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Supplementary Information

Unexpected Selective Alkaline Periodate Oxidation of Chitin for the Isolation of Chitin Nanocrystals

Peiwen Liu[1§], Huan Liu[1§], Timmy Schäfer[2], Torsten Gutmann[2], Holger Gibhardt[3], Houjuan Qi[1,4], Lin Tian[5], Xizhou Cecily Zhang[6], Gerd Buntkowsky[2], Kai Zhang[1*]

Address

1 Dept. Wood Technology and Wood-based Composites, Georg-August-University of Göttingen, Büsgenweg 4, D-37077 Göttingen, Germany

2 Technical University Darmstadt, Eduard-Zintl-Institute for Inorganic Chemistry and Physical Chemistry, Alarich-Weiss-Straße 4, D-64287 Darmstadt, Germany

3 Institute of Physical Chemistry, Georg-August-University of Göttingen, Tammannstrasse 6, D-37077 Göttingen, Germany

4 Key Laboratory of Bio-based Material Science & Technology（Northeast Forestry University）, College of material science and engineering, Ministry of Education，Harbin 150040, China

5 Institute for Material Physics, Georg-August-University of Göttingen, Friedrich-Hund-Platz 1, 37077 Göttingen, Germany

6 NMR-based Structural Biology, Max-Planck-Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany

*Corresponding author. E-mail: kai.zhang@uni-goettingen.de.

[§]These authors contributed equally.

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Methods

General. Practical grade of chitin from shrimp shells and crab shells, orthoperiodic acid (H_5IO_6), and deuterium oxide (D_2O, 99.9 atom % D) were purchased from Sigma-Aldrich. Aqueous hydrochloric acid (HCl, 37 %), potassium hydroxide (KOH), hydroxylamine hydrochloride, sodium periodate ($NaIO_4$), ammonia solution ($NH_3•H_2O$, 25 %) and sodium hydroxide (NaOH) were purchased from VWR. All chemicals were of analytical grade or higher. Deionized water (DI water) was used throughout the experiments.

Preparation of PO-ChNCs by alkaline periodate oxidation. 1 g of chitin was soaked in 3 wt.% KOH solution for 24 hours (35 mL). Then, the soaking solution was added into a reaction bottle, and 7 g of H_5IO_6 was added into the reaction bottle. The pH of these mixtures was adjusted to the wanted value by adding bases (NaOH for adjusting pH to 4, $NH_3•H_2O$ for adjusting pH to 7.5, and KOH for adjusting pH to 10 and 12) and the total volumes were adjusted to 150 mL with DI water. The reactions were performed in dark at room temperature under continuous stirring for a certain time. At the end of the reaction, the solid parts were separated by centrifugation (14000 rpm, 20 min, 4 °C, Thermo scientific Multifuge X3 FR, F15-6•100y), then the solid was re-suspended into DI water, and 2 mL of ethylene glycol was added before the suspensions were treated with ultrasonication (Elmasonic P 30 H, 37 kHz, 180 W) for one hour in ice-cold water. Thereafter, the solid products were thoroughly purified via dialysis in water using a dialysis membrane with a molecular-weight cut-off of 3500 Da (Thermo Fisher Scientific), and the volume of the obtained suspension was adjusted to 100 mL with DI water. The stable chitin nanocrystals in supernatant after 20 min centrifugation at 3000 rpm (20 °C) were used for yield calculation and further characterization.

Optimization of the reaction parameters for isolation of PO-ChNCs

In order to find the optimal condition for isolation of PO-ChNCs (the optimum condition is the condition to isolate the maximum amount of chitin nanocrystals in the minimum time), the parameters were optimized on the amount of periodate, the pH value, reaction time, temperature and the application of pretreatment as soaking. For the optimal amount of

periodate, the isolation processes were operated with 1g of soaked chitin at room temperature for 14 days at pH 10. Various amounts as 1.5 g, 3.5 g, 5.5 g, 7.0 g and 8.5 g of periodic acid were used for isolation. To obtain the optimal pH value for isolation, the isolation processes were operated with 1 g of soaked chitin and 7 g of periodic acid at room temperature for 14 days. pH values of 4, 7, 10 and 12 were investigated as reaction pH value. For the optimal time of isolation, the isolation processes were operated with 1g of soaked chitin and 7g of periodic acid at room temperature. The pH value of isolation was 10. The alkaline periodate oxidation on chitin was performed for 1, 3, 6, 10, 14, 30 days. For the optimal temperature of isolation, the isolation processes were operated with 1g of soaked chitin and 7g of periodic acid at pH 10. Here, the temperature was set as 10 °C, room temperature (nearly 25 °C), 35 °C and 45 °C.

Dynamic nuclear polarization (DNP) enhanced solid-state nuclear magnetic resonance (NMR) measurements.

Sample preparation

For DNP investigations, stable radicals which serve as spin polarization source have to be present in the sample. Thus, the sample systems were impregnated with a radical solution containing AMUPol[1] radicals in glycerol-d_8/D_2O/H_2O (hydrophilic).

All samples were prepared for DNP measurements by impregnation of 13.5 mg of each sample with 27 µL of a 15 mM AMUPol/glycerol-d_8/D_2O/H_2O (60:30:10 w:w:w) solution, respectively.

DNP enhanced solid-state NMR experiments

All measurements were performed on a Bruker Avance III 400 spectrometer system equipped with an Ascend 400 DNP magnet, a 9.7 T (263 GHz) Bruker gyrotron and a low-temperature ^1H/X/Y probe. Spectra were recorded at frequencies of 100.59 MHz for ^{13}C, and 40.54 MHz for ^{15}N, at nominally 120 K and 10 kHz spinning.

$^1H \rightarrow ^{13}C$ CP MAS spectra were referenced to TMS employing glycine (C=O signal at 176.5 ppm) as external standard. $^1H \rightarrow ^{15}N$ CP MAS spectra were referenced to ammonium chloride (0 ppm). The ramped CP MAS sequence[2] was utilized with a contact time of 2.0 ms for $^1H \rightarrow ^{13}C$ CP MAS and 3.5 ms for $^1H \rightarrow ^{15}N$ CP MAS. The recycle delay was set to 2 s and tppm decoupling[3] employing a 15 ° phase jump was

applied during data acquisition. The acquisition time for $^{1}H \rightarrow ^{13}C$ CP MAS was set to 40 ms and for $^{1}H \rightarrow ^{15}N$ CP MAS to 25 ms. All ^{13}C spectra were recorded with 512 scans and the ^{15}N spectra were recorded with 20480 scans.

Liquid-state ^{1}H-NMR and ^{13}C-NMR spectroscopy. The isolation of PO-ChNCs from shrimp chitin was performed in D_2O. 0.27 g of KOH was dissolved in 9 mL D_2O, and 0.25 g of shrimp chitin was soaked in this solution over 24 hours. Then, 1.76 g of H_5IO_6 was added in suspension and the pH of the reaction solution was adjusted to 10 by KOH (4 mol/L) which was dissolved in D_2O. The total volume of reaction was fixed to 38 mL. The reaction was performed at room temperature under continuous stirring for 14 days in dark. Immediately after the reaction, the solid was separated out with centrifugation (14000 rpm, 20min, 4 °C, Thermo scientific Multifuge X3 FR, F15-6•100y), then the supernatant was filtered for liquid-state ^{1}H-NMR, and ^{13}C-NMR measurement.

All spectra were obtained on a Bruker 300 MHz NMR spectrometer, a Bruker Avance III 500 MHz spectrometer or a Varian Inova 600 MHz spectrometer in D_2O at 25 °C. Chemical shifts are reported relative to the solvent peak (H_2O). Up to 128 and 2048 scans were accumulated for the ^{1}H-NMR spectra and ^{13}C-NMR spectra respectively.

Transmission electron microscopy (TEM). Dried PO-ChNCs were characterized with TEM, and the samples were prepared from their suspensions in water of 0.01 wt.%. The TEM observation was performed on a CM 12 Transmission Electron Microscope with a field-emission gun operated at 120 kV accelerating voltage. (Philips, Netherland). The specimens were stained by phosphotungstic acid solution (2 wt.% in water, pH was adjusted to 7.0 using 1 mol/L of aqueous NaOH), and the copper grids were treated with glow discharge (40 μA, 90 s).

Determination of the contents of aldehyde groups by titration. Before the titration, the pH values of the aqueous PO-ChNCs suspensions were adjusted to 3.5 using HCl solution (1 M). Then, 10 mL hydroxylamine hydrochloride solution (5% w/w) was added into the suspension. The pH of the solution was kept at 3.5 by adding NaOH solution

(0.05 M), until no decrease of pH was observed. The aldehyde group content was calculated by the consumption of the aqueous NaOH solution according to:

$$C = \frac{CNaOHVNaOH}{m} \quad (1)$$

Where C is the amount of aldehyde groups on ChNCs per gram, C_{NaOH} is the concentration of the aqueous NaOH solution, V_{NaOH} is the volume of the used NaOH solution, and m is the mass of dried ChNCs.[4]

Determination of carboxyl groups and amine groups by conductivity titration. Before the titration, the pH value of the ChNCs suspension was adjusted to 2.5 using an aqueous HCl solution (1 mol/L). Then, the suspension was titrated on a 665 Dosimat (Metrohm) employing a dosing rate of 0.01 mL/s up to pH 12, where the conductivity was recorded using the 865 Conductivity Module (Metrohm) with an interval of 2 s, and the pH changing also was recorded at same time. The calculation of the contents of both carboxylate content and C2 amino groups on PO-ChNCs are based on the conductivity and pH data.

The amounts of carboxyl groups and amino groups were calculated by the consumption of the aqueous NaOH solution according to:

$$n1 = CNaOHVNaOH \quad (2)$$

$$n2 = CNaOHVpINaOH \quad (3)$$

$$X - n2 = Y \quad (4)$$

$$X + Y = n1 \quad (5)$$

$$Ccarboxyl = \frac{n1 + n2}{2m} \quad (6)$$

$$Camine = \frac{n1 - n2}{2m} \quad (7)$$

where n1 is the total amount of carboxyl groups and amino groups, C_{NaOH} is the concentration of the aqueous NaOH solution (0.05 mol/L), V_{NaOH} is the volume of the used NaOH solution for neutralizing carboxylic groups and charged amino groups and m is the mass of PO-ChNCs. n2 is the amount for reaching the isoelectric point. VpI_{NaOH} is the volume of the used NaOH solution to reach the isoelectric point. X is the amount of

carboxyl groups. Y is the amount of amine groups. The isoelectric point can be obtained by the derivation of the pH value curve.[5]

Determination of the degree of deacetylation of chitin by titration.

Before the titration, the pH value of the chitin suspension was adjusted to 2.5 using an aqueous HCl solution (1 mol/L). Then, the suspension was titrated on the 665 Dosimat (Metrohm). The dosing rate was employed 0.01 mL/s till the pH value up to 12. The conductivity was recorded using the 865 Conductivity Module (Metrohm) with an interval of 2 s, and the pH changing also was recorded. The degree of deacetylation was calculated by the consumption of the aqueous NaOH solution according to:

$$\text{Degree of deacetylation \%} = \frac{\text{CNaOHVNaOH} \times 100}{\text{m} \times 4.9} \quad (8)$$

where C_{NaOH} is the concentration of the aqueous NaOH solution (0.05 mol/L), V_{NaOH} is the volume of the used NaOH solution for neutralizing the charged amino groups ($-NH_3^+$) and m is the mass of chitin. 4.9 (mmol/g) is the theoretical amine amount in chitosan with the full deacetylation.[5]

Raman spectroscopy. The Raman spectrometer was a T64000 from Jobin-Yvon with a focal length of 640 mm and a triple monochromator with three gratings, each with a groove density of 1800 lines per mm. The monochromator was operated in subtractive mode. The sample solutions placed in Hellma quartz cuvettes were measured in 90° scattering geometry. An Innova 90C argon ion laser with a wavelength of 514 nm was used as excitation source. The power was limited to 60 mW. A CCD camera was used for detection. The slits were set that the spectral resolution was adjusted to about 1.5 cm^{-1}.

Zeta potential. Zeta potential measurements were performed on a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). For the Zeta potential measurements, the PO-ChNCs suspensions were diluted to a concentration of ~0.5 mg/mL with DI water. 1 mL of PO-ChNCs suspension in DTS1070 disposable folded capillary cell was used for the measurement.

X-ray diffraction (XRD) patterns of the raw shrimp chitin and PO-ChNCs.

The original shrimp chitin and PO-ChNCs were air-dried. XRD measurements were conducted using the Cu-Kα line between 4° and 35° at room temperature with a step size of 0.05°. The crystallinity index was calculated according to:[6, 7]

$$\text{Crystallinity index} = \frac{I110 - I\text{am}}{I110} \quad (9)$$

where I_{110} is the peak intensity of the ordered domains of chitin at a diffraction angle of 19.6°, I_{am} is the peak intensity of the non-ordered domains of chitin at a diffraction angle of 16.0°.

The crystal sizes of the [020] and [110] directions were calculated according to the Scherrer's equation.[7] With the widths at half-heights of the diffractions peaks at 9.6° and 19.6°, respectively.

Supplementary Text and Figures

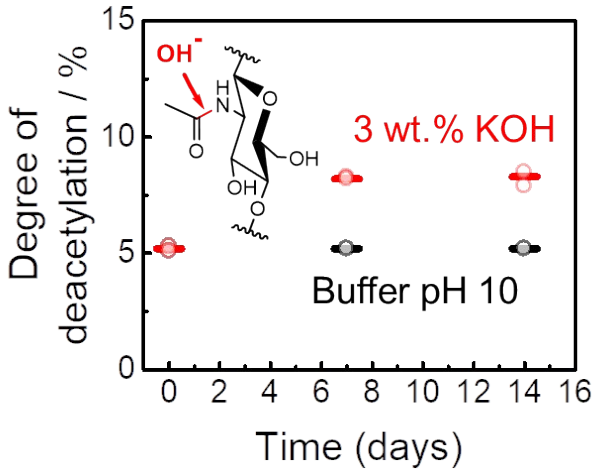

Figure S1. The limited deacetylation of chitin obtained at alkaline environment for different reaction times.

5 % of deacetylated chitin was generated from the production of chitin from native resources. The degree of deacetylation of chitin did not increase significantly by soaking in a buffer at pH 10 for 30 days. Even when soaking chitin in aqueous KOH (3 wt.%), only 8 ± 0.3 % of chitin was deacetylated within 14 days.

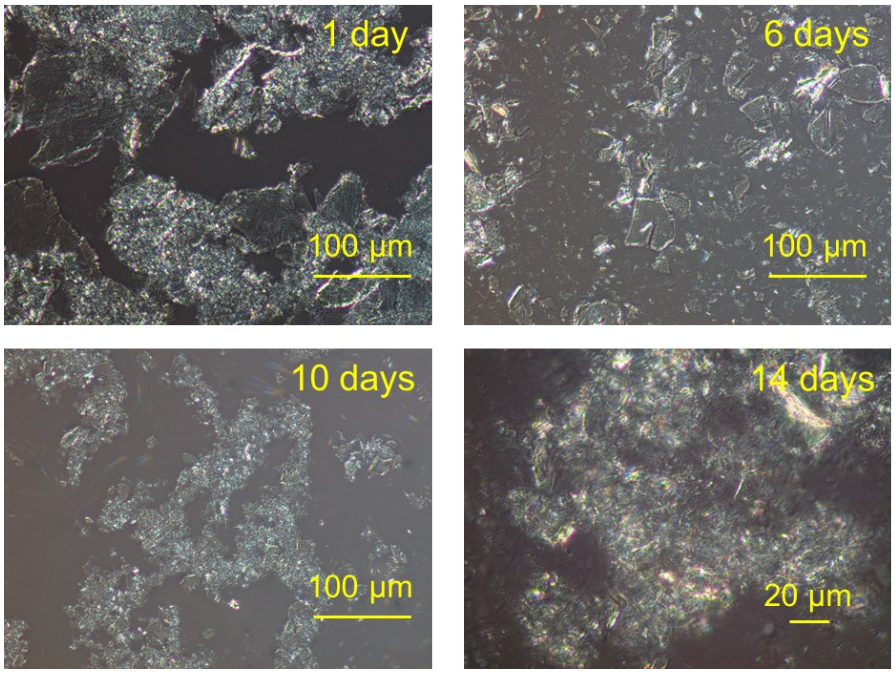

Figure S2. POM micrographs of the alkaline periodate oxidized chitins showing the strongly swollen structure as a function of the reaction time in days.

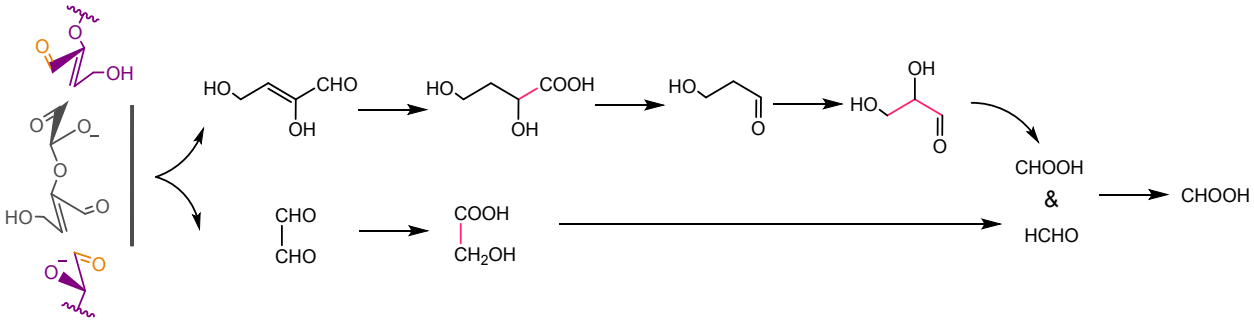

Figure S3. Proposed further oxidation and degradation on the products from β-alkoxy fragmentation.[8]

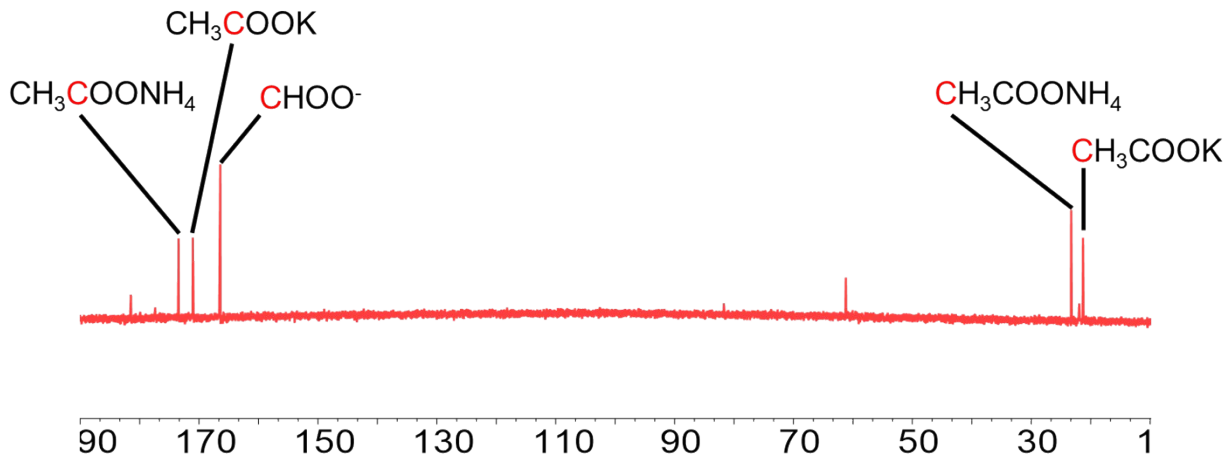

Figure S4. ^{13}C NMR spectra of soluble compounds after alkaline periodate oxidation of chitin in D_2O.

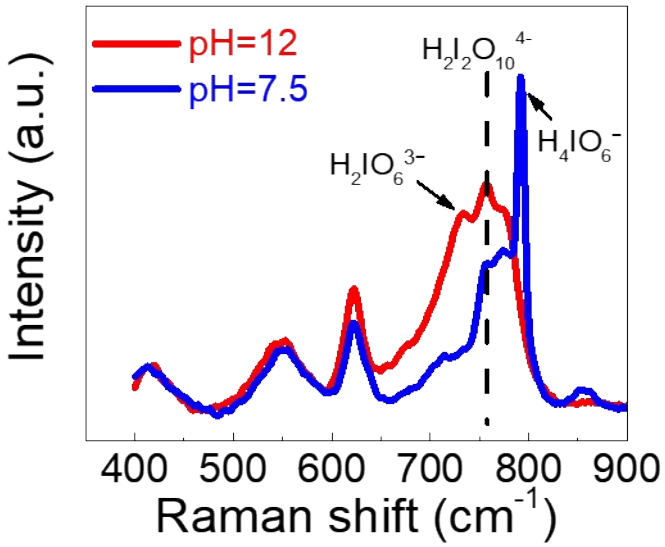

Figure S5. A representative Raman spectrum of the reaction solution at pH 7.5 and pH 12.[9-11]

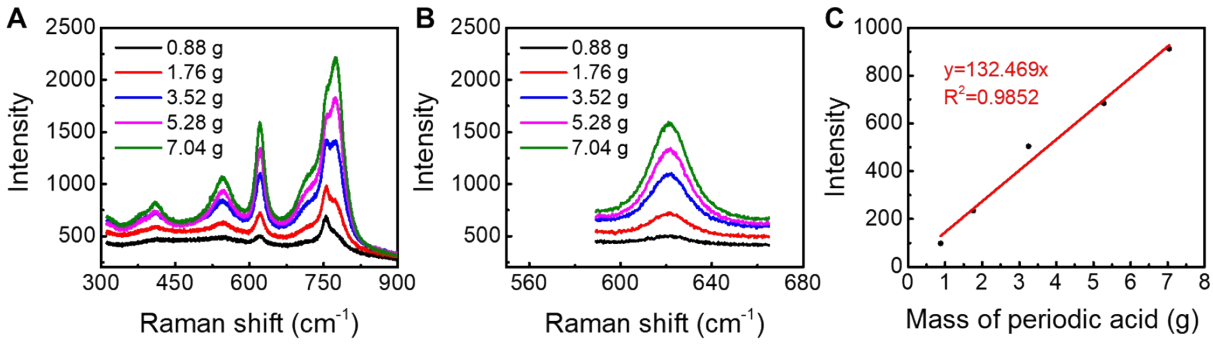

Figure S6. Calibration curve for calculating the amount of periodate in reaction solution. A) Representative Raman spectra of periodate solutions at pH 10 with various amount of periodic acid (the total volume of periodate solution is 150 mL). **B)** The intensity of the peak at 621 cm^{-1} was selected for creating the calibration curve **(C)** based on the amount of periodic acid.

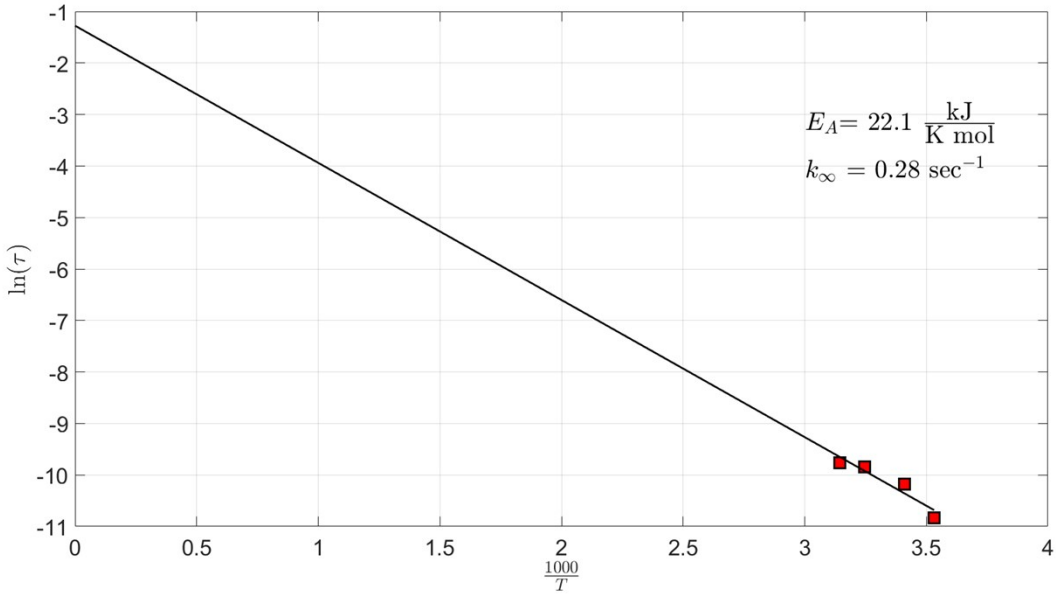

Figure S7. Arrhenius plot of the rate constants from Figure 2H.

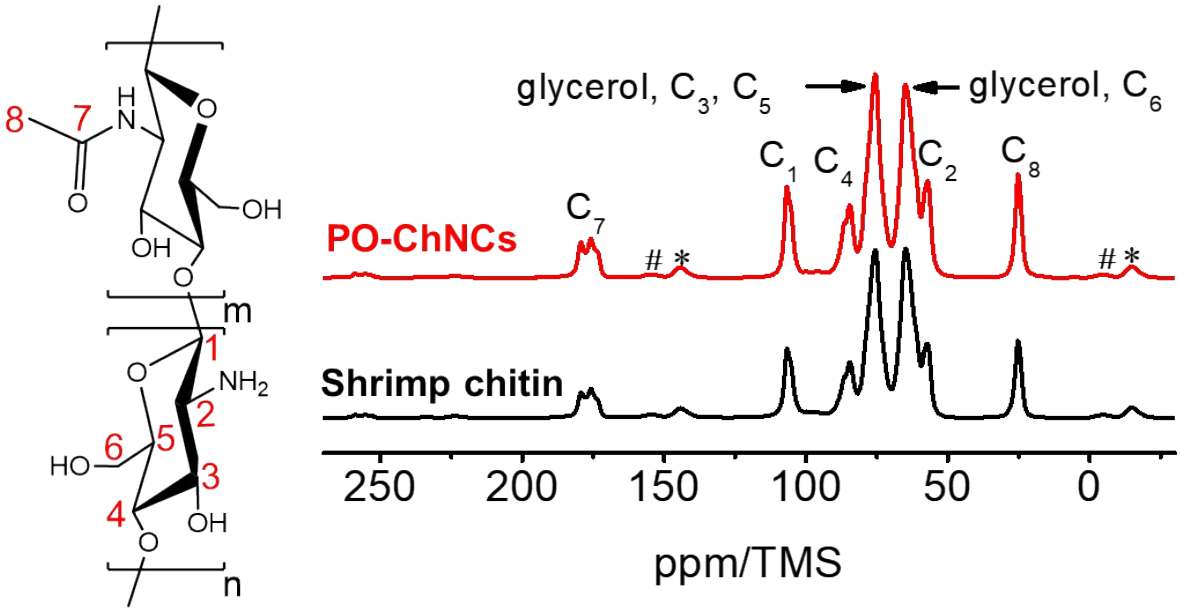

Figure S8. Dynamic nuclear polarization (DNP) enhanced solid-state $^{1}H \rightarrow ^{13}C$ CP MAS NMR spectra of shrimp chitin and PO-ChNCs. Signals marked with * and # are spinning side bands of glycerol.

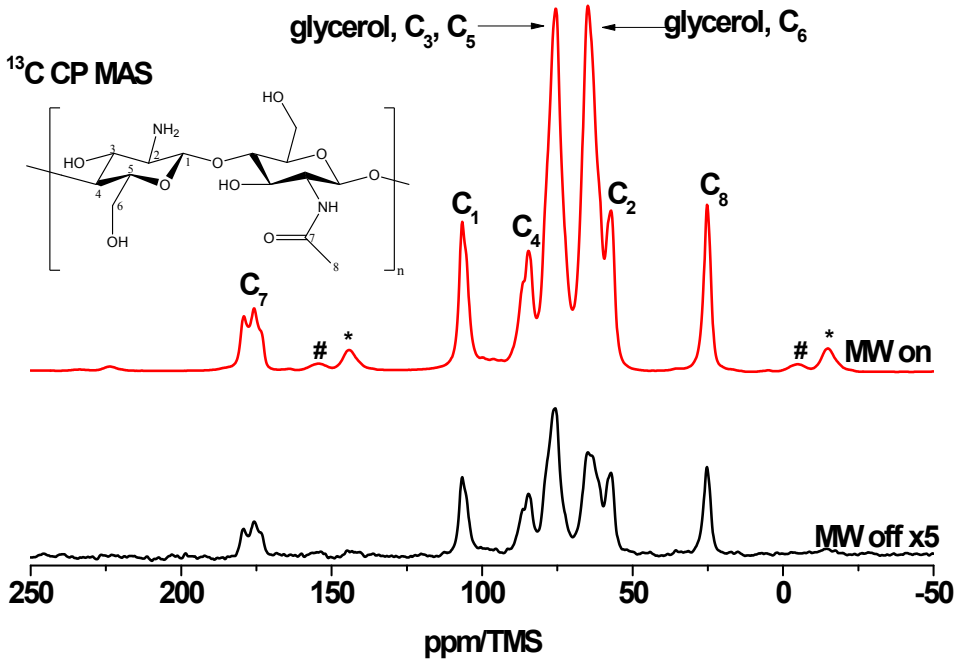

Figure S9. Signal assignment of the observed signals in the $^{1}H \rightarrow ^{13}C$ CP MAS spectra, measured with and without microwave irradiation (MW on/off). Note that the samples

were impregnated with a 15 mM AMUPol in glycerol-d_8/D_2O/H_2O solution. Signals marked with * and # are spinning side bands of glycerol.

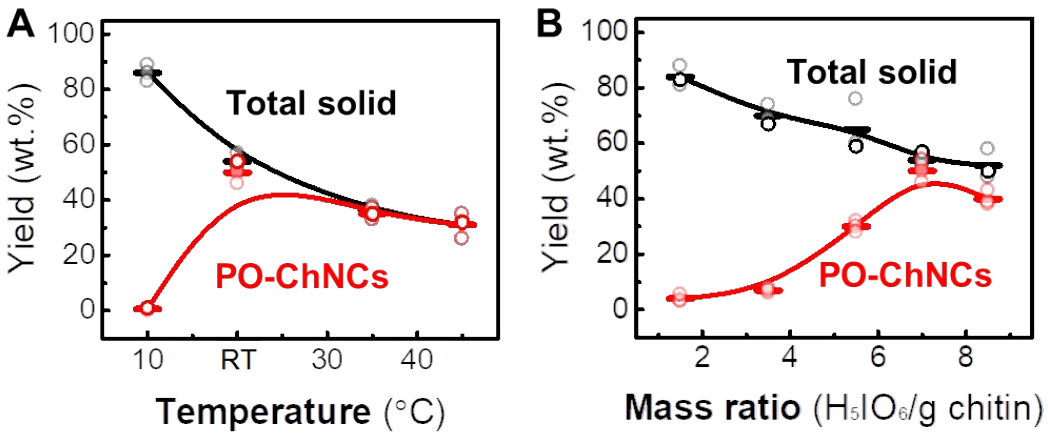

Figure S10. A) The effect of reaction temperature on the isolation process. B) The effect of the mass ratio of periodate and chitin on the isolation process.

The effects of some reaction parameters were investigated, which include the soaking as pretreatment, the amount of periodate, and the temperature.

Soaking chitin in 3 wt.% of KOH aqueous promoted the isolation process due to the cleavage of hydrogen bonds in non-ordered regions, which further accelerated the diffusion of periodate for isolation. To produce the same amount of ChNCs (~0.5 g) from 1 g of chitin without soaking, nearly 2 more weeks were needed

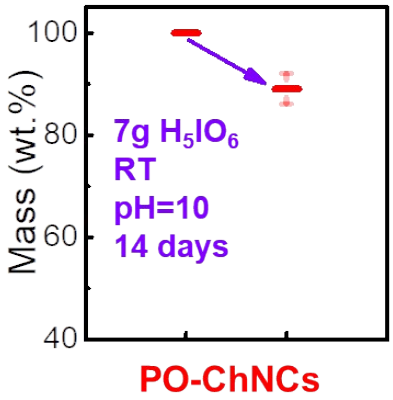

Figure S11. Mass change after 14 days alkaline periodate oxidation of PO-ChNCs.

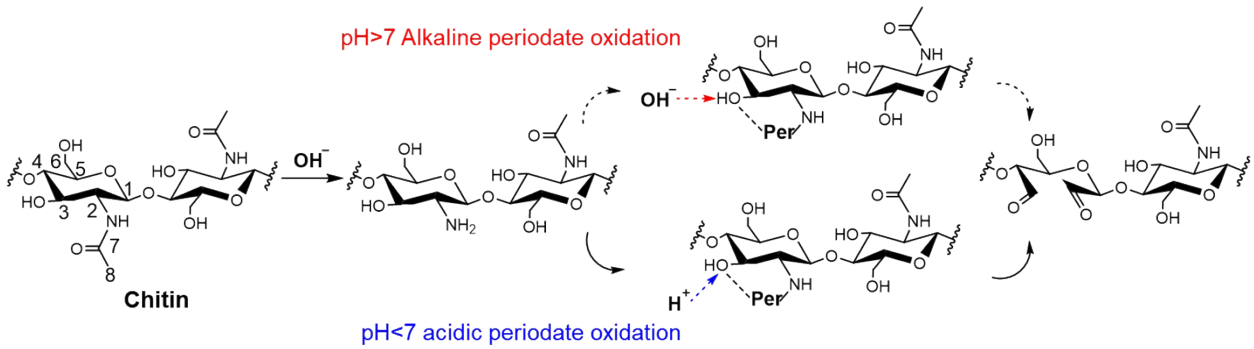

Figure S12. Alkaline periodate oxidation and acidic periodate oxidation on chitin.

Similar as the more widely accepted mechanism, periodate oxidation starts with the cyclisation of periodate ions and the hydroxyl groups and amine groups at C2/C3 positions of chitosan.[8] The cyclisation between periodate ions, hydroxyl groups and amine groups subject to general acid-base catalysis, which requires activated hydroxyl/amine groups on C2 or C3.[12, 13] In acidic periodate oxidation, the H^+ will activate the oxygen atoms in hydroxyl groups/nitrogen atom in amine groups on C2/C3. However, the protons in hydroxyl/amine groups on C2 and C3 will be activated in alkaline periodate oxidation by hydroxide.

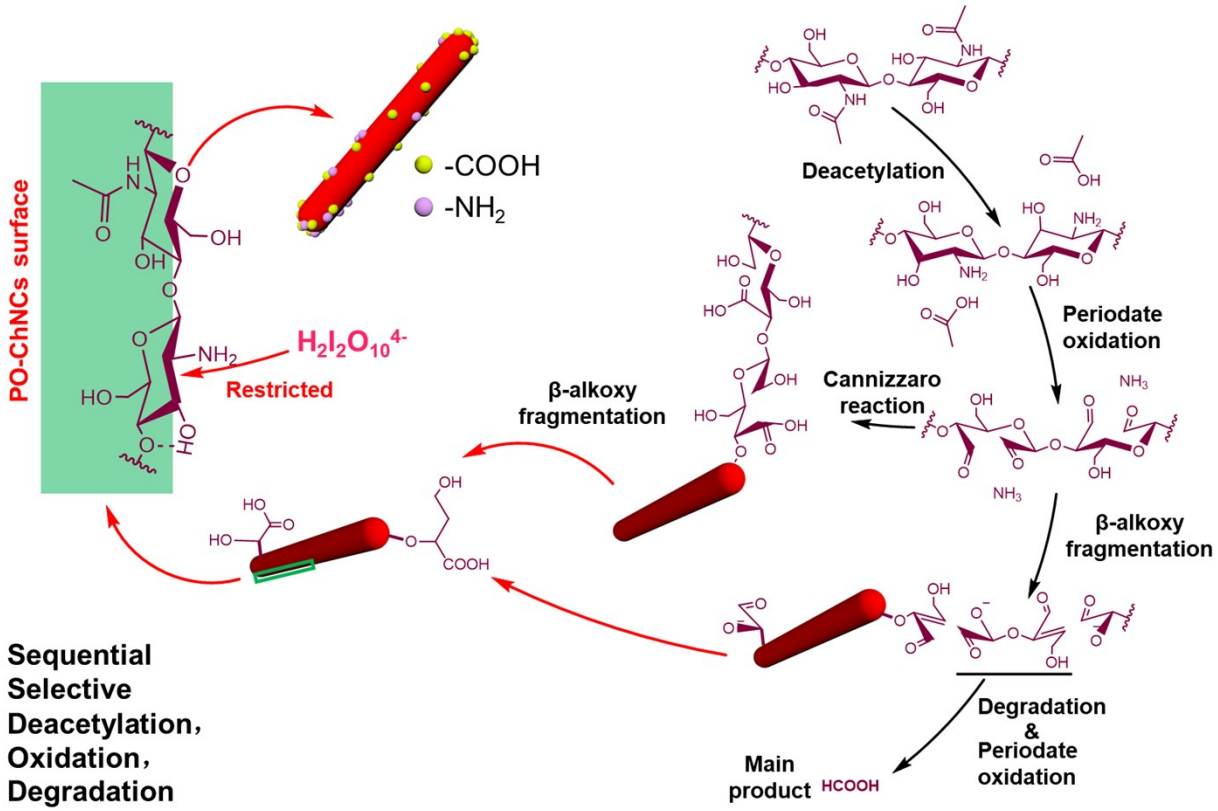

Figure S13. The proposed route for the sequential selective deacetylation, oxidation and degradation of chitin, which results in soluble compounds.

Table S1. Comparison between the alkaline periodate oxidation and existing methods for the isolation of ChNCs.[30-34]

Methods	Pre-treatment & requirement of substrate	Reaction process			Post-treatment	Size distribution[a]	Yield
		Temperature and time	Chemicals	Special treatment			
1 Alkaline periodate oxidation	Swollen in KOH (3 wt.%) for 24 hour	Ambient temperature/14 days	KOH&H_5IO_6 ($K_4H_2I_2O_{10}$, pH 10)	/	Ultrasonication, dialysis, centrifugation.	L: 241.8 ± 80 nm d: 12.2 ± 5 nm (shrimp) L: 227.1 ± 76 nm d: 11.7 ± 5 nm (crab)	50 wt.% (Shrimp) 40 wt.% (Crab)
2 Acid hydrolysis[14]	Purified fresh chitin	Boiling (100 ˚C)/1.5 hour	HCl (3N)	/	Dilution, centrifugation, dialysis, pH adjustment, ultrasonication, filtration.	L: 50 - 300 nm d: almost 10 nm	/
3 Partial deacetylation[15]	/	90 ˚C/2 - 4 hour	NaOH (33 wt.%), $NaBH_4$	Shaking every 15 – 20 min.	Wash with DI water, stirring at 1200 rpm for 5 days,[b] ultrasonication.	L: 250 ± 140 nm d: 6.2 ± 1.1 nm (crab)	85 – 90 % (Solid recovery ratios)
4 TEMPO-mediated oxidation[7]	/	Room temperature	TEMPO, NaBr, NaClO	The pH of the slurry should be maintained to be 10.	pH adjustment, centrifugation, purification, ultrasonication.	L: 50 - 500 nm d: 9 - 21 nm (crab)	90 % (water-insoluble fraction)
5 Ionic liquids[16]	Swollen in 1-allyl-3-methylimidazolium bromide for 24 hour	Boiling (100 ˚C)/48 hour	1-allyl-3-methylimidazolium bromide	/	Adding methanol, ultrasonication, purification.	L: several hundred nm d: 20 – 60 nm (crab)	/
6 APS oxidation[17]	Treatment with blender	75 °C/16 hour	Ammonium persulfate	/	Adding excess DI water, centrifugation, pH adjustment, high-intensity ultrasonication.	L: 400 - 500 nm d: average 15 nm (crab)	38 %

a: L = length, d = diameter.
b: rpm = revolutions per minute.

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