Pickering emulsions stabilised by hydrophobically modified cellulose nanocrystals: responsiveness to pH and ionic strength

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Abstract

The aims of this study were to hydrophobically modify cellulose nanocrystals (CNCs), investigate the ability of such modified CNCs (MCNCs) to stabilise Pickering oil-in-water (O/W) emulsions and understand their stability at different pHs (2.0–7.0) and ionic strengths (0–150 mM NaCl). Structural changes that resulted from esterifying CNCs with octenyl succinic anhydride (OSA) were determined using Fourier transform infrared (FTIR) spectroscopy, X-ray diffractometry (XRD), transmission electron microscopy (TEM) and wettability analysis. The stability of the Pickering O/W emulsions (20 wt% oil, 0.05–1.00 wt% MCNCs) was assessed using droplet sizing, microscopy, ζ-potential, apparent viscosity and oscillatory rheological measurements. FTIR spectroscopy confirmed a decrease in the intensity of the –OH-associated band because of reaction of the hydroxyl group with OSA. XRD indicated a lower (11.5%) crystallinity index in MCNCs. TEM revealed that there was no change in morphology of the needle-shaped CNCs upon OSA modification (length/diameter = 40–100 nm/2–4 nm). Hydrophobic modification of CNCs with OSA was evidenced by an increase in static water contact angle from 56^{o} (untreated CNCs) to 80.2^{o} (MCNCs) which allowed the MCNCs to be partially wetted by both the phases and stabilise O/W emulsions. The Pickering emulsions showed droplet flocculation at pH < 4.0 (without addition of NaCl) or ionic strength ≥ 20 mM NaCl (pH 7.0), with a predominant elastic gel-like behaviour observed at ≥ 20 mM NaCl. Resistance of MCNC-based Pickering emulsions to coalescence and responsiveness to flocculation at bio-relevant pHs and ionic strengths show promise in the design of delivery vehicles.

Keywords: Cellulose nanocrystal; Octenyl succinic anhydride; Pickering emulsion; Particle-stabilised emulsion; Electrostatic interaction

1. Introduction

Pickering emulsions, i.e. emulsions stabilised by solid particles, have gained significant research attention from food colloid scientists because of their combined advantages of high resistance to coalescence and the ability to delay lipid digestion (Dickinson, 2012; Sarkar, Zhang, Holmes, & Ettelaie, 2019). Pickering emulsions can be either oil-in-water (O/W) or water-in-oil (W/O) depending on the preferential wettability of the stabilising particles (Chevalier & Bolzinger, 2013). Hydrophilic particles favour the formation of O/W emulsions, whereas hydrophobic particles are generally suitable for W/O emulsions (Zembyla, Murray, & Sarkar, 2018). Pickering O/W emulsions using inorganic particles have been studied extensively, but the use of biocompatible particles, such as protein microgels (Destribats, Rouvet, Gehin-Delval, Schmitt, & Binks, 2014; Hu et al., 2016; Sarkar et al., 2016), modified starch and non-starch polysaccharide particles (Li et al., 2018; Marefati, Matos, Wiege, Haase, & Rayner, 2018; Tzoumaki, Moschakis, Kiosseoglou, & Biliaderis, 2011; Yusoff & Murray, 2011), protein–protein particle composites (Liu, Huang, Chen, Deng, & Yang, 2019), protein–polysaccharide particle composites (Doost et al., 2019; Sarkar et al., 2018a) and polysaccharide–polysaccharide particle composites (Li et al., 2019), for generating particle-stabilised interfaces is relatively recent. Such particles have been studied mainly to stabilise Pickering emulsions with the ultimate aim being to alter the kinetics of lipid digestion.

For the design of small-sized (1–10 μm) oil droplets, as solid stabilisers must be in the nanometric size range, modified starch granules might not be suitable Pickering stabilisers because they are generally several microns in size unless they have been modified by physical approaches (Yusoff & Murray, 2011). Although protein and inulin particles have been reported to have nanometric sizes, emulsions stabilised by protein/inulin composites or protein particles as the sole stabiliser had droplet sizes (d_{43}) of up to 24.7 μm (Sarkar, Li, Cray, & Boxall, 2018b) and larger than 100 μm in the case of zein particles (de Folter, van Ruijven, & Velikov, 2012). In addition, as protein-based particle-stabilised emulsions are generally sensitive to coalescence under physiological conditions because of their susceptibility to proteolytic enzymes (Sarkar et al., 2018a), there has recently been a demand for particles that are resistant to human gastrointestinal enzymes as potential Pickering stabilisers.

Interestingly, needle-shaped cellulose nanocrystals (CNCs) with a characteristic length of around 100–200 nm and a diameter of 8–86 nm, depending on their source (Chen et al., 2018; Lee et al., 2011; Yan et al., 2017), are human-enzyme-resistant particles that have attracted recent research attention for the production of stable Pickering emulsions. However, as CNCs are known to be highly hydrophilic (Cherhal, Cousin, & Capron, 2016), their partial wettability by a hydrophobic phase can be expected to be poor, which may limit their direct use as Pickering stabilisers. Hence, the first method to improve the wettability of these CNCs was to combine them with surfactants. For example, CNCs were blended with surfactants and these particle–surfactant mixtures were used to create the particle-laden interface (Hu, Ballinger, Pelton, & Cranston, 2015). Another approach was to use protein as a primary layer and then to add CNCs to the system as a second layer to form a composite protein–CNC interface (Sarkar et al., 2018b; Sarkar, Zhang, Murray, Russell, & Boxal, 2017); however, whether in such a system, a true particle-stabilization mechanism was there can be argued. In both approaches, as the emulsions were probably stabilised by surfactants and not by the solid CNCs, the high desorption energies associated with true Pickering stabilisers might not be achieved in these systems.

An alternative approach is hydrophobic modification of the CNCs with various substances, such as succinic anhydride (Liu, Sun, Zhang, Ren, & Geng, 2006), octenyl succinic anhydride (OSA) (Chen et al., 2018), phenyltrimethylammonium chloride (Gong, Wang, & Chen, 2017) and organic acids (hexanoic acid and dodecanoic acid) (Lee et al., 2011). Such modified CNCs (MCNCs) can be used as the sole stabiliser without the need for any surfactant or protein. Of all the chemicals mentioned above, OSA, which has been used for decades in the food industry for the modification of starch (Nilsson & Bergenståhl, 2007; da Silva et al., 2013), seems to be the most straightforward. Only one study has reported on the modification of CNCs using OSA to produce high internal phase emulsion gels (Chen et al., 2018). To our knowledge, there is no published literature on the creation of Pickering O/W emulsions using OSA-modified CNCs. In addition, there is no study to date that has systematically investigated the behaviour of OSA-modified CNC-stabilised Pickering emulsions when subjected to environmental stresses, such as pH and electrolytes. Reponses of the emulsions to such environmental stresses are crucial to understand

the fate of these emulsions during physiological transit when they are used for designing delivery systems.

Therefore, the aim of this study was to systematically characterise the modification of CNCs using OSA and investigate the physicochemical and microstructural behaviour of the O/W emulsions stabilised by OSA-modified CNCs as a function pH and ionic strengths. We used a combination of light scattering, confocal laser scanning microscopy, electron microscopy, electrophoretic mobility measurements, bulk rheology and spectroscopic techniques to examine the ability of these modified particles to create stable small-sized Pickering emulsion droplets and to study the stability of the droplets under various environmental conditions. To the best of our knowledge, this is the first work on the characterisation of MCNC-based Pickering emulsions and the responsiveness of these emulsions to pH and ionic strength. As this is the first study of a series of work being conducted by the authors on delivery of short chain fatty acids, a mixture of sunflower oil (SFO) with tripropionin [glyceryl tripropionate (TPP), a source of propionic acid] and tributyrin [glyceryl tributyrate (TBT), a source of butyric acid] was used as the dispersed phase for the production of the Pickering O/W emulsions.

2. Materials and methods

2.1. Materials

CNCs (94-96% sulphated CNCs) derived from sulphuric acid treatment of bleached Kraft pulp were purchased from CelluForce^{TM}, Montreal, Canada. The detailed chemical composition of CNCs can be obtained from a previous study (Reid et al., 2017). The CNCs used in this study was intended for research purposes and not for consumption. SFO was purchased from a local supermarket (Morrisons, Leeds, UK). Food-grade tripropionin (≥ 97.1% TPP), tributyrin (≥ 97.1% TBT), sodium azide and OSA were purchased from Sigma-Aldrich Company Ltd, Dorset, UK. All other chemicals were of analytical grade and were purchased from Sigma-Aldrich Company Ltd, Dorset, UK. Milli-Q water (ionic purity of 18.2 MΩ.cm at 25 °C) purified by a Milli-Q apparatus (Millipore Corp., USA) was used as the solvent for all experiments. Sodium azide (0.02 wt%) was used as a bactericide to prevent microbial growth during refrigerated storage (at 4 °C) of the samples.

2.2. Hydrophobic modification of CNCs

Hydrophobic modification of CNCs was conducted according to a method that has previously been used for inulin (Han, Ratcliffe, & Williams, 2015) with some modifications. All reactions were performed in a 500 mL round-bottomed flask at 25 °C. The CNC dispersion (3.0 wt% in water) was mixed with OSA at a ratio of 1:0.15 (w/w). The pH was adjusted to pH 8.30 ± 0.1 using 0.5 N NaOH. During the esterification reaction of hydroxyl groups on the CNC backbone with OSA, the pH was maintained by the continuous addition of 0.5 N NaOH using a pH-stat (TIM856, Radiometer Analytical, Hach Company, Loveland, CO, USA). The reaction was carried out until no further NaOH was needed to neutralise the acidic products to ensure that all the OSA had been consumed; typically, the time required was around 7.0 h. Once the reaction was complete, the resultant product was neutralised to pH 7.0 with 1.0 N HCl and then lyophilised, yielding a white powder. The powder was purified by Soxhlet extraction for 12 h using ethanol to remove any leftover OSA from the powder. Finally, it was air dried in an oven at 40 °C overnight to remove the ethanol. This powder is referred to as modified cellulose nanocrystals (MCNCs) and was used to create Pickering O/W emulsions.

2.3. Structural analysis of MCNCs

2.3.1. Fourier transform infrared (FTIR) spectroscopy

Before measurement, the MCNCs were ground sufficiently with a mortar and pestle; all samples were then pressed into pellets. The measurements were carried out using a Bruker ATR-FTIR spectrometer (Bruker Optics GmbH, Ettlingen, Germany) in the mid-IR region (400–4000 cm^{-1}), with a resolution of 4 cm^{-1} for at least 64 scans. The FTIR spectra of unmodified CNCs and MCNCs were collected as average values of 64 scans using Mobility Series™ software before being exported to Origin 2016 Sr2 (OriginLab Corp., Northampton, MA, USA) for peak fitting.

2.3.2. X-ray diffractometry (XRD)

XRD profiles of unmodified CNCs and MCNCs were obtained using a Bruker AXS D8 Advance diffractometer with LINXEYE detector using Cu K_α (λ = 0.154 nm). The XRD analysis was performed at room temperature at 40 kV and 40 mA, with 2θ ranging from 0 to 60°, at a scan rate of 0.02° min^{-1}. The crystallinity indexes (CIs) of the CNCs and MCNCs were calculated using the Segal method, as described previously (Lee et al., 2011):

$$CI = \left(\frac{I_{002} - I_{AM}}{I_{002}} \times 100\%\right) \quad (1)$$

where I_{002} is the peak intensity of plane 002 and I_{AM} is the minimum intensity between planes 002 and $10\bar{\imath}$ in the XRD profiles.

2.3.3. Wettability

Static water contact angles of unmodified CNCs and MCNCs were determined at 25 °C with the sessile drop method using an OCA25 drop-shape tensiometer (DataPhysics Instruments, Filderstadt, Germany) fitted with a microsyringe and a high speed IDS camera. Before measurement, both CNCs and MCNCs were pressed, under a weight of 6 tonnes for 30 s, between the plates of a hydraulic bench press (Clarke, Kempston, UK) into discs with diameters of 15 mm and thicknesses of approximately 2 mm, according to a previous method (Zembyla et al., 2018). A straight needle (0.52 mm outer diameter and 0.26 mm internal diameter) was used to produce 5.0 µL water droplets that formed sessile drops on the discs made from unmodified CNCs or MCNCs. The water droplet contour was recorded using a video camera and was immediately fitted using the SCA202 V5.0.15 software. Static water contact angles (θ_w) between the CNC or MCNC disc and the water were then measured. All measurements were done in triplicate and were reported as the mean and standard deviation.

2.3.4. Transmission electron microscopy (TEM)

TEM was used to observe the structural changes in the CNCs (if any) after modification with OSA. Both CNC and MCNC samples (10 µL) were stabilised with 2.5% (v/v) glutaraldehyde, fixed in 0.1% (w/v) osmium tetroxide and then embedded in araldite. Ultra-thin sections (80–100 nm) were then placed on 3.05 mm grids and stained with 8% (v/v) uranyl acetate and lead citrate. The imaging was carried out using a CM10 TEM microscope (Philips, Guildford, UK).

2.4. Preparation of Pickering O/W emulsions

The MCNCs were used as Pickering stabilisers at various concentrations to create O/W emulsions. The oil phase (TPP–TBT–SFO mixture with a weight ratio of 1:1:2) was pre-homogenised at an oil:aqueous phase ratio of 1:4 (w/w) using a high speed blender (D500 series, Biolab Ltd, Germany) at 10,000 rev min^{-1} for 3 min. The emulsions were prepared using various concentrations of MCNCs, i.e. 0.05, 0.10, 0.20, 0.50 and 1.0 wt% in the final emulsions, hereafter referred to as emulsions E0.05, E0.10, E0.20, E0.50 and E1.00 respectively. In the next step, the coarse emulsions were homogenised using a two-stage valve homogeniser (Panda Plus, GEA Niro Soavi, Parma, Italy) at pressures of 200/50 bar for three passes. The resulting emulsions were used for analysis of droplet size, ζ-potential, microstructure and stability. Control emulsions were also prepared from unmodified CNCs using 1.0 wt% CNCs.

2.5. Characterisation of Pickering O/W emulsion droplets

The emulsions were characterised for droplet size, *ζ*-potential and viscosity, and the microstructure was assessed using confocal laser scanning microscopy (CLSM). The samples were diluted to around 0.01% w/v for measurement of the droplet size and the *ζ*-potential. Droplet size distributions of the emulsions were determined at 25 °C by static light scattering using a Mastersizer (3000S series, Malvern Instruments Ltd, Malvern, UK). The relative refractive index, i.e. the ratio of oil (1.456) to that of the dispersion medium (1.33), was 1.095. The mean droplet size was reported as the Sauter-average diameter (d_{32}) and the volume-average diameter (d_{43}) from the size distribution results. Each individual d_{32} and d_{43} value was reported as the mean and standard deviation of at least three readings made on triplicate samples.

A zetasizer (ZS Nano, Malvern Instruments Ltd, Malvern, UK) was used to measure the electrophoretic mobilities of the MCNC-stabilised emulsion droplets. The diluted emulsion samples were transferred into DTS1070 folded capillary cells for measurement of the electrophoretic mobility, followed by 30 s of equilibration within the equipment. The mobilities were then converted to ζ-potential values using the classical Smoluchowski equation. Each ζ-potential value was reported as the mean and standard deviation of at least three reported readings made on triplicate samples.

The apparent viscosity, elastic modulus (G') and viscous modulus (G'') of the emulsions were determined at 25 °C using a Kinexus ultra rheometer (Malvern Instruments Ltd, Malvern, UK). Steady shear experiments were performed; apparent viscosities as a function of shear rate in the range from 2 to 1000 s^{-1} were recorded for the emulsions using a double gap geometry DG 24/27. Dynamic frequency sweep tests were then carried out at a strain amplitude of 1.0% and with an angular frequency range of 0.01–2.0 s^{-1}. The frequency-dependent curves of the storage (G′) and loss (G″) moduli were recorded. All measurements were done in triplicate and were reported as the mean and standard deviation.

CLSM images of the emulsions were taken using a Zeiss LSM 880 confocal microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Exactly 500 µL of emulsion was added to a 1.5 mL Eppendorf tube and mixed with 10 µL of Nile Red (0.1% w/v in dimethyl sulphoxide, excitation 514 nm, emission 539–648 nm) and then 100 µL of Calcofluor-white (1.0% w/v in Milli-Q water, excitation 405 nm, emission 410–523 nm). The mixture was vortexed for 10 s and equilibrated for 10 min, and then 30 µL was placed on to a concave slide. The sample was fixed by adding 50 µL of 1.0% w/w xanthan gum, was covered with a coverslip and was observed using a 63 × magnification oil immersion objective lens.

2.6. Stability of MCNC-based emulsions under different stresses

2.6.1. Storage at 4 ℃

The emulsions E0.05, E0.10, E0.20, E0.50 and E1.00 were stored at 4 °C and were monitored using visual observation and droplet sizing and ζ-potential measurements for 4 weeks.

279 2.6.2. pH and ionic strength conditions

280 Freshly prepared emulsions (E1.00) had a pH of approximately pH 6.8. For the pH
281 study, the emulsions were adjusted to pH 7.0 using 1 N NaOH and then to pH 6.0,
282 5.0, 4.0, 3.0 and 2.0 using 1 N HCl. For each pH study, 20 mL of emulsion E1.00 in a
283 50 mL beaker was adjusted to the desired pH by drop-by-drop addition of NaOH or
284 HCl under constant stirring at 500 rev min^{-1}. In separate experiments, emulsions
285 (E1.00) were adjusted to various ionic strengths from 10 to 150 mM NaCl by mixing
286 10 mL of emulsion with a suitable quantity of solid NaCl under constant stirring at
287 500 rev min^{-1}. Subsequently, samples were analysed for any change in droplet size,
288 ζ-potential and microstructure (CLSM). All measurements were done in triplicate and
289 were reported as the mean and standard deviation.

290

291 **2.7. Statistical analysis**

292 Analysis of variance was conducted using Minitab® version 17.3.1 to detect overall
293 significant differences ($p < 0.05$).

294

295 # 3. Results and discussion

296 ## 3.1. Characteristics of MCNCs

297 We first discuss the modification of the CNCs using OSA and any effects of this
298 hydrophobic modification on the particle behaviour and morphology, as measured for
299 the CNCs and MCNCs with a range of complementary techniques, such as FTIR
300 spectroscopy, XRD profiles, static water contact angles and TEM (Fig. 1). This sets
301 the scene for understanding the behaviour of the MCNCs when they are present at
302 an interface. As expected, the modification reduced the number of hydroxyl (–OH)
303 groups in the cellulose backbone because of their replacement by OSA in MCNCs;
304 this was clearly observed in the FTIR absorbance spectra (Fig. 1A). The intensity of
305 the band at 3400 cm^{-1}, which corresponds to the –OH groups, decreased by
306 approximately 14% in the MCNC sample, which was in agreement with a previous
307 study (Chen et al., 2018), in which the intensity of the same band also decreased
308 because of the substitution by OSA. There was a significant increase in the band at
309 1600 cm^{-1}; this corresponded to an increase in the water content of the MCNCs and

was associated with the addition of some water molecules during the modification process, which was also observed in the previous study (Chen et al., 2018).

Modification with OSA also led to changes in the crystalline form (allomorph) of cellulose I, the diffraction pattern of which was between planes 101 and 002, as indicated in Fig. 1B (Ciolacu, Ciolacu, & Popa, 2011). There was an increase in the intensity of the peak between diffraction planes 101 and $10\bar{1}$, which indicated increased amorphousness or decreased crystallinity. This observation was in contrast to previous studies on the OSA modification of cotton CNCs (Chen et al., 2018) and the organic acid modification of bacterial cellulose nanofibres (Lee et al., 2011), in which the intensity of the peak between planes 101 and $10\bar{1}$ decreased. In our study, the CIs of the CNCs and MCNCs were 77.7 and 68.8% respectively. That is, modification led to an around 11.5% decrease in the CI of the cellulose structure. This change was nearly three times higher than that reported by Chen et al., (2018) and Lee et al., (2011), which was around 4.5% and 5.0%, respectively. This discrepancy in the crystallinity of the MCNCs in the present study versus previous studies (Chen et al., 2018; Lee et al., 2011) might be attributed to the differences in source of CNCs used and the modification methods. For instance, Lee et al. (2011) used bacterial cellulose nanofibres while we used a commercial CNCs synthesised by sulphuric acid treatment of cellulose derived from bleached kraft pulp (Reid et al., 2017); both materials have different original crystallinity indexes (CIs), i.e. 90.2 and 77.7 % for cellulose nanofibres and CNCs in the current study, respectively. The differences might affect their behaviour and reactions during the subsequent modification process. In the present study, we controlled the pH at a constant value for at least 7 h, which was an important factor in improving the degree of substitution of OH by OSA. In contrast, Chen et al., (2018) did not control the pH and mixing with OSA was carried out for 1 min only.

As a result of this OSA substitution, the hydrophobicity of the MCNCs was significantly enhanced; this was clearly demonstrated by the dramatic increase in the water contact angle from 56.0 ± 0.3^{o} (CNCs) to 80.2 ± 0.8^{o} (MCNCs) (Fig. 1C). The differences in the organisation of the crystal as shown in XRD data in Fig.1B upon modification with OSA might also explain the shifting of static water contact angle in Fig.1C towards more hydrophobicity. That is, MCNCs will be wetted preferentially by water and only partially wetted by the oil phase; thus, they will stabilise O/W

emulsions, as opposed to unmodified CNCs, which are too hydrophilic and will probably remain in the aqueous phase (Cherhal, Cousin, & Capron, 2016; Kalashnikova, Bizot, Cathala, & Capron, 2012). Changes in the surface charge of the CNCs were also determined, by comparing the ζ-potentials of CNCs and MCNCs in 1 wt% solution at pH 7.0. The CNCs had a high negative charge of –39.3 ± 1.0 mV because of the presence of sulphated groups in the cellulose backbones. In addition, the ζ-potential (–39.1 ± 2.1 mV) of the MCNCs was similar to that of the unmodified CNCs. Although some changes in crystallinity were evident in the XRD profiles, the TEM images (Fig. 1D) of the CNCs and the MCNCs were indistinguishable, showing a needle-like shape and a similar aspect ratio (length:diameter) of 20:1, with a diameter of 2–4 nm and a length of 40–100 nm.

3.2. Characteristics of MCNC-based Pickering O/W emulsions

MCNCs at various concentrations (0.05–1.00 wt%) were used to produce 20 wt% Pickering O/W emulsions. As a control, emulsions were also prepared using 1.00 wt% unmodified CNCs. Supplementary Fig. S1 confirms that the unmodified CNCs did not have emulsifying capacity; phase separation with a clear oil layer occurred almost immediately after preparation of the emulsions, and exceptionally large oil droplets were observed in the confocal micrographs. This is in agreement with the low water contact angle observed in Fig. 1C and again confirms the need for hydrophobic modification to enable the formation of stable oil droplets. The MCNC-stabilised emulsions were characterised by droplet size distribution (Fig. 2A), confocal microscopy (Fig. 3) and ζ-potential (Table 1).

Fig. 2A clearly shows that all emulsions had bimodal distributions, with similar first peaks in the range 0.01–0.3 µm. As the MCNC concentration increased, the first peak became more prominent and the second peak narrowed and shifted to a lower size range. In all emulsions, the first peaks were too small to represent emulsion droplets; they probably represented the MCNCs, which had a needle-like shape with a length of 40–100 nm (Fig. 1D). Hence, a possible explanation for the first peak was the presence of unadsorbed MCNCs in the aqueous phase. In a previous study on emulsions stabilised by CNC–protein composites, a similar small-sized peak was observed; it was hypothesised to be unadsorbed CNCs (Sarkar et al., 2018b). To prove this hypothesis, we centrifuged an emulsion at 14,500 g for 40 min at 4 °C,

collected the cream layer, diluted it in Milli-Q water to a droplet concentration similar to that of the original emulsion and analysed it for droplet size distribution. The droplet size distributions of emulsion E0.50 and the cream phase of emulsion E0.50 confirm the hypothesis that the first peak was unadsorbed MCNCs, as it did not appear in the diluted cream phase in Fig. 2B. In addition, we also measured the particle size distribution of the MCNC solution using dynamic light scattering (DLS) (Supplementary Fig. S2). The MCNC solution had a multimodal distribution, in which the main peak was in the size range 100–300 nm, closely resembling that of the first peak of the droplet size distribution in Fig. 2A. Although the DLS size is in agreement with the characteristic length of the MCNCs (Fig. 1D), caution should be used in interpreting the DLS data, recognising the limitation that DLS assumes particles to be spherical; MCNCs are rod shaped (Boluk & Danumah, 2014). Therefore, in this study, the calculation of average particle sizes d_{32} and d_{43} were done after removal of the first peak in the range 0.01–0.3 µm (the peaks were separated by a dashed line, see Fig. 2A) similarly to a previous study (Zembyla, Murray, & Sarkar, 2018). In addition, d_{32} and d_{43} of the first peaks of all the freshly prepared emulsions were quantified and presented in Supplementary Table S1. As can be clearly seen in Supplementary Table S1, the d_{32} and d_{43} of all the first peaks were around 33–38 and 58–86 nm, respectively, which were within the range of lengths observed for the needle-shaped MCNCs (40–100 nm) TEM (see Fig. 1D). This analysis again confirmed that the first peak was due to the presence of free MCNCs in the continuous phase.

The results also confirmed the nature of the Pickering emulsions, in which the diameter of the droplets was around 10–100 times larger than that of the solid particles, which has been reported in many previous studies (Hu et al., 2015; Marefati et al., 2018; Sarkar et al., 2016). Furthermore, in our study, the average droplet size (d_{43} = 2.29 µm) obtained at low MCNC concentration (0.20 wt%) was smaller than that of most Pickering droplets that have been reported (Li et al., 2018; Marefati et al., 2018; Tzoumaki et al., 2011; Yusoff & Murray, 2011). This indicates the excellent partial wetting of these MCNCs by either of the two phases.

Confocal micrographs of the Pickering emulsions are shown in Fig. 3, in which A represents the Calcofluor-white channel (MCNCs) and B illustrates the merge channel of Calcofluor-white and Nile Red (oil droplets). It can be seen clearly that

the amount of MCNCs in emulsion E0.05, with an oil volume fraction of 20 wt%, was not high enough to prevent coalescence. This is in agreement with a previous study on emulsion gels stabilised by OSA-modified CNCs, which reported substantial coalescence when low concentrations (less than 0.3 wt%) were used (Chen et al., 2018). However, the 'clear blue ring' in Fig. 3 (zoomed image) highlights the signature of the MCNCs acting as Pickering stabilisers. An increase in the MCNC concentration from 0.05 to 0.10 wt% resulted in a 1.97-fold decrease in average droplet size (d_{43}) to 4.02 μm (Table 1). In addition, the MCNC coverage on the interface was evident in the confocal images (Fig. 3), with smaller droplets being formed at higher MCNC concentration (0.05–0.10 wt%) and with a greater amount of unadsorbed MCNCs being present in the continuous phase, in agreement with the size distribution results (Fig. 2A).

Another study on 20 wt% O/W emulsions stabilised by carboxylated CNCs (0.1 wt%) reported similar droplet sizes (Mikulcov, Bordes, Minarik, & Kasparkov, 2018) to those obtained in our study. However, in their study, the microscopic structure of the emulsions was not examined. In our study, there was significant improvement when the concentration of MCNCs was further increased to ≥ 0.20 wt%. Most droplets in these emulsions appeared to be fully covered by MCNCs and, even in a populated area, the droplets were well separated with distinctive layers of MCNCs on the interface without any sign of uncoated droplets. Also, there was no sign of droplet aggregation. This may have been associated with the higher negative surface charge (–68.7 mV) obtained at higher MCNC concentration (Table 1), which prevented the droplets from coming in close vicinity of each other. In comparison with the surface charge of the aqueous dispersion of MCNCs (–39.1 ± 2.1 mV), as reported in Section 3.1, the surface charges of all emulsions were significantly higher. This might be due to the difference in the arrangement of the MCNCs between bulk solution and the oil droplet surface, which needs to be investigated in future using XRD. The MCNCs were more densely packed at the surface of the droplets than in the solution. A similar difference in particle charge between in the bulk phase and at the interface has been reported previously (Sarkar et al., 2016). Electrostatic repulsion is generally common in Pickering emulsions when the particles carry a surface charge (Araki, 2013; Binks, 2002; Ridel, Bolzinger, Gilon-Delepine, Dugas, & Chevalier, 2016). Therefore, the MCNCs allowed the formation

of Pickering O/W emulsion droplets that carried large desorption energies, because of their particle-stabilised interface, and at the same time were electrostatically stabilised, because of the high charge densities of the MCNCs at the interface.

3.3. Stability of MCNC-based Pickering O/W emulsions

The emulsions were also examined for their microstructural stability and their physicochemical properties when subjected to storage at 4 °C and to different pHs (pH 2.0–7.0) and ionic strengths (0–150 mM NaCl).

3.3.1. Storage for 4 weeks at 4 ℃

Fig. 4 shows the appearance of freshly prepared emulsions and emulsions after 1, 2 and 4 weeks of storage under refrigerated conditions. Apart from emulsion E0.05, in which a thin oil layer had separated from the remaining emulsion, all emulsions were stable, without any signs of creaming or separation. After 1 week of storage, there was clear phase separation in emulsions E0.05 and E0.10. Instability of the emulsions was due to there being an insufficient amount of emulsifier to stabilise all interfacial area at the chosen oil content (20 wt% oil).

This phenomenon was also in line with the CLSM images (Fig. 3), which showed large droplets in emulsions E0.05 and E0.10. In contrast, the droplet sizes of emulsions E0.20, E0.50 and E 1.00 changed slightly (Table 1) but the appearance of the emulsions was rather homogeneous. The ζ-potentials of emulsions E0.20, E0.50 and E1.0 remained constant after 1-week storage, suggesting that the high net negative surface charges were providing electrostatic repulsive forces to prevent flocculation. After 2 weeks of storage, emulsion E0.20 showed almost an order of magnitude change in droplet size. The zeta-potential of E0.20 significantly decreased, i.e. –60.9 ± 2.6 and –50.7 ± 7.1 mV after 1 and 2 weeks respectively ($p<0.05$). This was in agreement with increase in droplet size (3.55 times) and associated emulsion instability. Although creaming was clearly seen in both emulsion E0.50 and emulsion E1.00, both emulsions returned to a visually homogeneous appearance after a gentle shake, and there were no signs of coalescence. In addition, there was no significant change in droplet size and ζ-potential in emulsions E0.50 and E1.00 after 4 weeks of storage.

In a previous study (Mikulcov et al., 2018), emulsions stabilised by less than 0.3 wt% carboxylated CNCs showed clear separation of bulk oil from the remaining emulsion, indicating coalscence. In our study, for MCNC concentrations higher than 0.20 wt%, creaming was detected only after 14 days. Overall, this suggests that 0.50–1.00 wt% of MCNC was sufficient to create fine droplets with sufficient particle coverage, as shown in the confocal micrographs (Fig. 3), a high negative charge (Table 1) and resistance to coalescence over the storage period.

3.3.2. pH conditions

The emulsions subjected to different pH conditions (pH 2.0–7.0) were characterised using droplet sizing (Fig. 5A), flow curves (Fig. 5B) and confocal micrographs (Fig. 5C), with mean sizes and ζ-potentials being reported in Table 2. Fig. 5A and Table 2 show that decreasing the pH from pH 7.0 to pH 5.0 slightly affected the sizes (1.3 times) and net negative surface charge of the emulsions. However, when the pH was decreased to ≤ pH 4.0, the droplet size increased significantly, by approximately 2.7 times, and the magnitude of the ζ-potential decreased from –30.4 mV at pH 4.0 to –20.5 mV at pH 2.0. In addition, when the pH was ≤ 4.0, the first peak of the size distribution decreased in width and the second peak shifted to a larger droplet size range.

For understanding of the role of MCNCs when present at the particle-stabilised interface, we first evaluated the effects of pH on the behaviour of aqueous dispersions of MCNCs (1.0 wt%) (Supplementary Fig. S3), which demonstrated that the ζ-potential became significantly less negative as the pH was decreased from pH 5.0 to pH 4.0. Behaviour of MCNCs in the aqueous dispersions and in the emulsions was not in complete agreement but the changes in ζ-potential were similar within the pH range 4-5. This phenomenon might be due to the difference in alignment and concentrated coverage of the MCNCs at the emulsion droplet surface as compared to that in the bulk aqueous dispersions. In addition, as discussed in Section 3.2, the high surface charge of the emulsions contributed by the MCNCs would guarantee strong electrostatic repulsion to prevent droplet aggregation. At lower pH, the net negative charge was reduced, resulting in insufficient electrostatic repulsion to prevent closer approach of the oil droplets. As a consequence, there was droplet aggregation. The confocal images of the emulsion at pH 3.0 in Figs. 5C1 and 5C2

clearly illustrate emulsion flocculation. Aggregation of the emulsion droplets was associated not only with the MCNCs on the droplet surface but also with the unadsorbed MCNCs in the aqueous phase. This might explain the decrease in the first peak of the size distribution, which represented the unadsorbed MCNCs, as explained in Section 3.2. Interestingly, there was no sign of coalescence in the emulsions on pH adjustment. That is, weak electrostatic interaction of the droplets at low pH induced aggregation but did not cause desorption of the MCNCs.

Studies on Pickering emulsions stabilised by CNCs from various sources have also reported the same trend, in which the changes in the magnitude of the ζ-potential were a function of pH (Liu et al., 2018; Mikulcov et al., 2018; Wen, Yuan, Liang, & Vriesekoop, 2014). However, in these studies, the authors did not characterise the microscopic structure of the Pickering emulsions under various pH conditions.

For a better understanding of the effects of pH, the bulk rheological properties of the emulsions at pH 7.0 and pH 3.0, hereafter referred to as emulsion E7.0 and emulsion E3.0 respectively, were determined by applying shear rates from 2 to 1000 s^{-1} and then from 1000 to 2 s^{-1} to the emulsions to observe hysteresis (if any) (Fig. 5B). In general, the viscosity of E3.0 was higher than that of E7.0 due to droplet flocculation at lower pH. In addition, the viscosities of the emulsions at both pHs decreased as the shear rate increased within the investigated shear rate ranges, exhibiting a slight shear-thinning behaviour. Interestingly, both emulsions demonstrated negligible hysteresis within the range of shear rate investigated, indicating their stability during the shearing. In a previous study on Pickering emulsions stabilised by fumed silica, it was reported that the emulsions were stable against coalescence at a shear stress up to 1000 Pa and the emulsions reversed to their original state after the removal of the stress (Lee et al., 2014). However, this observation was in contrast to some other findings on Pickering emulsions stabilised by silica particles (French, Taylor, Fowler, & Clegg, 2015; Whitby & Krebsz, 2014; Whitby, Fischer, Fornasiero, & Ralston, 2011), where shearing from 0.1 to 1000 s^{-1} resulted in droplet coalescence (Whitby, Fischer, Fornasiero, & Ralston, 2011). In our study, no such shear-induced droplet coalescence was observed. In our study, emulsions E7.0 and E3.0 had ζ-potentials of –64.3 and –29.1 mV respectively. Sufficient negative charge of the emulsion droplets possibly prevented the droplets

from coming in close vicinity and the higher mechanical strength of the MCNC-laden interface limited shear-induced coalescence.

3.3.3. Ionic strength conditions

On exposure to different ionic strengths (0–150 mM NaCl), the emulsions were assessed using droplet sizing (Fig. 6A) and apparent viscosity (Fig. 6B) measurements to understand the flocculation behaviour. In addition, the frequency dependence of the curves of storage modulus (G′) and loss modulus (G″) (Fig. 6C) together with their corresponding confocal images (Fig. 6D and 6E) gave an understanding of the ion-induced evolution in the material properties of these Pickering emulsions. The MCNC-stabilised Pickering emulsions were highly sensitive to changes in ionic strength at a critical electrolyte concentration (threshold) of $\geq$ 20 mM NaCl (Fig. 6A and Table 3). The volumetric proportion of the second peak increased significantly at the expense of the first peak. The average size d_{43} increased significantly from 1.21 µm in the absence of added NaCl to 2.04 and 76.86 µm at 20 and 100 mM NaCl respectively. Further increases in ionic strength did not lead to significant changes in the droplet size. In addition, the net surface charge of the emulsions decreased significantly from –64.3 mV in the absence of added NaCl to –34.6 and –18.6 mV at 20 and 100 mM NaCl respectively (Table 3). It can be clearly seen that within the NaCl concentration range of 20-100 mM, addition of more electrolytes resulted in higher degree of reduction of net surface charge. This change was due to salt induced electrostatic screening of droplet charge and eventually formation of aggregates, as clearly observed in Fig. 6D and 6E.

For comparative purposes, the ζ-potentials of aqueous dispersions of 1.0 wt% MCNCs at various NaCl concentrations (0–150 mM) were also determined (Supplementary Fig. S4). The net surface charge of the aqueous dispersion of MCNCs decreased as a function of NaCl concentration from 0 to 100 mM but a further increase in concentration did not lead to any significant changes in the ζ-potential. This trend agreed with the effects of ionic strength on the surface charge of the emulsion E1.00 droplets. This indicates that the behaviour of the MCNCs in the bulk phase at various ionic strengths may also influence the behaviour of the MCNCs at the droplet surface. The addition of electrolytes above the threshold of 20 mM NaCl will screen the charges associated with the cellulose molecules at the

surface. This might result in a reduction in electrostatic repulsion, and van der Waals' interactions might dominate, leading to aggregation (Boluk, Lahiji, Zhao, & McDermott, 2011; Chau et al., 2015; Prathapan, Thapa, Garnier, & Tabor, 2016; Zhong, Fu, Peng, Zhan, & Sun, 2012). Indeed, such aggregation of the MCNCs was evident in the confocal images of emulsion E1.00 at 20 mM NaCl (Fig. 6D). The decrease in the volumetric proportion of the first peak (Fig. 6A) was probably due to the utilisation of unadsorbed MCNCs to form bridges with the MCNCs adsorbed at the droplet surface, resulting in such aggregation. A further increase in the NaCl concentration to 100 mM led to the formation of a gel-like structure (Fig. 6E).

The apparent viscosities of the emulsions at 20 and 100 mM NaCl, hereafter referred to as emulsion E20 and emulsion E100 respectively, were also determined at shear rates from 2 to 1000 s^{-1} (ramp up) and then from 1000 to 2 s^{-1} (ramp down) (Fig. 6B). At both NaCl concentrations, the emulsions showed strong shear-thinning behaviour, with two orders of magnitude reduction in the apparent viscosity as a function of shear rate. This may have been due to reversible shear-induced breakdown of the flocs, supporting the gel-like behaviour observed in the confocal micrographs (Figs. 6D and 6E). The changes in the viscosity of emulsion E20 when the shear rate was ramped up and then down within the range 2–1000 s^{-1} were identical, showing no hysteresis (Fig. 6B). In contrast, the viscosity curve of emulsion E100 (ramp up) should be taken with some cautions as the emulsion E100 showed wall slip, most likely due to the higher stress required to shear this sample (data not shown). Particularly, during the ramp down, the apparent viscosity was higher in the region 10–100 s^{-1} but lower at 2–5 s^{-1}. At a shear rate of 2 s^{-1}, the apparent viscosity of emulsion E100 was about 50% lower at the end of the ramp down than at the start of the ramp up, largely associated with the slip, which leads to apparent decrease in the measured viscosity during ramp up (Franco, Gallegos, & Barn, 1998). Such pronounced slip might be associated with the emulsion E100 forming a gel-like network at low shear rate. A similar phenomenon has been reported in previous studies (Kim, Song, Lee, & Park, 2003; Nandi, Khakhar, & Mehra, 2001; Schokker & Dalgleish, 1998; Whitby et al., 2011). Strong shearing may destroy the gel network (Kim et al., 2003). Although at higher shear rates, 100 to 1000 s^{-1} and the corresponding downward shear rate sweep test overlap, the contribution of slip cannot be neglected. Such flow behaviour of E100 needs further attention in future studies using rheometer geometries with ridged or roughened surfaces that are

designed to mitigate wall slip (Sánchez, Valencia, Franco, & Gallegos, 2001). In our study, emulsion E20 was partially aggregated, i.e. some droplets were still well separated. In addition, emulsion E20 had a relatively high negative charge (–34.6 mV) (Table 3). In contrast, emulsion E100, as shown in Fig. 6E, was completely aggregated, meaning that the mobility of the droplets in the system was very restricted. Furthermore, emulsion E100 had quite a weak negative charge (–18.6 mV), approximately half that of emulsion E1.00 in the absence of NaCl. Therefore, during the shearing, the ion-induced droplet network of emulsion E100 was prone to breakdown, caused by the shear-induced disruption of flocs, but the effect was largely reversible.

To understand the gel-like behaviour of these flocculated Pickering emulsions in the presence of ions, dynamic oscillatory measurements were performed (Fig. 6C). The response of the Pickering emulsions to the applied frequency was determined at a constant strain (1.0%) within the linear viscoelastic region. The dominance of G′ over G″ in Fig. 6C convincingly confirmed the elastic gel-like structure of the emulsion at NaCl concentrations of ≥ 20 mM, which is in agreement with the network-like structure in Fig. 6E. Such observations can be attributed to the flocculation of droplets through the sharing of MCNC particles at the interface, as discussed above. The formation of an elastic network can also be explained by the tight packing of droplets that is associated with the charge screening by ions. As expected, the first peak in the size distribution almost disappeared when the NaCl concentration was ≥ 100 mM (Fig. 6A), which suggested that the unadsorbed MCNCs in the bulk phase associated strongly with the MCNCs at the interface, where the droplets behaved as ‘active fillers’ (Torres, Murray, & Sarkar, 2016). Interestingly, although sensitive to aggregation, the emulsions as a whole were resistant to coalescence. This might be associated with the fact that the rod-shaped MCNCs in the bulk phase had a strong tendency to collectively form a ‘space-filling isotropic gel’ (Oguzlua, Danumah, & Boluk, 2017) in the presence of ions that were somehow entrapping the droplets in a gel-like network, preventing further inter-droplet interactions.

4. Conclusions

This study showed that OSA-modified CNCs have excellent emulsifying capacity because of a significant increase in hydrophobicity, which resulted in partial wettability by an oil phase without the need for an additional surfactant. Pickering O/W emulsions (20 wt% oil) stabilised by 1.0 wt% MCNCs had very small droplet sizes (1.22 μm) relative to those in other studies, and they resisted phase separation for up to 4 weeks of storage under refrigerated conditions. The emulsions were sensitive to aggregation at pH < 4.0 and at ionic strength higher than ≥ 20 mM NaCl. The formation of aggregates under these conditions was associated with a reduction in electrostatic repulsive forces between the droplets. These aggregated emulsions at low pH and high ionic strength responded differently under shearing conditions; in particular, emulsions at higher ionic strength (≥ 20 mM NaCl) had a prominent gel-like character. Nevertheless, the emulsions were strongly resistant to coalescence under all pH and ionic strength conditions investigated, which might be associated with the formation of a thick and dense layer of MCNCs around the oil droplets as well as with the MCNCs in the bulk phase forming strong bridges with the MCNCs at the interface, resulting in a gel-like network that reduced the mobility of the droplets. Such unique responsiveness of MCNCs to elastic gel formation at acidic pH and in the presence of ions might be applied in the design of emulsion systems to target the delivery of bioactive compounds. Gastric destabilisation might be limited because of the gel-like structure formed at low pH, and because of the unresponsiveness of these particle-laden interfaces to proteolytic enzymes in the gastric phase. Future studies will be focused on understanding the fate of MCNC-stabilised droplets with encapsulated short chain fatty acids in an in vitro gastrointestinal model, in which these Pickering emulsion droplets will be subjected to a complex milieu of pH, divalent ions and biosurfactants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodhyd.2019.....

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