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Controlling the self-assembly behavior of aqueous chitin nanocrystal suspensions

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ABSTRACT

As with many other bio-sourced colloids, chitin nanocrystals (ChNCs) can form liquid crystalline phases with chiral nematic ordering. In this work, we demonstrate that it is possible to finely tune the liquid crystalline behavior of aqueous ChNC suspensions. Such control was made possible by carefully studying how the hydrolysis conditions and suspension treatments affect the colloidal and self-assembly properties of ChNCs. Specifically, we systematically investigate the effects of duration and acidity of chitin hydrolysis required to extract ChNCs, as well as the effects of the tip sonication energy input, degree of acetylation, pH and ionic strength. Finally, we show that by controlled water evaporation, it is possible to retain and control the helicoidal ordering in dry films, leading

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3 to a hierarchical architecture analogous to that found in
4 nature, e.g. in crab shells. We believe that this work serves as
5 a comprehensive insight into ChNC preparation and handling which
6 is required to unlock the full potential of this material in
7 both a scientific and industrial context.
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16 INTRODUCTION

17 Lyotropic liquid crystalline phases have been observed for many
18 biopolymers, from DNA and proteins to polysaccharides.¹ In
19 nature, these biological building blocks appear to directly
20 encode the functions and properties of incredibly diverse
21 hierarchical materials. As such, the ability to artificially
22 control such building blocks and their interactions at the nano-
23 scale is highly desirable for the fabrication of sustainable
24 functional materials.
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35 This is especially true in the case of the two most abundant
36 biopolymers available on the planet, cellulose and chitin. In
37 their colloidal form, they both spontaneously self-organize into
38 a chiral nematic liquid crystalline phase.^{2,3} However, while the
39 self-assembly of colloidal cellulose (a.k.a. cellulose
40 nanocrystals) has attracted significant attention in recent
41 years,^{2,4-6} liquid crystalline phases of chitin nanocrystals
42 (ChNCs) remain virtually unexplored. In fact, despite the
43 pioneering works by Revol *et al*³, Li *et al*⁷ and Belamie *et al*⁸
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3 that revealed the lyotropic behavior of ChNC in water, a
4 systematic assessment on the impact of each of the preparation
5 steps or post-treatments upon the ChNC suspension has yet to be
6 reported. Here, by carefully addressing all the steps required
7 to prepare an aqueous ChNC suspension; from initial hydrolysis
8 of chitin, to controlling the degree of acetylation, the extent
9 of tip sonication and the ionic strength or pH, we demonstrate
10 how to optimise the stability of the ChNC suspension, control
11 its self-organisation into a chiral nematic liquid crystal and
12 ultimately produce well-ordered helicoidal films with a defined
13 pitch, tuneable from a few hundred nanometres to several
14 microns.
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30 As a natural linear polysaccharide, chitin is an extremely
31 abundant biomaterial and is found in crustacean, insect, and
32 beetle exoskeletons, as well as the cell walls of fungi
33 (Figure 1A).⁹ In nature, chitin is structured into fibers that
34 are comprised of bundles of semi-crystalline microfibrils. Each
35 microfibril is composed of extended linear molecular chains of
36 an acetylglucosamine homopolymer with only the surface having
37 free amines (Figure 1B).⁹ Chitin microfibrils are used for
38 mechanical support in many living organisms, often in the form
39 of a finely-tuned helicoidal structure that serves to
40 significantly increase the mechanical robustness of e.g. crab
41 exoskeletons.¹⁰
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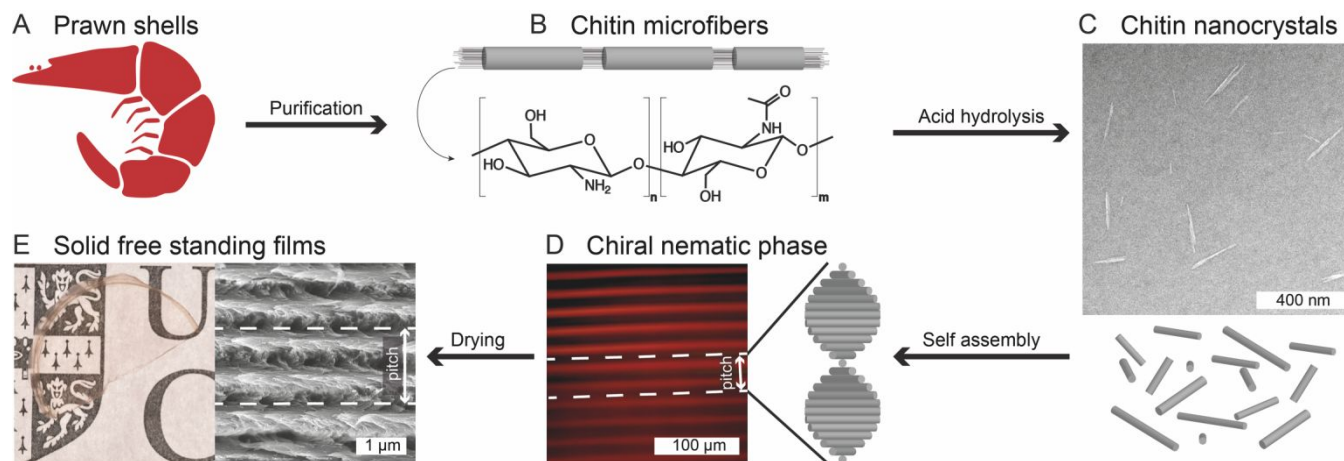


Figure 1. Overview of chitin nanocrystal preparation and the subsequent self-assembly into helicoidal films: Prawn shell chitin powder (A) is purified to yield chitin microfibrils (B), that upon acid hydrolysis form chitin nanocrystals (C). Under controlled conditions, colloidal chitin nanocrystal suspension (C) can self-assemble into a chiral nematic phase (D), that upon evaporation yields a transparent film that retains this helicoidal nano-architecture (E). The logo is used with permission from University of Cambridge.

ChNCs can be isolated from purified natural chitin by strong acid hydrolysis.¹¹ The resultant rod-shaped nanoparticles are highly polydisperse and their dimensions vary between 50 - 300 nm in length and 6 - 8 nm in width (Figure 1C).³ In water, ChNCs carry a positive surface charge, which originates from protonated amines at the ChNC surface, with an average pKa value of ~ 6.3.¹² At a pH below this pKa, i.e. acidic aqueous media, ChNCs are colloiddally stable. Similarly to cellulose

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3 nanocrystals,² colloidal ChNCs exhibit liquid crystalline
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5 behavior above a threshold concentration.^{3,8} This is an entropy
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7 driven process dictated by the anisotropic shape and the mutual
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9 interactions between the charged, rod-like ChNCs.¹³⁻¹⁶ This self-
10
11 assembly process starts by a nucleation and growth mechanism
12
13 until a macroscopic phase separation occurs.^{2,3} The anisotropic
14
15 ChNC phase has a chiral nematic order, as identified by the
16
17 characteristic 'fingerprint pattern' in polarized optical
18
19 microscopy (Figure 1D).^{3,8} In this structure, ChNCs locally align
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21 along a direction that spatially rotates in a left-handed
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23 fashion about an axis.¹⁷ The helix achieves a 360° rotation over
24
25 the distance which is defined as the chiral nematic pitch and
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27 corresponds to twice the periodicity of the measured fingerprint
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29 pattern. Upon evaporation this ordering can be retained giving
30
31 rise to films with a defined helicoidal nanoarchitecture
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33 (Figure 1E).
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43 **EXPERIMENTAL METHODS**

44 All reagents, unless stated otherwise, were acquired from Sigma
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46 Aldrich. Chitin (from shrimp shells, practical grade, powder)
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48 was purified by sodium hydroxide treatment (5 wt%, 80 °C, 3 h),
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50 followed by acidic bleaching in sodium chlorite (0.3 wt%, 80 °C,
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52 3 h).¹⁸ These purification steps were repeated three times each
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54 before dialyzing against Milli-Q water (Regenerated Cellulose
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3 dialysis tubing, MWCO 12-14 kDa), and subsequent isolation by
4 centrifugation to form a pellet (25,000g, 4 °C, 30 min), as
5 described in detail in the ESI.
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10 The purified chitin pellet (~10 wt% chitin in water) was
11 hydrolyzed in hydrochloric acid (HCl, 3.0 - 5.0 M) under reflux
12 (104 °C, 90 - 540 min, Table 1) as adapted from previously
13 reported protocols.^{3,18,19} The reaction mixture was quenched by
14 diluting with ice-cold Milli-Q water and washed twice by
15 centrifugation (as above). The produced ChNC suspension was then
16 dialyzed against Milli-Q water until the conductivity of the
17 surrounding bath remained stable for two days. The ChNC
18 concentration was measured by drying three samples at 45 °C
19 (~1 g suspension) and measuring the dry content. The ChNC
20 suspensions were diluted to 1.0 wt% and dialyzed against a
21 dilute HCl solution (1.0 mM) until stable at pH 3.0 for two
22 days. ChNCs were then dispersed using tip sonication. The
23 samples were subsequently vacuum filtered through nitrocellulose
24 filter paper with pore sizes of 8.0 µm and 0.8 µm. The final HCl
25 and NaCl concentration was adjusted by a further dialysis,
26 followed by concentration using rotary evaporation. Thus, all
27 the samples in this study have a fixed electrolyte ratio of 1.0
28 mM HCl to 1.0 wt% ChNC, except where otherwise stated. For
29 example, at 1.0 wt% ChNC the HCl concentration is 1.0 mM, while
30 at 10.0 wt% the HCl concentration is 10.0 mM. At 1.0 wt% ChNC
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3 the pH is 3.0 and decreases upon increasing ChNC concentration,
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5 reaching pH 2.0 at 10.0 wt% of ChNC. Other ionic strengths were
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7 prepared in an analogous fashion, as described in detail in the
8
9 ESI.
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12 Chitin and ChNC were analyzed for their chemical composition by
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14 freeze-drying samples, followed by FT-IR spectroscopy, solid-
15
16 state ^{13}C CP-MAS NMR spectroscopy (ssNMR) and PXRD analysis. All
17
18 characterization methods showed that purification has been
19
20 successful and that ChNC are chemically equivalent to chitin, as
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22 described in detail in the ESI.
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26 ChNC suspensions were assessed for their colloidal properties
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28 by means of dynamic light scattering (DLS), ζ -potential,
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30 transmission electron microscopy (TEM), UV-vis spectroscopy and
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32 conductometric titration, as described in detail in the ESI.
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36 ChNC suspensions were concentrated using a rotary evaporator at
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38 55 °C until a viscous fluid was attained (7-13 wt%). To assess
39
40 liquid crystalline behavior, ChNC concentration series were
41
42 produced through dilution with Milli-Q water. Phase separation
43
44 was then quantified by sealing the series of ChNC suspensions
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46 into round capillaries (inner diameter \approx 1.3 mm) and, after
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48 storing vertically for at least a month to ensure equilibration.
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50 Photographs between crossed polarizers were taken and the ratio
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52 between the height of the bright anisotropic phase to the whole
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54 suspension was defined as the volume fraction of the anisotropic
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3 phase. Pitch measurements were performed by placing capillaries
4 horizontally under a cross-polarized optical microscope in
5 transmission mode (SLWD objective, 20x magnification), as
6 described in detail in the ESI.
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15 RESULTS AND DISCUSSION

16 **Hydrolysis conditions.** The physical properties of ChNCs
17 produced from chitin hydrolysis depend on key reaction
18 conditions, such as duration and acidity (Figure 2). Therefore,
19 the effect of the hydrolysis time was examined by comparing ChNC
20 samples hydrolyzed for 90, 270 and 540 minutes in boiling
21 hydrochloric acid (HCl, 3.0 M). These samples are referred to as
22 3M90, 3M270 and 3M540, respectively. Similarly, the effect of
23 increased acidity was screened with 5.0 M HCl and are likewise
24 referred to as 5M90, 5M180, and 5M270. For consistency, an
25 equivalent tip sonication treatment of 20 kJ/g_{ChNC} was applied to
26 all the samples and the ionic strength was standardized to
27 100 $\mu\text{mol/g}_{\text{ChNC}}$ by dialysis against HCl.
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44 Figure 2A-C and Table 1 show that the hydrolysis time markedly
45 affects ChNC dimensions and their colloidal stability. 3M90
46 results in notably larger particles with significantly higher
47 polydispersity compared to the other samples. Therefore, it can
48 be concluded that 3M90 conditions are insufficient to hydrolyze
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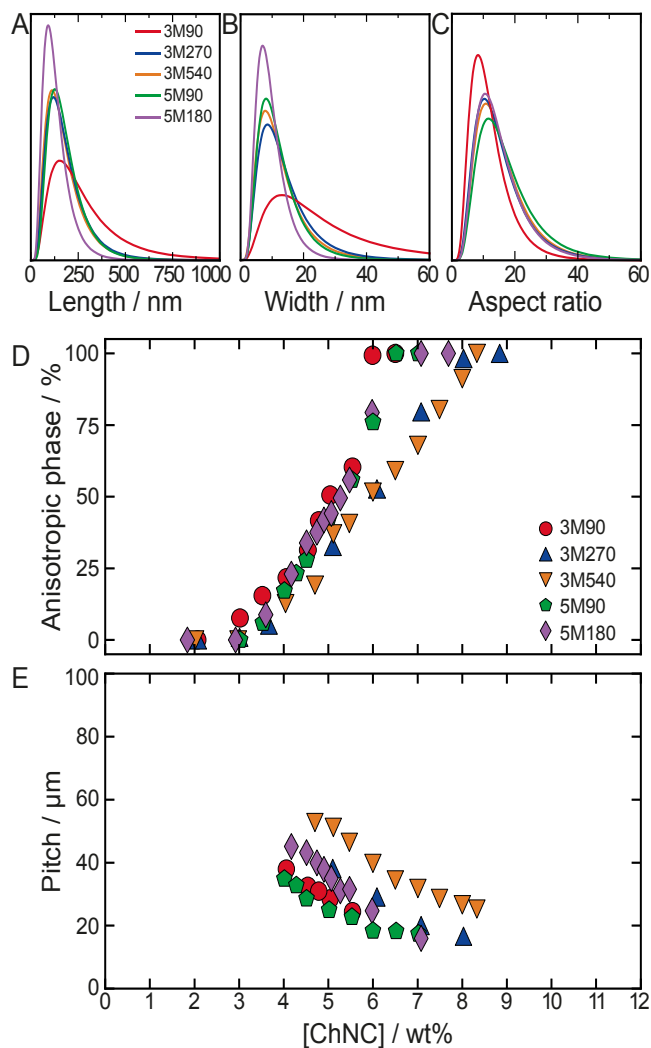


Figure 2. Effect of hydrolysis conditions on ChNC dimensions and self-assembly. (A-C) The fitted lognormal distribution curves from TEM measurements of ChNC (A) length, (B) width and (C) aspect ratio (Figure S9 provides full histograms). Plots showing (D) the increasing proportion of anisotropic phase and (E) decreasing chiral nematic pitch upon increasing ChNC concentration.

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3 chitin so as to achieve a well-dispersed stable ChNC suspension.
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5 These results agree with the lowest surface charge evaluated by
6 conductometric titration and ζ -potential values of the resulting
7 suspension (Table 1). In contrast, ChNC suspensions prepared at
8 3M270 and 3M540 displayed excellent colloidal stability. Both
9 ChNC suspensions remained sediment free over several weeks and
10 their turbidity was markedly lower when compared to 3M90 (Figure
11 S1A), indicating both smaller ChNCs and a notable absence of
12 aggregates. The slight decrease in turbidity, Z-average particle
13 size (as measured by DLS) and an increase in ζ -potential and
14 surface charge when 3M270 is compared to 3M540 are consistent
15 with prolonged acid hydrolysis slowly degrading the crystalline
16 part (Table 1). Moreover, the length and width distributions of
17 3M270 and 3M540 overlap (Figure 2A,B), thus it was concluded
18 that chitin is already sufficiently hydrolyzed within 270
19 minutes to achieve a stable and well-dispersed ChNC suspension
20 (Figure 2A,B).
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41 The effect of acidity on the hydrolysis process was also
42 evaluated. An increase in HCl concentration was found to simply
43 accelerate chitin hydrolysis and as a result ChNC suspensions
44 prepared at 5M270 were found to be dark grey/brown in appearance
45 (i.e. over hydrolyzed). As this is indicative of decomposition
46 processes, it was not studied further. 5M180, on the other hand,
47 displayed the smallest ChNC dimensions and lowest polydispersity
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3 of all ChNC samples prepared in this study (Figure 2A,B;
4 Figure S9). Despite the DLS measurements indicating it has
5 almost identical Z-average size and turbidity as compared to
6 3M540 (indicating similar degree of chitin hydrolysis), the
7 5M180 ChNC suspension exhibits a lower ChNC surface charge and
8 ζ -potential (Figure S1A; Table 1). ChNC following 5M90
9 preparation conditions were of dimensions similar to 3M270, but
10 again with a much smaller surface charge (Figure 2; Table 1).
11 Overall, it implies that increasing hydrochloric acid
12 concentration from 3.0 to 5.0 M accelerated chitin hydrolysis at
13 the glycosidic bond while having little effect on the hydrolysis
14 of surface amide groups. This observation is consistent with
15 reports showing that the amide hydrolysis rate plateaus at
16 similarly high hydrochloric acid concentrations.^{20,21} Thus, the
17 hydrolysis duration is the main parameter controlling the
18 maximum ChNC surface charge.

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39 The liquid crystalline behavior was analyzed by phase diagrams
40 obtained via serial dilution of each concentrated ChNC
41 suspension, allowing comparison of the proportion of the liquid
42 crystalline phase formed as a function of ChNC concentration. It
43 was observed that all ChNC suspensions, including 3M90,
44 macroscopically phase separated with increasing ChNC
45 concentration (Figure 2D) and gave rise to a chiral nematic
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3 phase, as quantified by polarized optical microscopy
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5 (Figure 2E).
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8 In order to discuss the variations observed between these
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10 samples, we first introduce some scaling arguments based on
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12 Onsager's theory of soft repulsing rods.^{13,22} The concentration at
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14 which the ChNC suspension separates into a biphasic (c_I) and then
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16 in a fully chiral nematic phase (c_A) can be correlated to the
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18 aspect ratios (L/D) of the ChNCs and also to their mutual
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20 interactions.¹⁴ To account for the latter, we can consider that
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22 each ChNC occupies an effective cylindrical volume that cannot
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24 overlap with other ChNCs, leading to an effective diameter D_{eff}
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26 and length L_{eff} . These effective dimensions, D_{eff} and L_{eff} , are
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28 expected to depend both on the thickness of the ionic double-
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30 layer surrounding the ChNCs (scaling as the Debye length κ^{-1} and
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32 controlled by the ionic strength as $\kappa^{-1}(\text{nm}) = 0.304/\sqrt{I(\text{M})}$ in aqueous
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34 medium) and the surface charge of the ChNCs (in a non-linear
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36 fashion).¹⁴
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42 This leads to an effective volume and thus concentration c_{eff} but
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44 also aspect ratio $L_{\text{eff}}/D_{\text{eff}}$. As a result, if the effective threshold
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46 concentrations scale as $c_{j,\text{eff}} \propto D_{\text{eff}}/L_{\text{eff}}$, the actual concentration c_j
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48 should scale as:
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$$51 \quad c_j \propto c_{j,\text{eff}} \left(\frac{V}{V_{\text{eff}}} \right) \propto c_{j,\text{eff}} \frac{D^2 L}{D_{\text{eff}}^2 L_{\text{eff}}} \propto \frac{D_{\text{eff}}}{L_{\text{eff}}} \frac{D^2 L}{D_{\text{eff}}^2 L_{\text{eff}}} \propto \frac{D^2 L}{D_{\text{eff}} L_{\text{eff}}^2}. \quad (1)$$

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3 Since the relative length variation is negligible compared to
4 the relative diameter variation ($L_{\text{eff}} \sim L$), this can be simplified
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$$c_j \propto \frac{D^2}{D_{\text{eff}}L} = \frac{D}{D_{\text{eff}}} \left(\frac{D}{L} \right), \quad (2)$$

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12 where the largest variations of c_j are expected for ChNCs of
13 small aspect ratios. In a polydisperse suspension, rods of
14 larger aspect ratios turn chiral nematic at a lower
15 concentration and thus are mostly responsible of the observed c_I ,
16 while the rods of smallest aspect ratios are expected to turn
17 chiral nematic at higher concentration, thus impacting mostly c_A .
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19 A direct consequence of this is that a variation in the
20 interactions between rods would cause more variation of the c_A
21 value (for which the associated rods have a smaller aspect
22 ratio) than the c_I value.¹⁵
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36 The observation of the phase diagrams in Figure 2D is in good
37 agreement with this description. The phase separation starts at
38 a similar ChNC concentration $c_I \sim 3$ wt% for all samples (Figure
39 2C,D), which suggests that ChNCs with a high aspect ratio (i.e.
40 long and thin) are present in all the samples. In contrast, the
41 second threshold concentration, c_A , varies much more between the
42 samples and increases with the hydrolysis time. This correlates
43 with the presence of shorter rods and agrees with the expected
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3 trend, however, its interpretation might also involve
4 differences in the ChNC colloidal stability.
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7 3M90 exhibited the lowest c_A and c_I concentration (Figure 2D;
8 Table 1), in agreement with a smaller proportion of short ChNCs
9 and overall lower colloidal stability. Closer inspection into
10 this sample revealed a sharp increase in the volume of the
11 anisotropic phase at ~ 6 wt% of ChNC, while at lower
12 concentrations a near linear relationship was observed. This
13 suggests an interruption of the macroscopic separation of the
14 two phases and can further be verified from the incomplete phase
15 separation just below this concentration (Figure S6, S7). The
16 lack of relaxation over time implied that a slow gelation
17 occurred in the suspension.²³⁻²⁵ Other samples in the series did
18 not exhibit such behavior due to higher colloidal stability.
19 3M270 and 3M540 both had high c_A thresholds in comparison with
20 the 5M90 and 5M180, and also more amine groups and thus a higher
21 structural charge when protonated (Figure 2D, Table 1).
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41 The pitch, in general, decreased with ChNC concentration
42 (Figure 2E) and is a consequence of ChNCs getting in closer
43 proximity with each other. Thus, they interact and twist more
44 with respect to each other, resulting in a lower chiral nematic
45 pitch. In addition, we relate the increase in pitch to the
46 surface charge, as exemplified by 3M540, where the highest
47 surface charge results in the largest D_{eff} and as such the largest
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3 pitch. However, to date no theory accounts for polydisperse
4 suspensions or the pitch evolution when chiral rods are
5 considered.
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10 We conclude that 3M270, 3M540, 5M90 and 5M180 prove to be
11 appropriate conditions to prepare stable self-assembling
12 colloidal ChNC suspensions, with 3M270 and 3M540 having a higher
13 surface charge. The use of 5.0 M HCl also corresponds to much
14 harsher reaction conditions, which are also harder to control
15 and less environmentally-friendly. For these reasons, 3.0 M HCl
16 was preferred in the following studies. More specifically, the
17 3M540 hydrolysis condition was chosen to test the effects of the
18 other parameters as these conditions yielded ChNC suspensions
19 with slightly smaller dimensions and higher surface charge, as
20 compared to 3M270.
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35 **Tip sonication.** Tip sonication is a common post hydrolysis
36 treatment that breaks apart large bundles of chitin
37 crystallites, resulting in well-dispersed ChNC suspensions. For
38 that reason, the application of tip sonication can have an
39 important impact on the final properties of the suspension and
40 should not be neglected.
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48 The influence of tip sonication energy input was studied by
49 preparing a new batch of 3M540 ChNC suspension. Three different
50 tip sonication conditions were then tested, namely, Ts4, Ts20,
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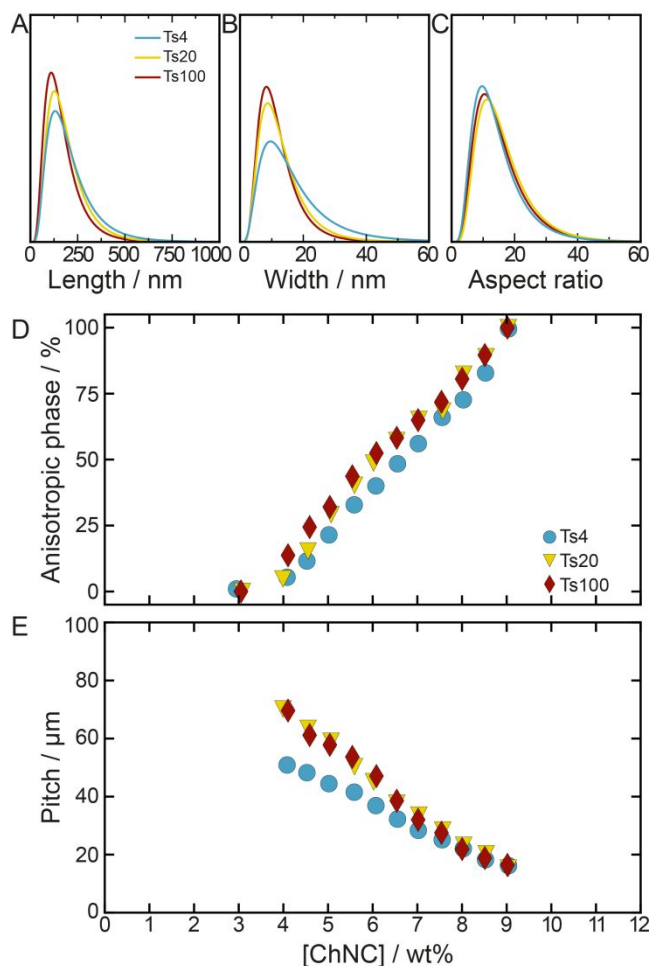


Figure 3. Effect of increasing tip sonication energy input on ChNC dimensions and self-assembly. (A-C) The fitted lognormal distribution curves from TEM measurements of ChNC (A) length, (B) width and (C) aspect ratio (Figure S10 provides full histograms). Plots showing (D) the increasing proportion of anisotropic phase and (E) decreasing chiral nematic pitch with increasing ChNC concentration.

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3 and Ts100 referring to the energy inputs of 4, 20, and
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5 100 kJ/g_{ChNC} respectively. Other conditions were kept constant,
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7 including the ionic strength and acidity, which were
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9 standardized to 100 μmol/g_{ChNC} by dialysis against HCl.

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12 As expected, we observed that tip sonication treatment reduced
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14 the dimensions and polydispersity of ChNCs (Figure 3A-C;
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16 Figure S10). The decrease in turbidity (Figure S1B) and Z-
17
18 average size (Table 1) both provide a robust confirmation that
19
20 the average ChNC dimensions decreased with tip sonication
21
22 energy. More precisely, TEM measurements indicate that Ts4 has
23
24 both the longest and thickest ChNCs when compared to Ts20 and
25
26 Ts100 (see *L*, *D* and *L* × *D* in Figure S10, S12 and Table S2). The
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28 latter two samples are very similar except that Ts100 has a
29
30 slightly lower polydispersity as seen from the shorter tails in
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32 the length and width distributions in the TEM histograms. Tip
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34 sonication, as expected, had no effect on the surface charge as
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36 measured by conductometric titration (Table 1).

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39 The phase diagrams of these ChNC suspensions are presented in
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41 Figure 3D. The first and second threshold concentrations, *c*₁ and
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43 *c*_A, are comparable for all three samples, in agreement with
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45 little variations in the aspect ratio and the surface charge
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47 (Figure 3C; Table 1). However, the chiral nematic pitch varies
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49 more significantly (Figure 3E) - samples Ts20 and Ts100 present
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51 a larger pitch at lower ChNC concentrations and, importantly,
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3 show a steeper decrease in pitch with increasing ChNC
4 concentration when compared to Ts4. The suspensions were
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6 dialyzed together after the tip sonication treatment to rule out
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8 the potential effect of ionic strength difference, which was
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10 indicated for a similar system of cellulose nanocrystals.²⁶ A
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12 more likely explanation would be that tip sonication breaks
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14 apart ChNC bundles with a strong chiral shape, the presence of
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16 which would provide a higher twisting power in the anisotropic
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18 suspension. As such, breaking these "colloidal chiral dopants"
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20 into smaller rod-like units should lead to a smaller twist
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22 between ChNCs and thus a larger pitch.²
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28 Notably, the sample prior to any tip sonication treatment did
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30 not display any sign of poor stability (no sedimentation
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32 occurred), however, the suspension appeared white due to the
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34 scattering of larger bundles. As such tip sonication was always
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36 applied to consistently disperse the particles prior to studying
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38 the liquid crystalline behavior of the system. This indicates
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40 that the tip sonication treatment investigated here is primarily
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42 breaking already colloidally stable ChNCs into smaller units,
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44 and that most of the loose bundles were already broken apart
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46 under Ts20, while Ts100 provided only marginal changes. However,
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48 Ts100 was chosen for further investigations as it results in
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50 slightly lower polydispersity (i.e. shorter tail in TEM
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Table 1. A summary of the conditions used to prepare ChNC suspensions and their resultant colloidal properties.

	[HCl] / M	Time / min	Tip sonication energy ^a	TEM length mean / nm	TEM width mean / nm	Z-average / nm	ζ-potential / mV	n(NH ₂) ^b	Yield / %
3M90	3	90	20	286 ± 192	28 ± 23	277 ± 112	39 ± 5	120	88
3M270	3	270	20	184 ± 98	14 ± 9	237 ± 101	45 ± 9	233	79
3M540 [†]	3	540	20	176 ± 95	12 ± 8	210 ± 85	55 ± 9	248	70
5M90	5	90	20	182 ± 87	13 ± 14	241 ± 95	50 ± 7	144	75
5M180	5	180	20	133 ± 66	10 ± 5	212 ± 94	48 ± 14	182	65
Ts4	3	540	4	211 ± 131	18 ± 14	291 ± 115	54 ± 12	248	-
Ts20 [†]	3	540	20	189 ± 99	13 ± 8	195 ± 115	56 ± 12	246	-
Ts100 [†]	3	540	100	166 ± 88	12 ± 7	175 ± 67	59 ± 13	247	-
Ac [†]	3	540	100	178 ± 88	18 ± 7	173 ± 73	57 ± 13	269	-
deAc	3	540	100	153 ± 78	12 ± 6	137 ± 58	56 ± 10	289	

^a Expressed in kJ per g_{ChNC}

^b Expressed in mmol (NH₂) per kg_{ChNC}

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3 Samples produced using comparable conditions are paired with †
4 and ‡ symbols (column 1).
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8 distribution data, Figure 3A,B; Table 1). It should also be
9 noted that the amount of tip sonication energy required to
10 achieve results analogous to Ts20 might differ depending on the
11 hydrolysis conditions.
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20 **Degree of acetylation.** Chitin is commonly treated with a strong
21 base, such as sodium hydroxide, to obtain chitosan - a fully
22 deacetylated form of chitin.²⁷ A controlled treatment can also
23 lead to a desired degree of acetylation (DA), which is defined
24 as the percentage of amide groups with respect to sum of all the
25 amine and amide groups. Thus, deacetylation offers a route to
26 control the potential maximum surface charge on ChNC and thus
27 their self-assembly behavior. In fact, it has been previously
28 reported that the DA of ChNC decreases when they are prepared
29 from deacetylated chitin.⁷ However, contrary to this finding, we
30 observed that the surface charge of ChNC prepared from
31 deacetylated chitin is rather unaffected and the differences in
32 ChNC self-assembly behavior arise instead from changes in ChNC
33 dimensions - a point which was not addressed in the previous
34 work.⁷
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3 The effects of the degree of acetylation were studied by
4 preparing two chitin samples: purified chitin (Ac-Chitin) and
5 deacetylated purified chitin (dAc-Chitin). The degree of
6 acetylation and surface charge were assessed by three methods:
7 solid-state NMR spectroscopy (ssNMR), which measures both the
8 core and the surface of the chitin nanocrystals, as well as
9 conductometric titration and ζ -potential measurements which
10 probe only the surface of the chitin nanocrystal. ssNMR
11 indicated that the deacetylation of purified chitin was
12 successful, with DA decreasing from 97% (Ac-Chitin) to 89 %
13 (deAc-Chitin). However, the produced Ac-ChNC and deAc-ChNC both
14 had a DA \approx 98 % after hydrolysis, similar to that recorded for
15 Ac-Chitin (Figure S4, Table S1). Furthermore, ζ -potential and
16 conductometric titration measurements support the fact that
17 deAc-ChNC and Ac-ChNC have almost identical surface charge
18 (Table 1).

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Chitin is insoluble in aqueous media and thus the surface of
the microfibers is readily available for deacetylation, whereas
the core would be largely unaffected. However, deacetylated
chitin is much more soluble in the acidic conditions present
during hydrolysis, allowing the deacetylated surface to be
easily removed, which would reduce the widths of the resulting
ChNCs. In contrast, the same treatment on less soluble Ac-Chitin

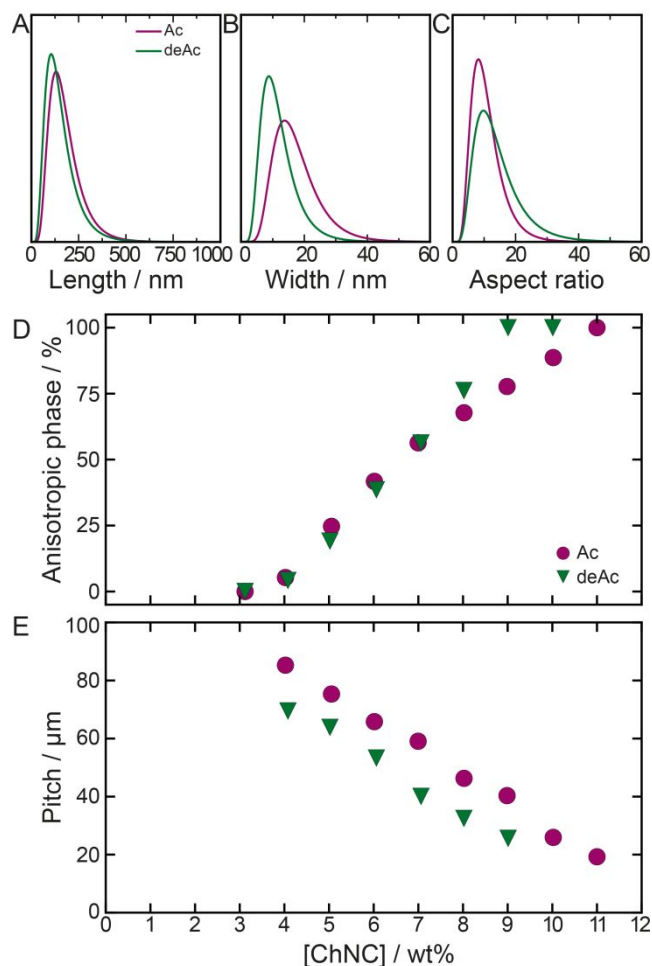


Figure 4. Effect of degree of acetylation on ChNC dimensions and self-assembly. (A-C) The fitted lognormal distribution curves from TEM measurements of ChNC (A) length, (B) width and (C) aspect ratio (Figure S11 provides full histograms). Plots showing (D) the increasing proportion of anisotropic phase and (E) decreasing chiral nematic pitch upon increasing ChNC concentration.

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3 would not lead to an identical etching. The surface charge on
4 both ChNC suspensions was found to be almost equivalent,
5 suggesting that most of the surface which was affected by sodium
6 hydroxide deacetylation, as in deAc-Chitin, was exfoliated
7 during the hydrolysis step. Thus, the apparent increase in the
8 DA of deAc-ChNC compared to deAc-Chitin is not because of
9 "reacetylation" but because of the "etching" of the deacetylated
10 surface during hydrolysis. Indeed, length and width histograms
11 from TEM analysis show that deAc-ChNC are shorter and thinner
12 than the Ac-ChNC and have a higher aspect ratio (Figure 4A-C;
13 Figure S11). This hypothesis is also supported by the DLS Z-
14 average size (Table 1) and turbidity values (Figure S1C),
15 indicating smaller ChNCs in the deAc-ChNC sample when compared
16 to Ac-ChNC. The changes in the aspect ratio influence ChNC self-
17 assembly, which results in deAc-ChNC reaching a fully
18 anisotropic phase at a lower concentration than Ac-ChNC (Figure
19 4D). Additionally, the pitch is only slightly smaller for the
20 deAc-ChNC suspension and it comparably decreases with ChNC
21 concentration for both suspensions. These observations are
22 consistent with the surface charge being effectively comparable
23 and the aspect ratio of deAc-ChNC being larger (Figure 4E).

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26 Chitin deacetylation prior to hydrolysis thus provides a way to
27 increase ChNC aspect ratio without a significant change in the
28 surface charge. This characteristic can potentially be repeated
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3 to obtain much thinner crystals and is unique to chitin, whereas
4 the physical aspect ratio of cellulose nanocrystals, as a
5 comparison, can only be addressed by changing the source and the
6 hydrolysis conditions, which provides less flexibility.²⁸
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14 **Ionic strength and pH.** The ChNC surface charge can be modulated
15 by controlling the pH. The surface-bound amine groups become
16 increasingly protonated at pH values below their pKa value.²⁹
17 Therefore, at lower pH, the surface charge is higher, which
18 favors repulsive interactions and thus a larger effective
19 diameter D_{eff} . However, reducing pH causes an increase in the
20 ionic strength of the medium, and consequently decreases the
21 Debye length, which favors a smaller D_{eff} . Therefore, the degree
22 of electrostatic repulsion between ChNCs is dependent on both
23 the explicit acid concentration and the overall ionic strength,
24 which produce opposite effects. Both parameters have to be
25 carefully considered to control their self-assembly while
26 maintaining the colloidal stability of the system.^{8,30}
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44 For the study on the interplay between acidity and ionic
45 strength on the self-assembly of ChNC, a 3M540 Ts100 ChNC
46 suspension was used. Different ionic strengths and acid
47 concentrations were achieved by dialysis against NaCl and HCl
48 solutions followed by a concentration step using a rotary
49 evaporator. Therefore, the ratio between the electrolyte and
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3 ChNC concentrations was maintained which would mimic the ChNC
4 suspension behavior upon drying.
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7 The effects of HCl concentration and total ionic strength can
8 be partly decoupled by preparing a series of ChNC suspensions
9 with increasing HCl concentration while adjusting the total
10 ionic strength to be equivalent with NaCl. As such, three
11 samples were prepared with increasing HCl concentration of 1, 10
12 and 100 $\mu\text{mol}/g_{\text{ChNC}}$, but an equivalent total ionic strength of 100
13 $\mu\text{mol}/g_{\text{ChNC}}$. Therefore, this experimental design allows an increase
14 ChNC surface charge when HCl concentration is raised, resulting
15 in a larger effective diameter D_{eff} . As a consequence, the
16 effective aspect ratio is reduced, and a higher threshold
17 concentration is expected. This agrees well with the
18 observations of the second threshold concentration shifting to
19 higher ChNC concentrations with increasing HCl concentration
20 (Figure 5A). These changes are only limited by complete
21 protonation of the surface amines.
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24 The increase in HCl concentration also led to higher chiral
25 nematic pitch values (Figure 5C), since larger D_{eff} results in the
26 ChNC to be spaced further apart and thus weakening any chiral
27 interactions.³¹ However, when considering these results, it is
28 important to consider that the surface charge rises non-linearly
29 with increasing HCl concentration resulting in the observed
30 liquid crystalline behavior (Figure 5A,C).
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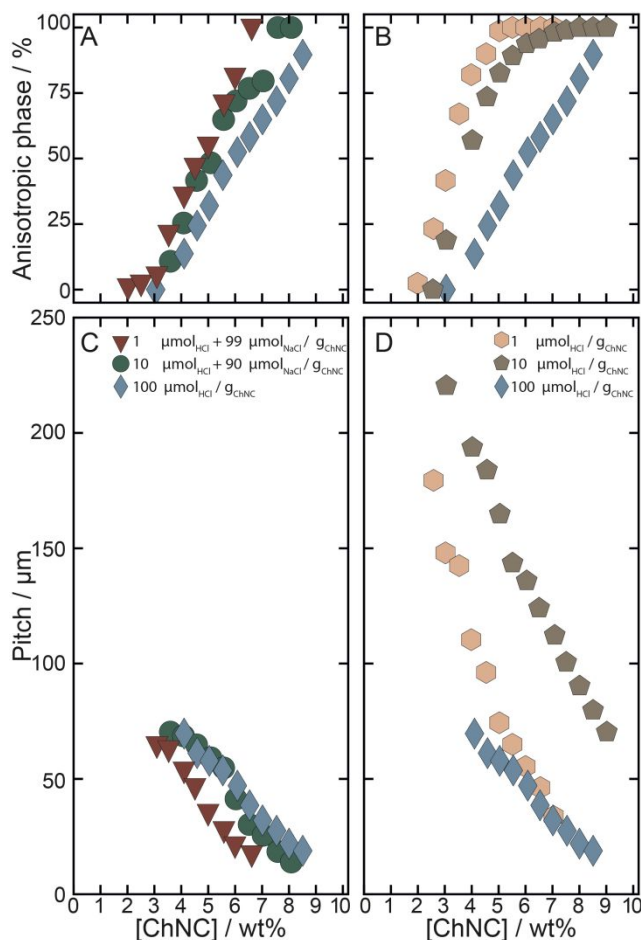


Figure 5. Effect of acidity and ionic strength on ChNC self-assembly. (A, B) Phase diagrams and (C, D) corresponding pitch diagrams showing the ChNC dependence with varying HCl concentration from 1-100 $\mu\text{mol/gChNC}$, with the total ionic strength (A, C) standardized to 100 $\mu\text{mol/gChNC}$ with NaCl or (B, D) uncorrected.

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3 The influence of HCl concentration was also tested without
4 compensating for changes in the ionic strength. Therefore, the
5 D_{eff} would respond to the increase of both the surface charge and
6 total ionic strength. In this case, three samples with HCl
7 concentration of 1, 10, and 100 $\mu\text{mol/g}_{\text{ChNC}}$ were prepared and so
8 the total ionic strength is also different in each sample. The
9 phase diagrams indicate that the first and second threshold
10 concentrations increased with the total HCl concentration
11 (Figure 5B). This can be explained by a higher ionic strength
12 leading to a smaller Debye length and D_{eff} , in agreement with Eq.
13 (1). The deviation from a linear trend when approaching the
14 second threshold concentration is observed at the two lower HCl
15 concentrations and it is possible that the chitin nanocrystal
16 contribution becomes non-negligible at these relatively low
17 ionic strength conditions.

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37 The chiral nematic pitch reported in Figure 5D shows more
38 dramatic variations. Increasing the ionic strength from 1 to 10
39 $\mu\text{mol/g}_{\text{ChNC}}$ resulted in a larger pitch but with a further increase
40 from 10 to 100 $\mu\text{mol/g}_{\text{ChNC}}$ the pitch substantially dropped. The
41 initial increase of the pitch corresponds to the increase of the
42 surface charge of the ChNCs as more amine groups are protonated,
43 while the ionic strength remains very low (at 10 $\mu\text{mol/g}_{\text{ChNC}}$ and
44 10 wt% ChNCs, $I = 1 \text{ mM}$). Thus, the increase in the surface
45 charge had a dominant effect, compared to the increase in the
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3 ionic strength. This led to ChNC being spaced apart more and
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5 thus weakening their chiral interactions, ultimately producing
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7 bigger chiral nematic pitch. However, when the amount of HCl was
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9 elevated from 10 to 100 $\mu\text{mol/g}_{\text{ChNC}}$, the surface charge of the
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11 ChNCs only slightly increased, thus the increase in the ionic
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13 strength became the dominant factor. Therefore, the Debye length
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15 strongly shrank (e.g., from ~ 9.6 nm at 1 mM to ~ 3.0 nm at
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17 10 mM). This caused a strong decrease in D_{eff} so that the ChNCs
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19 could come in closer proximity and so strengthening their chiral
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21 interactions.
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26 **Solid films.** The chiral nematic order can be preserved in the
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28 solid-state by evaporating the ChNC suspensions, giving rise to
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30 transparent, nanostructured films (Figure 1E). Cross-sectional
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32 SEM analysis revealed a well-ordered helicoidal structure with
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34 characteristic Bouligand arches (Figure 6A, 6B).³²
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37 The pitch of the helicoidal structures in dry films is
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39 determined by two processes: the self-assembly of ChNC in the
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41 chiral nematic phase, and the distortion experienced upon
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43 further drying after the onset of the kinetic arrest.^{2,33,34} The
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45 former depends primarily on the ChNC concentration and the
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47 parameters explored above, whereas the factors affecting the
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49 onset of the kinetic arrest are less clear. Nevertheless, at a
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51 given ChNC concentration, the system will turn from liquid-like
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53 to solid-like, preventing ChNC rods from rearranging
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3 collectively.² Past this point, further water evaporation leads
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5 to a vertical compression of the suspension³⁵, forcing the ChNCs
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7 to get closer and reducing drastically the pitch. Since both the
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9 ionic strength and the pH influence the pitch before the kinetic
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11 arrest as well as the onset of the kinetic arrest itself, they
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13 represent the two main parameters controlling the final pitch of
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15 the film. Combining various pH and ionic strengths, it is
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17 possible to produce ChNC films with a helicoidal arrangement and
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19 pitch values ranging from 650 nm to 3720 nm. Interestingly, ChNC
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21 suspensions could even be dried in a few hours at 90 °C and
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23 still yield films with a well ordered helicoidal structure
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28 (Figure S10).

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30 The correspondence between the pitch in ChNC suspensions and in
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32 dry films is presented in a log-log plot (Figure 6C).³³ The pitch
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34 in the liquid phase is measured from ChNC suspension in
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36 capillaries (*cf.* Figure 5) while the solid state (*i.e.* 100 wt%
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38 ChNC concentration) is measured by cross-sectional SEM analysis
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40 of dry films. This plot shows that in general the ChNC
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42 suspensions that exhibit the largest pitch in solution
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44 correspond to the largest pitch in the solid state. However, to
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46 have full control over the pitch in the dry film, a better
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48 understanding of the onset of the kinetic arrest should be
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50 investigated in the future.
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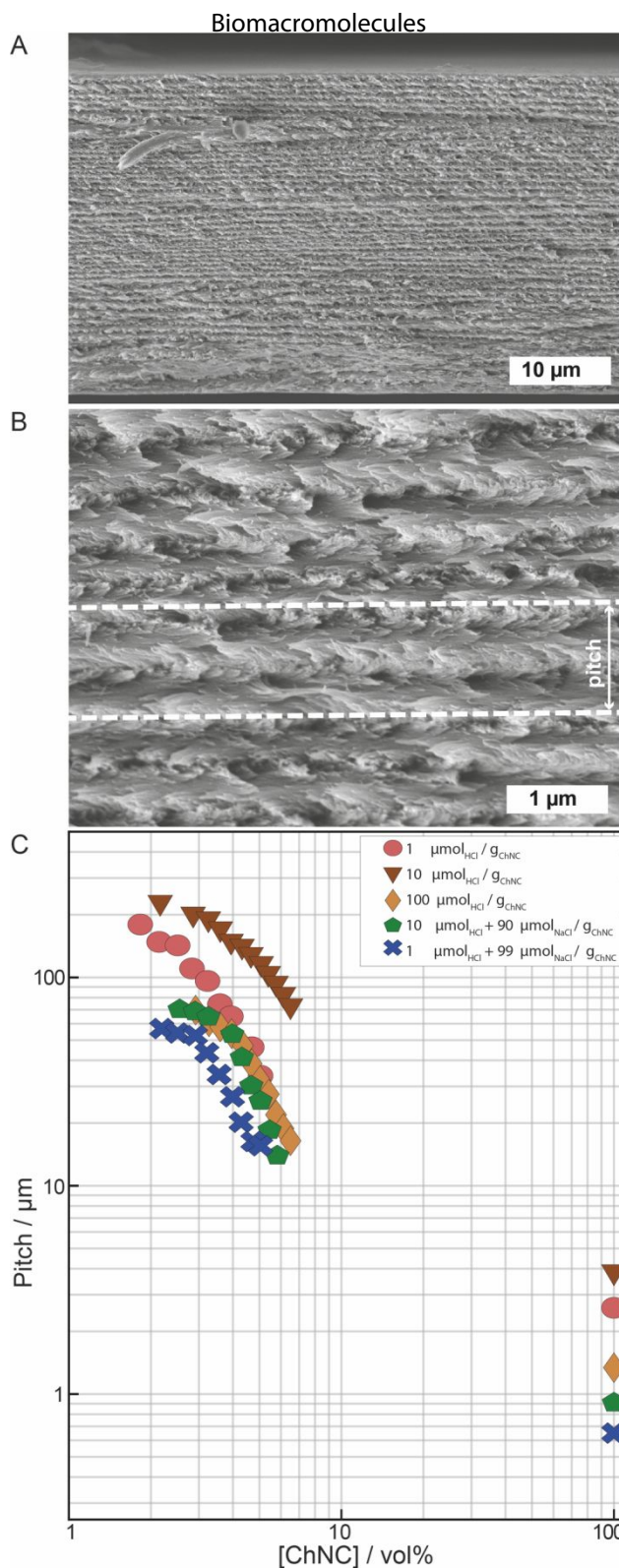


Figure 6. Cross-sectional SEM analysis of ChNC films at different conditions. Films prepared by drying chiral nematic ChNC suspension retain a helicoidal architecture (A), with Bouligand arches clearly seen at higher magnification (B). The chiral nematic pitch in suspension (as measured in capillaries) and in solid state (i.e. 100 wt% ChNC concentration) can be tuned by varying the HCl and NaCl concentration (C).

CONCLUSIONS

Here we demonstrated that a careful investigation of ChNC extraction methods, namely, chitin hydrolysis duration and acidity, tip sonication energy, degree of acetylation, ionic strength and pH led to a better understand of their colloidal properties and in turn allowed for control over their liquid crystalline behavior. Using this understanding, we were able to produce well-ordered helicoidal ChNC films with tunable pitch spanning an order of magnitude.

More specifically, we evaluated how the ChNC colloidal properties are affected by the preparation method and demonstrated that 3.0 M HCl at reflux for 270 min is sufficient to hydrolyze chitin to achieve a stable ChNC suspension, with higher HCl concentrations only accelerating the reaction speed, while longer hydrolysis time, as in 3M540, yielded smaller, more charged and less polydisperse particles. Additionally, we addressed the effects of tip sonication energy, which assists in dispersing ChNC in a controlled manner and showed influence on the chiral nematic phase. The degree of acetylation prior to hydrolysis proved to be a way to reduce ChNC width rather than increase the surface charge, while the ionic strength and pH allowed to finely tune ChNC self-assembly reaching chiral nematic pitch values as high as 250 μm . This understanding led to a controlled production of dried ChNC films that retained a

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3 helicoidal nanostructure. The pitch of dry helicoidal ChNC films
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5 can be tuned independently from the preparation conditions, *via*
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7 ionic strength or pH.
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10 In conclusion, we believe that this comprehensive study with
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12 detailed information on the characterization and preparation of
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14 ChNC will serve well in promoting this exciting field and thus
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16 unlock the potential of chitin as a truly valuable colloidal
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18 system, due to its abundance and renewability as well as for its
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20 fascinating surface chemistry and liquid crystalline behavior.
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26 **ASSOCIATED CONTENT**

27 28 29 **SUPPORTING INFORMATION**

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31 The Supporting Information is available free of charge on the
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33 ACS Publications website.
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36 An extensive description of all the experimental methods.
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38 Supplementary results: FT-IR, ssNMR, PXRD data showing chemical
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40 composition; conductometric titration, UV-Vis spectroscopy,
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42 extensive TEM data analysis supporting claims about ChNC
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44 colloidal properties; SEM images of ChNC suspensions dried at
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46 elevated temperature (Supporting_Information.pdf).
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51 Additional data relating to this publication is available from
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53 the University of Cambridge data repository
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55 (<https://doi.org/10.17863/CAM.40425>).
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Notes

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