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Supporting Information

High-Humidity Shaker Aging to Access Chitin and Cellulose Nanocrystals

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Materials

PG chitin, microcrystalline cellulose (MCC), ammonium persulfate (APS), butylated hydroxytoluene (BHT), hydrochloric acid, sodium hydroxide and potassium sulfate were all purchased from Sigma Aldrich (St. Louis, MO).

Methods

Charaterization

Fourier transform infrared (FTIR) spectra were collected from 4000 to 400 cm^{-1} for 100 scans at a resolution of 4 cm^{-1} using a Bruker Tensor 27 FTIR spectrometer. Samples were run in the ATR mode over a ZnSe crystal.

X-ray diffraction spectra were acquired using a Bruker D8 Advance X-ray diffractometer equipped with a CuKα filament, scanned with a 2θ range between 5-60° with an increment of 0.02°.

The crystallinity index was calculated using the DiffractSuite Software by Bruker after removing the low-angle signal produced from the zero-background sample holder. Measurements were taken from a 2θ range of 5° to 50°.

Transmission electron microscopy (TEM) micrographs were taken on a Thermo Scientific Talos F200X G2 S/TEM equipped with a Ceta 16M CMOS camera. Sample preparation for TEM were done on freshly glow-discharged grids using EMS GloQube-D, Dual chamber glow discharge system (Electron Microscopy Sciences, PA) operating in negative mode and applying the plasma current of 25 mA during 45 s. Then, 1 mg/mL suspensions of samples were pipetted onto the TEM grids and subsequently dried.

Atomic force microscopy (AFM) images were taken on a Nanoscope IV (Digital Instruments, Veeco, Santa Barbara, CA) with a silicon tip operated in tapping mode. Particle analysis of the micrographs was performed using Scion Image (http://www.scioncorp.com/pages/scion_image_windows.htm). The width of chitin nanocrystals (ChNCs) and cellulose nanocrystal (CNCs) were estimated as the height of AFM micrographs.

Dynamic light scattering and the zeta potential of the samples (2 mg/mL) in water were determined using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) in triplicate. PDI is the standard deviation (or half-width max) compared to the mean:

$$PDI = \left(\frac{std.dev.}{mean}\right)^2$$

Thus, for colloidal or nanomaterial suspensions, a ‘perfect’ monodisperse solution would be close to 0, with 0.1 – 0.4 PDI being a generally monodisperse suspension with little to no aggregation.

Conductometric titrations were conducted to determine the carboxylic acid content of the CNCs. Briefly, ChNCs (50 mg) were suspended in 10 mL of 0.01 M HCl and sonicated for 2 min to

disperse the nanocrystals. The ChNC suspensions were then titrated against 0.01 M NaOH and DO is calculated using the following equation:

$$DO = \frac{203(V_2 - V_1)C}{w - 36(V_2 - V_1)C}$$

where *C* is the NaOH concentration (M), *w* is the weight of ChNC sample (g), and V_2 and V_1 are the volumes of NaOH (L). The molecular weight of an N-acetylglucosamine unit is 203. The molecular weight difference between an N-acetylglucosamine unit and its COONa derivative is 36.

The equation for determining cellulose DO is[1]:

$$DO = \frac{162(V_2 - V_1)C}{w - 36(V_2 - V_1)C}$$

Solid-state ^{13}C NMR spectra were collected on a Varian 400 MHz VNMRS widebore spectrometer operating at a 1H frequency of 399.76 MHz with a 4 mm double-resonance probe spinning at 13 kHz. The 13C multiple-cross polarization (multiCP) Experiments were performed using similar conditions described previously to measure the purity of chitin. Initially, standard ^{13}C CP experiments were acquired on a sample of ChNC to assess the CP build-up properties. Each point of the build-up curve used a 4.13 μs π/2 on ^{1}H with a ramped Hartman-Hahn match on 13C and TPPM ^{1}H decoupling ($\gamma B1/2\pi$=61 kHz). Each data point was acquired using a 3 s recycle delay, 512 co-added transients and contact times varying between 0.06 - 8 ms. The optimized contact time was determined to be 1 ms and was used for the $^{13}C\{1H\}$ multiCP experiments. The number of CP loops used in the $^{13}C\{^{1}H\}$ multiCP experiment was optimized and set to a value of 8 loops with a 1 s repolarization period for all subsequent samples. The π-pulse (echo) performed at the end of the ^{13}C sequence had a pulse length of 8 μs. All other parameters were analogous to the CP set up experiment. A total of 640 scans (~2 h) were acquired for each $^{13}C\{^{1}H\}$ multiCP experiment on the ChNCs. Samples were ground and placed into 4 mm o.d. zirconia rotors and sealed using Kel-F® drive caps. All ^{13}C NMR spectra were referenced to the C=O of α-glycine at 175.7 ppm and a MAS frequency of 11 kHz was employed throughout.

The thermal stability of the samples was tested using a Mettler-Toledo STARe DSC/TGA. Approximately 5 mg of each sample was analysed in air through a temperature range of 30 to 500 ˚C at a ramp rate of 10˚C/min using alumina crucibles. The difference in mass was then calculated using STARe Evaluation software to determine the total mass loss during the temperature ramp, as well as the inflection temperature of the sample.

Ball milling experiments

Initial mechanochemistry conducted by ball-milling were conducted by a Retsch MM 400 mixer mill, with jars made of polytetrafluoroethylene (PTFE 20 mL), equipped with 1 ball of zirconia.

Standard Accelerated aging experiments

For a standard accelerated aging experiment, controlled humidity chambers were used. The chamber consisted of a standard Tupperware container. Within the chamber resides a glass dish filled with 20 mL of a saturated aqueous solutions of K_2SO_4, to access 98% relative humidity (RH) within the chamber, confirmed by a digital hygrometer (ThermoPro TP50). A 50 mL beaker, containing the solid-state sample of mixed biomass and ammonium persulfate, was placed inside the chamber. The overall chamber could be heated by placing in a Fisher Scientific Isotemp oven.

For a standard experiment to access ChNCs from PG chitin, 300 mg of PG chitin and 4 mass equivalents (1.2 g) of APS were mixed by either an agate mortar and pestle (3 min) or ball mill (30 min, 29.5 Hz). The solid mixture was then placed within the 50 mL beaker inside the controlled humidity chambers and aged for 3 days at 50 °C and 98 % RH. CNC from MCC were also accessed in this way. Raw biomass samples such as green crab shells or commercial Kraft soft wood pulp derived from red pine and spruce were also accessed with similar masses (300 mg of biomass).

High-Humidity Shaker Aging experiments, purification, and yield collection

For a standard shaker aging experiment, a shaker incubator (Innova 4080 incubator shaker) was employed. The relative humidity chamber consisted of a standard Tupperware container that was placed inside the shaking incubator. Within the chamber resides a glass dish filled with 20 mL of a saturated aqueous solutions of K_2SO_4, to access 98% relative humidity (RH) within the chamber, confirmed by a digital hygrometer (ThermoPro TP50). A 50 mL beaker, containing the solid-state sample of mixed 300 mg biomass and 1.2 g APS, was placed inside the chamber. This preparation is the same for all PG chitin, microcrystalline cellulose, soft wood pulp, and green crab shells. The shaking incubator was shaken at 150 rpm for the prescribed amount of aging time and 50 °C. Yields are found within Table S1. The yields are calculated relative to the starting raw biomass material. A picture of the set up is below:

All samples underwent the same cleaning procedure. DI water was used to dilute the solid mixture and transferred into a Vulcan 50 mL centrifuge tube. The suspension was homogenized with mild agitation *via* ultrasonication for 30 s at 20 % amplitude between centrifugation cycles. The suspension was then centrifuged at 12 000 rpm for 10 min and the supernatant was decanted. This process was repeated 2 times to ensure all reagent and potentially solubilized chitin oligomers are washed off. After the final centrifugation cycle, the sample was ultrasonicated a final time of 30 s at 20 % amplitude. The product was then lyophilized to obtain the yield. A control experiment was conducted in which no aging and milling was done on a mixture of 1.2 g APS and 0.3 g chitin in 40 mL water, to which was ultrasonicated for 3 min at 20 % amplitude. Negligible liberation of nanocrystals was observed.

Negative Control experiments

Listed below are two pathways that are proven to happen with APS in *aqueous solution* under heating:

$$S_2O_8{}^{2-} + heat \rightarrow 2SO_4{}^{\cdot -} \quad (1)$$

$$SO_4{}^{\cdot -} + H_2O + heat \rightarrow HSO_4{}^{-} + OH^{\cdot} \quad (2)$$

Over 50°C, persulfates decompose into sulfate-free radicals ($SO_4^{\bullet -}$) which can penetrate the amorphous regions in chitin and hydrolyze the β-1,4 glycosidic linkages of the polysaccharide backbone (eq. 1).[2] $SO_4^{\bullet -}$ also oxidizes the C6 primary hydroxyl into an aldehyde, while the $OH^{\bullet -}$ formed under rapid decomposition of the sulfate-free radical can further oxidize the aldehyde into the carboxylic acid species (eq. 2).[3] We believe this mechanism to also exist under our HHSA conditions as well.

This is not the first time APS has been used as an oxidant in the solid phase. For example, Fukuoka and coworkers used APS for the solid-state oxidation of steam activated carbon using a planetary mill with promising effect, with an increase in oxygen content as confirmed by elemental analysis as compared to other solid oxidants such as potassium peroxymonosulfate (oxone).[4]

a. Control experiment with BHT as a radical scavenger

For a standard experiment to access ChNCs from PG chitin, 300 mg of PG chitin, 4 mass equivalents (1.2 g) of APS and 300 mg of BHT were mixed by an agate mortar and pestle (3 min). The solid mixture was then placed within the 50 mL beaker inside the controlled humidity chambers and aged for 3 days at 50 °C and 98 % RH. Cleaning was performed with the procedure above.

The subsequent product showed no signs of reactivity through qualitative observation of the suspension itself after allowing to settle overnight, which displayed no colloidal suspension.

b. Control experiment with initial addition of water

300 mg of PG chitin and 4 mass equivalents (1.2 g) of APS were mixed by an agate mortar and pestle (3 min). The solid mixture was then placed within a 50 mL round bottom flask along with

1.5 mL of DI water and a stir bar. The mixture was stirred mildly (~100 rpm) and heated in an oil bath at 50 °C for 3 days under a condenser. Cleaning was performed with the procedure above.

The subsequent product showed no signs of reactivity through qualitative observation of the suspension itself after allowing to settle overnight, which displayed no colloidal suspension.

Moisture content monitoring during accelerated aging

A standard shaker aging experiment was followed like what is described above however at allotted times, 20-25 mg of the solid mixture was taken out and immediately analyzed *via* TGA to measure the water content.

Ball milling with liquid-assisted grinding (LAG)-inspired water additive and aging

Ball-milling was conducted with a Retsch MM 400 mixer mill, with jars made of polytetrafluoroethylene (PTFE 20 mL), equipped with 1 ball of zirconia for 30 min. The same amounts of chitin and APS were used (300 mg chitin, 1.2 g APS) and supplemented with DI water to reach the specific η value. For η = 0.2, 0.3 mL of DI water was added. For η = 0.4, 0.6 mL of DI water was added. After ball milling, the jars were sealed entirely with parafilm and electrical tape before being placed in the shaker incubator at 50 °C and shaking at 150 rpm. The relative humidity was not adjusted. This is to ensure to the best of our capabilities that the water wt. % content does not change over the course of 3 days.

Process Mass intensity (PMI) calculation comparing the synthesis of carboxylated CNCs using HHSA vs. solution

The standard PMI equation is used:

$$PMI = \frac{\sum Mass\ of\ reagants\ in\ the\ process}{Product\ yield}$$

For the solution-based APS method,[1] a 1:123 w:w ratio of microcrystalline cellulose: 1 M aqueous APS is used. With a product yield of 61 %, and using 1 g of starting cellulose, the PMI is (not including purification):

$$PMI = \frac{1\ g\ (cellulose) + 123\ g\ (water + APS)}{0.61} = 203$$

For the HHSA method, the CNC yield was 60 %, using 1 g of starting cellulose:

$$PMI = \frac{1\ g\ (cellulose) + 4\ g\ (APS) + 2.5\ g\ (deliquescence)}{0.60} = 12.5$$

Supplemental Figures

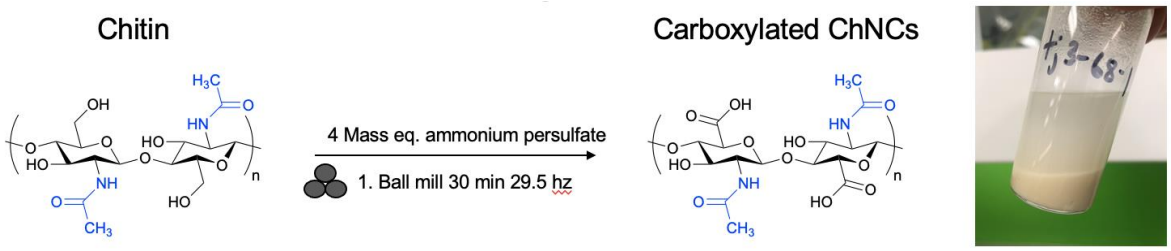

Figure S1: Standard ball milling experiment with 1 ZrO_2 ball. Photograph depicts what the suspension of the cleaned product looks like after leaving overnight.

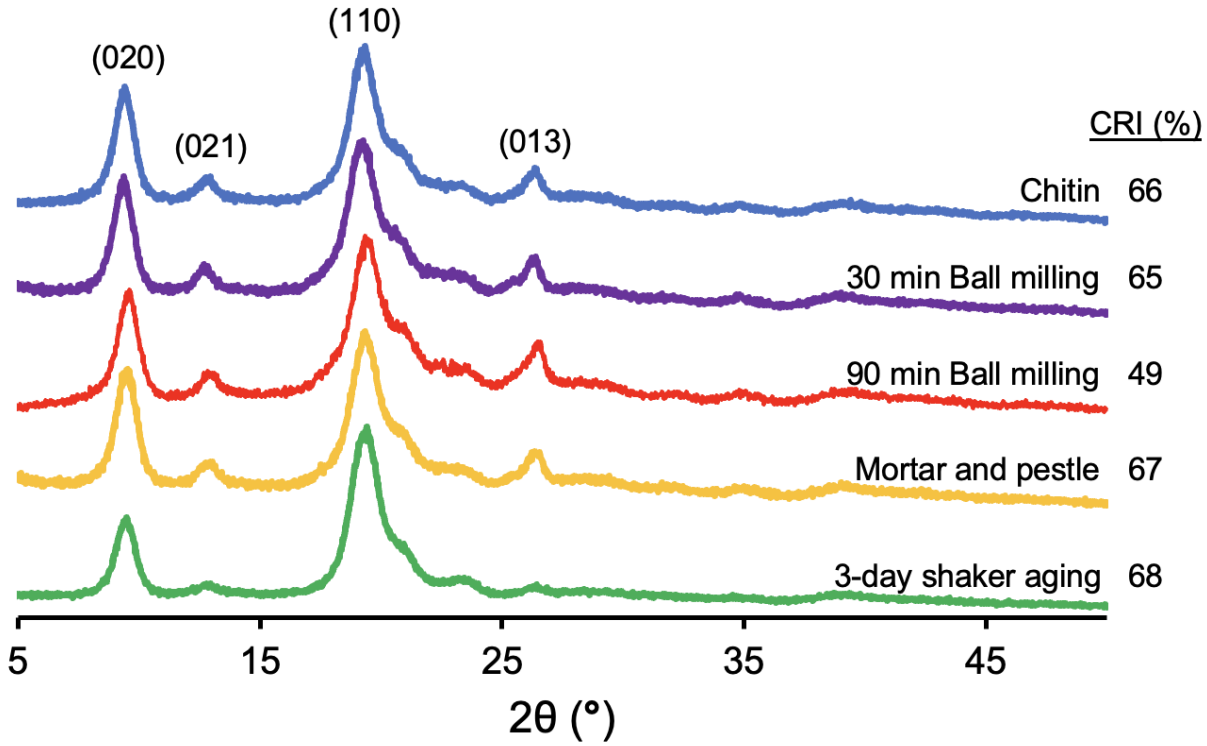

Figure S2: X-ray diffractograms of the starting chitin material compared to various mechanochemical treatment methods and shaker-aging technique and the major reflections for α-chitin: (020), (021), (110), and (013) at 9.5°, 13.4°, 19.6°, and 26.7°. All the samples were conducted with APS at 4 mass eq. like the standard aging experiment. Mortar and pestle sample was done by hand (TJ) for 3 min. Interestingly, 30 min of ball milling did not adversely affect the chitin CrI value of 66 %, presumably due to the abundance of APS acting as a solid lubricant for chitin. Comparing this to 90 min ball milling, the CrI value decreases to 49 %, outlining the harsh treatment amorphizing the chitin.

Table S1: Dynamic light scattering (DLS) and degree of oxidation (DO) measurements of ChNC and CNC suspensions prepared with various methods

Entry	Type	Method	Z-average (nm)	Polydispersity Index	Degree of oxidation (DO)	Yield (%)
1	ChNC	Ball milling + 3 days aging	270.4	0.548	0.20	58
2	ChNC	Mortar and pestle + 3 days aging	250.2	0.541	0.18	56
3	ChNC	HHSA 1 day	267.2	0.436	0.12	70
4	ChNC	HHSA 2 days	159.9	0.235	0.23	63
5	ChNC	HHSA 3 days	156.7	0.268	0.25	60
6	ChNC	HHSA 5 days	197.0	0.225	0.25	45
7	ChNC	LAG aging η = 0.4	736.7	0.285	0.22	61
8	ChNC	LAG aging η = 0.2	274.2	0.268	/	59
9	ChNC	Slurry synthesis	240.0	0.313	/	63
10	CNC from MCC	HHSA 3 days	202.1	0.192	0.20	61
11	CNC from soft wood pulp	HHSA 3 days	278.4	0.321	0.27	48
12	ChNC from green crab shells	HHSA 3 days	380.2	0.413	/	/

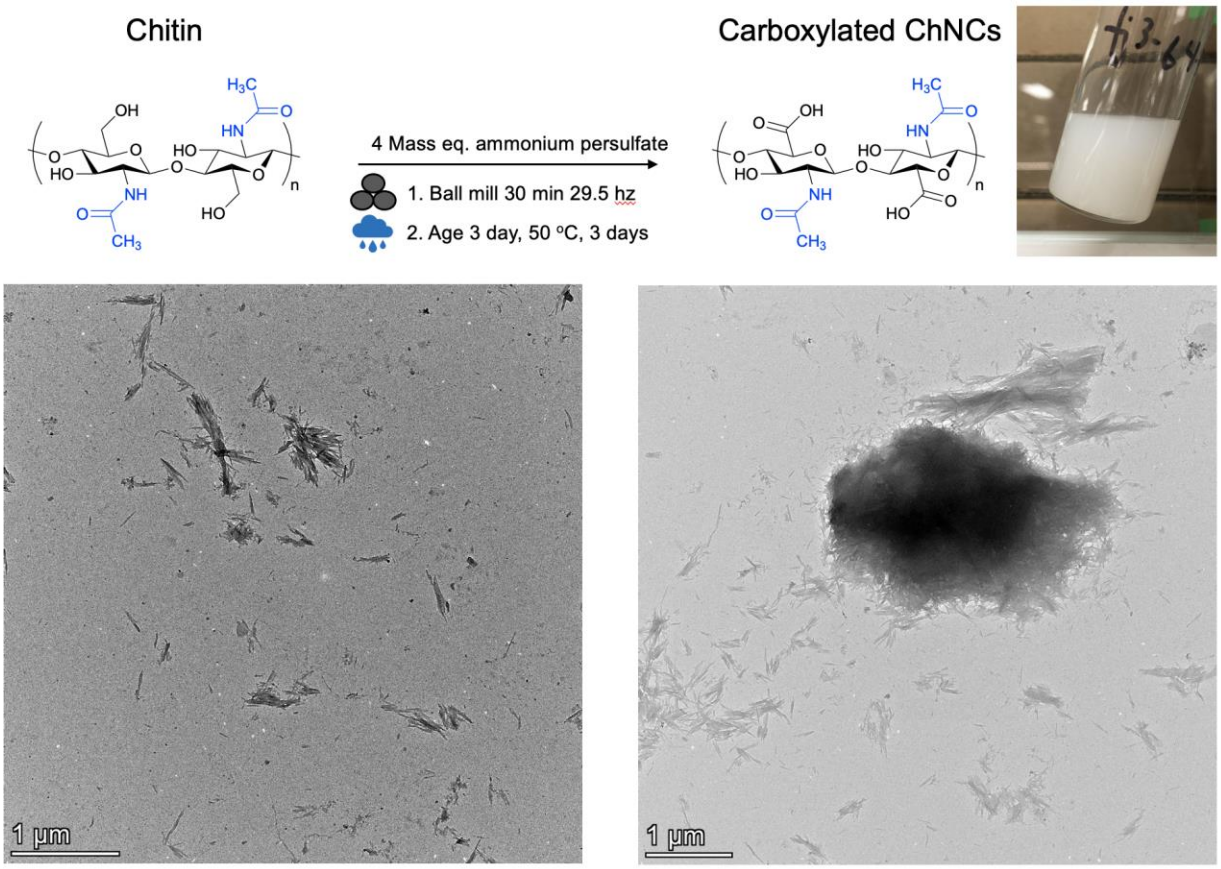

Figure S3: Ball milling blank experiment with 1 ZrO_2 ball followed by subsequent accelerated aging. Photograph depicts what the suspension of the cleaned product looks like after leaving overnight. Below shows 2 TEM micrographs of both individual nanocrystallites as well as large micron-sized aggregates still present after accelerated aging. A further experiment with 6 days of aging time showed similar results. It is to be noted ultrasonication (refer to cleaning procedure in **methods** section) was done prior to make this suspension. DO is 0.20. Yield is 58 %.

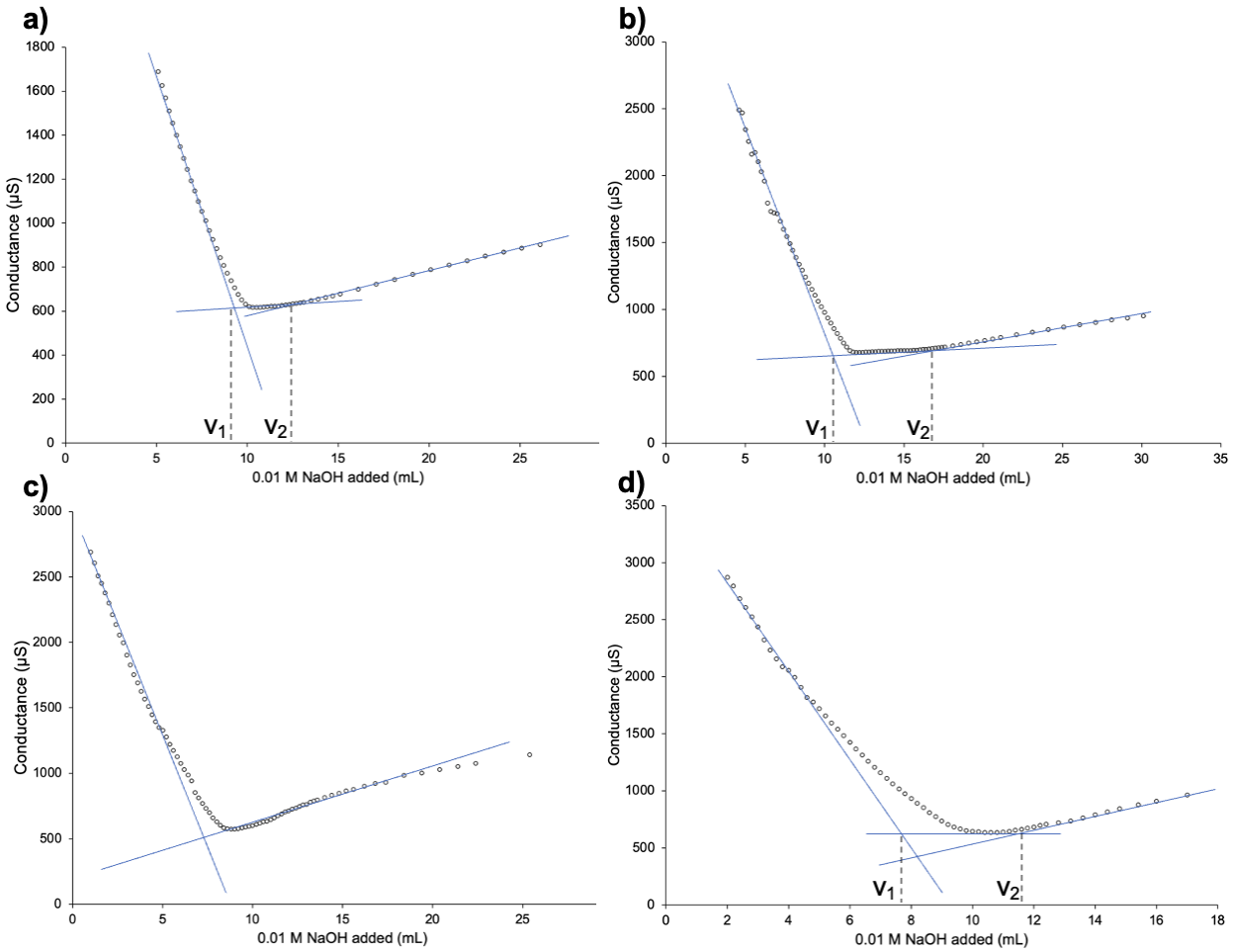

Figure S4: Representative conductometric titration curves of (a) 1-day HHSA ChNCs (b) 3-day HHSA ChNCs, (c) PG chitin and (d) 3-day HHSA CNCs in suspension. It can be seen the PG chitin has minimal weak-acid strong base neutralization effect, demonstrating minimal effect of amines on the conductometric titration and degree of oxidation measurements.

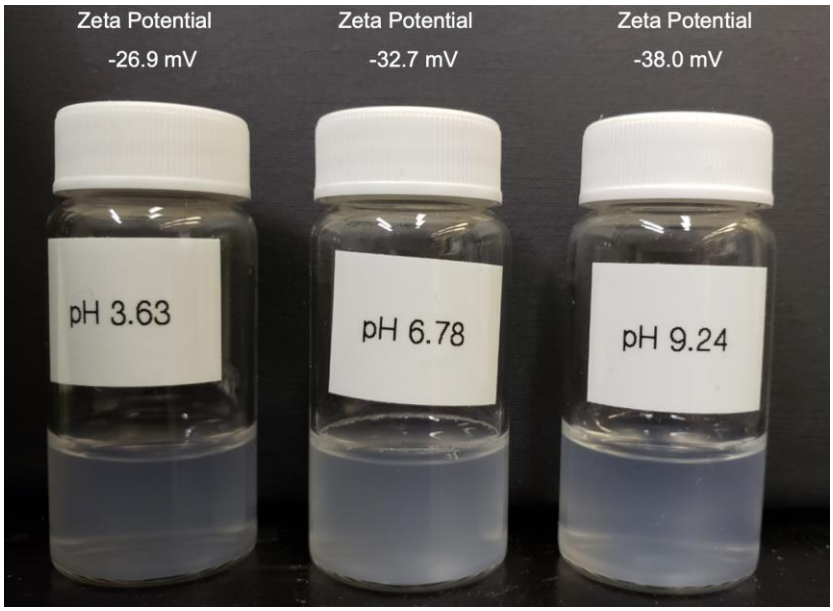

Figure S5: Zeta potential measurements of 2-day HHSA ChNCs and their optical photographs of 0.1 wt. % in aqueous suspension. At all three pH values tested, colloidally stable suspension were produced. Furthermore, as the pH is increased, the zeta potential value increases (negatively), indicating that it is the carboxylate functionality that is dominating the charge density on the ChNCs.

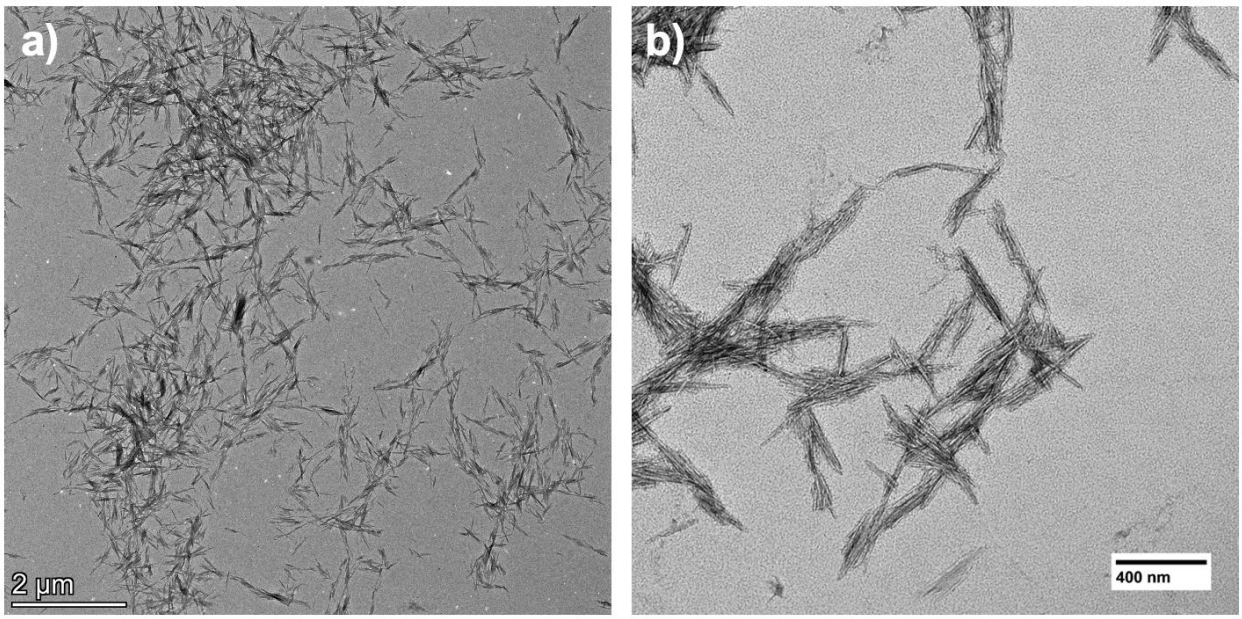

Figure S6: TEM micrograph of a) unstained ChNCs and b) stained ChNCs prepared using optimized 3-day HHSA. A 1 % uranyl acetate stain was used in the sample preparation.

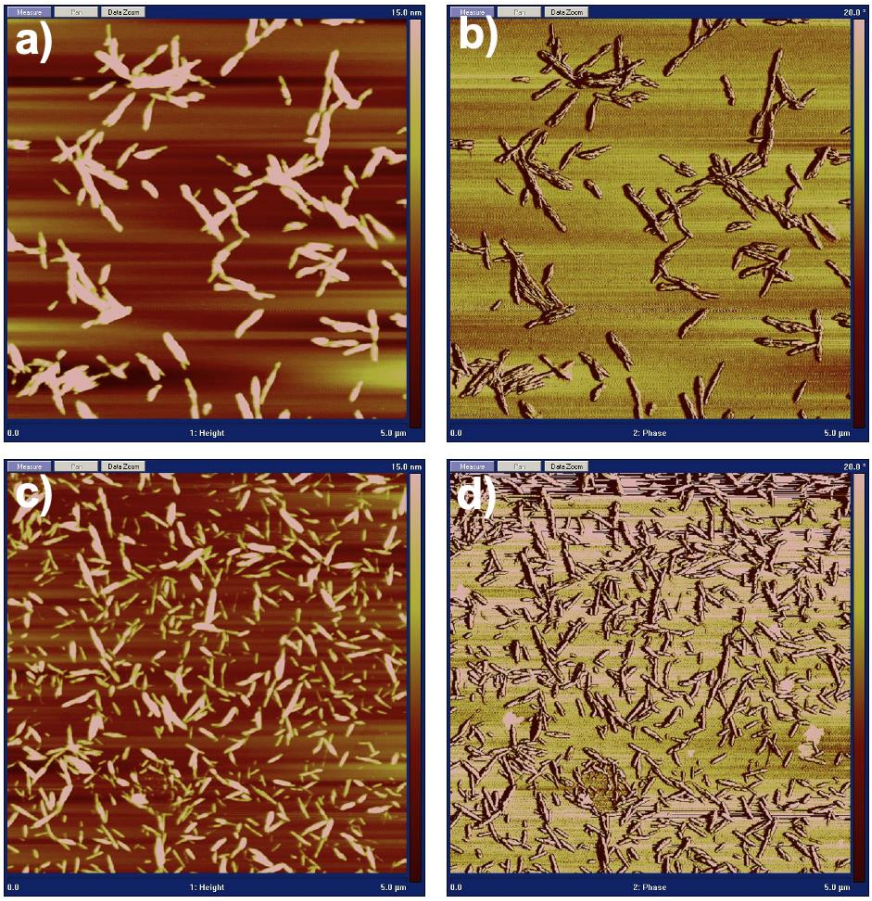

Figure S7: Atomic force microscopy images of the (a) ChNC height profile and (b) ChNC phase profile, as well as (c) CNC height profile and (d) CNC phase profile after performing the optimized 3-day HHSA procedure.

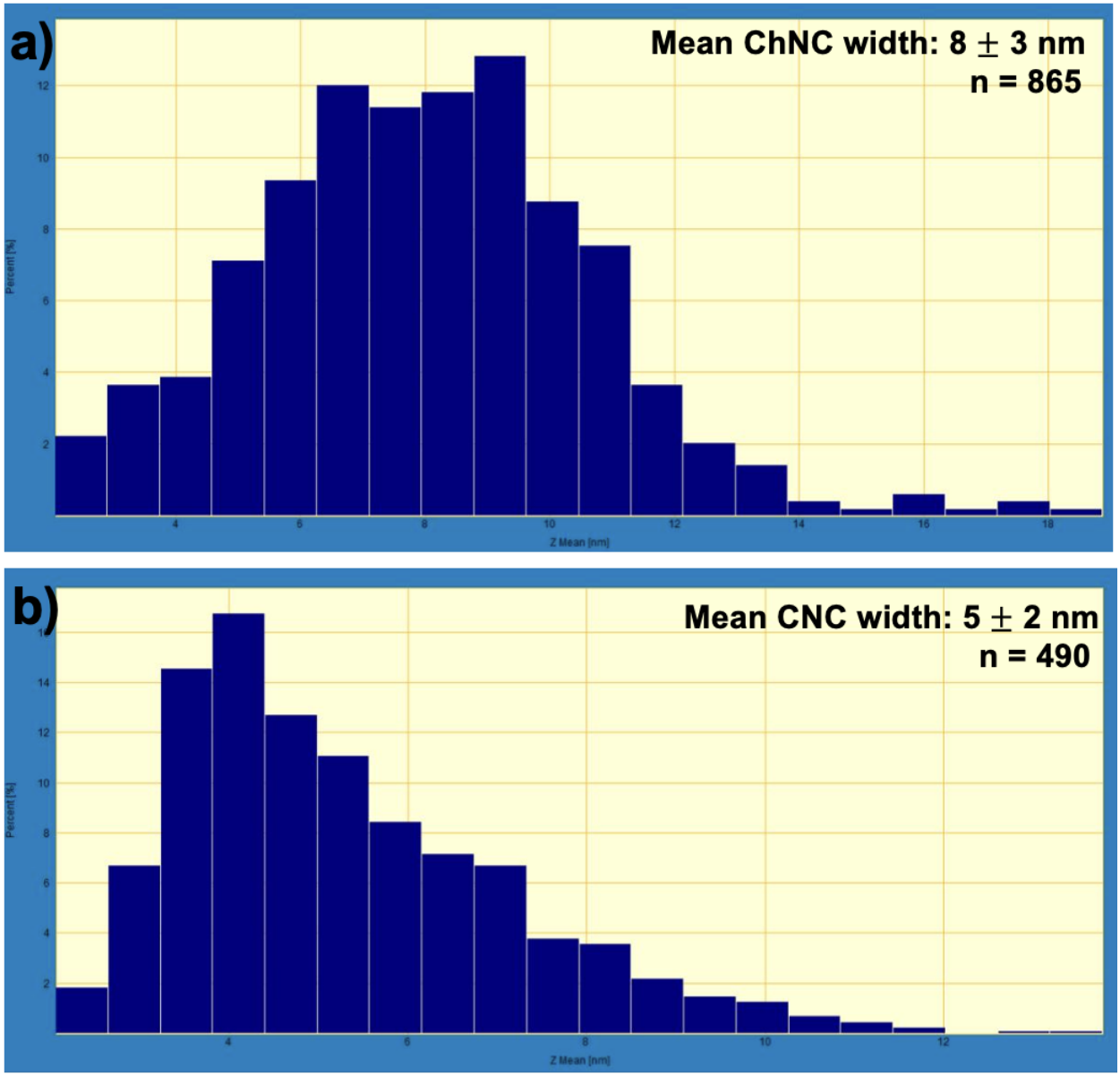

Figure S8: Width measurement histogram from AFM measurements for (a) ChNCs and (b) CNCs made through the optimized 3-day HHSA procedure.

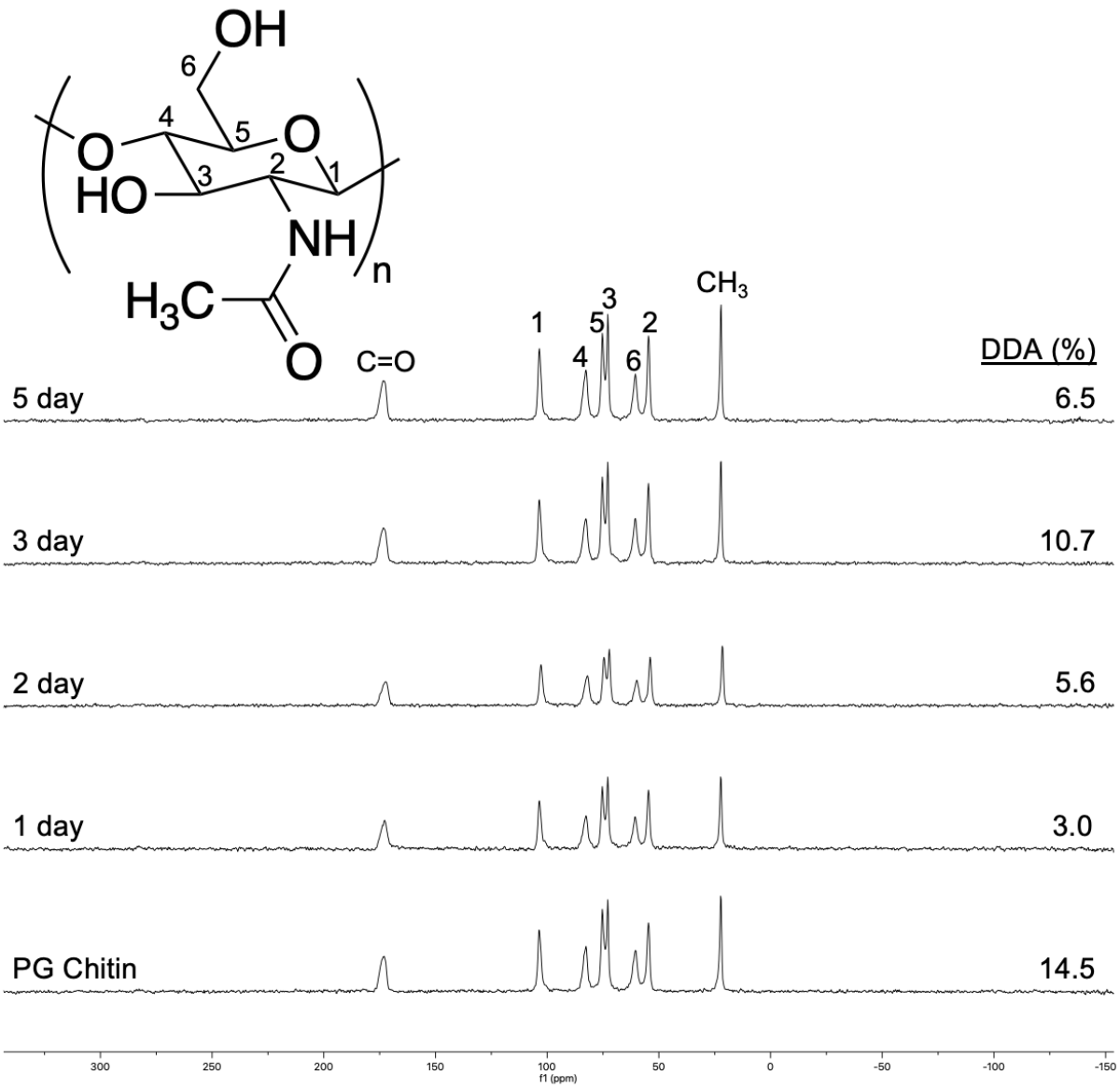

Figure S9: ^{13}C CPMAS NMR of PG chitin along with varied shaker aging time samples of ChNCs. DDA is calculated by taking the integral of the peak at 24 ppm and dividing over 1/6 the area of the polysaccharide backbone from 50 – 120 ppm. The corresponding peaks of the C1 through C6 are given, along with the peak labelling of the C=O and CH_3 of the acetylamide.

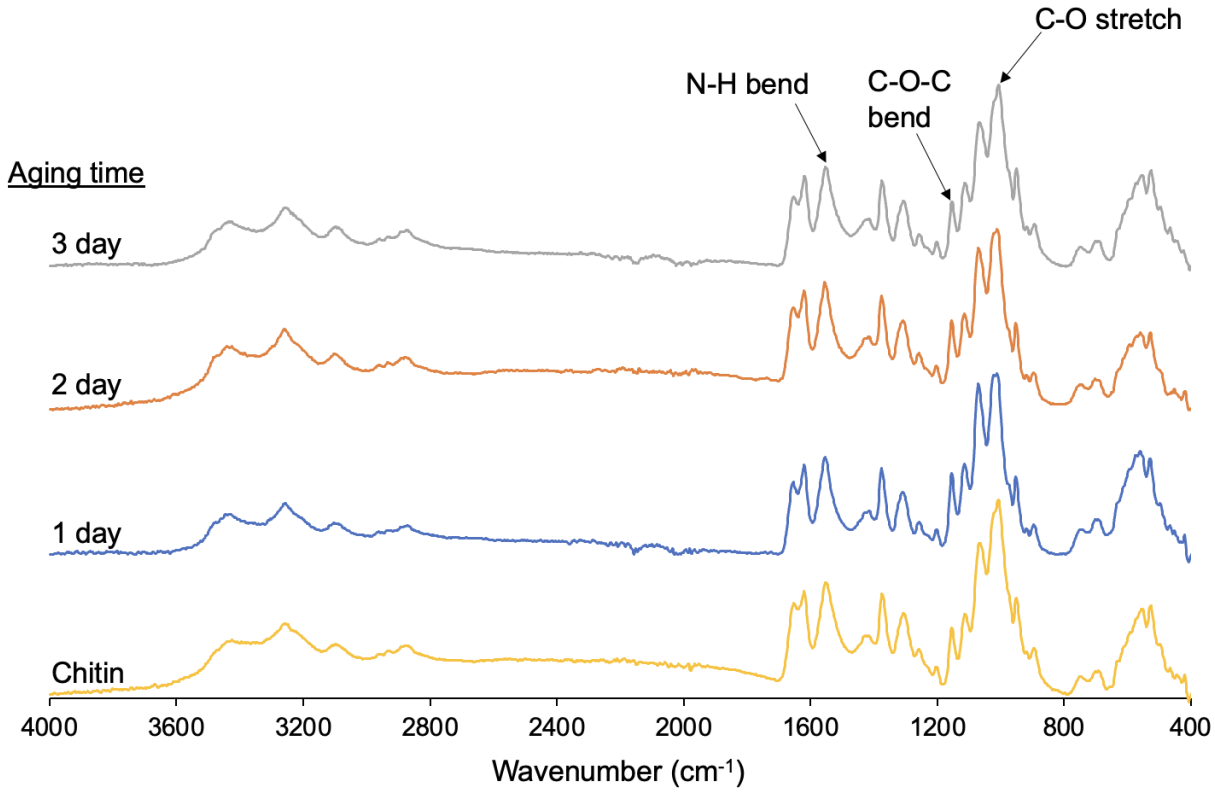

Figure S10: ATR-FTIR spectra of chitin and varied shaker aging time of ChNCs. A more detailed analysis and peak assignment can be found in [5]. It can be seen very minimal changes occur after HHSA, indicating little change occurs in the polysaccharide backbone. In particular, the N-H bend and C-O stretch indicate the presence of the acetamide, while the C-O-C bend indicates minimal polysaccharide degradation during the aging process. In fact, the N-H bend and C-O stretch absorbance ratio can be used to calculate the degree of acetylation.[6]

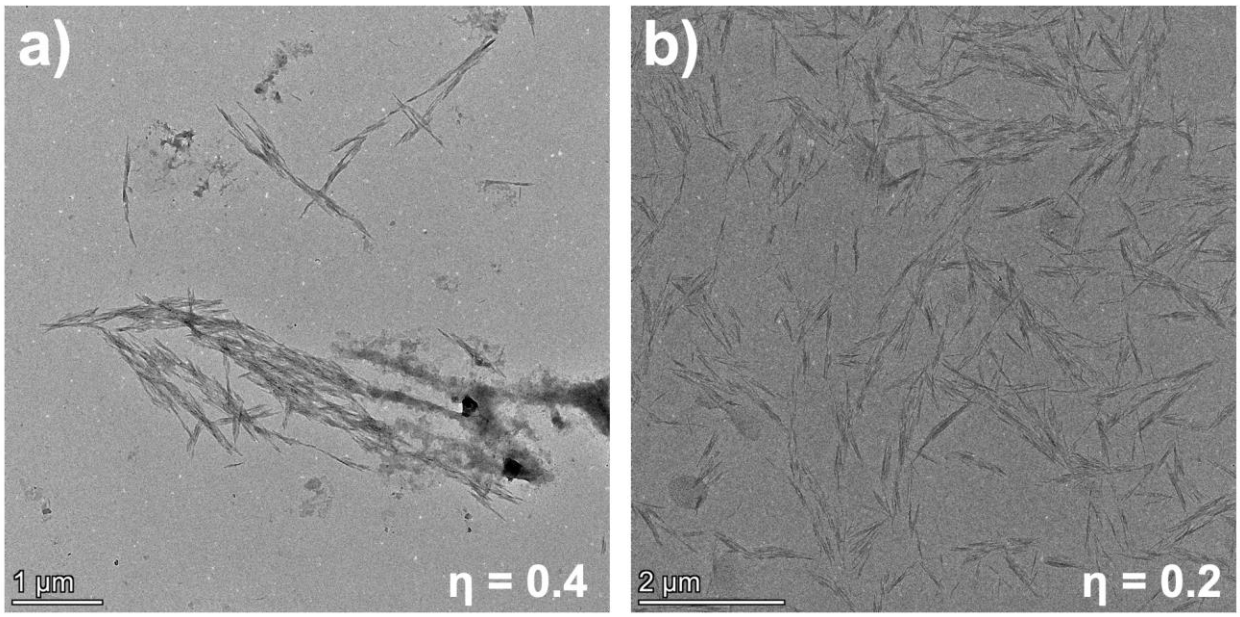

Figure S11: TEM images of (a) η = 0.4 and (b) η = 0.2 LAG aging blank reactions. DO for η = 0.4 is 0.22 with a yield of 61 %. η = 0.2 afforded a yield of 59 %.

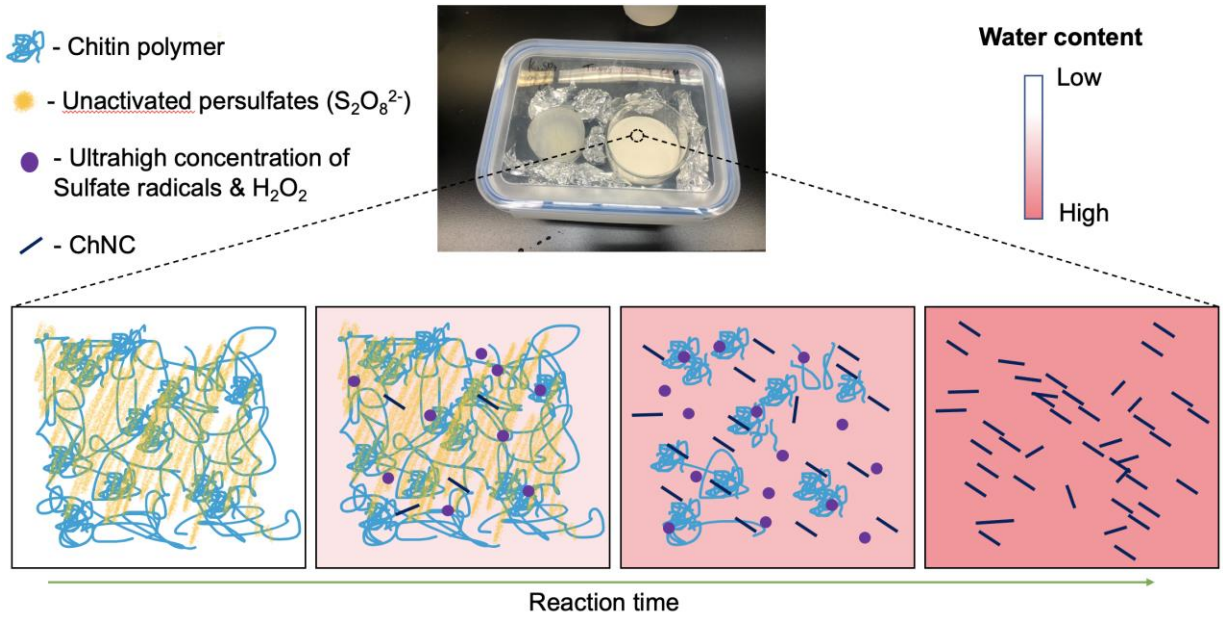

Figure S12: Schematic depiction of the proposed impact that deliquescence plays within the shaker aging method for chitin depolymerization to make carboxylated ChNCs.

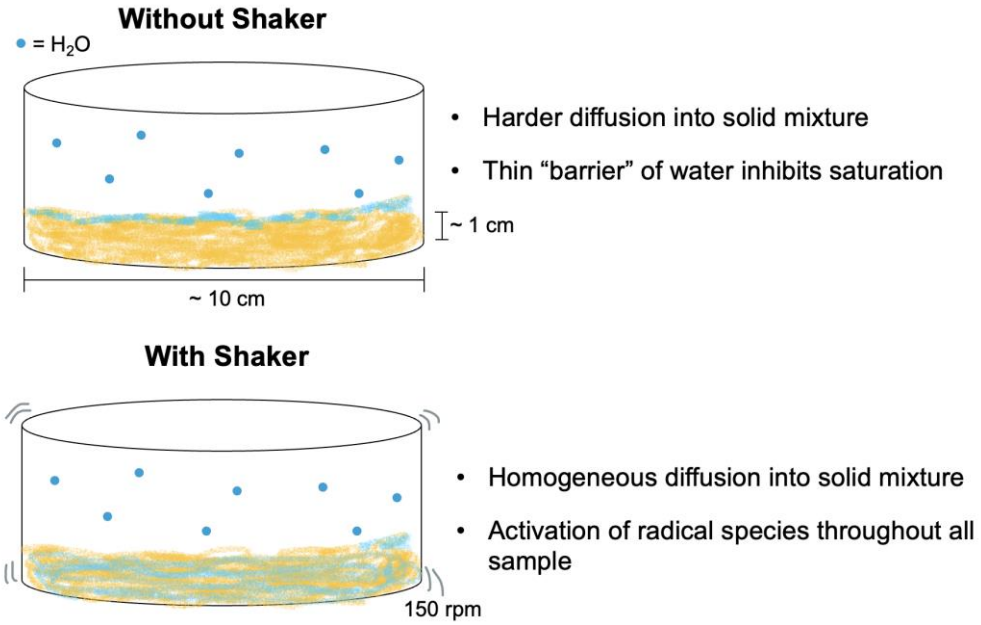

Figure S13: Schematic depiction of the effect of aging with and without a shaker.

Table S2: Comparison of 3-day HHSA ChNCs from solution-based processes in the literature.

Method	Conditions	Yield	Average Length (nm)	Average Width (nm)	Reference
3-day HHSA	4 mass eq. APS	60	175	8	*This work*
HCl acid hydrolysis	3 M HCl at reflux (104 ° C) for 9 h	70	176	12	[7]
TEMPO oxidation	TEMPO-mediated oxidation at 60 ° C for 4 h at 10 mmol NaClO per g chitin	40	270	13	[8]
Alkaline periodate oxidation	0.55 M KOH + H_5IO_6 (excess) at room temperature for 14 days	50	242	5	[9]
APS oxidation	1 M APS at 60 ºC for 16 h	53	149	6	[10]

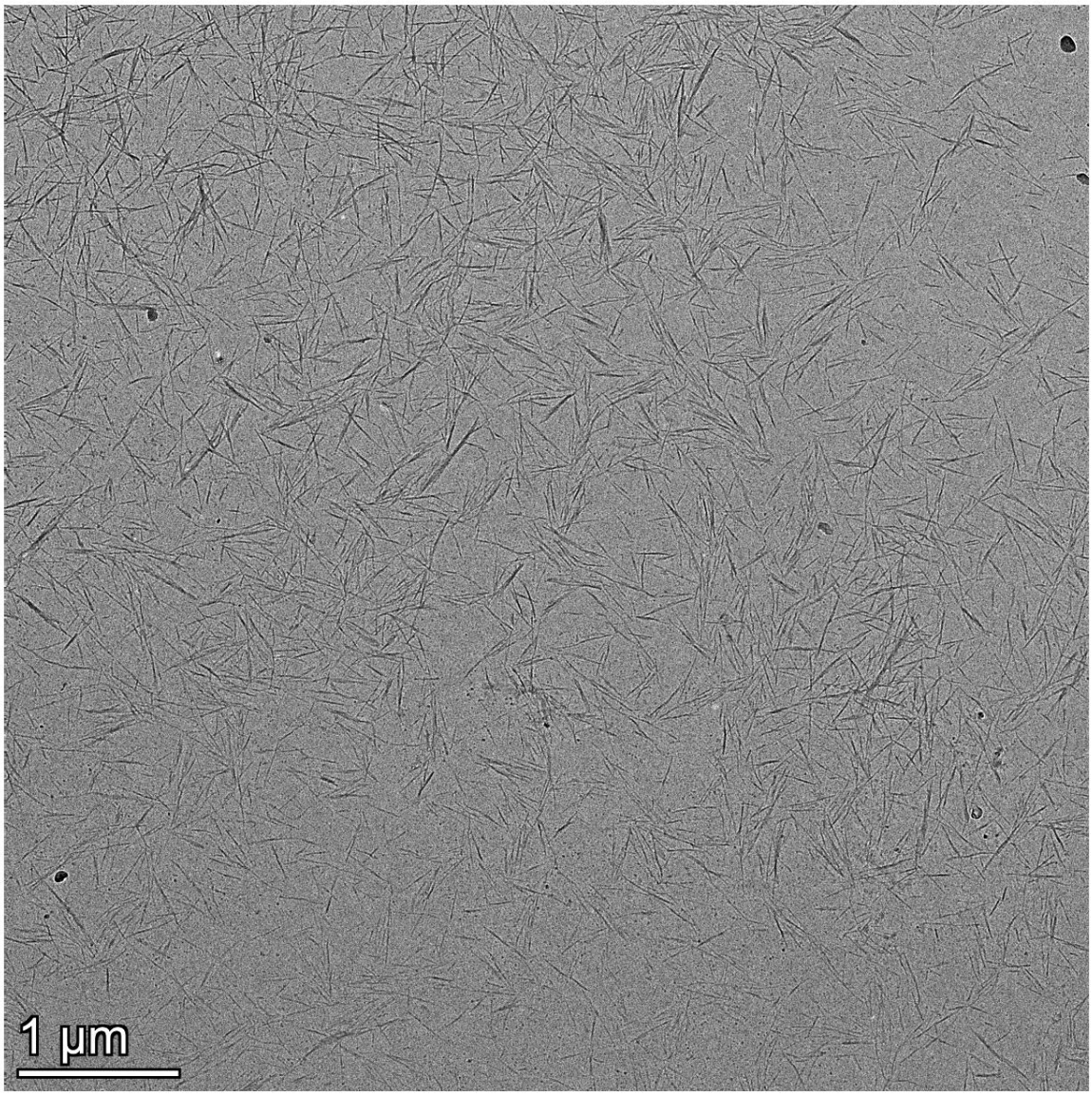

Figure S14: TEM micrographs of carboxylated cellulose nanocrystals from soft wood pulp after 3 days of shaker aging. DO is 0.27. Yield is 48 %.

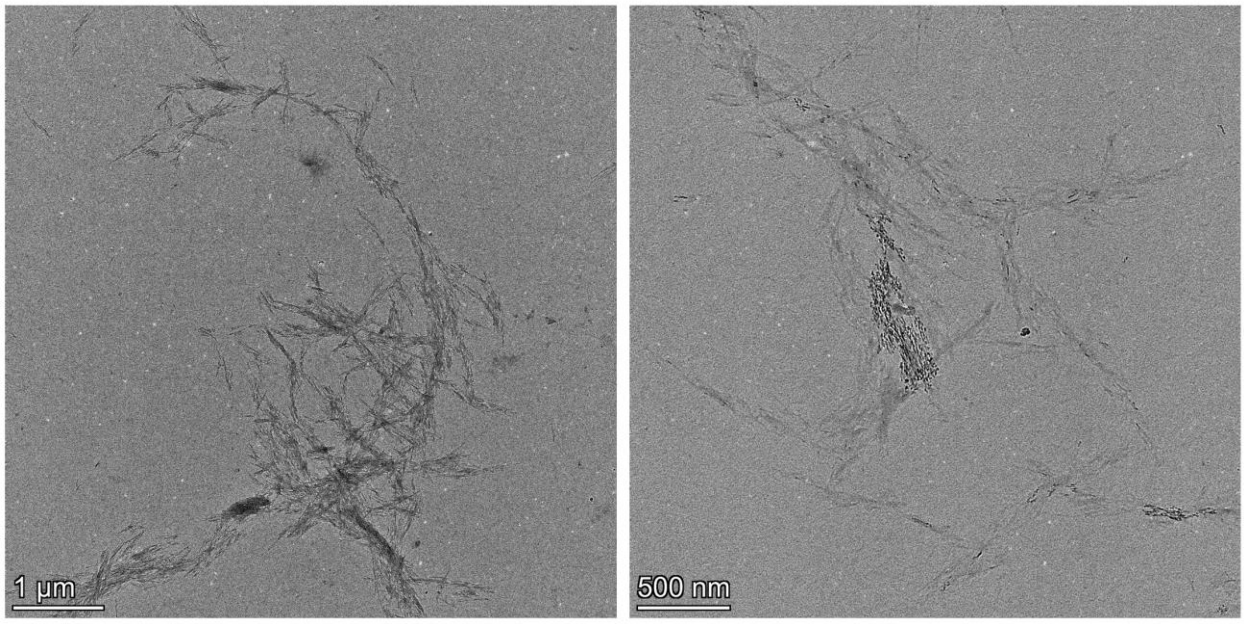

Figure S15: TEM micrographs of ChNCs liberated directly from green crab shells. DO could not be calculated due to inconclusive conductometric titration data, presumably due to aggregation and calcium carbonate impurities. Yield is 57 %.

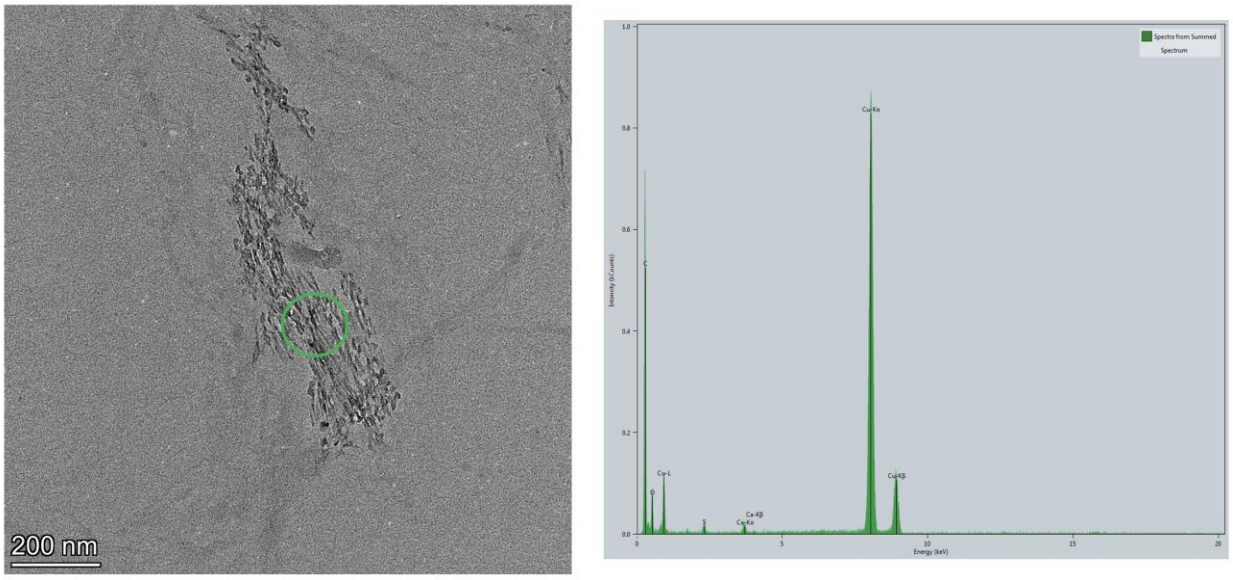

Figure S16: Energy dispersive X-ray spectrum (right) of the segment of the TEM micrograph (left) highlighted by the green circle. The sample is ChNCs from green crab shells.

References

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