy transfer from the donor to the acceptor fluorophore is reduced. With an increase in donor fluorescence and reduction in acceptor emission, the donor/acceptor ratio increases with disruption of the emulsion interface during digestion.

Bile salts and pancreatic lipase have an essential role in the digestion of lipids [5, 16, 28]. Bile salts are natural bio-surfactants present in the gut lumen [5] and pancreatic lipases are water soluble enzymes that catalyzes hydrolysis of lipids [28]. Pancreatic lipases are activated upon binding to insoluble emulsified substrate and the extent of binding is related to the properties of the interface [28]. During intestinal digestion of emulsions, bile salts present in the digestive environment (above the critical micelle concentration) partially or completely displace the emulsifier from the droplet surface [16]. The original emulsifiers may leave the emulsion interface in a form of micelles or vesicles as a result of these interactions [5]. The displacement

of interface by bile salts allows binding of pancreatic lipase to the bile salts covered interface and facilitate lipid hydrolysis [5]. This caused a rapid increase in donor/acceptor ratio of the FRET pair dyes immediately after adding only bile salts or a combination of bile salts and pancreatic lipase, suggesting instantaneous changes in the organization of the phospholipids at the interface (**Fig. 2**). However, pancreatic lipase itself has limited efficacy in binding to the phospholipid interface as tight packing of the phospholipids at the interface limit accessibility of pancreatic lipase to emulsified lipids. [5, 16, 29]. The role of bile salts in facilitating the binding of pancreatic lipase may explain the lower donor/acceptor ratio observed in emulsion samples incubated with only pancreatic lipase compared to emulsion samples treated with bile salts and pancreatic lipase (**Fig. 2**). Results in **Fig. 2** are also in agreement with the interfacial tension measurement results from the previous studies [11, 16, 30, 31]. These prior studies suggest that the adsorption of bile salts onto the phospholipids stabilized emulsion interfaces resulted in an instant and rapid decrease in the interfacial tension between the oil-water interface [16, 31]. The results in this study are also in agreement with the observations in the literature that suggests that bile salts can rapidly extract phospholipids from the interface of lipid particles such as liposomes [32, 33].

Interface design and lipid digestibility

Fig. 5 of this study show that a layer of EPL or chitosan did not significantly influence the initial interactions of the bile salts or bile salts and pancreatic lipase with the emulsion interface. The results indicate that during an initial period (~10 minutes), in which there was no significant difference in the rate of release of FFA between the primary and secondary emulsions. Also with FRET measurements, changes in D/A ratio observed upon incubation of the secondary emulsion with bile salts or lipase or the combination of bile salts and lipase were

rapid and similar to the trend observed in **Fig. 2a**. The findings suggest that bile salts can freely diffuse across a monolayer coating and the net charge on the surface, electrostatic shielding of the interface or steric hindrance from these coatings did not significantly hinder their interactions with the lipid interface. The results also indicate that after adding a layer of EPL or chitosan at lecithin-bile emulsion interface, the extent of disruption of emulsion interface during simulated intestinal digestion was significantly depressed compared to the lecithin-bile primary emulsion. Based on the kinetic analysis of FFA release, the results indicate that chitosan based coatings reduced the overall rate of FFA release by 50%, while the EPL coating did not significantly influence the rate of FFA release but impacted the total % of the FFA released during the simulated intestinal digestion. These results are similar to some prior studies showing that lipase activity was reduced in lecithin–chitosan emulsion [34]. Based on the data set, the EPL or chitosan coating could have an inhibitory effect on bile salts and pancreatic lipase, resulting from their biochemical interactions with pancreatic lipase or a combination of bile salts and pancreatic lipase. The results also indicate the differences between the EPL and chitosan coatings in influencing FFA release from the secondary emulsions, further indicating the significance of polymer chain lengths and their biochemical interactions with the simulated intestinal digestion. The influence of polymer chain length is also indicated by the significant differences in the net zeta potential of the emulsion interface coated with chitosan or EPL. This is due to differences in the molecular chain length and the charge density on EPL polymer as compared to chitosan. Due to a weak net charge, it is expected that the electrostatic repulsion between emulsion droplets in the case of lecithin-EPL emulsions will be limited. These indications are in agreement with the results presented in a study using lecithin emulsion coated with EPL polymers [35]. Further studies are required to specifically monitor the interactions of these selected polymers

with the lipase or its combination with bile salts. These studies may include FRET measurements between the biopolymers and the phospholipids and/or other complementary measurements to characterize interactions of the biopolymers with the digestive enzymes.

To evaluate the effect of a layer of EPL or chitosan coating at lecithin-bile primary emulsion interface on lipid susceptibility to hydrolysis, release of FFA from lecithin-bile primary emulsion and secondary emulsions coated with EPL and chitosan during simulated intestinal digestion was measured over time (**Fig. 5**). After the initial period, a significantly slower rate of release rate of FFA from EPL or chitosan coated emulsion was observed compared to the primary emulsions. Furthermore, the final level of FFA generated from the chitosan coated emulsion was same as the level of FFA released from the primary emulsion, while the EPL coated secondary emulsion inhibited hydrolysis of the encapsulated lipids. These results indicate that the layer-by-layer coatings may not inhibit the initial displacement of the interface by bile salts, but may inhibit the progress of lipase enzyme in hydrolysis of encapsulated lipids. The overall trend observed based on the release of FFA is correlated with the FRET measurements in these emulsions during simulated digestion. Together, these results indicate that a layer of EPL or chitosan at lecithin-bile primary emulsion interface could decrease the extent of interface disruption by bile salts and pancreatic lipase and FFA release during simulated intestinal digestion. Further studies are required to characterize the dynamics of these interactions and the role of biopolymer mediated inhibition of lipid digestion. These results also indicate that this inhibition may not result simply based on the gel forming ability or enhanced viscosity of chitosan coatings as has been suggested in some of the prior studies [21, 22]. Detailed understanding of the role of biopolymers in controlling the rate of lipid hydrolysis and

potentially the release of encapsulated compounds may impact absorption of fat and nutrients and provide rational approaches in design of colloidal carriers.

CONCLUSIONS

A novel method for real-time quantitative measurement of structural changes in emulsion interface during simulated intestinal digestion was developed. The measurements demonstrate that the disruption of phospholipids at the interface and the displacement from the interface was instantaneous upon addition of bile salts. Incubation of emulsions with pancreatic lipase could only displace some of the emulsifier from the interface. Using FRET based approach, the effect of electrostatic coating (EPL or chitosan) on dynamics of phospholipid interface was measured. The results demonstrate that by depositing a layer of biopolymers at emulsion interface, the phospholipid interface was more resistant to displacement by bile salts and a combination of bile salts and pancreatic lipase, although the initial rate of displacement was similar to the primary emulsion. The structural changes in emulsion interface were correlated with the release of free fatty acids. Similar to the FRET results, the release of FFA from lecithin-bile-EPL and lecithin-bile-chitosan emulsions was at a relatively lower rate compared to the primary emulsion. Overall, the unique approaches developed in this study will lead to rational design of encapsulation systems to control the release of encapsulated compounds.

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List of tables

Table 1: Number based droplet size and zeta potential of lecithin-bile primary emulsion and secondary emulsions coated with EPL and chitosan before digestion and after 3 h of digestion. Each value is an average of three independent measurements $\pm$ standard deviation. Different letters represent statistically significant difference among the three emulsions under each category ($p < 0.05$).

615 **List of figures**

616 **Figure 1:** (a) Fluorescence images showing the co-localization of OG-ph and TR-ph at the
617 lecithin-bile emulsion interface. (I) A representative fluorescence image of OG and TR dual
618 labeled interface with direct excitation of OG. (II) A representative fluorescence image of OG
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622 The excitation and emission maxima of OG and TR dyes are 501/526 nm and 595/615 nm,
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624 **Figure 2:** (a) Changes in donor/acceptor ratio of OG and TR dual labeled lecithin-bile primary
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628 Different letters represent statistically significant difference ($p < 0.05$).

629 **Figure 3:** (a) Fluorescence emission spectra of OG, TR and OG and TR dual labeled secondary
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633 **Figure 4:** Donor/acceptor ratio of OG and TR dual labeled lecithin-bile primary emulsion and
634 secondary emulsions with EPL and chitosan coatings after 3 h of simulated intestinal digestion.
635 Each value is an average of three independent measurements $\pm$ standard deviation. Different

636 letters represent statistically significant difference among the emulsions at the same treatment
637 conditions ($p < 0.05$).

638 **Figure 5:** Kinetics of FFA release from the lecithin-bile primary emulsion and secondary
639 emulsions with EPL and coatings during simulated intestinal digestion. Each value is an average
640 of three independent measurements $\pm$ standard deviation.

Table 1

	Number weighted droplet size before digestion (nm)	Number weighted droplet size after digestion (nm)	Zeta potential before digestion (mV)	Zeta potential after digestion (mV)
Lecithin-bile	166.4 ± 10.7 ^a	2420 ± 335 ^a	-78.3 ± 1.0 ^a	-12.1 ± 1.1 ^a
Lecithin-bile-EPL	196.1 ± 15.0 ^a	2414 ± 490 ^a	-7.1 ± 0.4 ^b	-5.2 ± 0.5 ^b
Lecithin-bile-chitosan	195.1 ± 26.3 ^a	2208 ± 784 ^a	76.0 ± 1.6 ^c	6.7 ± 0.4 ^c

Figure 1(a)

Figure 1(b)

Figure 2(a)

Figure 2(b)

Figure 3(a)

Figure 3(b)

Figure 4

Figure 5

*Graphical Abstract