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Heteroaggregation effects on Pickering stabilization using oppositely charged cellulose nanocrystal and nanochitin

Published in: Carbohydrate Polymers

DOI: 10.1016/j.carbpol.2022.120154

Published: 01/01/2023

Document Version Publisher's PDF, also known as Version of record

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Please cite the original version:

Guo, S., Zhu, Y., Xu, W., Huan, S., Li, J., Song, T., Bai, L., & Rojas, O. J. (2023). Heteroaggregation effects on Pickering stabilization using oppositely charged cellulose nanocrystal and nanochitin. *Carbohydrate Polymers*, 299, Article 120154. https://doi.org/10.1016/j.carbpol.2022.120154

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Contents lists available at ScienceDirect

# Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol



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ARTICLE INFO

Keywords: Cellulose nanocrystals Nanochitin Pickering emulsions Heteroaggregation

## ABSTRACT

Pickering emulsions are stabilized using complexes of cellulose nanocrystals (CNC) and nanochitin (NCh). The colloidal behavior and heteroaggregation in aqueous media are studied in relation to complex formation and net charge. The complexes are remarkably effective in stabilizing oil-in-water Pickering emulsions under conditions of slightly net positive or negative charges, as determined by the CNC/NCh mass ratio. Close to charge neutrality (CNC/NCh ~5), large heteroaggregates form, resulting in unstable emulsions. By contrast, under net cationic conditions, interfacial arrest of the complexes leads to non-deformable emulsion droplets with high stability (no creaming for 9 months). At given CNC/NCh concentrations, emulsions with up to 50% oil fraction are produced. This study shows how to control emulsion properties beyond consideration of the typical formulation variables, for instance, through adjusting CNC/NCh ratio or charge stoichiometry. We highlight the possibilities that are available for emulsion stabilization by using a combination of polysaccharide nanoparticles.

## 1. Introduction

An emulsion is a thermodynamically unstable mixture of two immiscible liquids, in which one phase is dispersed in the other one (Bai, Huan, Rojas, & McClements, 2021; McClements & Jafari, 2018). When used in formulating emulsions, different from molecular-based surfactants, colloidal particles can irreversibly adsorb at the liquid/liquid interfaces during homogenization, resulting in the so-called Pickering stabilization, following the observations by Ramsden and Pickering (Pickering, 1907; Ramsden, 1903).

The demands for sustainable colloidal materials (renewable, biocompatible, and biodegradable polymeric structures) are growing in the areas relevant to cosmetics, pharmaceutical, and food industries. Therein, cellulose is a promising candidate given its wide availability, which includes sourcing from agricultural waste and other biomass (Li et al., 2021; Zhu et al., 2021). Cellulose presents a nanoscale

hierarchical structure with long-range order (Ajdary, Tardy, Mattos, Bai, & Rojas, 2021), which can be disassembled into fibrils that are ultimately composed of ordered linear β-D-glucopyranose units linked together by β-1,4-linkages (Habibi, Lucia, & Rojas, 2010; Zhang, Chen, et al., 2020). Among various types of cellulosic particles, cellulose nanocrystals (CNC) are obtained via a top-down approach by controlled acid hydrolysis of the cellulose source (Thomas et al., 2018). CNC can adsorb efficiently at the oil/water interface given its anisotropic and rod-like morphology, amphiphilicity, and surface charge (Bai et al., 2020; Capron, Rojas, & Bordes, 2017; Varanasi et al., 2018). Unmodified, highly-charged CNC has been extensively used in stabilizing Pickering emulsions with different preparation methods, such as sonication (Kalashnikova, Bizot, Cathala, & Capron, 2011) and microfluidization (Bai, Lv, et al., 2019; Chen, Liu, & Tang, 2019). Moreover, it has been demonstrated that a less charged CNC can be better adsorbed at the interfaces and stabilize oil droplets of small size (Torlopov et al., 2021;

https://doi.org/10.1016/j.carbpol.2022.120154

Received 21 July 2022; Received in revised form 6 September 2022; Accepted 21 September 2022 Available online 27 September 2022

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Wen, Yuan, Liang, & Vriesekoop, 2014). However, owing to the fact that the smallest droplet size reported is in the micron-range, irrespective of the surface charge density of CNC (Bai, Huan, et al., 2018), the main issue for CNC-stabilized Pickering emulsion applications is the lack of stability, via gravitational separation, especially in long-term storage. Hence, surface modification or hydrophilization has been used to improve CNC affinity with the oil/water interfaces, expanding CNC adoption in Pickering stabilization (Liu et al., 2017; Tang et al., 2018; Zhang, Karimkhani, Makowski, Samaranayake, & Rowan, 2017). Unfortunately, most processes used for these purposes reduce the sustainable prospects of CNC. Therefore, a significant challenge that needs to be addressed is to endow CNC with a high interfacial affinity while retaining its green nature.

There has been a growing interest in the utilization of structural design to improve functional attributes of Pickering emulsions, for instance, by combining particles (Cerbelaud, Aimable, & Videcoq, 2018; Liu et al., 2021; Tao et al., 2020). In this context, heteroaggregation is a phenomenon whereby colloidal particles of different types come together to form aggregates (Islam, Chowdhry, & Snowden, 1995). The particles may differ in a variety of ways, for example, composition and shape, size, surface potential and charge (Binks, Liu, & Rodrigues, 2008). The aggregated particles can be held together by strong attractive electrostatic interactions between oppositely charged particles, for instance, between CNC and cationic surfactants or soy proteins (Bai, Xiang, et al., 2018; Zhang, Luo, et al., 2020). Considering label-friendly products, one natural polymer that can be used in combination with cellulose is chitin, whose repeating unit is  $\beta$ -(1,4)-N-acetyl-D-glucosamine (Duan, Huang, Lu, & Zhang, 2018; Liu et al., 2019). In living organisms, chitin nanofibrils are tightly bonded through hydrogen bonding, van der Waals forces and other interactions. Similar to the case of CNC, rod-like nanochitin (NCh) can be easily disassembled under acidic conditions by mechanical processing (Bai et al., 2022; Fan, Saito, & Isogai, 2008). Besides to the increased surface hydrophobicity of the system when CNC electrostatically interacts with NCh, the advantage of using NCh to modify CNC is the possibility to maximize the stabilizing capability of the complexes. For instance, rod-shaped particles display superior interfacial stabilization compared to other morphologies (e.g., spherical particles) (Bai et al., 2022).

Several nanopolysaccharides have been considered for their assembly at the interfaces in Pickering emulsions, driven by electrostatic interactions, for instance, cellulose nanofibrils and nanochitin (Huan et al., 2021; Lv, Zhou, Bai, Rojas, & McClements, 2021). However, related studies focus on the complexation of NCh with long, flexible, less-charged cellulose nanofibrils (CNF) rather than the short, rigid, highly-charged CNC. Notably, the complexation mechanism and the role as a Pickering stabilizer are expected to be quite different. Herein, we consider the interactions between CNC and NCh to stabilize emulsions by tailoring their interfacial adsorption using a given CNC concentration and varying NCh loading. We investigate the stabilizing mechanisms underlying Pickering stabilization with a dual system and rational design of emulsion-based products with improved physicochemical and functional properties. The results of this work are relevant to the design and construction of sustainable, green Pickering emulsions using widely available natural resources, allowing for desired applications in the area of food, cosmetics, and pharmaceuticals.

#### 2. Materials and methods

## 2.1. Materials

Ashless cotton fiber paper (Whatman 1) was used as raw materials to produce CNC according to a previous method (Bai et al., 2018a). Briefly, the cotton fiber paper was hydrolyzed by 64 wt% sulfuric acid for 45 min at 45 °C, and then the precipitate was rinsed, centrifuged and dialyzed until the effluent conductivity was lower than 5  $\mu$ S·cm<sup>-1</sup>. The prepared CNC aqueous suspension (Fig. S1a) was stored at 4 °C after filtration and

sonication (Sonifier 450, Branson Ultrasonics Co., Danbury, CT, USA). During sulfuric acid hydrolysis, sulfate half esters are introduced on the surface of CNC, providing negative surface charges to enable colloidal stabilization in water. Sunflower oil was purchased from a local supermarket and used without further purification. Calcofluor white stain, Nile red, 100% acetic acid, HCl, NaClO<sub>2</sub>, NaOH, styrene (St), and azobis (isobutyronitrile) (AIBN) were purchased from Sigma-Aldrich (Helsinki, Finland). Milli-Q water was used throughout the experiments from a Millipore Synergy UV unit (MQ, 18.2 MΩ·cm).

## 2.2. Nanochitin

Fresh crabs (*Callinectes sapidus*) were purchased from a local market in Helsinki harbor. The purification steps on isolating  $\alpha$ -chitin present in crab shells follows our previous work (Bai, Huan, et al., 2019). Briefly, the crab shells were immersed successively in 1 M HCl and 1 M NaOH for 24 h, following at least three cycles. The obtained solids were decolorized by soaking in 0.5 wt% NaClO<sub>2</sub> solution (pH 5.0, acetic acid) for 2 h at 70 °C. Purified chitin was obtained after thoroughly washing with Milli-Q water in the form of chitin flakes that were crushed by household blender and stored at 4 °C for further use.

Chitin nanofibers, herein termed as nanochitin (NCh), were obtained from mechanical disintegration, as reported previously (Bai, Huan, et al., 2019). Briefly, a given amount of purified chitin was deacetylated with 33 wt% NaOH solution at 90 °C for 3.5 h. Then, partially deacetylated chitin was washed with water until the pH was constant, following an overnight dialysis to further remove impurities. The deacetylation degree of chitin was about 27%, similar to our early report (Huan et al., 2021). The partially deacetylated chitin was re-dispersed in water (0.3 wt%), following pH adjustment using acetic acid to 3 under continuous stirring. The obtained suspension was passed through a microfluidizer (M-110P, Microfluidics In., Newton, MA, USA) with 400 and 200  $\mu$ m chambers at a pressure of 1500 bar after prehomogenization (T-25, Digital Homogenizer, IKA, Germany) at 10000 rpm for 5 min. The obtained NCh suspension (Fig. S1b) was stored at 4 °C prior to use.

## 2.3. CNC/NCh complexes

The CNC stock suspension was diluted to 0.5 wt% and NCh suspensions were prepared at various concentrations (0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 wt%). Briefly, the complexes were prepared by mixing 0.5 wt% CNC dispersion with NCh at different concentrations at a mass ratio of 1:1 for a total of 10 g. Vortexing and bath sonication (DT 52/H, Sonorex Digitec, Germany) were used sequentially to ensure full mixing. The suspensions composed of CNC/NCh complexes and produced under given conditions are listed in Table 1, with CNC concentration in the suspension being kept at 0.25 wt%. Therein, CNC/NCh complexes are named as "CNx", wherein x represents the

# Table 1

Composition and zeta ( $\zeta$ ) potential of CNC/NCh aqueous suspensions and emulsions.

Sample	C <sub>f-CNC</sub> <sup>a</sup> (wt%)	C <sub>f-NCh</sub> a (wt%)	Mass ratio (CNC/NCh)	ζ <sub>suspension</sub> (mV)	ζ <sub>emulsion</sub> (mV)
CN1	0.25	0	-	$-59\pm7.8$	$-62\pm1.7$
CN2	0.25	0.0005	500 (500/1)	$-48 \pm 1.6$	$-64 \pm 1.5$
CN3	0.25	0.0025	100 (100/1)	$-41 \pm 1.2$	$-59\pm0.5$
CN4	0.25	0.005	50 (50/1)	$-36\pm1.2$	$-54 \pm 2.8$
CN5	0.25	0.025	10 (10/1)	$-36\pm0.6$	$-48 \pm 1.8$
CN6	0.25	0.05	5 (5/1)	$-1.8\pm0.2$	$26 \pm 1.9$
CN7	0.25	0.1	2.5 (5/2)	$82\pm13$	$59\pm0.2$
CN8	0.25	0.15	1.66 (5/3)	$92\pm17$	$62\pm0.3$
CN9	0.25	0.2	1.25 (5/4)	$92\pm1.3$	$65 \pm 5.0$
CN10	0.25	0.25	1 (1/1)	$86 \pm 4.2$	$61\pm0.4$

 $^{\rm a}$  C<sub>f-CNC</sub> and C<sub>f-NCh</sub> represent the concentration of respective nanomaterial in the final suspension.

respective composition. A reference sample was obtained by diluting 0.5 wt% CNC suspension to 0.25 wt% (CN1, Table 1). Digital photos of the systems were taken after storage for at least 12 h.

## 2.4. Characterization of CNC/NCh complexes

#### 2.4.1. $\zeta$ -potential

The electrophoretic properties of neat CNC and NCh, as well as CNC/ NCh complexes, were determined using a ZetaSizer unit (ZS-90, Malvern Instruments, UK). Each sample was diluted using Milli-Q water prior to measurement. Freshly prepared samples were used for  $\zeta$ -potential tests in triplicate.

# 2.4.2. Turbidity

The turbidity of CNC/NCh suspensions at room temperature was determined using UV-vis spectrophotometer (UV-2600, SHIMADZU, Japan) at a wavelength of 600 nm. An optical cell with 1 cm path length was used to place the suspensions. The turbidity was calculated according to below equation (Huan et al., 2021):

Turbidity =  $2.3 \times log \frac{1}{T}$ 

where T stands for the transmittance of the suspension. The results were averaged by measuring three freshly prepared samples.

# 2.4.3. Morphology

The microstructure of CNC, NCh, and CNC/NCh complexes was characterized using atomic force microscope (AFM, Bruker Inc., Germany). Briefly,  $20 \ \mu$ L of the diluted sample was spin-coated on freshly cleaved mica plate at 2000 rpm for 2 min using spin coater (Laurell Technologies Corporation, North Wales, USA). High-resolution AFM images were obtained in air using tapping mode. Research NanoScope 8.15 software (Bruker) was applied for image analysis, processing, and correction. The width of CNC and NCh was analyzed using ImageJ software (1.53e, Java 1.8.0\_172) by counting at least 100 particles from different AFM images (Schneider, Rasband, & Eliceiri, 2012).

To acquire images of highly aggregated complexes (CN6), a transmission electron microscope (TEM, H7600, Hitachi, Japan) was used with an acceleration voltage of 100 kV. 5  $\mu$ L of the suspension (0.2 wt%) was deposited on a copper grid for 30 s, and extra amount of water was removed with filter paper. Negatively stain was proceeded with 2% uranyl acetate solution (5  $\mu$ L) for 30 s, following removal of excess liquid by blotting. The stained sample was dried at room temperature.

## 2.5. CNC/NCh-stabilized Pickering emulsions

Pickering emulsions were prepared using sunflower oil as the oil phase (10 to 50 wt%) and given CNC/NCh suspensions (CN1-CN10) as the water phase. Briefly, a certain amount of sunflower oil was added to CNC/NCh suspension in a plastic tube followed by vortexing for 30 s. Afterwards, the pre-emulsified emulsion was sonicated (microtip sonicator, Sonifier 450, Branson Ultrasonics, Danbury, CT) by placing the tip close to the top surface of the sample. The sonication was applied by alternating 3 s–2 s on-off cycles for total 60 s at 40% input power (the maximum power of the sonicator was 550 W). The tube was placed in ice-water bath to minimize overheating throughout sonication. After preparation, visual appearance of emulsions was captured within 24 h. The long-term stability was recorded by taking photos during storage at room temperature.

To access the surface morphology and identify the heteroaggregates adsorbed at the droplet surface, CNC/NCh-stabilized styrene (St)-inwater Pickering emulsions were prepared, wherein St droplets were polymerized to polystyrene (PS) beads via radical polymerization. Briefly, the CNC/NCh complexes were used as the aqueous phase and St (mixed with AIBN, oil-soluble initiator, at AIBN-to-St ratio of 1/100 w/ w) was used as the oil phase. The oil-to-water ratio was set as 10/90, wherein the mixture was emulsified following previously introduced procedure. The polymerization of obtained emulsion was initiated at 65 °C after degassing with nitrogen for 10 min to remove air trapped in the system. The polymerization of St into PS was performed for 24 h at 65 °C without stirring. The insight on adsorption of complexes at the interfaces were assessed using scanning electron microscopy (SEM, Zeiss Sigma VP, German), even if assessed with solid beads that originated from a different organic phase, given limitations to directly obtain the same information in the fluid system (Guo et al., 2021). Briefly, a drop of diluted emulsion (100-fold) was deposited onto the sample holder, following overnight drying at room temperature. The dried PS beads were sputter-coated with platinum/palladium prior to observation, and observed under an accelerated voltage of 1-2 kV.

## 2.6. Characterization of Pickering emulsions

## 2.6.1. Droplet size

The particle size of emulsion was characterized by static light scattering (Mastersizer 2000, Malvern Instruments, U.K.) equipped with a He-Ne laser. 500 mL Milli-Q water was used to dilute samples to avoid effects of multiple scattering. To facilitate comparison between multiple histograms shown in the figures, the distribution profiles were shifted vertically in the size plot, and the relative volume was defined to clearly show the height of the peak. The refractive index (RI) of aqueous and oil phases were set to be 1.33 and 1.47, respectively. The mean droplet size was calculated as the Sauter mean diameter ( $D_{32} = \Sigma n_i d_i^3 / \Sigma n_i d_i^2$ ) using the full-size distribution range. The measurements were conducted in triplicate.

## 2.6.2. Microscopy

The microstructure of emulsion samples was examined by a confocal laser scanning microscope (CLSM, Leica DMRXE, Leica, Germany). The top layer of emulsion was collected and dyed using Nile red (emulsion-to-Nile red ratio of 10/1, v/v) prior to observation. After homogeneously mixing, the dyed sample was equilibrated for at least 10 min at room temperature. 7 µL of the stained sample was dropped on a microscope slide followed by a coverslip immediately with no extra load. Nail polish was used to fix the coverslip as soon as the emulsion was fully spread. The excitation/emission spectra for Nile red used in this study were 488/ 539 nm, respectively.

Florescent optical microscope (Olympus BX53, Japan) was used to image CNC/NCh complexes and sunflower oil simultaneously. Emulsification was done using sunflower oil dyed with Nile red and CNC/NCh complexes stained with Calcofluor white. The procedures for both emulsification and sample preparation for fluorescent optical microscopy analyses were the same as above. The excitation/emission spectra for Calcofluor white used in this study were 365/435 nm, respectively. The obtained fluorescent images were processed with ImageJ (1.53e, Java 1.8.0\_172).

## 2.6.3. Rheological behavior

The dynamic measurements of emulsions were measured by a rheometer (MCR 302, Anton Paar, Germany) using the parallel plate geometry (PP25) with a gap distance fixed at 0.5 mm. The linear viscoelastic range was determined using a strain sweep (1 to 100%) at a fixed frequency (10 rad s<sup>-1</sup>). Thereafter, a constant strain (0.5%) was applied, within the nearly linear region, and the viscoelasticity was determined over a frequency range between 1 and 100 rad s<sup>-1</sup>. From the dynamic mechanical spectra, the storage (G') and the loss (G'') moduli were determined as a function of frequency. All the measurements were performed at 25 °C.

## 3. Results and discussion

## 3.1. CNC/NCh heteroaggregation

The binary suspensions of negatively-charged CNC and positivelycharged NCh showed different stability levels against phase separation, flocculation and sedimentation, depending on the mass ratio. Thus, turbidity and  $\zeta$ -potential were carried out to monitor the association between CNC and NCh. Visual appearance of CNC/NCh complexes with fixed CNC (0.25 wt%) and varied NCh concentrations is showed in Fig. 1a. Phase separation occurred after storing the suspensions for 24 h, which became apparent that increased NCh ratio induced destabilization at low concentration while re-stabilization at higher concentrations. These results are likely related to relatively strong inter-particle electrostatic repulsion in aqueous media, in accordance with the net electrostatic surface charge, Fig. 1b. In more detail, the neat CNC suspension was stable because of its strong negative surface charge (-59 mV, Table 1). The addition of positively charged NCh to CNC suspension caused electrostatic adsorption and charge neutralization, leading to aggregation, colloidal destabilization and sedimentation. According to Table 1 and Fig. 1b, the  $\zeta$ -potential of CN2 was -48 mV, which is slightly lower than that of neat CNC suspension (-59 mV). The net anionic surface charge is sufficiently high to ensure electrostatic stabilization with no signs of phase separation. Sedimentation started to occur in sample CN3 and increased gradually with increasing NCh loading until a condition such as that of CN5. The top layer of the complex suspensions presented blue color indicating the presence of CNC (Fig. 1a), which was evidenced by similar magnitude of  $\zeta$ -potential (<-36 mV, Fig. 1b). The reason for sedimentation in this concentration range relates to particle flocculation owing to charge neutralization. However, further increasing NCh (CN6), the upper layer became transparent, indicating the absence of free CNC, that is, CNC and NCh were fully complexed. Indeed, the  $\zeta$ -potential was almost zero for CN6, which is in contrast

compared to a previous study where NCh formed complexes with CNF (Huan et al., 2021), owing to the differences in morphology and surface properties of such system. After crossing the transition point for charge compensation (CN6), the CNC/NCh complexes became recharged and homogeneously dispersed (CN7 to CN10, Fig. 1a). In these cases, charge reversal led to positive  $\zeta$ -potential, which dramatically increased to +80 mV for CN7 and reached a value of +90 mV for CN8, CN9 and CN10. The positive charge of CNC/NCh complexes was mainly due to free NH<sub>3</sub><sup>+</sup> groups in NCh. The turbidity was in accordance with ζ-potential trending, wherein an increased turbidity occurred at low NCh loading, reaching a maximum value at neutral charge, and decreased with further increasing NCh concentration. A small increase of NCh concentration in the anionic system did not produce a significant change in the net electrostatic charge (Fig. 1c, left), resulting in no signs of sedimentation. At near-neutral condition (CN6, Fig. 1c, middle), large-sized complexes formed, which is easy to sediment. Thereafter, additional NCh loadings promote redispersion of the complexes, rather than further aggregation (Fig. 1c. right).

Typical morphology and size of CNC and NCh are shown in Figs. S1 and S2. Compared to NCh, CNC presented a larger lateral size and a more uniform length distribution. Fig. 1d shows the morphology of CNC/NCh complexes, below, at and above the neutralization condition. The relative size of the particles determines the morphology of aggregates formed. The larger and rigid CNC were surrounded by the thinner NCh through electrostatic interaction and were homogeneously dispersed with no evident aggregation in aqueous media (CN5, below charge neutralization and for a net anionic system), Fig. 1d. Significant aggregation occurred in the net neutral system (CN6) (Fig. 1d), which was consistent with TEM images (Fig. S2c) and turbidity measured in the aqueous suspension (Fig. 1b). At above charge neutrality condition (CN7), excess NCh led to redispersion and a homogeneous dispersion with free NCh (of narrower diameter) was clearly seen, Fig. 1d. In sum, colloidal heteroaggregates show surface charges going from -59 to +86



**Fig. 1.** (a) Visual appearance after 24 h and (b) variations of the  $\zeta$ -potential and turbidity of aqueous CNC (0.5 wt%) with increased addition of NCh (samples CN1-CN10, Table 1). All samples were stored at ambient temperature. (c) Schematic illustration (not to scale) of NCh adsorption on CNC resulting from possible electrostatic binding. (d) AFM images of CNC/NCh complexes in samples CN5, CN6, and CN7. The scale bar is 500 nm.

mV, depending on the CNC/NCh ratio, wherein a consequence of CNC and NCh complexation was the progressive homogenization of particle size and, above the neutralization condition (CN7–CN10), a better dispersed state was attained (Aberkane, Jasniewski, Gaiani, Scher, & Sanchez, 2010). Indeed,  $\zeta$ -potential of the complexes remained fairly similar until reaching the maximum NCh loading tested (CN10), where the deviation in  $\zeta$ -potential was reduced, which explains the decrease in turbidity at this stage.

# 3.2. Pickering emulsions stabilized by CNC/NCh complexes

Sunflower oil-in-water Pickering emulsions were produced by using 10 wt% oil and the respective CNC/NCh aqueous suspensions. As shown in Fig. 2a and b, Pickering emulsions with no obvious oiling-off were formed using various CNC/NCh ratios. The emulsions were relatively stable, with no obvious phase separation within one day after emulsification. However, creaming layers started to form on top of the emulsions prepared with CN1 (neat CNC), CN2, CN3 and CN4, with similar height after 1-day storage. The creaming layers became more apparent after 7 days, Fig. 2a. The height of the creaming layer became almost double when the emulsion was prepared with CN5. As further increasing addition of NCh, different phase behaviors took place. For instance, the most creamed sample was that stabilized by CN6. This is attributed to the charge condition, closer to neutrality of CN6, which resulted in the largest droplet size for all of the samples (Figs. 1b, 2b and Table 1). Nevertheless, it should be noted that the  $\zeta$ -potential of CN6-stabilized Pickering emulsion was +26 mV; it was the smallest absolute value for all of the emulsions but not close to neutral, which may be caused by the adsorption of free, positively-charged NCh onto the surface of the droplets during ultrasonication.

As shown in Fig. 2b, the mean droplet diameter  $(D_{32})$  of emulsions could be tailored by adjusting the NCh concentration, from CN1 to CN10. Herein, the net  $\zeta$ -potential of CNC/NCh complexes was considered as a parameter to compare emulsion properties. When emulsions

were stabilized by CNC/NCh complexes showing net negative charges (CN2 to CN5), the droplet size was slightly larger compared to that of emulsion produced with neat CNC (Fig. 2c). Under near-neutral condition (CN6), the droplets showed extremely larger diameter (>30  $\mu$ m) (Fig. 2b and c). Two primary factors are likely responsible for the result. First, the large size of CNC/NCh complexes formed under near-neutral conditions limited their transport from continuous, aqueous phase to newly formed oil/water interfaces. Consequently, the oil droplets formed upon sonication remarkably coalesced soon after preparation, leading to large droplets. Moreover, while complexes with lower surface charges may promote their adsorption at the oil/water interfaces, a balance regarding to reduced inter-particle electrostatic repulsion and limited transport should be considered. On the other hand, coalescence of droplets occurred during formation and storage because of weak electrostatic repulsion between the droplets, given their low surface charge. The emulsions stabilized with CNC/NCh systems with a net cationic charge (CN7) had droplets with substantially reduced size (5 μm), which slightly increased with further adding NCh (CN8, CN9 and CN10). Although the droplets presented similar size to that of emulsions stabilized with CN1 to CN5, the phase behavior was distinctly different: stable and homogeneous with no signs of creaming and separation after 7 days (Fig. 2a). These results imply the beneficial effect of incorporating NCh in the complexes, mainly attributing to the screened surface charge and excellent emulsifying ability brought by NCh (Bai, Huan, et al., 2019; Huan et al., 2021), which prevented coalescence and restricted oiling-off during storage.

The size distribution of emulsion droplets that were stabilized using CNC/NCh complexes is illustrated in Fig. 2c. All size distributions of freshly prepared emulsions were monomodal and uniform. By contrast, the mean droplet sizes, as well as size polydispersity, increased in emulsions prepared with net anionic complexes at low NCh loadings (CN1 to CN6) after 1 week (Fig. S3), which indicates low stability. When using highly charged cationic complexes (CN7 to CN10), the size of droplets kept a distribution with only apparent peaks, pointing to higher



**Fig. 2.** (a) Visual appearance, (b) mean droplet diameter ( $D_{32}$ ), and (c) size distributions of CNC/NCh-stabilized Pickering emulsions containing 10 wt% sunflower oil with given CNC/NCh ratios in the aqueous phase at 0-day. The number 1 to 10 in (a) refers to the Pickering emulsions that are stabilized by CN1 to CN10, from left to right. To facilitate comparison in (c), the profiles for the emulsions at different NCh loadings were shifted in the vertical axis.

stabilization capability of NCh in Pickering emulsions.

We used confocal imaging to evaluate the morphology of CNC/NChstabilized Pickering emulsions (Fig. S4). The oil phase was stained using Nile red and the results show similar trends as discussed above (Fig. 2b). Emulsions stabilized with anionic CNC/NCh complexes (CN2 to CN5) showed well-dispersed droplets compared to those produced with neat CNC, while emulsions originated from the net-zero complexes (CN6) presented large droplets and irregular shapes. The emulsions prepared with the cationic complexes presented a reversed evolution of oil droplet diameter, namely, the droplets became smaller and more uniform. This result indicates the capability of such complexes in stabilizing Pickering emulsions. In sum, incorporation of NCh in the system improves the emulsifying ability of CNC, enabling stable Pickering emulsions with tunable droplet properties.

We identified the CNC/NCh complexes in Pickering emulsions through a dual-channel fluorescent microscope, wherein the sunflower oil was dyed in red and the nanoparticles were stained in blue. The blue channel and merged images are illustrated in Fig. 3a (see the blue, thin contour surrounding the droplets). It should be noted that although

multiple dye reflection took place from the aqueous phase, the CNC/ NCh complexes were found to be distributed at the oil/water interfaces regardless of the droplet size. The droplet size of CN5-stabilized Pickering emulsions was uniform, wherein bright fluorescence (blue) was observed at the perimeter of the emulsion droplets (at the oil/water interface). However, the CN6-stabilized Pickering emulsions presented a variable size with weak blue fluorescence (Fig. 3b), which means limited CNC/NCh adsorption at the oil/water interface. This result is consistent with Figs. 1 and 2 where the  $\zeta$ -potential of CN6 is close to zero and contains large aggregates, preventing its adsorption. Fig. 3c and d showed uniform droplets size in CN7- and CN10-stabilized Pickering emulsions, which displayed positive  $\zeta$ -potential value. In addition, there was less agglomeration of CN10-stabilized Pickering emulsions compared to that with CN7, which indicated that viscous, nanofibril networks of free CNC/NCh complexes formed in the continuous phase, contributing to droplet stabilization. Although the droplet sizes of emulsions stabilized by CN5, CN7, and CN10 were much smaller and more uniform than that of the emulsion stabilized by CN6, similar adsorption behaviors confirmed that CNC/NCh complexes stabilized

> Fig. 3. Fluorescent micrographs of CNC/NChstabilized Pickering emulsions containing 10 wt% sunflower oil and CNC/NCh complexes of (a) CN5, (b) CN6, (c) CN7, and (d) CN10. The left and middle rows are the images obtained from the stained oil phase and the dyed CNC/NCh, respectively. The right row is a composed image after merging the others. The oil phase and the CNC/NCh complexes were stained following the standard procedure using Nile red and Calcofluor white, respectively. The scale bars correspond to 20  $\mu$ m.



Pickering emulsions via interfacial adsorption. Therefore, a question that arises is how NCh enables adjustable adsorption of CNC/NCh complexes at the interfaces, from randomly dispersed nanofibrils in the continuous, aqueous phase to tightly adsorbed heteroaggregation at the oil/water interfaces. This question will be considered in the following section (Figs. 4 and 5).

We evaluated the surface coverage of complexes using CNC/NChstabilized styrene (St)-in-water Pickering emulsions, wherein St was polymerized to PS beads, which allowed observation of the nanoparticles at the interfaces (Figs. 4 and S5). Free CNC dispersed in the continuous, aqueous phase was the result from the large (negative)  $\zeta$ -potential (Fig. 1a and b). When neat CNC was used for emulsion preparation, PS beads were sparsely covered with CNC, which showed a loose and random organization at the droplet surface (Fig. 4a). By adding NCh, the complexation via electrostatic interactions between CNC and NCh gradually led to a partial coverage of CNC by NCh (Fig. S5a). In other words, smaller NCh particles adsorbed on the surface of larger CNC particles (heteroaggregation) driven by electrostatic attraction (Fig. 1b). Possible physicochemical mechanism at low NCh addition is shown in Fig. 5a. CNC surfaces were still exposed, favoring the CNC to remain partially dispersed in the continuous, aqueous phase. In the meantime, the acetyl groups on the surface of NCh that retained in the complexes could act as hydrophobic sites with a better affinity to the oil phase, presumably improving interfacial adsorption of the complexes, with their hydrophilic groups interacting with the water phase (Fig. 1c). As a result, when increasing NCh loading until reaching net neutrality (CN6), CNC/NCh bundles were identified on the surface of PS beads (Fig. 4b). A possible reason for this phenomenon could be the flocculation between cationic and anionic nanoparticles in the aqueous phase or at the oil/water interfaces due to electrostatic neutralization. Thus, such a system was less efficient for interfacial adsorption of CNC/ NCh, resulting from aggregates that were too large to stabilize oil droplets and leading to an obviously increased droplet size (Fig. 5b). The large dimension and hydrophilicity of CNC/NCh complexes limited their diffusion from the continuous, aqueous phase to the oil/water interfaces and their subsequent adsorption under weak electrostatic repulsion.

When  $\zeta$ -potential of CNC/NCh complexes shifted to positive, the CNC surface was considered to be fully covered by NCh and complexes of CNC/NCh (Figs. 4c and S5b). Therefore, replying on the NCh's nature, it is reasonable to consider that a more complete adsorption of the complexes at the interfaces can be obtained by means of hydrophobic acetyl groups of NCh, close to the droplet surface (Fig. 5c). Therefore, complexation between oppositely charged CNC and NCh is considered

as an efficient system for green Pickering emulsions. It should be noted that small and neat PS spherical beads (Fig. 4) can be explained by the polymerization of St monomer in the aqueous phase, in which AIBN initiated the reaction thermally and free CNC/NCh complexes could function as nucleation sites (Bai, Huan, et al., 2019).

## 3.3. High oil fraction Pickering emulsions

Visual image, size distribution, and CLSM images of emulsions with different oil fractions (WOR) are shown in Fig. 6. The droplet diameter and distribution indicated that oil droplets gradually increased in size with increasing oil loading (Fig. 6b), reaching 28.5  $\mu$ m for WOR = 50/ 50, which is consistent with the CLSM images (Fig. 6d). Since the concentration of CNC/NCh complex in the emulsions system remained unchanged, there is an indication that a higher sunflower oil fraction increased the total interfacial area that was not sufficiently covered by the available particles (Fig. S6) (Binks & Whitby, 2004), which increased the frequency of drop collisions during ultrasonication. Thus, the extent of coalescence led to increased drop size. Interestingly, there was a bimodal size distribution at lower oil loading, which may result from the presence of CNC/NCh clusters in the aqueous phase, and free NCh was quickly adsorbed at the oil/water interface. In contrast, at high oil fraction, low coverage ratio was not sufficient to stabilize the newly generated interfaces, leading to coalescence, and a relatively large oil droplets were formed (28.5 µm in size). The rheological properties of the emulsions with varying oil fractions showed an elastic behavior (Fig. 6c). The high NCh loading in the complex and free NCh in the aqueous phase promoted the formation of physical networks (Zhu et al., 2020); on the other hand, the increased oil fractions may increase interdroplet interactions. This explains the high stability of the emulsions against creaming and oiling-off upon storage, indicating that the CNC/ NCh complexes (CN10) are superior stabilizers of Pickering emulsions, even for systems with high oil loading. More significantly, the emulsions that were stabilized with the CNC/NCh complex (CN10) at different oil fractions (WOR up to 50/50, w/w) were stable after storage for 7 days and 1 month (Fig. S6).

## 3.4. Long-term stability of Pickering emulsions

The stability of emulsions determines their shelf life, which is a crucial factor in practical applications, in particular for applications of food, pharma, and cosmetic (McClements, 2007). Consequently, long-term stability of Pickering emulsions was evaluated using CLSM after



Fig. 4. Scanning electron microscope (SEM) images of polymerized PS beads that were stabilized by CNC/NCh complexes of (a) CN1, (b) CN6, and (c) CN10. Small spherical beads in the images are PS formed during polymerization.



Fig. 5. Possible stabilization mechanisms (not to scale) involving Pickering emulsions in the presence of CNC and NCh.



**Fig. 6.** (a) Visual appearance, (b) mean droplet diameter ( $D_{32}$ ) and size distribution, and (c) modulus for emulsions at different WORs. Open and filled symbols in (c) are used to indicate storage (G') and loss (G'') moduli, respectively. (d) CLSM images of CNC/NCh-stabilized Pickering emulsions at different WORs. The concentration of CNC and NCh in the aqueous phase was 0.25 wt%, respectively. The scale bar is 30  $\mu$ m in (c).

storage for 30 days at room temperature. Fig. 7 shows that creaming with oil coalescence occurred when Pickering emulsions were stabilized with net-negative or net-zero CNC/NCh complexes (CN1–CN6). The net-positive systems (CN7–CN10) were stable after 1-month storage, showing similar visual appearance as the freshly prepared samples, which is attributed to adsorption of CNC/NCh on the oil droplets that protected them from aggregation by electrostatic and steric effects (Lv et al., 2021). In addition, the network structure formed by free nanoparticles present in the aqueous phase arrested droplet movement and aggregation (Fig. 3d), restricted or at least delayed creaming or coalescence (Zhu et al., 2020), particularly for emulsions stabilized with CN9 and CN10. The latter produced emulsions that showed no creaming after 9-month storage (Fig. S7).

# 4. Conclusions

We investigated the influence of nanoparticle type (CNC and NCh) and concentration as well as oil fraction on the formation, structure, and stability of Pickering emulsions with sunflower oil. The mechanism of emulsion stabilization was tailored by changing the CNC-to-NCh mass ratio. CNC/NCh complexes efficiently stabilized Pickering emulsions and prevented oiling-off. In addition, at a mass ratio of CNC/NCh < 1.25, the emulsions showed long-term storage stability, at least 9 months under ambient condition. The droplet diameter was large at charge neutrality condition (CNC/NCh = 5), where large hetero-aggregates were ineffective to stabilize the oil phase. We postulate that the CNC/NCh complexes adsorbed to oil droplets and the acetyl groups in NCh improved interfacial adsorption. A combination of CNC and NCh



Fig. 7. (a) Visual appearance and (b) CLSM images of CNC/NCh-stabilized Pickering emulsions after 1-month of storage. The number 1 to 10 in (a) refers to the Pickering emulsions that are stabilized by CN1 to CN10, from left to right. The emulsions contained 10 wt% sunflower oil. The scale bar is 20 µm. All the samples were stored at room temperature.

endowed Pickering emulsions with stability against coalescence at different sunflower oil fractions, up to 50 wt%. This study establishes the basis for the preparation of Pickering emulsions stabilized by natural binary particles via heteroaggregation, which shows promise for the formulation of sustainable plant-based multiphase systems for food, cosmetics, and pharmaceuticals.

## CRediT authorship contribution statement

All authors contributed equally to this manuscript.

## Declaration of competing interest

The authors declared that they have no conflicts of interest to this work.

## Data availability

Data will be made available on request.

## Acknowledgements

This work was supported by the Canada Excellence Research Chair Program (CERC-2018-00006), the Canada Foundation for Innovation (Project 38623) and the European Research Council under the European Union's Horizon 2020 research and innovation program (ERC Advanced grant no. 788489, "BioElCell"). This work was also part of the Academy of Finland's Flagship Program (Competence Center for Materials Bioeconomy, FinnCERES) under project no. 318890 and 318891. L.B. was supported by National Natural Science Foundation of China (32201483). L.B. and S.Q.H. were also supported by the Natural Science Foundation of Heilongjiang Province (YQ2021C009 and YQ2021C008) and the China Postdoctoral Science Foundation (2021M700734). S.S.G. is thankful to the Overseas Study Program of Guangzhou Elite Project (JY201936) and Bin Zhao for help with SEM imaging.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2022.120154.

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