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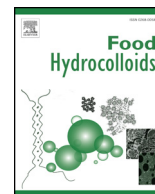David Julian McClements
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Oil-in-water Pickering emulsions *via* microfluidization with cellulose nanocrystals: 1. Formation and stability

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A B S T R A C T

Oil-in-water Pickering emulsions were successfully prepared *via* high-energy microfluidization using cellulose nanocrystals (CNC) as interfacial stabilizers. The influence of microfluidization pressure, CNC concentration, and oil type on droplet size and emulsion stability was determined. Under optimized homogenization conditions, CNC formed and stabilized emulsions based on corn, fish, sunflower, flax, orange, and MCT oils. The droplet size decreased with increasing microfluidization pressure from 9 to 17 kpsi, but then increased slightly at 19 kpsi. The creaming stability of the emulsions increased with CNC concentration, which was mainly attributed to the decrease in droplet size (mean particle diameter $< 1\,\mu m$ at CNC-to-oil ratios greater than 1:10) and slightly increased viscosity. The Pickering emulsions were stable to droplet coalescence, presumably due to strong electrostatic and steric repulsions between the lipid droplets carrying adsorbed nanoparticles. The Pickering emulsions had good stability over a range of environmental stresses: pH 3 to 10; NaCl $\leq 100\,mM$; temperature from 30 to 90 °C. Droplet flocculation was, however, observed under more acidic conditions (pH 2) and at high ionic strength (200–500 mM NaCl), owing to electrostatic screening. Our results indicate that microfluidization is an effective method for forming CNC-stabilized Pickering emulsions suitable for utilization in the food industry.

1. Introduction

Oil-in-water emulsions are colloidal dispersions containing small droplets of an oily disperse phase distributed throughout a watery continuous phase (McClements, 2015). These systems are thermodynamically unstable because of the positive free energy associated with the oil-water interface. Consequently, they tend to breakdown over time to reduce the contact area between the oil and water phases. For this reason, emulsifiers are required that adsorb to the oil droplet surfaces and generate strong repulsive forces to prevent the droplets from coming close together (Jafari, Assadpoor, He, & Bhandari, 2008). Numerous natural and synthetic molecular-based emulsifiers have been identified that are commonly used in the food industry to form and stabilize emulsions, including small-molecule surfactants, phospholipids, and surface-active biopolymers (Bai, Huan, Gu, & McClements, 2016; McClements, Bai, & Chung, 2017).

Recently, there has been growing interests in the utilization of particle-based stabilizers to form and stabilize emulsions (Berton-Carabin & Schroën, 2015; Dickinson, 2012, 2017; Fujisawa, Togawa, & Kuroda, 2017; Linke & Drusch, 2018). However, the discovery that small solid particles could stabilize emulsions *via* Pickering stabilization was discovered over a century ago (Pickering, 1907; Ramsden, 1903). The colloidal particles used in Pickering emulsions require a balanced interfacial wettability that can present affinity for both phases (Wu & Ma, 2016). Droplets coated with particles typically exhibit much greater stability to droplet coalescence than those coated with molecular-based emulsifiers because of their irreversible adsorption and greater steric repulsion (Bai, Greca et al., 2019; Bai, Huan, Xiang, & Rojas, 2018; Dickinson, 2017). This attribute may be beneficial for certain types of commercial applications of emulsions, for instance, in the food, pharmaceutical, petrochemical, or cosmetics industries.

Pickering emulsions can be fabricated from particles made from a wide range of materials, including silica (Binks, Philip, & Rodrigues, 2005), metals (Chen, Colver, & Bon, 2007), and chemically-modified biopolymers (Zoppe, Venditti, & Rojas, 2012). However, in certain commercial applications, particularly the food industry, it is desirable

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to use emulsifiers that are derived from natural and renewable materials when formulating emulsion-based products (Bai, Huan, Li, & McClements, 2017; Bai, Greca, et al., 2019, Bai, Huan, et al., 2019). Consequently, there has been great interest in identifying and characterizing the performance of natural particles that can be used to form and stabilize food-grade Pickering emulsions (Berton-Carabin et al., 2015). These particles must have appropriate physicochemical and structural characteristics, such as size, shape, surface energy, and charge, as well as being safe, economical, stable, and label-friendly. So far, only a few food-grade particles have been reported to be efficacious at forming and stabilizing Pickering emulsions (Bai, Greca, et al., 2019; Berton-Carabin et al., 2015). Particles fabricated from cellulosic materials are one of the most promising forms of Pickering stabilizers due to their sustainability (Serpa et al., 2016), biodegradability (Huan, Bai, Cheng, & Han, 2016; Huan, Liu, Cheng, Han, & Bai, 2018), and non-toxicity (Pitkänen et al., 2014).

In particular, cellulose nanocrystals (CNC), which are produced by acid hydrolysis of cellulose fibers (Habibi, Lucia, & Rojas, 2010), have great potential for application as Pickering stabilizers. Compared to emulsions formed with other types of cellulosic nanomaterials, such as cellulose nanofibrils, which have a larger axial aspect and higher dimensional variability (Costa, Gomes, Tibolla, Menegalli, & Cunha, 2018), the main advantage of CNC in Pickering systems stems from the better control in morphology and dimensions as well as surface properties, which allows more reproducible formation of emulsion droplets (Bai, Greca, et al., 2019; Bai, Huan, et al., 2018; Habibi et al., 2010). Recent studies have explored the fabrication and performance of Pickering emulsions formed from CNC using both non-food (Kalashnikova, Bizot, Cathala, & Capron, 2011a) and food-grade (Bai, Xiang, Huan, & Rojas, 2018; Hu, Marway, Kasem, Pelton, & Cranston, 2016) oils. CNC-stabilized emulsions have also been developed for particular applications in the food industry, such as antimicrobial agents and nutraceutical delivery systems (Mikulcová, Bordes, & Kašpárková, 2016; Sarkar, Li, Cray, & Boxall, 2018). However, one of the limitations of using CNC as a stabilizer is that the smallest oil droplet size achievable in emulsions is rather large (~ 2 μm), irrespective of CNC's aspect ratio (Kalashnikova, Bizot, Bertoncini, Cathala, & Capron, 2013), surface charge density (Kalashnikova et al., 2011a), or oil type (Bai, Huan, et al., 2018). For certain applications, it would be advantageous to have smaller droplets to improve the stability or modulate the functional properties of the emulsion. Another problem is that the homogenization methods normally used to generate small droplets in CNC-based Pickering emulsions, such as sonication, lead to a wide range of droplet size (Abismail, Canselier, Wilhelm, Delmas, & Gourdon, 1999; Wang et al., 2016), again impairing emulsion performance. Capron et al. reported that the limited coalescence during droplet generation was a main reason for the large size observed and for the uneven distribution of CNC-based emulsions that were prepared *via* sonication (Kalashnikova, Bizot, Cathala, & Capron, 2011b). This leads to the fact that the initial state of CNC-stabilized droplets (e.g., initial size upon sonication) is a key factor that determines the performance of the obtained emulsions. Therefore, an approach that can generate small uniform droplets in CNC-stabilized Pickering emulsions would be highly desirable to increase their range of applications.

A variety of high-energy approaches are commercially available to prepare emulsions (Håkansson & Rayner, 2018), but microfluidization appears to be the most efficient at producing small droplets with uniform distributions (Bai & McClements, 2016a). Emulsion formation using a microfluidizer usually involves two separate steps (Jafari, He, & Bhandari, 2007). First, a high-shear blender is used to form a coarse emulsion by mixing oil, emulsifier, and water together. Second, this coarse emulsion is forced through the microfluidizer using pneumatic pressure. After the coarse emulsion enters the microfluidizer, it is split into two separate streams that are made to collide with each other at high velocities (Mahdi Jafari, He, & Bhandari, 2006). This process generates intense disruptive forces, such as cavitation, turbulence and

shear, that efficiently disrupt the large droplets in the coarse emulsion, thereby leading to very fine oil droplets (Håkansson et al., 2011). Previous studies have shown that compared to other homogenization devices, smaller droplets with narrower size distributions can be produced using a microfluidizer (Bai & McClements, 2016a; Perrier-Cornet, Marie, & Gervais, 2005). However, it is also important that sufficient emulsifier is present in the system, and that it rapidly adsorbs to the oil droplet surfaces and stabilizes them against aggregation.

High-energy microfluidization is commonly used as an efficient homogenization technique for producing emulsions from molecular-based emulsifiers (Bai et al., 2016; Bai, Huan, et al., 2017; Mahdi Jafari et al., 2006). However, only few attempts are reported on their use to prepare Pickering emulsions. One recent study used a microfluidizer to produce soybean oil-in-water Pickering emulsions stabilized by nano-fibrillated cellulosic particles (NFC) (Winuprasith et al., 2018). However, the droplets produced in this study were relatively large (> 10 μm) and polydisperse even at high NFC concentrations. Moreover, the emulsions had gel-like characteristics, which would limit their application in some products (Li et al., 2019). These emulsions had to be stabilized against droplet coalescence and creaming by using a combination of interfacial coating (Pickering mechanism) and thickening (aqueous phase network) (Bai, Huan, et al., 2018).

In this study, we investigated the possibility of using CNC to produce low viscosity (liquid-like), oil-in-water Pickering emulsions *via* a high-energy microfluidizer. Compared to other high-energy emulsification systems used to produce CNC-based Pickering emulsions, microfluidization allows control over the emulsion properties upon its generation, which leads to more consistent droplet sizes and uniformity. To the best of our knowledge, this is the first study on the microfluidization of oil and water phases in the presence of CNC to produce Pickering emulsions comprising highly uniform, micron-size droplets. Moreover, we develop for the first-time food-grade, fluid CNC-stabilized Pickering emulsions with tunable droplet sizes. Our findings demonstrate a new pathway for manufacturing food-grade Pickering emulsions with small droplet size that might be suitable for a range of applications in the food and other industries.

2. Materials and methods

2.1. Materials

Cellulose nanocrystals (CNC) were isolated by sulfuric acid hydrolysis of dissolving-grade wood fibers. After dilution and membrane filtration the resulting system consisted of a concentrated suspension of CNC that had typical dimensions of around 5–20 nm in width and around 150–200 nm in length and contained 0.95 wt% sulfur. The CNC was produced as an aqueous suspension (12.1 wt%) at the USDA's Forest Products Laboratory (FPL, Madison, WI) and acquired through the Process Development Center (University of Maine, Maine). Sodium chloride (NaCl), Calcofluor white stain and Nile red were purchased from the Sigma-Aldrich Co. (Helsinki, Finland). Medium chain triglycerides (MCT, Miglyol 812N) were purchased from the Warner Graham Co. (Cockeysville, MD). Fish oil was from DSM Inc. (Columbia, MD). Orange oil (4-fold) was from International Flavors and Fragrances (Union Beach, NJ). Corn oil, sunflower oil and flaxseed oil were purchased from a local supermarket. Double distilled water (Milli-Q) was used throughout the experiments.

2.2. CNC-stabilized Pickering emulsions

CNC-stabilized oil-in-water emulsions were prepared by homogenizing 10 wt% oil phase (corn oil, fish oil, sunflower oil, MCT, flaxseed oil or orange oil) with 90 wt% aqueous phase (containing 0.05 to 2.0 wt% CNC). Aqueous phases were prepared by diluting concentrated CNC suspension with Milli-Q water, and then adding 0.06 wt% NaCl, followed by 2 h vigorous stirring. The addition of NaCl was to partially

screen the surface charge of CNCs, thereby promoting their interfacial packing. The coarse emulsions were prepared by blending the oil and aqueous phases together using a high-shear blender (Bamix, Biospec Products, Bartlesville, OK) for 2 min at ambient temperature. Pickering emulsions were formed by forcing the coarse emulsions through an air-driven microfluidizer equipped with Y- and Z-type disruption chambers (M110P, Newton, MA, USA). Coarse emulsions were fed into the microfluidizer through a 100 mL glass reservoir (pressurized by an intensifier pump), then passed through the instrument three times at different homogenization pressures (9–19 kpsi). With the exception of those used in studies on the impact of microfluidization pressure, the emulsions were prepared at an operating pressure of 13 kpsi. A pressure of 13 kPa was used since it produced relatively small droplets while avoiding the penalty of a high energy consumption, among others. Emulsion storage stability was determined by visual inspection and droplet size analysis after 1- and 14-day storage.

2.3. Emulsion stability

Freshly prepared CNC-stabilized emulsions containing 0.75 wt% CNC and 10 wt% corn oil were subjected to a series of environmental stresses including a range of temperature, pH, and ionic strength conditions. All the samples were stored for 24 h at ambient temperature prior to analysis.

Thermal processing: Emulsions (10 mL) were placed into glass test tubes, incubated in water baths (30–90 °C) for 30 min, and then cooled to ambient temperature.

pH and ionic strength: Emulsions were placed in 20-ml beakers, adjusted to different pH values (2–10) using NaOH and/or HCl solutions or salt levels (100–500 mM) by adding NaCl, and then transferred into glass test tubes (10 mL sample).

2.4. Surface coverage

The percentage surface coverage (*C*) of the CNC-stabilized droplets was determined according to the method described in a previous study (Bai, Xiang, et al., 2018):

$$C = 100 \times \frac{m_p D_{32}}{6h\rho V_{oil}}$$

where m_p is the mass of adsorbed CNC (kg), D_{32} is the mean droplet diameter (m), h is the thickness of the adsorbed CNC layer (m), ρ is the density of CNC (1600 kg/m³), and V_{oil} is the volume of corn oil present in the emulsion (m³). The density of the corn oil was 920 kg/m³ and the thickness of the CNC layer determined by AFM was 7± 2 nm.

2.5. Droplet sizing

The mean droplet diameter and size distribution of Pickering emulsions were measured using static light scattering (Mastersizer 2000; Malvern Instruments, Malvern, UK). Emulsions were diluted with Milli-Q water prior to analysis to avoid multiple scattering effects. The refractive indices (RI) of the aqueous and oil phases used by the instrument to calculate the particle size are listed in Table 1. The mean droplet diameter of each sample was represented as the Sauter mean diameter $(D_{32} = \sum n_i d_i^3 / \sum n_i d_i^2)$ that was calculated from the full droplet size distribution. The D_{32} Sauter diameter was used because it relates to the specific surface area of the oil droplets, which determines the amount of CNC needed to cover the droplet surfaces. To determine the width of the particle size distribution, the span was calculated from the following equation:

$$Span = \frac{D_{90} - D_{10}}{D_{50}}$$

where D_{90}, D_{10}, and D_{50} are particle diameters at 10%, 50%, and 90% cumulative volume, respectively.

Table 1
The RI and density of different oils used in this study.

Oil type	RI[a]	Density (g/cm³)
Water	1.330	–
Corn oil	1.472	0.93
Fish Oil	1.481	0.93
Sunflower oil	1.474	0.92
MCT	1.445	0.95
Flaxseed oil	1.480	0.93
Orange oil	1.472	0.84

[a] The RI of the oils was measured at ambient temperature.

2.6. ζ-Potential

The droplet charge (ζ-potential) of Pickering emulsions was measured using particle micro-electrophoresis (Zetasizer Nano ZS-90, Malvern Instruments, Worcestershire, UK). The samples were diluted either with Milli-Q water or corresponding buffer solutions (same pH as the samples) prior to measurements to avoid multiple scattering effects.

2.7. Shear viscosity

The apparent shear viscosity of CNC-stabilized Pickering emulsions was characterized with a dynamic shear rheometer (MCR 302, Anton Paar, Germany), using parallel plates (PP25) measurement cell with a gap fixed at 0.5 mm. A constant shearing rate (10 s⁻¹) and test time (3 min) were used for all the measurements. All the measurements were performed at ambient temperature (25 °C). This shear rate was selected so that all samples could be compared under similar conditions at a value that is within the range experienced by foods in multiple industrial processes (Bai, Liu, et al., 2017).

2.8. Emulsion morphology

The morphology of the cream layer (when creaming occurred) or the top layer (when no creaming occurred) was examined using confocal laser scanning microscopy (CLSM) with a 63 × oil immersion objective (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, USA). 100 μL of sample was stained with 10 μL of Nile red solution (1 mg/mL ethanol) prior to observation. After homogeneously mixing with a pipette and equilibrating for 10 min at ambient temperature, 6 μL of the dyed sample was placed on a microscope slide and covered with a glass coverslip (Assistent, Sondheim, Germany). The coverslip was quickly fixed by nail polish to avoid evaporation. The excitation and emission spectrum used for Nile red detection were 543 and 605 nm, respectively. Note: the morphology of the observed droplets may be affected by the limited maximum magnification of the confocal microscope used in the current study. Therefore, in order to compare the droplet sizes, an additional characterization technique is necessary, such as those based on light scattering.

The simultaneous observation of CNC and corn oil in the emulsions was achieved using fluorescent microscopy (Zeiss Axio Observer, Zeiss, Germany) with a 100 × oil immersion objective. For sample preparation, 100 μL of emulsion was first stained by adding 10 μL Nile red (for the oil phase) and then by adding 10 μL of Calcofluor white (for the CNCs). 4 μL of prepared sample was used for observation following the CLSM procedure. The excitation and emission wavelengths used for Calcofluor white stain were 365 and 435 nm, respectively. Merged fluorescent images were processed by ImageJ (Schneider, Rasband, & Eliceiri, 2012). Note: since the sample volume in the glass slide was larger than that needed to observe a single droplet layer, droplet overlapping often occurred, obscuring the interpretation (artifact images that appear to occur from flocculation). Therefore, the droplet distribution was also assessed with simultaneous experiments by confocal microscopy.
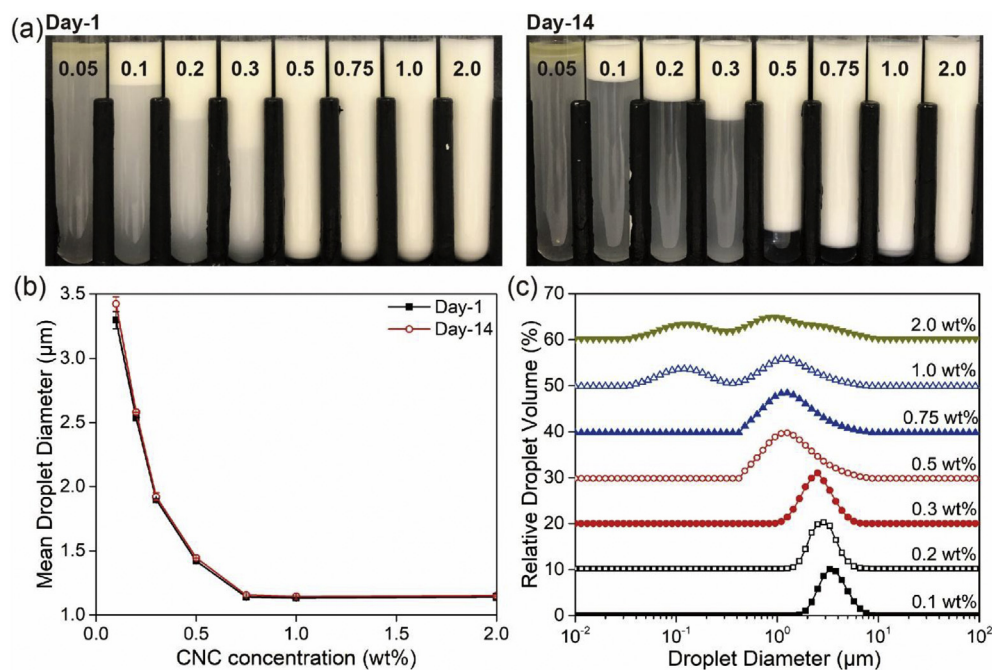
Fig. 1. (a) Visual appearance, (b) mean droplet diameter (D_{32}) and (c) droplet size distributions (Day-1) of oil-in-water Pickering emulsions (10 wt% corn oil) stabilized by CNC at concentrations of 0.05, 0.1, 0.2, 0.3, 0.5, 0.75, 1.0, and 2.0 wt%, as indicated. The visual appearance in (a) and droplet size in (b) were characterized after emulsion storage for 1 and 14 days, respectively. The emulsion containing 0.05 wt % CNC was phase-separated soon after preparation.

2.9. Statistical analysis

All measurements were performed on at least two freshly prepared samples with three measurements per sample and are reported as means and standard deviations. Analysis of variance (ANOVA) was used via the Duncan's new multiple range test (Minitab 16.2.4, Minitab Inc., State College, PA). A p-value of < 0.05 was considered as statistically significant.

3. Results and discussion

3.1. CNC-stabilized Pickering emulsion via microfluidization

3.1.1. Formation and stabilization of Pickering emulsions

Pickering emulsions containing 10 wt% corn oil and different CNC levels were prepared using microfluidization at 13 kpsi (Fig. 1). As shown in Fig. 1a, the Pickering emulsion containing 0.05 wt% CNC exhibited extensive oil separation after storage, which is probably because of insufficient CNC present to cover the oil droplet surfaces that were formed during homogenization. The extent of oiling-off in the emulsions decreased as the CNC level was increased, but creaming was observed in all the samples after 14-days storage (Fig. 1b). However, the thickness of the serum layer at the bottom of the emulsions decreased as CNC concentration was increased from 0.05 to 2.0 wt%, which showed that the emulsions became more stable to creaming at higher CNC levels. The observations can be attributed to two effects: (i) the decrease in droplet size (Fig. 1b) and (ii) the slight increase in aqueous phase viscosity (Fig. 2). Even so, it should be noted that the emulsions were still fluid, even at the highest CNC concentration (insert, Fig. 2).

The dimensions and organization of the oil droplets in the emulsions were assessed by fluorescent microscopy after 1-day storage (Fig. 3). The Nile red images show that the size of individual oil droplets decreased with increasing CNC concentration, which supports the light scattering data. The Calcofluor white images indicate that CNC was mainly present in the same location as the oil droplets, which was confirmed in the merged images (see the blue contour surrounding the oil droplets). This result suggests that most of CNCs were adsorbed to the oil-water interfaces, highlighting the fact that CNC-stabilized Pickering emulsions were produced by high-energy microfluidization.
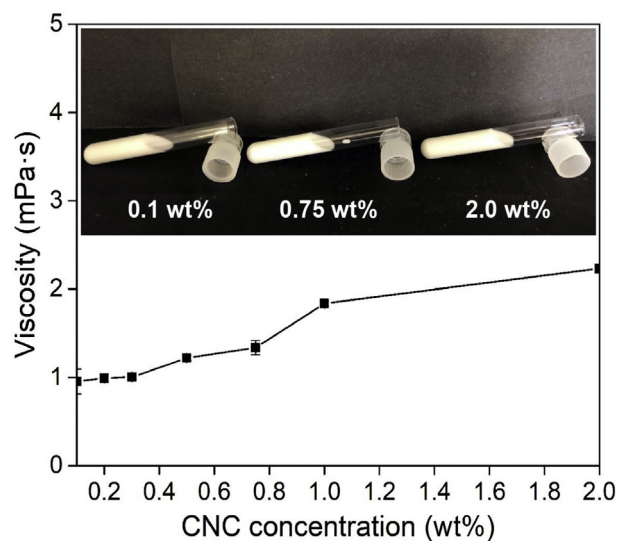

Fig. 2. Apparent shear viscosity of CNC-stabilized Pickering emulsions (10 wt% corn oil) at different CNC concentrations. The shear rate was 10 s^{-1} and measuring time was 3 min. Inserted image displays the visual appearance of the emulsions containing 0.1, 0.75 and 2.0 wt% CNC, respectively. The emulsion containing 0.05 wt% CNC separated during shearing. All the measurements were performed within 3 h after sample preparation.

The mean droplet diameter (D_{32}) decreased with increasing CNC concentration from 0.05 to 0.75 wt% but then reached a plateau value of ~1 μm from 0.75 to 2.0 wt% (Fig. 1b and Table 2). The particle size versus stabilizer concentration relationship observed therefore followed the typical two-regime profile seen for high-pressure homogenization (McClements, 2015). This kind of behavior is because the size of oil droplets produced by homogenization is limited by the amount of stabilizer present at low CNC levels, but by the disruptive energy of the microfluidizer at high CNC loadings. The particle size distribution was monomodal from 0.1 to 0.75 wt% CNC but bimodal at 1.0 and 2.0 wt% CNC (Fig. 1c). Based on the location of the smaller peak in the particle size distribution curves (~200 nm), these particles can be attributed to free CNC dispersed in the aqueous phase. The presence of free CNC was
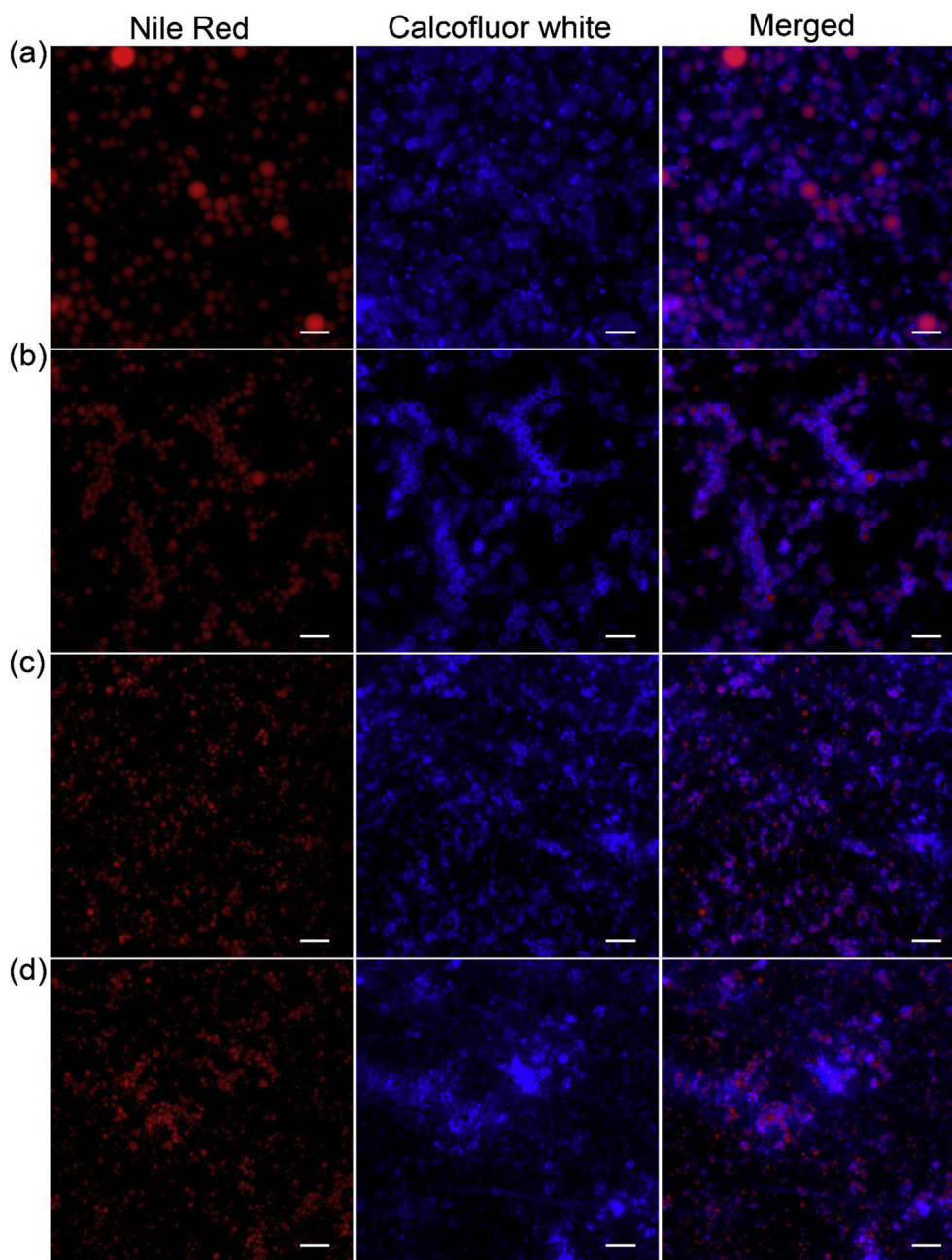
Fig. 3. Fluorescent micrographs of Pickering emulsions (10 wt% corn oil) stabilized with CNC at concentrations of (a) 0.1, (b) 0.3 (c) 0.75, and (d) 1.0 wt%. The left, middle and right rows correspond to the stained oil phase, dyed CNC, and the merged images, respectively. Prior to observation, the oil phase was stained with Nile red, and CNCs were dyed with Calcofluor white. The scale bar is 10 μm. All the samples were stored at ambient temperature for 1 day before observation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Calculated surface coverage (C) of CNC on the oil droplet surfaces.

Concentration (wt%)	D_{32} (μm)	Coverage (C, %)
0.1	3.296	40.6
0.2	2.537	62.5
0.3	1.899	70.2
0.5	1.420	87.5
0.75	1.140	> 100[a]
1.0	1.134	> 100[a]
2.0	1.141	> 100[a]

[a] Free CNCs were observed in the aqueous phase.

also confirmed by the fluorescent microscopy images, which showed the presence of bluish regions in the aqueous phase surrounding the lipid droplets at high CNC loading (Fig. 3c and **d**). Moreover, the calculated surface coverage values for the oil droplets were greater than 100% for CNC > 0.75 wt% (Table 2), which suggests that either multilayers were formed at the droplet surfaces or some of excess CNC was in the aqueous phase.

The mean droplet diameter of CNC-stabilized Pickering emulsions produced by microfluidization in this study (Fig. 1b) were considerably smaller than those produced under similar conditions by using sonication (Bai, Huan, et al., 2018). This result is explained by the higher disruptive energy applied during the microfluidization. It should be noted, however, that compared to the particle-based stabilizers considered here, both microfluidizer and sonicator produce smaller droplet

sizes when using molecular-based emulsifiers, such as small molecule surfactants or proteins. This suggests that the presence of CNC may have interfered with droplet disruption within the homogenizer or that some droplet coalescence occurred before the oil droplets were fully covered with CNC. Since CNC is a rod-like, relatively rigid nanoparticle, it may interfere with the flow profile of the streams generated inside the microfluidizer. Moreover, depending on its concentration, it may also increase the viscosity of the aqueous phase, which would also alter droplet formation and coalescence. Our calculations of the percent surface coverage show that the droplets were relatively stable to coalescence when only about 40% of their surface was covered with CNC (Table 2). This indicates that the relatively thick and charged CNC nanorods are able to generate a strong electrosteric repulsion between the droplets, even when their surfaces were not saturated with such nanoparticles.

After storing for 14 days, the mean droplet diameter of the emulsions was similar to that observed after 1-day storage (Fig. 1b), indicating that no droplet coalescence occurred during storage. It should be noted that prior to analysis, all the emulsions were vortexed to ensure homogeneity during the measurements. The confocal images showed that the oil droplets remained as individual entities and were evenly spread (Fig. 4). The good long-term stability of the CNC-based Pickering emulsions can be attributed to three mechanisms: (1) strong *electrostatic repulsion* due to the high surface potential of the CNC-coated oil droplets, *i.e.* ζ-potential of $\sim$ -55 mV (Fig. 7); (2) strong *steric repulsion* due to the formation of a thick CNC assembly around the droplet surfaces; and (3) the *irreversible adsorption* of the CNCs at the oil droplet surfaces based on the Pickering stabilization.

3.1.2. Influence of oil type on CNC-stabilized Pickering emulsion formation

Emulsion-based foods are usually formulated with different types of oils, depending on the intended use. Therefore, a range of oils, including those derived from corn, fish, sunflower, MCT, flaxseed and orange were selected as references for a wide range of oils that are of commercial relevance. It should be noted that the triglyceride oils (corn oil, fish oil, sunflower oil, MCT and flaxseed oil) are all non-polar, while the orange oil is relatively polar. Stable emulsions could be successfully

produced using corn oil, fish oil, sunflower oil, MCT, flaxseed oil and orange oil at 0.75 wt% CNC using the microfluidizer (Fig. 5). The mean droplet diameters of the emulsions produced from the corn, fish, sunflower and MCT oils were fairly similar, while that of the orange oil emulsion was a little larger (Fig. 5a). Interestingly, the emulsions formulated using flaxseed oil had the smallest mean droplet diameter ($\sim 0.8 \ \mu m$). In general, the size of the droplets in an emulsion may depend on oil type due to differences in their viscosity, polarity, surface tension, and water-solubility (McClements, 2015). In particular, flavor oil (orange oil) is known to be highly susceptible to Ostwald ripening due to its relatively high polarity, which may have led to the larger droplet size observed in this study. It is not clear why flaxseed oil would lead to a lower droplet size than the other oils used. One possibility is that it may have contained some surface-active impurities that rapidly adsorbed to the oil droplet surfaces during homogenization and therefore led to smaller droplets (Liu, Singh, Wayman, Hwang, & Fhaner, 2015).

After storage at ambient temperature for 14 days, the overall appearance of the emulsions fabricated from different oils was fairly similar – there was no evidence of oiling-off but some evidence of creaming (Fig. 5b). There was little or no increase in the mean particle diameter of the emulsions after 14 days storage, with the exception of the orange oil system (Fig. 5a). As mentioned earlier, the instability in the orange oil emulsions was most likely a result of droplet growth due to Ostwald ripening (Wooster, Labbett, Sanguansri, & Andrews, 2016). Orange oil is known to have a much higher water-solubility than medium and long chain triglyceride oils, which makes it most susceptible to Ostwald ripening (Lim et al., 2011). For all the triglyceride oils, the emulsions appeared to have good stability to droplet coalescence. In summary, our results show that CNC is capable of forming stable Pickering emulsions from a wide variety of oils that typically used in food formulations. We note that Ostwald ripening could be minimized by mixing the orange oil with a water-insoluble oil, such as a triglyceride oil, before homogenization (Lim et al., 2011).
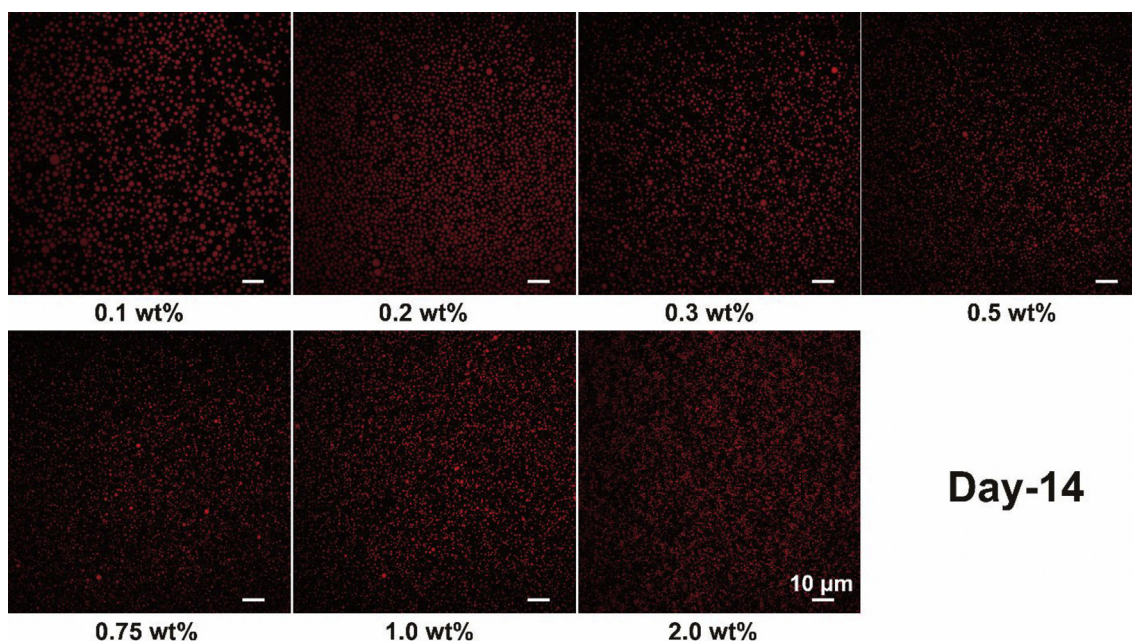

Fig. 4. CLSM images of CNC-stabilized Pickering emulsions with different CNC concentrations after 14-day storage. The emulsions contained 10 wt% corn oil and CNC concentration of 0.1, 0.2, 0.3, 0.5, 0.75, 1.0, or 2.0 wt%, as indicated. The oil phase was stained with Nile red prior to observation. The emulsion containing 0.05 wt% CNC was phase-separated soon after preparation. The scale bar is 10 μm. All the samples were stored at ambient temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
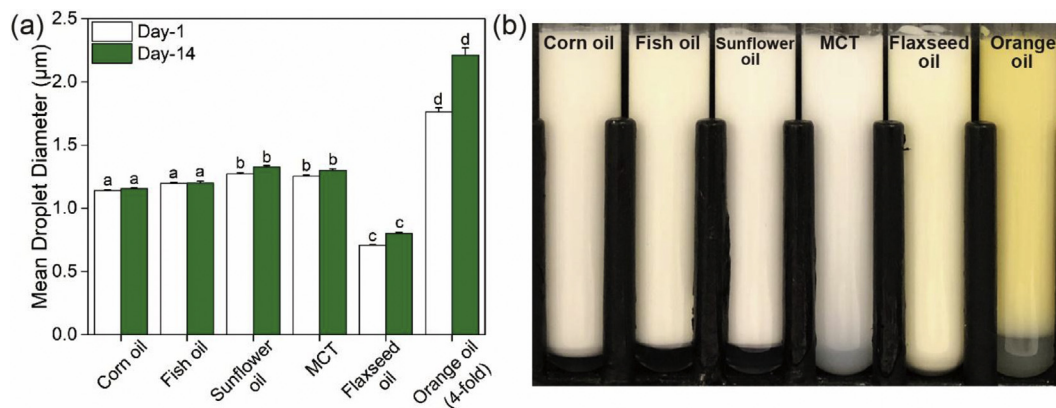
Fig. 5. (a) Mean particle diameter (D_{32}) and (b) 14-day storage stability of CNC-stabilized oil-in-water Pickering emulsions with different oil types. Emulsions contained 0.75 wt% CNC and 10 wt% oil. All the emulsions were stored at ambient temperature. The different lowercase letters used (a to d) indicate significant differences (p < 0.05) in the values of D_{32} at a given oil concentration (i.e., the effect of oil type).

3.2. Impact of microfluidization pressure

Previous studies have shown that the size of oil droplets produced using a microfluidizer depends on the operating pressure used (Bai et al., 2016), which is because of the respective changes in the disruptive forces generated (Schultz, Wagner, Urban, & Ulrich, 2004). This effect suggests that it may be possible to tune the size of the droplets produced in CNC-stabilized Pickering emulsions by varying the operating pressure of the microfluidizer.

Fig. 6 shows the influence of various microfluidization pressures on the mean droplet diameter produced. Initially, there was a gradual decrease in droplet size as the operating pressure was increased from 9 to 17 kpsi (Fig. 6a), with a minimum mean droplet diameter of ~0.92 μm being achieved. Moreover, the droplet size distributions of the emulsions were monomodal at all pressures tested, even at the lowest (9 kpsi) (Fig. 6b). The polydispersity (span) of the emulsions also decreased from 2.8 to 1.6 with the increased microfluidizer pressure. This indicates that all of the oil droplets had passed through the disruptive zone inside the homogenizer. The decrease in droplet size with increasing operating pressure can be attributed to the increase in the magnitude of the disruptive energy generated inside the microfluidizer, which leads to more efficient droplet disruption. The slope of a plot of logarithm (mean droplet diameter) *versus* logarithm (pressure) was found to be −0.87, which was slightly outside the range reported for emulsions produced using high-pressure homogenizers and molecular-based emulsifiers (−0.6 to −0.8) (Walstra, 2002). This result may occur for two reasons: (i) droplet breakup in CNC-based systems was not primarily governed by turbulent-inertial forces in the microfluidizer; and (ii) the CNC retarded droplet breakup. Surprisingly, the

droplet size increased when the operating pressure was raised from 17 to 19 kpsi, which may be attributed to over-processing at high pressures (Jafari et al., 2007). This effect could have occurred because there was insufficient CNC present to cover all the very small oil droplets formed at the highest pressure or because the CNC itself was damaged at higher pressures. However, given the concurrent and complex physicochemical phenomena that take place during microfluidization, including droplet disruption and coalescence, CNC adsorption, disruption, and interference with fluid flow, among others, further studies are required for a better elucidation of the mechanisms involved. In summary, and most remarkably, this is the first report to show the possibility of producing CNC-stabilized Pickering emulsions containing submicron droplets with narrow particle size distributions.

3.3. Effect of environmental stresses on Pickering emulsion stability

Practically, food emulsions are exposed to a range of environments during their preparation, storage, transportation, and utilization. To be commercially successful, it is important that emulsions are robust enough to resist alterations in their properties when exposed to these changing conditions. It is therefore important to explore the influence of different environments on the stability and properties of CNC-stabilized Pickering emulsions (Fig. 7).

3.3.1. Thermal processing

For commercial applications, it would be advantageous to test the stability of the emulsions under thermal processing profiles that are used in given products, e.g., cooking, pasteurization, or sterilization conditions. However, it should be noted that the temperatures used in
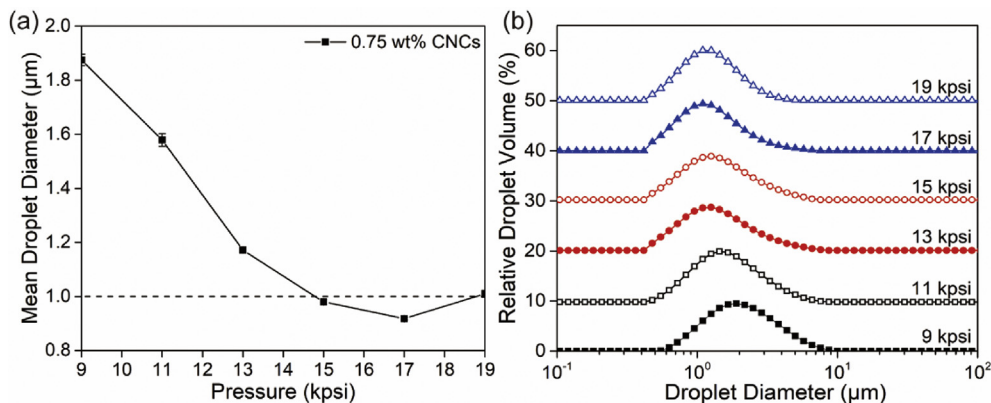

Fig. 6. (a) Mean droplet diameter (D_{32}) and (b) droplet size distributions of CNC-stabilized oil-in-water Pickering emulsions produced with varying microfluidization pressures (9–19 kpsi). Emulsions contained 10 wt% corn oil and 0.75 wt% CNCs. The dash line in (a) represents the droplet diameter of 1 μm.
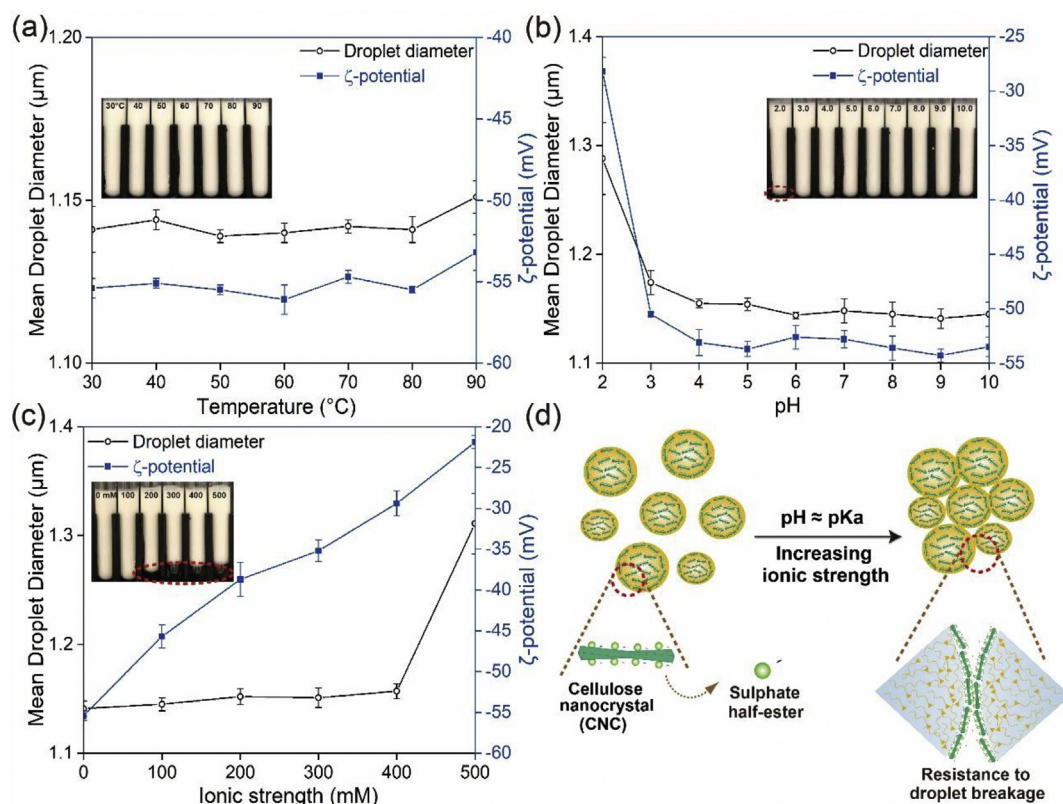
Fig. 7. (a) Thermal processing (30–90 °C, 30 min), (b) pH value (2–10), and (c) ionic strength (0–500 mM NaCl) on the mean particle diameter (D_{32}) and ζ-potential of CNC-stabilized Pickering emulsions. The emulsion contained 0.75 wt% CNC and 10 wt% corn oil. All the samples were stored at ambient temperature for 24 h before analysis. Inserted photographs in (a), (b) and (c) were taken after 24 h storage. (d) Schematic showing for the high stability of Pickering droplets at different environment stresses enabled from electrostatic repulsion and/or steric hindrance of CNCs.

this study (30–90 °C) are common in several commercial applications (Bai & McClements, 2016b). Fig. 7a shows the influence of thermal processing on the stability of Pickering emulsions, and corresponding ANOVA data are listed in Table S1. Across the range of temperatures studied, there was little change in the mean droplet diameter ($\sim$ 1.14 μm) of the emulsions. Moreover, no creaming or oiling-off was observed from visual appearance of the emulsions (insert, Fig. 7a). Taken together, these results suggest that Pickering emulsions were resistant to thermal processing, which may be because temperature did not have a major influence on the strong steric or electrostatic repulsion between the droplets. Indeed, the CNC-coated lipid droplets maintained a high surface potential (-55 mV) after thermal treatment (Fig. 7a).

3.3.2. pH

Fig. 7b shows the influence of the pH value on the stability of

Pickering emulsions, and corresponding ANOVA is summarized in Table S2. The emulsions contained relatively small oil droplets that were visibly stable to creaming and oiling-off from pH 3 to 10. Moreover, the confocal images confirmed that the oil droplets were stable to flocculation and coalescence over this pH range (Fig. 8). This effect can be attributed to the fact that the ζ-potential of the oil droplets remained relatively high and negative across this pH range (Fig. 7b). As a result, the droplets were stabilized against aggregation by a combination of strong electrostatic and steric repulsions.

At pH 2, however, the droplet size increased slightly and creaming was observed (Fig. 7b), which can be attributed to a limited amount of droplet flocculation rather than coalescence (Fig. 8). This effect can be due to changes in the electrostatic interactions in the system (McClements, 2015). As shown in Fig. 7b, ζ-potential of the emulsions became less negative (-25 mV) at pH 2, which may be ascribed to
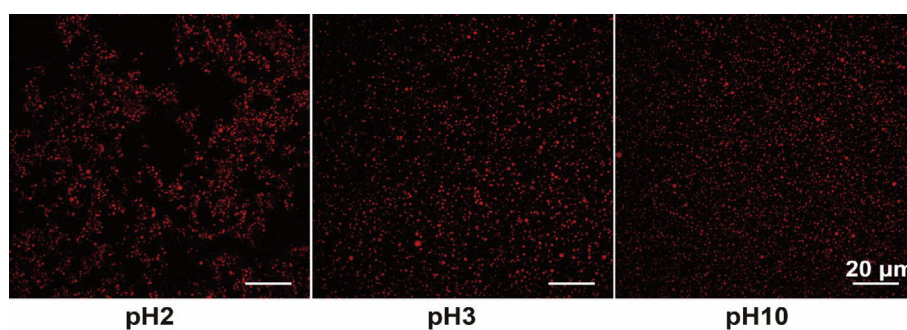

Fig. 8. CLSM images of CNC-stabilized Pickering emulsions at pH value of 2 (left), 3 (middle), and 10 (right). The emulsions contained 0.75 wt% CNC and 10 wt% corn oil. The oil phase was stained with Nile red prior to observation. The scale bar is 20 μm. All the samples were stored for 24 h at ambient temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 9. CLSM images of CNC-stabilized Pickering emulsions at ionic strength of 0, 100, 200, 300, 400 and 500 mM NaCl. The emulsions contained 0.75 wt% CNC and 10 wt% corn oil. The oil phase was stained with Nile red prior to observation. The scale bar is 20 μm. All the samples were stored for 24 h at ambient temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
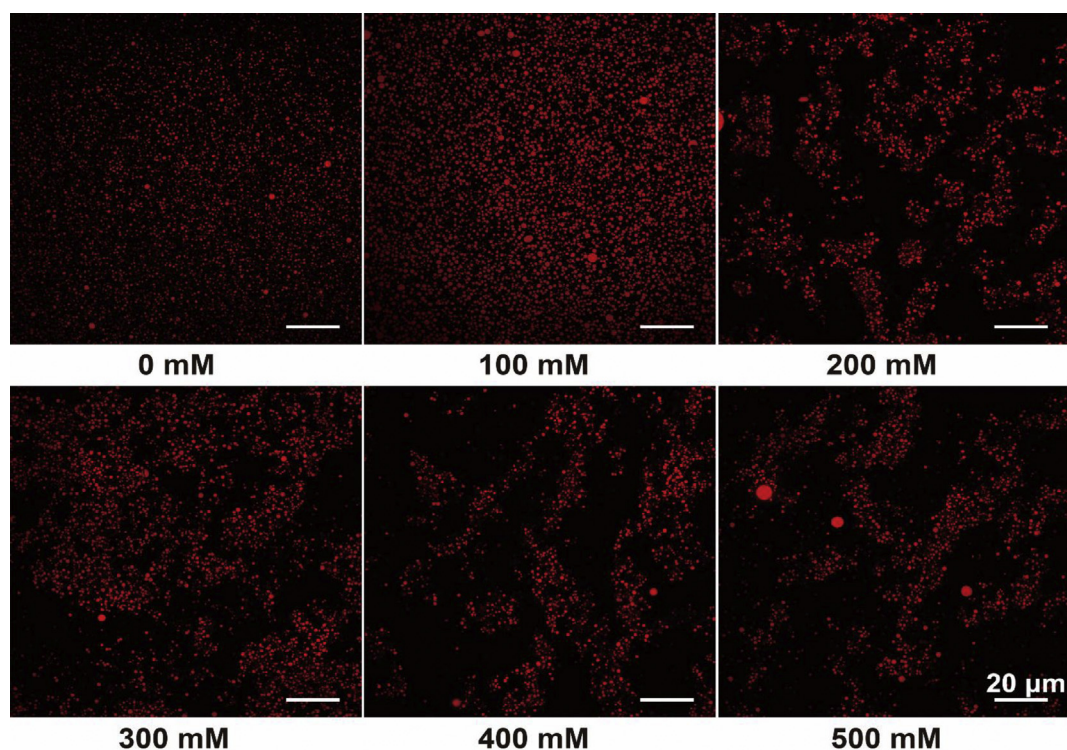
partial protonation of covalently bound sulfate ester groups (pK_a = 1.9) (Varanasi et al., 2018). As a result, there was a weaker electrostatic repulsion between the oil droplets. Since the range of the steric repulsion generated by the adsorbed CNC layer was not long enough compared to the range of the van der Waals attraction, droplet flocculation occurred. Coalescence, however, did not occur because there was still a strong steric repulsion at close range. A possible mechanism for this effect is shown schematically in Fig. 7d.

3.3.3. Ionic strength

The influence of ionic strength on the stability of the CNC-coated oil droplets was assessed by incubating them at different NaCl levels (Fig. 7c), and corresponding ANOVA analysis is included in Table S3. Visible observation of the emulsions indicated that creaming occurred at ≥ 200 mM NaCl but no oiling-off occurred at any salt level. Fig. 7c also shows that the mean particle diameter remained relatively low from 0 to 400 mM NaCl, but increased slightly at 500 mM NaCl. These results imply that the instability of the emulsions at elevated ionic strength was primarily due to weak droplet flocculation rather than coalescence. This observation was confirmed by the microscopy images (Fig. 9), which showed that increasing droplet flocculation occurred as the NaCl concentration was raised. Presumably, the large flocs were not seen in the light scattering measurement for the emulsions with high ionic strengths because they were diluted appreciably in Milli-Q water prior to analysis.

The tendency for droplet flocculation to occur in CNC-stabilized emulsions at high ionic strength can be attributed to electrostatic screening, *i.e.,* the tendency for counter-ions (in this case Na^+) to cluster around the surfaces of the anionic oil droplets. This effect was confirmed by the ζ-potential measurement (Fig. 7c), which showed a steep decrease in the magnitude of the surface potential with increasing salt concentration (from −55 to −20 mV). At low NaCl concentrations (0 and 100 mM), the electrostatic repulsion was sufficiently intense to overcome the van der Waals attraction, thereby preventing the

aggregation of the oil droplets. At higher NaCl levels (200–500 mV), the repulsive interactions were no longer stronger than the attractive interactions, leading to aggregation of the droplets. Again, no coalescence was observed after the oil droplets had flocculated because of the strong short-range steric repulsion generated by the adsorbed CNCs (Fig. 7d).

4. Conclusions

There is considerable interest in replacing synthetic particle stabilizers with natural ones suitable for food applications. However, currently information is lacking with regards to the ability of high-energy homogenization methods to produce this kind of Pickering emulsion. To our knowledge, this is the first detailed study of the preparation and performance of CNC-stabilized Pickering emulsions using microfluidization. We were able to fabricate food-grade Pickering emulsions that were fluid and had submicron droplet sizes from a wide variety of oil types. These emulsions were highly stable to coalescence but did exhibit flocculation and creaming under certain circumstances. The CNC-coated droplets presented good flocculation stability over a range of environmental conditions: 30–90 °C; 0–100 mM NaCl; pH 3 to 10. However, they did tend to flocculate and cream under strongly acidic conditions (pH 2) and at high ionic strength (200–500 mM NaCl), which is attributed to a reduction in the electrostatic repulsion between the droplets. This might limit their application in certain types of food products. Overall, our findings open a new avenue for the design and manufacture of natural particle-stabilized Pickering emulsions using microfluidization that have potential for application in food products.

The formation of a layer of indigestible dietary fiber around the lipid droplets may influence their behavior within the human gut. For instance, it may slow down lipid digestion, which may be useful for the development of functional foods that prevent lipid spikes in the bloodstream or that induce satiety. Conversely, an indigestible coating may inhibit the release of any encapsulated bioactive agents, such as oil-soluble vitamins, ω-3 oils, or nutraceuticals. For this reason, in a

companion study, we have investigated the impact of the CNC-coatings on the gastrointestinal fate of Pickering emulsions using a simulated digestion model.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2019.04.038.

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