Composite whey protein–cellulose nanocrystals at oil-water interface: Towards delaying lipid digestion

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Abstract

Lipid digestion is an interfacial process that is largely controlled by the adsorption of lipase + colipase + bile salts onto the surface of the emulsified lipid droplets. Therefore, engineering oil-in-water (O/W) interfaces that prevent competitive displacement by bile salts and/or delay the transportation of lipase to the hydrophobic lipid core can be effective strategies to delay lipolysis. In this study, we present such an interface using composite protein-particle system, consisting of whey protein isolate (WPI) (1 wt%) + cellulose nanocrystals (CNCs) (1-3 wt%). Droplet size, microscopy at various length scales (confocal, electron microscopy), ζ-potential and kinetics of fatty acid release were used to assess how the presence of CNCs impacted the microstructural stability of the emulsions in in vitro duodenal conditions (pH 6.8, 37 °C). Adding CNCs at sufficiently high concentrations (3 wt%) significantly decreased the rate and degree of lipolysis as compared to that of protein-coated emulsion droplets. The principal cause of this altered lipolysis profile was the binding of bile salts by CNCs, which restricted both the interfacial displacement and solubilization of lipid-digestion products by bile salts. The CNCs can be envisaged to be strongly bound to the protein-coated droplets by virtue of hydrogen bonding with the underlying protein. Furthermore, the ability of the CNCs in the continuous phase to bridge several protein-coated droplets reduced the overall surface area available for the lipolysis. Composite WPI + CNC interface holds promise in designing physiologically relevant emulsions to target satiety or delivery systems for sustained release of lipophilic components.

Keywords

Cellulose nanocrystals, emulsion; particle-protein interface; bile salts; pancreatin
1 Introduction

Obesity is a growing global health crisis, which has more than doubled in extent in the last 25 years. As of 2014, more than 1.9 billion adults were overweight globally, and of those over 600 million people were obese [WHO, 2015]. Among many strategies to achieve weight management, food scientists, psychologists and nutritionists are attempting to enhance the satiating and satiety-promoting properties of food in order to generate appetite suppression. In the case of lipids, satiety hormones can be triggered by the presence of undigested lipids in the ileum via the so-called “ileal brake” mechanism [Maljaars, Peters, Mela, & Masclee, 2008]. Hence, a delayed lipid digestion in the intestine that will release the lipids in a more sustained manner and has potential to promote the feeling of satiety has become a target of research in the literature.

Nearly 70–90% of the lipid digestion takes place in the duodenal i.e. in the upper part of the small intestine in healthy human adults. Lipid digestion is essentially an interfacial process that involves complex adsorption phenomenon of lipase/colipase and bile salts onto the surface of the oil droplets [Sarkar, Ye, & Singh, 2016c; Singh & Sarkar, 2011]. Bile salts are bio-surfactants that competitively push out the interfacial materials originally present at the surface, facilitating the adsorption of pancreatic lipase-colipase complex and subsequent lipolysis [Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011]. In the last decade two key food-structuring approaches have been investigated to delay intestinal lipolysis, which would allow the detection of undigested lipids in the distal parts of the intestine. The first one involved modulating the interfacial parameters of emulsion (size, charge, interfacial composition etc.) in order to prevent competitive displacement of the original interfacial materials by bile salts and adsorption of lipase thus delaying the process of
binding of lipase/colipase complexes to act on the bile-coated oil droplets (Corstens, et al., 2017; Golding, 2014; Golding & Wooster, 2010; Sandra, Decker, & McClements, 2008; Sarkar, Horne, & Singh, 2010c; Sarkar, et al., 2016a). The second approach included restricting the transport of lipase to the emulsified lipid droplets via encapsulation of the emulsion droplets within a gel system (Guo, Bellissimo, & Rousseau, 2017; Guo, Ye, Lad, Dalgleish, & Singh, 2014; Sarkar, et al., 2015). It is now clearly recognized that both ionic surfactants and biopolymers are generally easily displaced from the emulsion droplet surface by bile salts during duodenal digestion (Mackie, Gunning, Wilde, & Morris, 2000; Maldonado-Valderrama, et al., 2011; Sarkar, Horne, & Singh, 2010b; Sarkar, et al., 2016c). However, lately, it has been demonstrated that particle-laden interfaces, such as those created by chitin nanocrystals (Tzoumaki, Moschakis, Scholten, & Biliaderis, 2013), intact or fused whey protein microgel particles (Sarkar, et al., 2016a) were not displaced by bile salts, by virtue of high desorption energy of these particles from the interfaces.

One alternative strategy is to coat protein-stabilized interfaces with a layer of particles, which would provide a steric hindrance to the transport of lipase to hydrophobic lipid core. Recently, such protein-particles laden composite interfaces have shown promising effects on enhancing the gastric stability of oil-in-water (O/W) emulsions (Sarkar, Zhang, Murray, Russell, & Boxal, 2017). In this study, cellulose nanocrystal particles (CNCs) were used to create O/W emulsions with composite protein-CNC interfaces by carefully exploiting the electrostatic attraction between cationic whey protein and negatively charged CNCs at pH 3 (Sarkar, et al., 2017). The presence of higher concentration of particles (3 wt%) increased the interfacial shear viscosity of the underlying protein film by almost 40 times and thus, increased the resistance of the interfacial protein film to subsequent rupture by pepsin in the gastric
regime at pH 3 (60% intact interfacial protein remained after gastric digestion). The composite WPI-CNC interfacial layer inhibited droplet coalescence in the gastric phase. Such droplet coalescence would have occurred rather spontaneously in an emulsion stabilized by WPI alone, where almost no intact interfacial protein tends to remain after gastric digestion [Sarkar, Goh, & Singh, 2010a; Sarkar, Goh, Singh, & Singh, 2009b; Sarkar & Singh, 2016b; Sarkar, et al., 2017; Singh, et al., 2011]. Besides formation of rigid composite interface, the network formation by the CNCs in the bulk (continuous) phase was also hypothesized to reduce the overall kinetics of interfacial proteolysis [Sarkar, et al., 2017].

In this study, we have focused on duodenal lipolysis of those emulsions stabilized by composite WPI-CNC interfacial layer, latter created by interfacial electrostatic complexation of mutually complimentarily charged species at pH 3. We hypothesize that the presence of such unmodified CNCs at the WPI-stabilized O/W interface could prevent the competitive displacement by bile salts via steric and/or electrostatic effects and thus might contribute to delaying the lipid digestion. To our knowledge, this is the first study that reports the impact of composite protein-particle laden O/W interface on kinetics of lipid digestion in simulated duodenal conditions.

2 Materials and Methods

2.1 Materials

Whey protein isolate (WPI) powder containing 96.3 wt% protein was kindly gifted by Fonterra Limited (Auckland, New Zealand). Cellulose nanocrystal powder (CNC, sulphated) was purchased from CelluForce™, Canada. Sunflower oil was purchased from a local supermarket (Morrisons, UK). Porcine bile extract B8631, porcine pancreatin (P7545, 8 × USP) and sodium azide were purchased from Sigma-Aldrich.
Company Ltd, Dorset, UK. All other chemicals used were of analytical grade and were obtained from Sigma-Aldrich Chemical Company unless otherwise specified. Milli-Q water having an ionic purity of 18.2 M\(\Omega\) cm at 25 °C (water purified by treatment with a Milli-Q apparatus, Millipore Corp., USA) was used for all the experiments.

2.2 Preparation of emulsions

Appropriate quantities of WPI were dispersed in 10 mM citrate buffer solution at pH 3 (adjusted using 0.1 M HCl) for 2 h to ensure complete dissolution. Oil-in-water emulsions (20 wt% oil) stabilized by WPI (1 wt%), hereafter cited as W1 were prepared by homogenizing 20.0 wt% sunflower oil and 80.0 wt% WPI solution using two passes through a two-stage valve homogenizer (Panda Plus 2000, GEA Niro Soavi Homogeneizador Parma, Italy) operating at first / second stage pressures of 250 / 50 bars, respectively at 25 °C. The maximum temperature reached by the emulsions during the homogenization step was 37 °C. For preparing the protein-particle-stabilized emulsions (schematic diagram shown in Figure 1), the primary emulsions (40 wt% oil, 2 wt% WPI) were dispersed in CNC dispersions (2-6 wt% in citrate buffer at pH 3) (1:1 w/w) to achieve final concentration of 20 wt% oil, 1 wt% WPI and 1 or 3 wt% CNCs, hereafter reported as W1C1 or W1C3, respectively. All the three emulsion samples were prepared in triplicates. Sodium azide (0.02 wt%) was used as a preservative for the emulsions during refrigerated storage at 4 °C.

2.3 Particle size analysis of emulsions

The droplet size distribution of each of the three emulsions before and after duodenal digestion was measured using static light scattering (Malvern MasterSizer 3000,
Malvern Instruments Ltd, Malvern, Worcestershire, UK). The absorbance of the emulsion droplets was set to 0.001. Refractive indices of 1.456 and 1.33 were selected for the sunflower oil and the continuous phase, respectively. Mean droplet size was reported as Sauter-average diameter ($d_{32}$) and volume-average diameter ($d_{43}$) calculated on five measurements on triplicate samples.

2.4 Zeta-potential

The $\zeta$-potential of each of the emulsions before and after duodenal digestion (180 minutes) with or without the addition of bile salts and/or pancreatin was measured using a Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Emulsions were diluted to 0.005 wt% droplet concentration in citrate buffer before digestion at pH 3 and simulated duodenal fluid (SDF) buffer after digestion at pH 6.8. The samples were equilibrated in respective buffers for 1 h and then were carefully transferred into DTS1070 folded capillary cells to measure the electrophoretic mobility following 30 seconds of equilibration within the equipment. The mobility recorded was then converted to $\zeta$-potential values using classical Smoluchowski equation. Each individual $\zeta$-potential data point was reported as the mean and standard deviation of at least five reported readings made on triplicate samples.

2.5 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was used to gain insights on the size of CNC and interfacial structure of the freshly prepared emulsions. Samples (10 μL) were fixed with 2.5% (v/v) glutaraldehyde, post fixed in 0.1% (w/v) osmium tetroxide and were exposed to serial dehydration in ethanol (20-100%) before being embedded in araldite. Ultra-thin sections (silver-gold 80-100 nm) were then deposited on 3.05
mm grids and stained with 8% (v/v) uranyl acetate and lead citrate. The sections were cut on an “Ultra-cut” microtome and imaged using a CM10 TEM microscope (Philips, Surrey, UK).

2.6 In vitro duodenal digestion

For in vitro duodenal digestion, freshly prepared emulsions were diluted with simulated duodenal fluid (SDF) buffer (without or with added bile salts and/or pancreatin) in 1:1 v/v at 37 °C under magnetic stirring at 350 rpm. The SDF was prepared using the harmonized INFOGEST digestion protocol described by Minekus, et al. (2014). The composition of SDF was 0.253 g L$^{-1}$ KCl, 0.054 g L$^{-1}$ KH$_2$PO$_4$, 3.57 g L$^{-1}$ NaHCO$_3$, 1.12 g L$^{-1}$ NaCl, 0.335 g L$^{-1}$ MgCl$_2$(H$_2$O)$_6$, 0.44 g L$^{-1}$ CaCl$_2$.2H$_2$O, 0.23 g L$^{-1}$ bile salts and 125 mg mL$^{-1}$ pancreatin (2800 U, 63 U/mL). The temperature was maintained at 37 °C and pH was adjusted to pH 6.8 and equilibrated for 1 h before the addition of pancreatin. Samples of emulsion-SDF mixtures were analysed for size, charge and microstructural changes.

2.7 Confocal laser scanning microscopy (CLSM)

The microstructural changes of the emulsions after in vitro duodenal digestion were imaged using a Zeiss LSM 880 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). A small quantity (100 μL) of emulsions after in vitro duodenal digestion in absence and presence of SDF containing bile-pancreatin mixture was mixed with 10 μL each of Nile Red staining oil, Fast Green staining WPI and Calcofluor White staining CNCs for 30 min. The stained samples were then put
into custom-made cavity microscopic slides, covered with cover slips and imaged using a 63×/1.4NA oil immersion objective lens.

2.8 Kinetics of free fatty acid release

The free fatty acids (FFAs) generated from emulsified lipids during the digestion of the emulsions in SDF containing bile salts and pancreatin were measured over 3 hours whilst maintaining the pH at 6.8 by addition of NaOH using a pH-Stat (TIM 854, Radiometer). The volume of 0.05 M NaOH consumed was used to calculate the concentration of free fatty acids (FFAs) generated from the triacylglycerols (assuming the generation of 2 FFAs per triacylglycerol molecule by the action of lipase) using Equation 1 (Li & McClements, 2010; Sarkar, et al., 2016a; Sarkar, et al., 2016c):

\[
\% \text{FFA} = 100 \times \left( \frac{V_{\text{NaOH}} \times M_{\text{NaOH}} \times M_{\text{W,Lipid}}}{2 \times W_{\text{Lipid}}} \right)
\]  

(1)

The kinetic parameters for the initial FFA release were calculated using Equation (2) (Sarkar, et al., 2016c; Ye, Cui, Zhu, & Singh, 2013):

\[
\ln \left[ (\Phi_{\max} - \Phi_t)/\Phi_{\max} \right] = -kt
\]  

(2)

where \( k \) is the first-order rate constant for FFA release (s\(^{-1}\)) and \( t \) is the duodenal digestion time (s). The lipolysis half time (t\(_{1/2}\), min) i.e. the time required to achieve half lipid digestion and the total FFA level (\( \Phi_{\max} \), %), were obtained from the FFA curves.
2.9 Statistical analysis

The results were statistically analyzed by analysis of variance (ANOVA) using SPSS software (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) and differences were considered significant when p < 0.05 was obtained.

3 Results and discussion

3.1 Characteristics of freshly prepared emulsions

In this study we briefly introduce the behaviour of aqueous dispersions of CNC particles, which sets the scene for understanding the characteristics of O/W emulsions stabilized by WPI-CNC before and after duodenal digestion. The CNCs used in this study (TEM image, Fig. 1) were crystalline, needle-like in shape with diameter of ~100 nm and high aspect ratio (i.e. the ratio of length to diameter (L/D)) of 10–50. This is in agreement with previous reports on nano-crystalline cellulose where sulfuric acid has been used as a hydrolysing agent (Ehmann, et al., 2013; Sarkar, et al., 2017; Scheuble, Geue, Windhab, & Fischer, 2014). As expected, grafting of numerous sulphate groups (Lin & Dufresne, 2014), which were created in the course of the preparation of these CNCs to remove the amorphous domains, led to significantly high negative surface charges in the CNC dispersion at pH 3. The ζ-potential values were −40 and −44 mV for 1 and 3 wt% CNC, respectively, and displayed higher magnitude of negative charge at pH 6.8 (~83 and −89 mV for 1 and 3 wt% CNC, respectively, data not shown). This is consistent with previous reports suggesting that CNCs beared HSO₄⁻ and SO₄²⁻ surface moieties at near neutral pH and the charge dissociation was almost independent of pH and ionic strength thereafter until pH 10 (Safari, Sheikhi, & van de Ven, 2014).
Three emulsions with or without containing CNCs were characterized using droplet size distribution and TEM (Figure 2). The W1 droplets were uniformly distributed in the range of 1-10 µm with $d_{4,3}$ value $\leq 5$ µm and the TEM image showed no obvious occurrence of flocculation or coalescence (Figure 2A). However, in presence of CNCs, both W1C1 (Figure 2B) and W1C3 (Figure 2C) showed bimodal and trimodal distributions, respectively with significant population of droplets in the range of 10-100 µm. The second peak in case of both W1C1 and W1C3 were diminished once they were dispersed in 2% SDS (data not shown) suggesting that such larger droplets were corresponding to flocculation of emulsion droplets rather than coalescence, as observed previously. One might argue that the first peak in the distribution W1C3 is too small ($\leq 0.1$ µm) (Figure 2C) to be composed of emulsion droplets, leading us to believe that these were most likely unabsorbed CNCs in the continuous phase. However, such hypothesis must be taken with caution as static light scattering assumes that all species as spherical, whereas CNCs were not spherical as can be observed in Figure 1.

The TEM images of the W1C1 and W1C3 (Figures 2B and 2C) revealed interesting information on the presence of CNCs at the O/W interfaces. Both W1C1 and W1C3 showed CNCs at the O/W interface which might be attributed to the electrostatic attractive forces and hydrogen bonding between WPI-coated droplets and CNC. At lower magnifications, both the W1C1 and W1C3 emulsions showed direct evidence of sharing layer of CNC particles between adjacent droplets, supporting bridging phenomenon. A closer look at the higher magnification TEM images revealed that in W1C1, the distribution of CNCs at the protein-coated O/W interface
seemed to be rather sparse and incomplete. However, in W1C3, a much denser network of CNCs was observed at the droplet interface. This is in line with the higher surface coverage (about 1.4 times) reported in case of 3 wt% as compared to that of 1 wt% CNC [Sarkar, et al., 2017]. The cohesion of CNCs forming such a dense network of particles at the W1C3 interface might be attributed to the inter-particle hydrogen bonding, capillary forces and attractive van der Waals forces (Moon, Martini, Nairn, Simonsen, & Youngblood, 2011).

It is also worth noting that in W1C3 (lower magnification, Figure 2C), there seemed to be significant level of CNC-CNC aggregation in the continuous phase. First of all, this supports the initial hypothesis of presence of unadsorbed CNCs in the continuous phase as mentioned before in frame of light scattering. Secondly, such CNC-CNC interactions in the continuous phase as well as interactions between CNC adsorbed to the protein-coated droplets and unadsorbed CNCs at the continuous phase might be further attributed to strong intermolecular hydrogen bonds [George & Sabapathi, 2015]. The combination of bridging of droplets and CNC-CNC interaction in the continuous phase can reduce the overall surface area of the droplets, and thus can potentially contribute to the slowing down of lipid digestion, latter being largely a surface-area dominated process.

3.2 Microstructural fate of the emulsions post duodenal digestion

The emulsions were digested in an in vitro duodenal model in the presence of ions, bile salts and pancreatin, latter composed of amylase, protease (trypsin) as well as lipase. As can be observed in Figure 3, the droplet size distribution of W1 droplets shifted dramatically after 3 h of duodenal digestion, with significant proportion of the droplets being observed in the size range of 10–100 μm (p<0.05). Interestingly, in
case of W1C1, there was appearance of another small peak in the range of 100-1000 μm. However, in case of W1C3, no such second peak was observed. Irrespective of the original interfacial composition of the emulsions, the $d_{4,3}$ values ranged from 50-60 μm for all the emulsions after duodenal digestion ($p > 0.05$), which indicates that the samples were either severely aggregated or had a certain degree of droplet coalescence.

To investigate the microstructural behaviour, confocal laser scanning microscopy images were taken at 0-180 min (Figure 4). In the case of W1, droplets underwent strong floc formation at 0 min (absence of pancreatin and bile salts), which clearly suggests the charge screening effects of the Na$^+$ and Ca$^{2+}$ ions present in SDF. In presence of pancreatin and bile salts, individual W1 droplets were coalesced to extremely large oil droplets [Sarkar, et al., 2010c]. It therefore appears that the dramatic increase in $d_{4,3}$ values of these emulsions under simulated intestinal digestion was linked to the digestive action of both proteases and lipase within the pancreatin. The action of trypsin might have cleaved the proteinaceous interfacial layer generating peptides, which lacked the cohesiveness of the parent protein layer to prevent droplet coalescence. Furthermore, due to the action of pancreatic lipase, surface-active free fatty acids (FFAs) and mono-acylglycerols (MAGs) were possibly generated at the droplet surfaces. These FFAs and MAGs are known to be comparatively less effective at protecting the oil-in-water emulsion droplets against coalescence as compared to that of a protein film present in the original emulsion [Sarkar, et al., 2010c].

In the case of W1C1, the emulsion droplets showed limited aggregation in the presence of SDF (Figure 4). This suggests that the CNC-coated droplets were not severely affected in their microstructural integrity by charge screening or ion binding.
effects. In the presence of pancreatin and bile salts, there was gradual appearance of large coalesced droplets after 30 min. However, the W1C1 micrograph still showed considerable amounts of intact CNC-coated droplets (blue stained by Calcofluor White) of 5-30 µm diameter at 30 min, the population of which gradually diminished as digestion time progressed to 180 min with subsequent fusion of droplets. This suggests that lipid digestion did occur in W1C1 generating FFAs and MAGs leading to subsequent droplet coalescence, however, it appears that there were few CNC-coated droplets, which remained intact even after 180 min of digestion.

The behaviour of W1C3 emulsion in presence of SDF without pancreatin/ bile salts did not show any prominent aggregation behaviour, quite similar to that observed in case of W1C1 (Figure 4). Although W1C3 also showed a small degree of coalesced droplets in presence of pancreatin and bile salts, the majority of the population of emulsion droplets were the ones coated by CNC (10-30 µm) that appeared to retain the microstructural intactness. A closer look at these CNC-coated droplets in case of W1C1 and W1C3 revealed that these were actually not individual droplets coated by CNC, but were rather several small emulsion droplets bridged together in a “raspberry-like” floc, resembling emulsion microgel particles. This indicates that this relatively large fraction of droplets, which were rather encapsulated by and within a CNC particulated layer (3 wt%) might not have had direct access to be cleaved by the enzymes and thus the coalescence was rather diminished in case of W1C3 (Figure 4).
3.3 ζ-potential

To provide indirect quantitative insights into the droplet behaviour, ζ-potential values are reported at pH 3 (freshly prepared emulsions), pH 6.8 (pH of SDF) and in presence of SDF without/with the addition of bile salts and/or pancreatin (Figure 5). Freshly prepared W1 droplets were positively charged (~ +41 mV), which is expected as the WPI at the interface was below its isoelectric point (pI) \([\text{Sarkar, et al., 2017}]\). However, WPI eventually reversed its charge at pH 6.8, with WPI being above its pI \([\text{Sarkar, et al., 2009b}]\). On addition of SDF, W1 showed a strong charge screening effect due to the presence of mono-and divalent cations supporting the microstructural behaviour in Figure 4. In presence of bile salt (Figure 5), W1 showed a rapid rise in magnitude of negative charge (p<0.05), which might be attributed to the displacement by negatively charged bile salts. It is noteworthy that bile salt-stabilized oil-in-water emulsion has a ζ-potential value of nearly -50 mV \([\text{Sarkar, et al., 2016c}]\), which suggests that in this study, the original whey protein layer was almost completely displaced by bile salts (~ -48 mV), achieving an almost bile salt-stabilized interface. This is congruent with the previous findings of orogenic displacement of β-lactoglobulin network at O/W interface by bile salts using interfacial measurements and atomic force microscopy \([\text{Maldonado-Valderrama, et al., 2008}]\). In addition, gradual increase of negative charge as well as desorption of β-lactoglobulin from the O/W interface to the continuous phase in presence of bile salts observed using SDS-PAGE support such bile salts-mediated competitive displacement \([\text{Sarkar, et al., 2010b}][\text{Sarkar, et al., 2016c}]\). In presence of bile salts and pancreatin, W1 showed significant rise in negative surface charge, which might be attributed to the generation of FFAs and MAGs at the interface, supporting the laser diffraction data (Figure 3) and confocal micrographs showing coalesced droplets (Figure 4).
At pH 3, W1C1 containing 1 wt% CNC had a substantial reduction of positive charge as compared to that of W1 (p<0.05), which might be attributed to the electrostatic binding of anionic CNC to positively charged protein-coated droplets (Figure 5) [Sarkar, et al., 2017]. Since, the coverage by CNC at 1 wt% was not sufficiently complete as shown in the TEM image (Figure 2b), the W1C1 did not show a negative charge at pH 3. At pH 6.8, the samples showed a significantly high magnitude of negative charge, which might be attributed to the sulphated CNCs being highly ionized at pH 6.8, as indicated before, and bound to positive patches of WPI coated emulsions. Furthermore, the strong inter-molecular hydrogen bonding between CNCs and WPI [Qazanfarzadeh & Kadivar, 2016] at the interface also might have led to the presence of CNCs at the interface at pH 6.8, where both WPI and CNC were mostly anionic. The salt-induced screening effects were rather negligible in the case of W1C1 (p>0.05). The W1C1 showed almost two-times higher negative charge in presence of bile salts as compared to that in presence of SDF (p<0.05), which might suggest bile-mediated displacement. The increase in negative charge was substantially high in the presence of pancreatin, supporting the release of lipid digestion products, such as FFAs and MAGs supporting the coalescence behaviour (Figure 4).

The W1C3 was originally negatively charged (Figure 5) due to rather higher coverage of anionic CNC at the WPI-coated droplet surface (Figure 2C, TEM image). At pH 6.8 and in presence of SDF containing no bile salts or pancreatin, the W1C3 showed similar behaviour with accumulation of negative charges when compared to W1C1. Interestingly, W1C3 had no change in ζ-potential value after treatment with bile salts (p>0.05). These results suggest that bile salts might not have been able to displace completely the thick viscous layer of CNC particles from the WPI-coated interface supporting our hypothesis. Another possibility might be that the CNC
particles were highly negatively-charged and retarded the anionic bile salts from the vicinity of the interface. However, one might argue that SDS was able to displace the CNCs from the interface as mentioned in the light scattering data above, but the displacement by bile salts in case of 3 wt% CNC appeared to be rather restricted. This can be explained based on structural differences between SDS and bile salts. In case of bile salts, instead of the classical hydrophilic head–hydrophobic tail geometry like SDS, bile salts have a flat steroidal structure with four rings attached to a short and flexible tail [Vila Verde & Frenkel, 2016]. The hydrophilic character of one of the steroid faces arises from the presence of two or three hydroxyl groups. It is highly plausible that CNCs were sequestering bile salts via hydrogen bonding with these hydrophilic groups and thus the interfacial displacement by bile salts were rather restricted. Such sequestering of bile salts by dietary fibres has been previously reported as one of the key mechanism behind the hypercholesteremic properties of dietary fibres [Vahouny, Tombes, Cassidy, Kritchevsky, & Gallo, 1980].

In presence of pancreatin, W1C3 droplets became more negatively charged (p<0.05). However, it is worth recognizing that the $\Delta\zeta$-potential i.e. the difference in magnitude between $\zeta$-potential of samples containing bile salts-pancreatin and bile salts alone was significantly less in W1C3 (6.22 mV) as compared to W1C1 (13.9 mV) and W1 (18.1 mV), which might justify the limited degree of droplet coalescence in W1C3 (Figure 4).

3.4 Kinetics of lipid digestion

The lipolysis profiles of the emulsions were assessed in the presence of bile salts and pancreatin using a pH-stat method as shown in Figure 6. As expected, in W1, there was steep rise in FFA release with half of the digestion achieved within the first
4 min (Table 1). The W1 generated approximately 46% of FFAs derived from the long-chain FFAs from sunflower oil, which tend to assemble at the oil–water interface and impede further lipid digestion as can be observed by the plateau. In the case of W1C1, the shape of the kinetic plot showed a slight tendency to shift towards the right (Figure 6) leading to a significant decrease in rate and extent of digestion (p<0.05) with more than doubled t₁/₂ as compared to that of W1 emulsion (Table 1).

The W1C3 containing 3 wt% CNC showed a dramatic decrease in the rate (i.e. kₗip and t₁/₂ ~ 8-times lower) and degree of lipid digestion (i.e. φ ~ 3-times lower) as compared to W1. This suggests that higher concentration of CNC was capable of forming a rather rigid network at the interface and was not completely displaced by bile salts. The second mechanism can be that higher concentrations of CNCs was capable of sequestering bile salts as discussed above. This might have partially hindered the action of the lipase–colipase complex via restricting its transport to the close proximity of the emulsified lipids, although lipid digestion was not completely prevented.

It is well known that the interfacial process of lipolysis involves two key roles of bile salts: anchoring of the bile salt–lipase/colipase complex to the oil/water interface as well as solubilisation of these lipolytic products to continue the digestion [Singh, et al., 2011]. Besides restricted interfacial displacement by bile salts as discussed before, the insufficient amount of bile salts (due to CNC-mediated sequestering) available for solubilisation and removal of inhibitory digestion products (e.g. FFAs, mono- and/or di-acylglycerols) also cannot be ignored. Accumulation of such lipolysis inhibitory products at the interface might also have impeded further hydrolysis of the emulsified lipids by pancreatin.
Interestingly, in spite of such proposed sequestering of bile salts by CNCs, lipid digestion took place with release of 16% FFA. It should be recognized that CNCs are needle-shaped particles with high aspect ratio, hence close packing cannot be achieved and one can expect relatively large gaps in between the CNC particles. This justifies that lipid digestion still took place as lipase-colipase complex (2.5 nm) could easily squeeze in through such gaps reaching the O/W interface and initiated lipolysis (Sarkar, et al., 2016a). As these droplets were not completely bile-coated and the digestion products were not continuously solubilised by bile salts due to binding by CNCs, the lipolysis was eventually delayed. Furthermore, the reduced surface area of the W1C3 droplets due to the floc formation bridging several droplets together would have significantly reduced the available surface area for the lipolytic enzymes to bind further contributing to the reduction in the rate of lipid digestion.

Conclusions

In this study, we demonstrated that it is possible to alter the lipolysis rate by creating a composite interfacial layer with WPI and CNCs, latter formed via electrostatic attraction and hydrogen bonding. The presence of WPI with higher concentration of CNCs (3 wt%) can act as a steric and possibly electrostatic barrier to the displacement by bile salts. The presence of insufficient bile salts for solubilisation of lipolysis end-products due to sequestering of bile salts by CNCs also impeded the degree of lipolysis. Furthermore, the substantial lowering of surface area in W1C3 due to the CNC bridging several emulsion droplets together, almost encapsulating them within CNC-shells led to delaying the digestion of lipids in an in vitro duodenal model. These results together with future in vivo validation realization of such delaying lipid digestion might have potential implications in the designing of physiologically
relevant emulsions, for targeting satiety. These composite protein-particle interfaces might be also useful in designing delivery vehicles for lipophilic drugs and bioactive nutrients, where sustained release of lipids is a key requirement.
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