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Modulating in vitro gastric digestion of emulsions using composite whey protein–cellulose nanocrystal interfaces

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Abstract

In this study, we designed emulsions with an oil-water interface consisting of a composite layer of whey protein isolate (WPI, 1 wt%) and cellulose nanocrystals (CNCs) (1-3 wt%). The hypothesis was that a secondary layer of CNCs at the WPI-stabilized oil-water interface could protect the interfacial protein layer against in vitro gastric digestion by pepsin at 37 ^{0}C. A combination of transmission electron microscopy, ζ-potential measurements, interfacial shear viscosity measurements and theoretical surface coverage considerations suggested the presence of CNCs and WPI together at the O/W interface, owing to the electrostatic attraction between complementarily charged WPI and CNCs at pH 3. Microstructural analysis and droplet sizing revealed that the presence of CNCs increased the resistance of the interfacial protein film to rupture by pepsin, thus inhibiting droplet coalescence in the gastric phase, which occurs rapidly in an emulsion stabilized by WPI alone. It appeared that there was an optimum concentration of CNCs at the interface for such barrier effects. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) results further confirmed that the presence of 3 wt% of CNCs reduced the rate and extent of proteolysis of protein at the interface. Besides, evidence of adsorption of CNCs to the protein-coated droplets to form more rigid layers, there is also the possibility that network formation by the CNCs in the bulk (continuous) phase reduced the kinetics of proteolysis. Nevertheless, structuring emulsions with mixed protein-particle layers could be an effective strategy to tune and control interfacial barrier properties during gastric passage of emulsions.

Keywords

Cellulose nanocrystals, emulsion; in vitro gastric digestion; particle-protein interface; whey protein; pepsin

1 Introduction

Emulsions stabilized by biopolymer-based particles, such as those derived from proteins [1-4] and polysaccharides [5-7] have attracted a lot of attention recently owing to the demand for ultra-stable emulsions and biocompatible 'clean-label' emulsifiers that are immediately suitable for use in food, pharmaceutical, cosmetics, and other allied soft matter applications [8-10]. Besides their unique interfacial stabilizing properties, particles, such as whey protein microgel particles [2] and chitin [11] have also shown abilities to modulate digestion of emulsified lipids by restricting the access of lipase to the hydrophobic lipid substrate. Such lipid digestion modulating properties might be exploited to enhance satiety or used for targeted release of bioactive components within the gastrointestinal tract [2, 11-16].

However, it is worth recognizing that biochemical processes occurring in the gastric regime might hinder such impact owing to the hydrolysis of the Pickering stabilizers by pepsin, as previously reported for protein-based particles [2, 17]. Such rupture of the interfacial particle layers may induce gastric instability, such as flocculation and coalescence [2, 18-20]. In view of such possible gastric destabilization studies, it might be useful to create a much more complex interface to help protect the emulsions against pepsin attack. In this regard, cellulose nanocrystals (CNCs) are interesting candidates for creating an interfacial barrier surrounding the protein interface, since human enzymes cannot digest cellulose.

Solid rod-like cellulose nanocrystals (CNCs) derived from world's most abundant biopolymer are a biocompatible and renewable source material. They are typically 5–70 nm in width and between 100 nm and several micrometers in lengths [21]. Biopolymer-based particles derived from proteins are intrinsically surface-active, but most CNCs, widely manufactured via sulphuric acid treatment, create

strong sulphate charges on the CNC particles, increasing their hydrophilicity so that they are not wetted by oil unless chemically modified [12, 22], i.e. their surface activity is low.

In this study, we utilized the negative charge on CNCs to create composite WPI + CNCs interfaces at pH 3. The hypothesis was that the presence of unmodified CNCs at the WPI-stabilized O/W interface could enhance the gastric stability of the corresponding emulsions by acting as a barrier to the pepsin attacking the whey protein at the interface. Although there have already been recent reports of mixed protein-polysaccharide particle interfaces, such as chitin nanocrystals + β-lactoglobulin [23], cellulose particles + sodium caseinate [24], to our knowledge, this is the first study that reports the effect of combining whey protein + unmodified CNC particles synergistically at interface and discover the influence of such composite layers on enhanced gastric stability in a simulated gastric condition. The properties of freshly prepared emulsions and pepsin-digested emulsions were measured using particle sizing, microscopy at various length scales (confocal laser scanning microscopy, transmission electron microscopy), SDS PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) analysis, ζ-potential and interfacial shear viscosity measurements.

2 Materials and Methods

2.1 Materials

Cellulose nanocrystal powder (CNCs) was purchased from CelluForce™, Canada According to the manufacturer; it contained of 100% sulphated CNCs. Whey protein isolate (WPI) powder containing 96.3 wt% protein was kindly gifted by Fonterra Limited (Auckland, New Zealand). Microcrystalline cellulose (MCC), produced by

acid hydrolysis of cellulose, product code 310697 and pure β-lactoglobulin (β-lg), ≥ 90% (PAGE), product code L3908, were purchased from Sigma Aldrich, (New Jersey USA). Sunflower oil was purchased from a local supermarket (Morrisons, UK). Pepsin enzyme (P7000-25G, actual activity: 474 U mg^{-1}) was purchased from Sigma-Aldrich Company Ltd, Dorset, UK. All other chemicals used were of analytical grade unless otherwise specified. Mini-Protean Precast TGX Gels (8-16%) and Precision Plus Protein All Blue Standards were purchased from Bio-Rad Laboratories, Inc, USA. Milli-Q water having an ionic purity of 18.2 MΩ·cm at 25 °C (water purified by treatment with a Milli-Q apparatus) was used for all the experiments.

2.2 Preparation of emulsions

Oil-in-water emulsions stabilized by WPI and/or CNCs were prepared with 10 mM citrate buffer solution at pH 3 (adjusted using 0.1 M HCl). Whey protein isolate (WPI) solution was prepared by dispersing appropriate quantities of WPI in citrate buffer and stirring for 2 h at room temperature to ensure complete dissolution of the protein. Oil-in-water emulsions (20 wt% oil) stabilized by WPI (1 wt%), hereafter reported as W1 were prepared by homogenizing 20.0 wt% sunflower oil and 80.0 wt% WPI solution using a Leeds Jet Homogenizer at 300 bar pressure at 25 °C. For preparing the protein-particle-stabilized interfaces. primary emulsions (40 wt% oil) were prepared first using WPI by passing through the Jet Homogenizer. Secondary emulsions were prepared by dispersing the primary emulsions into CNCs 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 cited as W1C1 or W1C3, respectively. Sodium azide (0.02 wt%) was added to the emulsions to prevent microbial growth during refrigerated storage at 4 °C.

2.3 Particle size analysis of emulsions

A Malvern MasterSizer 3000 (Malvern Instruments Ltd, Malvern, Worcestershire, UK) was used to measure the droplet size distribution of each of the three emulsions before and after gastric digestion. The relative refractive index, i.e., the ratio of sunflower oil (1.456) to that of dispersion medium (1.33) was 1.095. Droplet size measurements were reported as Sauter-average diameter (d_{32}) and volume-average diameter (d_{43}) from the particle size distributions, using equations 1 and 2, respectively:

$$d_{32} = \frac{n_i d_i^3}{n_i d_i^2} \quad (1)$$

$$d_{43} = \frac{n_i d_i^4}{n_i d_i^3} \quad (2)$$

where, n_i is the number of particles with diameter d_i. Mean and standard deviations were calculated on five measurements on triplicate samples.

2.4 Interfacial shear viscosity (η_i)

Simple measurements of interfacial shear rheology at pH 3 were used to test for the presence of attractive interactions between anionic cellulose and whey proteins. Interfacial shear rheology is particularly sensitive to the composition of and interactions at an interface [25, 26]. In addition, interfacial shear viscosity (η_i) has been shown to be sensitive to the accumulation of particles at interfaces in the presence of protein. For example, authors [27] have demonstrated a significant

increase in η_i at the air-water interface in the presence of stable O/W emulsion droplets when sodium caseinate was also adsorbed, whilst even larger increases in η_i were seen [28] in the presence of hydrophobically modified cellulose and starch granule particles. Safouane, Langevin & Binks (2007) [29] have reported extremely stiff films of partially hydrophobic particles at the air-water interface.

Because the CNC sample from CelluForce™ used in this study was relatively expensive and the supply very limited, we chose to use the MCC sample since this was relatively cheap and readily available. As described in the Results and Discussion section, the MCC and the CNC are expected to have similar zeta potentials and aspect ratio (as derived from optical micrograph) as a function of pH. A two dimensional Couette-type interfacial viscometer [26], was operated in a constant shear-rate mode, as described in recent studies [27, 30]. Briefly, a stainless steel biconical disc (radius 14.5 mm) was suspended from a thin torsion wire with its edge in the plane of the air-water (A–W) or oil-water (O–W) interface of the solution contained within a cylindrical glass dish (radius 72.5 mm). The constant shear rate apparent interfacial viscosity, η_i, is given by the following equation:

$$\eta_i = \frac{g_f}{\omega} K(\theta - \theta_0) \qquad (3)$$

where K is the torsion constant of the wire; θ is the equilibrium deflection of the disc in the presence of the film; θ_0 is the equilibrium deflection in the absence of the film, i.e., due to the bulk drag of the sub-phase on the disc; g_f is the geometric factor and ω is the angular velocity of the dish. A fixed value of $\omega = 1.27 \times 10^{-3}$ rad s^{-1} was employed throughout, for comparison with previous measurements of proteins + particles [28]. For experiments at the O–W interface, a layer of pure n-tetradecane was layered over the aqueous solution within 1 min of adding the aqueous phase to

174 the dish. Pure β-lg at a concentration of 10^{-3} wt% was used as the aqueous phase,
175 representative of the main component of WPI and again for comparison with previous
176 measurements with hydrophobically modified cellulose, with or without added MCC
177 (1 wt% or 3 wt%) at pH 3 or 7. Experiments were repeated at least three times and
178 the η_i results are reported as the mean values ± the range about the mean.
179
180 2.5 Zeta-potential
181 A Zetasizer Nano ZS (Malvern Instruments Ltd, Malvern, Worcestershire, UK) was
182 used to measure the ζ-potential of each of the emulsions before and after gastric
183 digestion (120 minutes). Emulsions were diluted to 0.005 wt% droplet concentration
184 and the solution was transferred into a DTS1070 folded capillary to measure the
185 electrophoretic mobility, which was converted to ζ-potential using classical
186 Smoluchowski equation. Each individual ζ-potential data point was reported as the
187 average and standard deviation of at least five reported readings made on triplicate
188 samples.
189
190 2.6 Transmission electron microscopy (TEM)
191 Transmission electron microscopy (TEM) was employed to observe the structure of
192 the CNCs and the original emulsions. Samples (10 μL) were fixed with 2.5% (v/v)
193 glutaraldehyde and post fixed in 0.1% (w/v) osmium tetroxide 32. Then, the samples
194 were subjected to serial dehydration in ethanol (20-100%) before being embedded in
195 araldite. Ultra-thin sections (silver-gold 80-100 nm) were deposited on 3.05 mm grids
196 and stained with 8% (v/v) uranyl acetate and lead citrate. The sections were cut on an
197 "Ultra-cut" microtome. Images were recorded using a CM10 TEM microscope
198 (Philips, Surrey, UK).

2.7 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) of the emulsions before and after in vitro gastric digestion were imaged using a Zeiss LSM 880 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Nile Red was used to stain oil (Excitation 514 nm, Emission 539-648 nm), Fast Green was used to stain WPI (Excitation 633 nm, Emission 657-755 nm) and Calcofluor White was used to stain CNCs (Excitation 405 nm, Emission 410-523 nm). A small quantity of emulsion before and immediately after gastric digestion (30 min, 120 min) was placed on a concave microscope slide. About 10 μL each of Nile Red (0.1% w/v in dimethyl sufoxide), Fast Green (0.1% w/v in Milli Q water) and Calcofluor White (0.1% w/v in Milli Q water) was added to the samples and stained for 30 min. Finally, the sample was covered with a cover slip and imaged using a 63× magnification oil immersion objective lens.

2.8 In vitro gastric digestion

Emulsions were digested by mixing them with simulated gastric fluid (SGF) with pepsin using the harmonized digestion protocol (Minekus et al., 2014) at 37 °C. Briefly, 20 mL of the emulsions (20 wt% oil) were incubated for 2 hours in 20 mL of SGF, which consisted of 0.514 g L^{-1} KCl, 0.123 g L^{-1} KH_2PO_4, 0.042 g L^{-1} $NaHCO_3$, 0.06 g L^{-1} NaCl, 0.0004 g L^{-1} $MgCl_2(H_2O)_6$, 0.0009 g L^{-1} $(NH_4)_2CO_3$ and 3.2 g L^{-1} pepsin. The pH value of SGF was adjusted to pH 3 using 0.1 M HCl to simulate after meal ingestion conditions. To observe the change of emulsions during digestion, aliquots were collected at different time intervals between 0 and 120 min for analysis.

2.9 SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis)

To determine the influence of CNCs on digestion of the adsorbed WPI at the O/W interface, the cream phase of W1, W1C1 and W1C3 emulsions sampled at various time intervals during in vitro gastric digestion was analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Samples (1 mL) were heated at 95 °C for 5-10 min to stop digestion. Control experiments were also carried out where pepsin activity was stopped in the three emulsions by raising the pH to pH 7 using 0.5 M ammonium bicarbonate from 0-60 minutes and then using 0.1 N NaOH after 60 min. The aliquots were centrifuged for 40 min at 14500g and 20 °C using a table-top micro-centrifuge (Eppendorf MiniSpin plus, Scientific Laboratory Supplies. Ltd. UK). A certain amount of cream layer was carefully collected, mixed with 50 μL SDS buffer (1 M Tris, pH 6.8) and again heated at 95 °C for 5-10 min. The SDS-PAGE was carried out by loading 5 μL of protein marker and 10 μL of digested samples + loading buffer mixtures into precast gels, then placed in Mini-PROTEAN II system (Bio-Rad Laboratories, Inc, USA). The running process had two stages: 100 V for 10 min followed by 200 V for 20 min. The gels were then stained for 2 hours with 90 wt% ProtoBlue Safe Colloidal Coomassie G-250 stain and 10 wt% ethanol. After staining, the gels were destained using distilled water overnight and then scanned using a ChemiDoc™ XRS+ system with image LabTM Software (Bio- Rad Laboratories, Inc, USA). The SDS-PAGE experiments were repeated three times.

2.10 Statistical analysis

The results were statistically analyzed by analysis of variance (ANOVA) with Tukey's post-hoc test using Graphpad 5 Prism software and differences were considered significant when $p<0.05$ were obtained.

Results and discussion

Firstly, CNCs were charcaterized in order to understand better the properties of the emulsions stabilized by protein plus CNCs. The secondary coverage of protein-stabilized interface by different concentrations of CNCs was calculated. The behaviour of these emulsions (W1, W1C1 and W1C3) during in vitro gastric digestion conditions was then assessed.

3.1 Characteristics of CNC and MCC

The TEM image (Figure 1) suggests that the CNCs were stiff, needle-like particles of a nearly perfect crystalline structure with a diameter of ~ 100 nm, very similar to those reported by Scheuble, et al., 2016 using atomic force microscopy images [12]. A percolated network-type architecture was observed at 3 wt% CNC (Figure 1A). This is expected due to the high aspect ratio of CNCs, i.e. the ratio of length to diameter (L/D), which was within the range of 10 to 50 (Figure 1B), consistent with previous reports [31]. Dispersions of CNCs at 1 and 3 wt% were anionic at pH 3 with ζ-potential values of –39.6 and –43.8 mV, respectively (data not shown). Such negative charge has been attributed to processing conditions using sulphuric acid hydrolysis resulting in negatively charged sulphate groups grafted to the surface of the individual cellulose chains [31].

The nominal particle size of the MMC cellulose used in the interfacial rheology experiments was 20 μm according to the supplier, but light microscopy revealed a broad range of a particle sizes between 1 and 50 μm (data not shown). The ζ-potential of the MCC was therefore not measured because this size range was too large for the NanoSizer. However, many authors [32, 33] report negative values of ζ-potential in the pH range 3 to 7 for all types of MCC produced via acid or alkaline

hydrolysis of cellulose, typically in the range of -20 mV to – 30 mV for pH 3 to 7, respectively, depending upon the salt composition of the buffer (which can lead to ion binding). Therefore, it was assumed that the MCC was also negatively charged.

3.2 Properties and structure of emulsions with WPI + CNCs

Figure 2A shows a typical particle size distribution, as determined by static light scattering, for the three emulsions. In absence of CNCs, W1 emulsion had a monomodal droplet size distribution with the majority of droplets being in the range of 0.3–5.0 μm, with an average droplet size (d_{32}) of ~0.45 μm (Table 1), consistent with the TEM image. However, emulsions containing CNCs (W1C1 and W1C3) showed bimodal and trimodal distributions, respectively. In particular, both the emulsions containing CNCs showed a larger proportion of droplets within the 10-100 μm size range, with a significant increase in d_{43} > 25 μm (Table 1) as compared to that of W1 emulsion (d_{43} = 2.4 μm) ($p < 0.05$). When the W1C1 and W1C3 emulsions were mixed gently with 2% SDS, the distributions reverted to being similar to that of the W1 emulsion (data not shown), which indicated that the emulsions had not coalesced and the several peaks in absence of SDS treatment were most likely due to droplet flocculation [34]. The smaller peak area of the W1C3 emulsion within the 0.01-0.1 μm size range logically corresponds to the free CNCs in the continuous phase rather than emulsion droplets and thus, the d_{32} value of the emulsion has been re-calculated removing this peak area from the distribution (Table 1).

The morphology of the adsorbed particles was examined by visualisation of the droplet interfaces via negative staining and TEM observations of the emulsions. Figure 2B clearly show CNCs adsorbed on the surface of the W1C1 and W1C3 emulsion droplets. However, the secondary surface coverage by clearly discernible

CNCs appeared to be rather incomplete in case of W1C1 (Figure 2B). The W1C3 emulsion showed more aggregated CNCs closely associated with droplet surfaces forming a rather continuous particulate layer. In both emulsions, the secondary layer of CNCs seemed to be shared between neighbouring emulsion droplets, in agreement with suggestion of droplet flocculation from the size distribution data (Figure 2A). Bridging phenomena of entangling adjacent droplets has been observed with CNCs of this aspect ratio elsewhere [7]. Furthermore, in both the emulsions, a significant degree of particulate network could be observed at the droplet surface (Figure 2B). This might be hypothesized due to CNCs-CNCs aggregation via van der Waals forces, as well as intra- and inter-molecular hydrogen bonds [31] or possible electrostatic complexation between sulphate-bearing CNCs and cationic protein layer at pH. 3 [34]. The size evolution of the CNC aggregates was found to be related to the CNC concentration with more prominent interfacial clusters in W1C3 as compared to W1C1 emulsions, as revealed by the TEM images.

The ζ-potential results shows that W1 emulsion was considerably cationic at pH 3 (Table 1), which was expected as whey protein is a zwitterionic polyelectrolyte emulsifier with pI 5.1.With the addition of anionic CNCs (1-3 wt%), the ζ-potential of the whey protein-coated emulsion droplets gradually decreased from +42 to –16 mV. This is obviously most likely due to the increased binding of CNCs to the oppositely charged WPI adsorbed at the oil droplet surface as CNC concentration is increased. This is also consistent with the laser diffraction results showing bridging flocculation in case of W1C1 (Figure 2A), typical with low biopolymeric surface coverage in protein-polysaccharide systems where interactions are net attractive at both the interface and in the bulk phase [35, 36]. In order to confirm this low surface coverage,

the degree of secondary surface coverage of CNCs, Γ_{sat} (mg m^{-2}) was calculated using equation (4) [37]:

$$\tau_{sat} = \frac{c_{sat} d_{32}}{6\phi} \quad (4)$$

where, Φ is the droplet volume fraction (= 0.2) and c_{sat}, the so-called saturation concentration of CNCs i.e. the mass of CNCs adsorbed to cover 95% of the droplet surface per unit volume of emulsion (kg m^{-3}), is given by equation (5) [38]:

$$\frac{\zeta_c - \zeta_{sat}}{\zeta_0 - \zeta_{sat}} \approx e^{\left(-\frac{c}{3c_{sat}}\right)} \quad (5)$$

where, ζ_c is the ζ-potential of emulsion at CNC concentration c, ζ_0 is the ζ-potential without the addition of CNCs (W1 emulsion) and ζ_{sat} is the ζ-potential at c_{sat}. The ζ_{sat} (≈ –55.82 mV) was measured using a control emulsion (3 wt% CNC-coated emulsion droplets, without added WPI). The overall change in droplet ζ-potential at saturation coverage ($\Delta\zeta_{sat} = \zeta_0 - \zeta_{sat}$) was 97.42 mV, which provides an estimate of the amount of charge associated with the adsorbed CNCs molecules upon saturation. For this study, Γ_{sat} calculated using equation (4) was in the range 6–10 mg m^{-2} (Table 1) depending upon the CNC concentration. Such values are lower than the range typically found for surface loads of particles adsorbed to the interfaces [1, 2], but higher than those for adsorbed biopolymer molecules [38]. With the increase of the CNC concentration to 3 wt%, the layer at the interface became denser, with 36% higher surface coverage in W1C3 as compared to W1C1 (Table 1).

346 3.3 Interfacial rheology

347 Interfacial viscosity of adsorbed protein films shows long time dependence due to
348 slow unfolding and cross-linking of proteins and/or changes in interfacial composition
349 due to slow protein desorption. Therefore, for the sake of brevity, we present the
350 measured η_i values at just a 'short' (2 h) and a 'long' (24 h) adsorption time, in Table
351 2. Firstly some measurements were made at the A–W interface at pH 7 to check the
352 correct operation of the instrument and procedures. Although measurements at the
353 A–W interface at first sight might seem not relevant to the O/W emulsions, removing
354 the oil removes complications of interactions between the protein and particles with
355 the oil, which may contain surface active impurities. The value of η_i for β-lg at the
356 A–W interface was seen to increase considerably between 2 and 24 h, from 51 ± 8 to
357 388 ± 72 mN s m^{-1}. Interfacial rheology is very sensitive to the composition and
358 history of the adsorbed film and the reproducibility of values obtained for these
359 conditions are in agreement with previous work [26]. Some experiments were
360 performed with 1 wt% MCC at the A–W interface for interest. Although η_i after 2 h
361 in the presence of 1 wt% MCC was the same (within experimental error) as without
362 cellulose, after 24 h, the MCC apparently caused η_i to decrease to zero (or at least < 2
363 mN s m^{-1}, the limit of detectability with the torsion wire used). This decrease was
364 most likely due to the presence of surface active impurities in the MCC.

365 As mentioned before [28], a significant increase in η_i was shown at the A–W
366 interface on addition of hydrophobically modified cellulose [12]. Additionally, η_i is
367 sensitive to pH, generally reaching a maximum at the isoelectric pH (pI), as long as
368 solubility is maintained. It is seen that η_i (A–W) for β-lg on its own at pH 3 after 2 h
369 was similar to the value at pH 7, but after 24 h, η_i had again apparently decreased to
370 zero, which might be ascribed to greater protein repulsion on the positive side (pH 3)

of the protein isoelectric pH (pI, = 5.4) than on the negative (pH 7) side of the pI, or substantial differences in unfolding and cross-linking behaviour at long time, or even acid hydrolysis. Notwithstanding the lack of a completely satisfactory explanation for this decrease, the more important result is that in the presence of 1 wt% MCC this trend was entirely reversed. At pH 3 after 24, β-lg + 1 wt% MCC gave η_i = 130 ± 9 mN s m^{-1}, the highest value being measured at the A–W interface at this pH (pH 3) and MCC concentration. This is presumably due to the still negatively-charged MCC particles somehow getting trapped in the film of net positively charge β-lg molecules, without the MCC particles necessarily adsorbing to the A–W interface directly. However, the η_i results at the O–W interface at pH 3 did not suggest any significant strengthening of the interfacial film by addition of 1 wt% MCC; in fact all values were zero at 2 or 24 h on addition of 1 wt% MCC. The lower values of η_i may be due to oil molecules or impurities in the oil affecting the surface properties of both the MCC particles and the protein. However, increasing the MCC concentration to 3 wt% apparently swamped any such effects, since once again the adsorbed film was significantly strengthened: the η_i values at 2 and 24 h being 136 and 383 mN s m^{-1}, respectively. These results fit in with the much greater accumulation of CNCs apparently observed at the interface of the emulsions with 3 wt% versus 1 wt% cellulose, as observed in calculated surface coverage (Table 1) and TEM images (Figure 2B).

3.4 Microstructural changes during in vitro gastric digestion

As can be observed in Figure 3, the droplet size distribution of W1 emulsion droplets shifted markedly within the first 30 min of gastric digestion, with a considerable proportion of the droplets being in the size range of 10–100 µm. This result is

congruent with the visible observation of pronounced creaming and some degree of coalescence, which is also consistent with previous studies [18, 19, 39]. From 30 to 120 min, the area of the peak at 10–100 µm remained steady, which might suggest that the proteolysis of the interfacial layer by pepsin progressed relatively rapidly and might be almost complete within the first 30 min.

In the case of W1C1 emulsion, the area of the peak at 10 µm increased considerably with parallel decrease of the area of the peak at 1 µm during the first 30 min of gastric digestion. During 60–90 min, the area of the peak at 10 µm decreased gradually and area of the peak at 100 µm appeared to increase, suggesting droplet flocculation, which was consistent with the visual creaming. At 120 min, the area of the peak at 100 µm gradually increased to a maximum value indicating that the proteolysis was continuing even at 120 min, which was not the case in case of W1 emulsion. In case of W1C3 emulsion, the peak from 0.01–0.1 µm disappeared within the first 30 min, whilst the area of the peak at 1 µm increased. During 30-120 min, digesta mainly consisted of almost equal proportions of two peaks at ~ 1 µm and 10–100 µm, with no visual creaming. Overall, the d_{43} values for both the W1 and W1C1 emulsions were markedly higher on completion on 120 min of gastric digestion than those obtained without the addition of pepsin ($p < 0.05$) (Table 1). However, the W1C3 emulsions showed no significant change in d_{43} values during the entire gastric digestion time ($p > 0.05$).

The emulsions in the absence and presence of pepsin showed distinctly different arrays of microstructures depending on the concentration of CNC (Figure 4). Prior to addition of pepsin, emulsions showed no clear signs of aggregation. The confocal micrograph of the W1 emulsion illustrates the large-scale microstructural changes observed with droplet flocculation and some degree of coalescence. This might be

attributed to weakening of the viscoelastic protein layer at the oil-water interface, after its digestion to lower molecular weight peptides. This probably also explains the substantial loss of surface charge (Table 1), where the ζ-potential fell to near zero ($p < 0.05$), after digestion, which is in line with previous findings [18, 19, 39].

As digestion of W1C1 emulsion progressed, a gradual appearance of well-connected networks of agglomerates was observed, illustrated by the confocal micrograph of the W1C1 emulsions, with no discernible large droplets (Figure 4), in agreement with the laser diffraction results (Table 1). This suggests bridging flocculation via CNC inter-connecting WPI-coated coated droplets rather than their coalescence. Flocculation was considerably more pronounced in W1C1 samples in presence of pepsin (30–120 min) as compared to the some samples in the absence of pepsin (0 min). It therefore appears that the instability of this emulsion under simulated gastric digestion was also associated with the digestive action of the pepsin with significant change in ζ-potential ($p < 0.05$) (Table 1). This suggests that pepsin possibly gained access to the adsorbed protein through the insufficiently complete secondary adsorbed CNC layer, cleaving the WPI proteins and making the residual surface charge of the sulphated CNC particles more prominent. Interestingly, there was still a significant proportion of relatively small droplets that appeared completely coated by CNCs (i.e., droplets apparently stained blue by calcofluor white) (Figure 4). This indicates that the CNCs might form an effective barrier to pepsin attack on some droplets i.e. those that are more completely covered.

In the case of W1C3 emulsions, the presence of droplets apparently completely coated with CNCs (i.e. droplets stained blue by calcofluor white) was even more prominent. In fact, a closer look at the micrograph after 30 min of digestion (Figure 4) highlights that the CNCs were not only coating single emulsion droplets but also

networking several droplets together within a CNC shell, resembling emulsion microgel particles [40]. This suggests that the attractive forces between CNC and WPI existed not only around individual droplets but also between droplets. Such rigid 'shells' might be attributed to a combination of extensive hydrogen-bonding arising from the glucan residues of CNC-CNC [41] as well as the net electrostatic attractive protein-particle interactions [12] as described previously. Even after 120 min of digestion, droplets fully coated by CNCs and not exhibiting significant flocculation or droplet coalescence were observed. Correspondingly, in W1C3, there was no alternation in charge on pepsin digestion (Table 1) ($p > 0.05$), which again suggests that a rigid layer of negatively charged CNC that remains intact and significantly restricts the access of pepsin to the inner-adsorbed protein layer.

3.5 Hydrolysis of the interfacial protein layer

In order to understand the link between the gastric stability of the emulsions and pepsinolysis of the adsorbed protein layer, hydrolysis patterns of the interfacial whey protein (cream phase) from the three emulsions are presented in Figure 5. In either of the protocols (heat treatment or raising pH) that were used to stop the activity of pepsin after gastric digestion, no difference in band patterns was observed (data not shown). In the W1 emulsion, β-lg and α-lactalbumin (α-la) were rapidly digested, with no intact whey proteins remaining after 30 min, as previously reported by Sarkar [18] in β-lg emulsions. The β-lg protein appeared to be digested significantly more slowly in the emulsions containing the WPI + CNC composite layers, particularly during the first 60 min (see the bands at 30 and 60 min (Figure 5A). Interestingly, considerable quantities (40 and 60%) of intact β-lg bands were observed even after 120 min of digestion in case of W1C1 and W1C3 emulsions, respectively (Figure

5B). Clearly, the electrostatic binding of CNC to WPI at the interface had a prominent effect in diminishing the rate and extent of interfacial proteolysis. Besides the formation of a rigid composite protein-particle layer, electrostatic repulsion between pepsin and CNC layer might have also played an important role, particularly in case of W1C3 emulsions. As the net charge of both pepsin [42] and the original W1C3 emulsions (Table 1) were negative at pH 3, the mutual electrostatic repulsion might have contributed to restricted access of the pepsin to the underlying positively charged binding points of the whey protein layer.

Golding and Wooster [39] showed that the rate of emulsion digestion was largely controlled by the ability of enzymes to bind to emulsion interfaces, which was determined by emulsion droplet size and interfacial composition. In this study, gastric stability of emulsions was apparently controlled by careful structuring of the interfacial composition. Schematic representations of the possible interactions are given in Figure 6. Although whey protein forms a stable emulsion at pH 3, it could not reduce the digestion rate in gastric conditions due to pepsin-induced rupture of the interface and generation of peptides <10 kDa, which do not provide a sufficiently viscoelastic adsorbed film [18, 19].

Binding of CNC to WPI as a secondary layer, probably via attractive electrostatic interactions, produced significant resistance to the breakdown of WPI interfacial layer by pepsin. This is in slight contrast to the results of previous work [12], where neutron reflectivity measurements showed that addition of the non-surface active CNC (0.01 wt%) served as steric barrier to physicochemical stresses at pH 4. However, the CNC in the previous study did not give sufficient protection to the interfacial β-lg layer from pepsin attack unless the CNC particles were methylated [12]. This might be attributed to the lower pH (pH 3) and higher (3 wt%) CNC concentrations used in our

study, which led to stronger composite formation and a pronounced decrease on the extent and rate of proteolysis.

The concentration of CNC and subsequently the secondary surface coverage was found to be a significant factor in dictating the gastric stability of the emulsions. When the concentration of CNC was low (1 wt%), it was insufficient to cover all the droplet surfaces as shown by residual positive charge on the droplets. However, at 3 wt%, sufficient coverage of the droplets by a rigid layer of CNC provided steric (mechanical) stabilization. Interestingly, the interfacial viscosity measurements also revealed a large increase in interfacial strength on increasing the MCC concentration from 1 to 3 wt%. Furthermore, the electrostatic repulsion between pepsin and anionic CNCs probably accounted for reduced access of pepsin to the proteinaceous interface. This validates our hypothesis that the presence of CNCs can act as a barrier to pepsin–induced digestion of the WPI at the interface and provide better emulsion gastric stability than oil-in-water emulsions stabilized by WPI alone. However, quantification of network formation of CNCs in the bulk phase versus at the interface require further investigation.

3 Conclusions

The influence of molecular architecture and charge of WPI + CNC composite interfaces on gastric stability has been investigated by means of an array of complimentary physicochemical techniques and microstructural analysis. Our study confirms for the first time that the presence of CNCs decreases the degree and extent of in vitro gastric digestion of the proteinaceous interface by pepsin using SDS-PAGE. This occurs by formation of strong protein-particle composite adsorbed layers at pH 3 exhibiting effective electrostatic and steric repulsion that slows the access of

pepsin to the protein interface. At present, we cannot also rule out the possibility of network formation by the CNC in the bulk (continuous) phase and encapsulation of several emulsion droplets together in a rigid CNC shell that also reduces the kinetics and extent of proteolysis. Nevertheless, the present work has uncovered an interesting link between the fundamental interfacial properties of protein-particle composite layers and enhanced gastric stability, which could act in the design of physiologically relevant emulsions. In addition, these findings could have important implications for the design and delivery of lipophilic drugs and bioactive compounds in foods.

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