Supporting information

Chitosan Nanocrystals *Via* Aging and its Application Towards Alginate Hydrogels for Sustainable Drug Release

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Materials

All reagents and solvents were of reagent grade and used as received. Water used was Milli-Q 18 MΩ. Chitin (from shrimp shells), sodium hydroxide, phosphate buffered saline (7.4), calcium chloride, and Albumin–fluorescein isothiocyanate conjugate (BSA-FITC, A9771) were obtained from Sigma Aldrich. Ammonium persulfate was obtained from FMC Corp. (Philadelphia, PA). Sodium alginate（I-1G）was obtained from KIMICA Corportation.

ChNC Synthesis

ChNCs were prepared using a procedure previously reported by our group.[1] Shrimp chitin (27.2 g) was added to ammonium persulfate (APS, 1 M, 540 mL) and stirred at 60 °C for 16 h. The suspension was then centrifuged at 10,000 rpm (RCF = 15,300 g) for 10 min, and the pellet was washed six times with water. ChNCs (14 g) were isolated via lyophilization as a white solid.

ChsNCST Solvothermal Synthesis

ChsNCsST were prepared using a procedure previously reported by our group.[1] ChNCs (50 g) and $NaBH_4$ (2.5 g) in NaOH (2.5 L, 40 wt% solution in water) were mixed and heated to reflux at 117 °C for 18 h. The suspension was centrifuged at 10,000 rpm for 10 min, the supernatant was decanted, and the pellet was resuspended in NaOH (2.5 L, 40 wt% solution in water) and heated under the same conditions. This process was repeated two to three times, centrifuging the suspension followed by resuspending the pellet in fresh NaOH solution. A small sample of solid was taken after each reflux step and the DDA was determined by monitoring the FTIR absorbance peaks at 1030 and 1560 cm^{-1} according to a characterization method outlined by Shigemasa. Once the DDA was above 80%, the ChsNC pellet was suspended in deionized water and centrifuged in the same conditions as above. The centrifugation/washing cycles were repeated eight times until the solution conductivity was below 400 $\mu S \cdot cm^{-1}$. The purified pellet was resuspended in water, and HCl (1 M) was added until the solution became slightly acidic pH 5. The ChsNC product was lyophilized to yield a white powder (29.0 g, 58% yield).

Milling Reactions

ChNCs and a prescribed amount of NaOH micropearls were put into a 10 ml zirconia milling jar with one 10mm zirconia milling ball, and milled for 5 min. using a Retsch Oscillating MM 400 mill. The product was rinsed with excess methanol until the pH reached ~ 7.0.

ChsNC^{Ag} Synthesis *via* Aging Reactions

To a standard Tupperware glass container was charged 200 mg of ChNCs within a 1-dram vial and a petri dish of super-saturated K_2SO_4 solution to obtain r.h. = 98 %. The glass container was then allowed to incubate at 50 °C. The as-obtained product was then rinsed with methanol until the pH reached ~ 7.0. Average yield from three replicates was 75 % yield. The scale-up reaction up to 2 g was done using the same method as above, with 60 % yield. Low r.h. control reactions were done at 43 % relative humidity using a super-saturated K_2CO_3 solution using the same method as above. Room temperature control reactions were done using the same method as above,

but on the counter with a temperature reading of 23 °C. Disperse solutions of the as-synthesized aged ChsNCs were facilitated through the use of ultrasonication.

Characterization

The Transmission Electron Microscopy (TEM) images were taken on a Thermo Scientific Talos F200X G2 S/TEM equipped with a Ceta 16M CMOS camera, with energy dispersive X-ray (EDX) spectroscopy done on a Super-X EDS detector system. 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. The ChNC and ChsNC samples were subsequently stained twice for 14 s and 45 s with 1 % uranyl acetate, then dried at room temperature prior the TEM observation.

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°.

Dynamic light scattering and the zeta potential of the samples (2 mg mL^{-1}) in water were determined using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) in triplicate.

Solid-state nuclear magnetic resonance (NMR) spectroscopy

Solid-state ^{13}C NMR spectra were collected on a 9.39 T (ν_L (^1H) = 400.1 MHz, ν_L (^{13}C) = 100.6 MHz) Bruker AVANCE HD spectrometer equipped with a 4 mm double resonance (H-X) magic-angle spinning (MAS) probe. The ^{13}C multiple-cross polarization (mulitCP).[2] Experiments were performed using similar conditions described previously to measure the purity of chitin.[3] Initially, standard ^{13}C CP experiments were acquired on a sample of chitin nanocrystals to assess the CP build-up properties. Each point of the build-up curve used a 4.13 μs π/2 on ^1H with a ramped Hartman-Hahn match on ^{13}C and TPPM ^1H decoupling ($\gamma B_1/2\pi$=61 kHz).[4] 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 $^{13}C\{^1H\}$ 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 hrs) were acquired for each $^{13}C\{^1H\}$ multiCP experiment on the aged chitosan nanocrystal series and solution-made chitosan nanocrystals. 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.

ChsNC and alginate hydrogel formation

The Alginate-ChsNC hydrogel was formed using a syringe mixing technique. To a 5 mL syringe (Air-TITE Products Co., INC., Virginia Beach, VA) was charged 1 mL of 4 % (w/v) sodium alginate solution. To a separate 5 mL syringe was charged 1 mL of 4 % (w/v) nanocrystal solution. A connecting syringe tube was used to connect the two syringes together and the two solutions were mixed until homogeneous. The mixture was then ejected onto the bottom plate of a TA Instrument DHR2 rheometer equipped with 20 mm flat upper plate geometry. Mineral oil was applied to the gel periphery to prevent the hydrogel from dehydration during test. Gelation kinetics of the samples was measured using 0.1% strain at 1Hz.

Synthesis of Ca^{2+} crosslinked alginate with ChsNC incorporation

To a 5 mL syringe was charged 2 mL of 4 % (w/v) sodium alginate solution. To a separate 5 mL syringe was charged 1 mL of 6 mg mL^{-1} nanocrystal solution. The two syringes were connected *via* syringe connecting tube and mixed until homogeneous. The resulting solution was then added drop-wise by hand through a 20-guage needle into a bath of 10 mL of 0.2 M $CaCl_2$ aqueous solution. The needle tip was held horizontal and roughly 10 cm above the surface of the $CaCl_2$ solution. The resulting hydrogel beads were then washed three times in distilled water before being dried for use in swelling tests. Below is a photo of the two syringes connected with the syringe connector:

Swelling ratio tests

Ca^{2+} crosslinked alginate with ChsNC incorporation hydrogel beads were dried by being placed in an oven held at 50 °C overnight. The dried hydrogel beads were then weighted and placed into 20 mL of phosphate-buffered saline (PBS) held at 7.4 pH and room temperature. The swelling ratio is calculated as follows:

$$Swelling\ ratio = \frac{S_w - S_i}{S_i}$$

Where S_w is the weight of the hydrogel beads at the time of weighing and S_i is the initial weight of the dried hydrogel beads.

Loading of drug into Ca^{2+} crosslinked alginate hydrogel with ChsNC incorporation

To a 5 mL syringe was charged 2 mL of 4 % (w/v) sodium alginate solution. To a separate 5 mL syringe was charged 1 mL of 6 mg mL^{-1} nanocrystal solution and 15 mg of BSA-FITC. The two syringes were connected *via* syringe connecting tube and mixed until homogeneous. The resulting

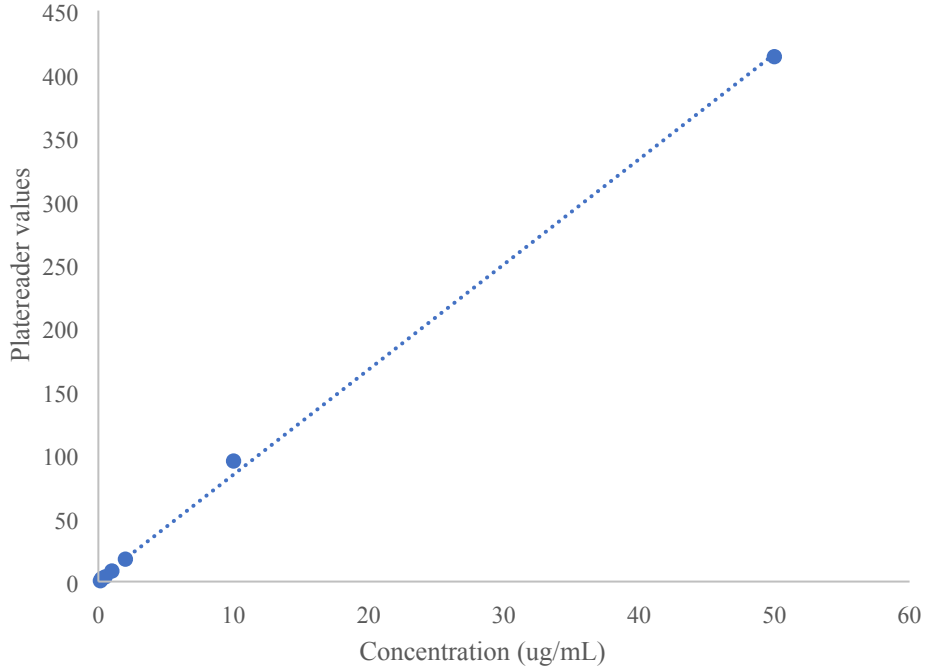
solution was then added drop-wise by hand through a 20-guage needle into a bath of 10 mL of 0.2 M $CaCl_2$ aqueous solution. The needle tip was held horizontal and roughly 10 cm above the surface of the $CaCl_2$ solution. The resulting hydrogel beads were then washed three times in distilled water and quickly transferred into 20 mL of pH 7.4 PBS solution (pH 7.4) that were incubated at room temperature. The distribution of drug within the hydrogel beads was visualized using a confocal laser scanning microscope (Zeiss, LSM710). Since the BSA-FITC is directly incorporated within the hydrogel itself through mixing and homogenisation, the loading efficiency is 100 %. The amount of total drug loaded into the hydrogel beads were calculated as follows:

$$Total\ drug\ amount\ in\ bead\ (mg) = mass\ of\ hydrogel\ bead\ (mg) \times 0.0048$$

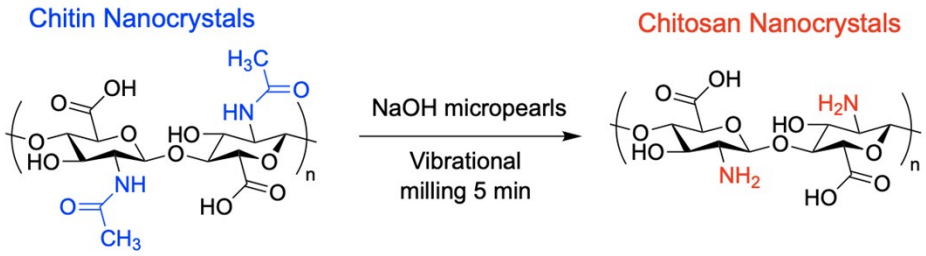
0.0048 is the weight ratio of BSA-FITC within the homogeneous mixture of sodium alginate, nanocrystals, and water, as specified above.

Release profile of BSA-FITC

Each release profile for each hydrogel with nanocrystal incorporation was done with 4 replicates at room temperature in the dark. Aliquots of 0.2 mL of solution were taken from each replicate and replaced with 0.2 mL of fresh PBS. The fluorescence intensity of the solutions was determined using a BioTek Synergy HTX multi-mode microplate reader (λ_{ex} = 485/20 nm, λ_{em} = 528/20 nm). A standard calibration curve was made as follows in order to determine cumulative drug release:



Supplementary Figures



Reaction method	NaOH eq. (w/w% rel. to ChNC)	DDA (%)
Vibrational milling	1	3
	2	3
Manual grinding	1	2
	2	2

Figure S1: DDA values from control milling reactions using a Retsch Oscillating MM 400 mill, a 10 ml zirconia milling jar and one 10mm zirconia milling ball for the vibrational milling, and an agate mortar and pestle for the manual grinding.

Aging Time (days)	Degree of Deacetylation (%)	ζ-potential (mV)	Crystallinity index (%)
0	0	10.1 ± 0.3	72
0.5	8.7	24.1 ± 2.4	51
1	32	32.3 ± 1.0	42
3	52.4	36.3 ± 1.0	40
6	65.5	35.9 ± 1.6	36
10	67.1	33.5 ± 1.1	33
$ChsNC^{ST}$	88.0	53.2 ± 0.8	24

Table S1: Table of values for the aged $ChsNC^{Ag}$ samples. The last row depicts the values seen for the $ChsNC^{ST}$ made through the solvo-thermal method.

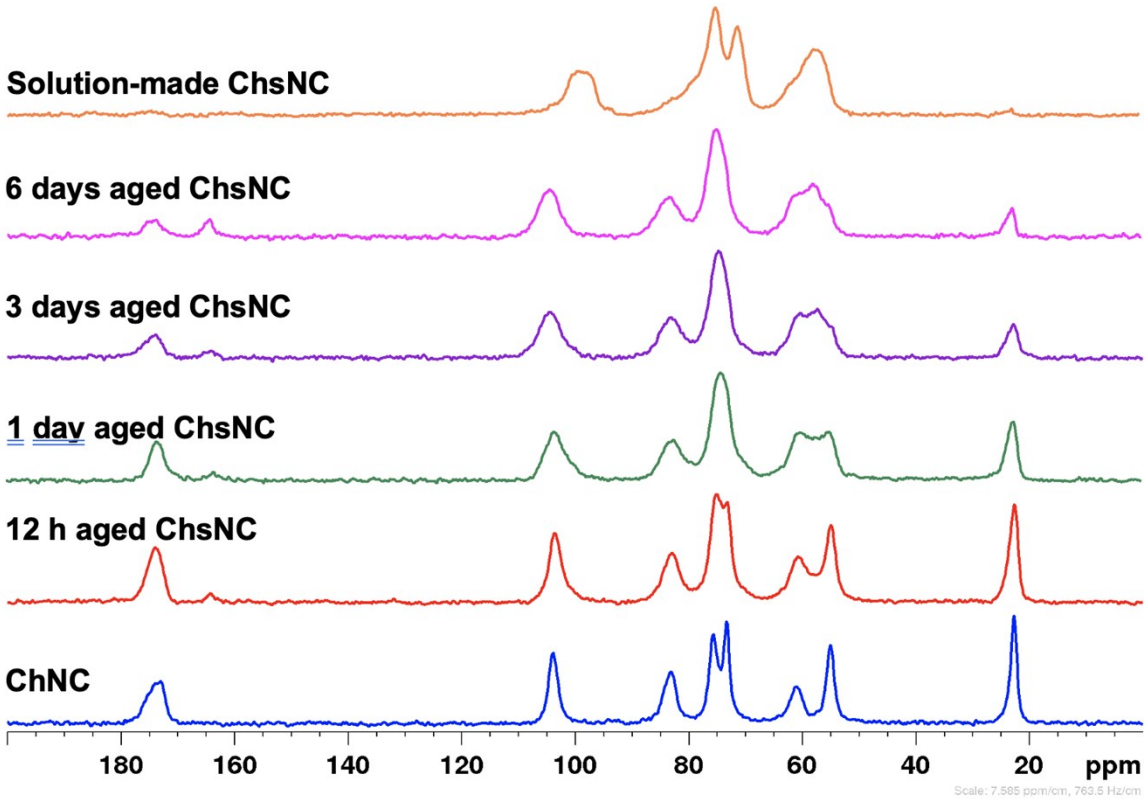


Figure S2: $^{13}C\{^{1}H\}$multiCP/MAS solid-state NMR spectra of aged ChsNC samples in comparison with solution-made ChsNCs and ChNCs.

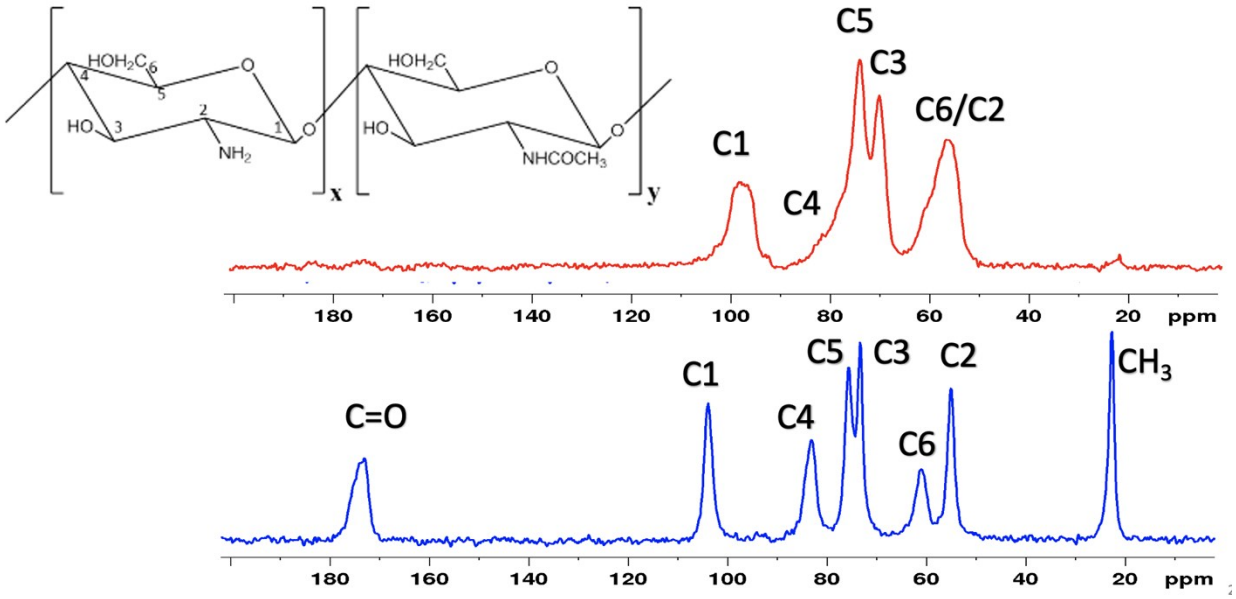


Figure S3: Peak labelling of $^{13}C\{^{1}H\}$multiCP/MAS spectra seen for chitosan (red, upper) and chitin (blue, lower) nanocrystals.

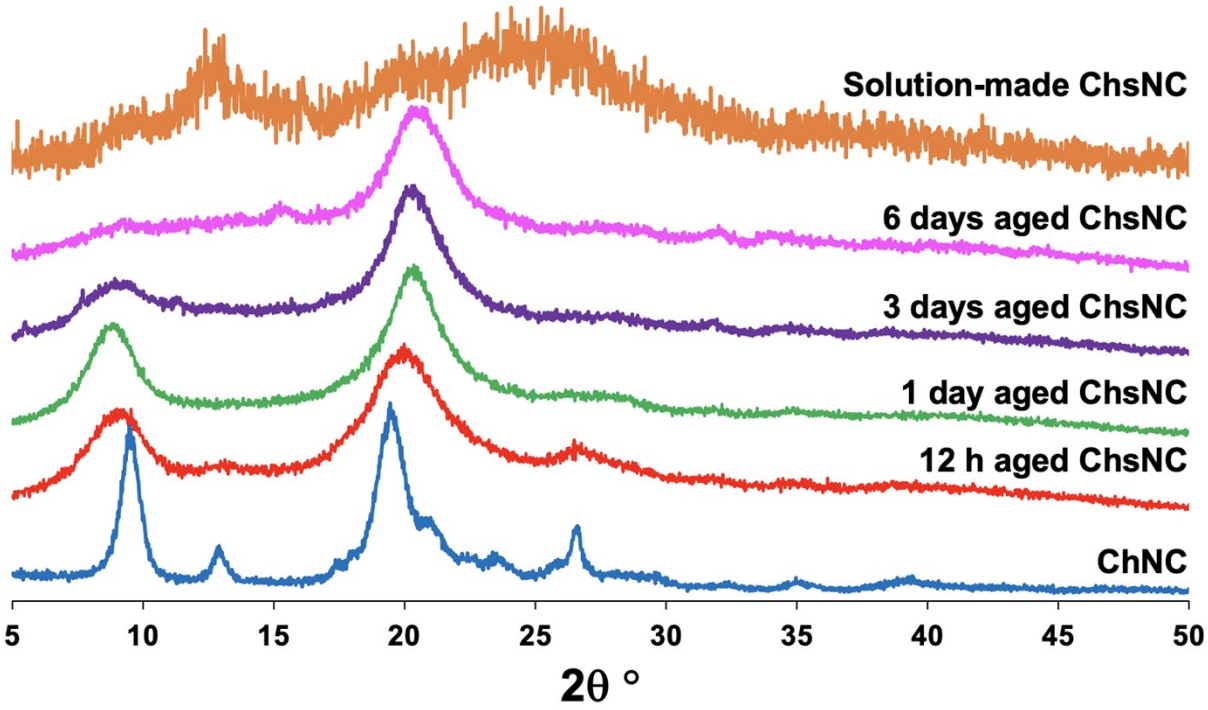


Figure S4: Powder X-ray diffraction patterns of aged ChsNC samples in comparison with solution-made ChsNCs and ChNCs.

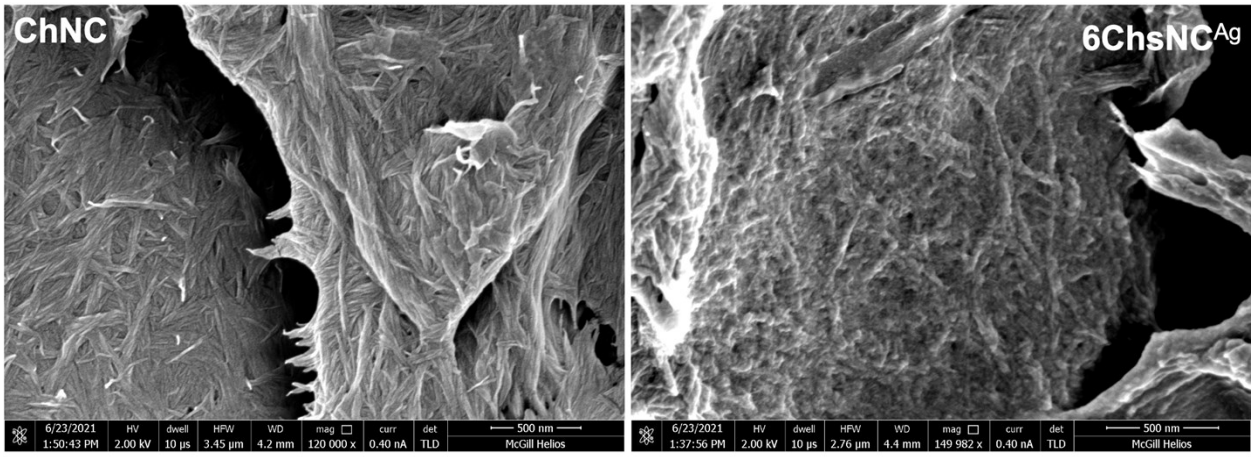


Figure S5: Scanning electron microscopy images of ChNC (left) and 6 days aged ChsNC^{Ag} (right).

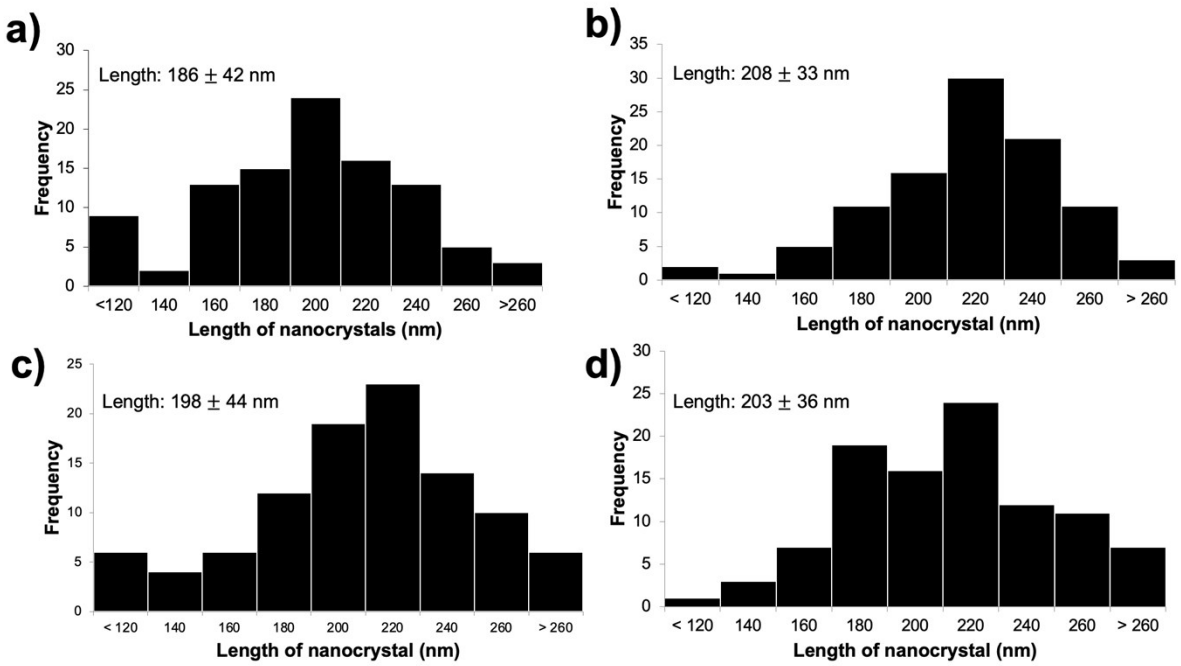


Figure S6: Size histograms (n = 200) including the mean and standard deviation of the lengths of aged ChsNCs aged for (a) 12 h, (b) 1 day, (c) 3 days and (d) 6 days.

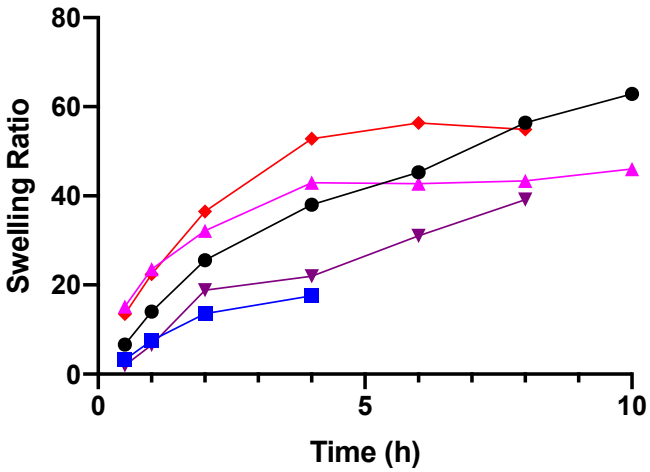


Figure S7: Swelling tests for Ca-alginate beads with 0.2 wt % incorporation of (blue) ChNC, (dark-purple) 6-day aged ChsNC, (light-purple) 3-day ChsNC, (red) solution-made ChsNC, and (black) control with no nanocrystal incorporation.

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3. King, C.; Stein, R. S.; Shamshina, J. L.; Rogers, R. D. *ACS Sustain. Chem. Eng.* 2017**, 5**, 8011-8016.
4. Bennett, A. E.; Rienstra, C. M.; Auger, M.; Lakshmi, K. V.; Griffin, R. G. *J. Chem. Phys.* 1995**, 103**, 6951-6958