Controlled Chitosan Molecular Weight Reduction by Mechanochemical and Aging-based Phosphoric Acid Hydrolysis

*Galen Yang[a], Edmond Lam[a,b] and Audrey Moores[a,c]**

[a] Centre in Green Chemistry and Catalysis, Department of Chemistry, McGill University, Montréal, Québec H3A 0B8, Canada

[b] Aquatic and Crop Resource Development Research Centre, National Research Council of Canada, Montreal H4P 2R2, Canada

[c] Department of Materials Engineering, McGill University, 3610 University Street, Montreal, Quebec H3A 0C5, Canada

*Audrey Moores, E-mail: Audrey.moores@mcgill.ca

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ABSTRACT: Chitosan is typically produced by the deacetylation of biopolymer chitin. Its molecular weight (Mw) is a function of the chitin source and the deacetylation conditions. It remains a challenge to both control the Mw of chitosan and obtain good polydispersity, essential for optimal function of chitosan in different applications. This study explores the use of mechanochemistry and aging as effective methods towards the controlled reduction of the Mw of commercial high Mw chitosan (HMwChs) with phosphoric acid where Mw was measured by gel permeation chromatography (GPC). In comparison to conventional solution-based methods, this new mechanochemical process features improved process mass intensity in comparison to conventional techniques and affords low Mw chitosan (LMwChs) samples in a range of 200 to 19.5 kDa, with a polydispersity of 1.8.

INTRODUCTION: Chitin is a biopolymer composed of N-acetylglucosamine units, which can be extracted from various biomass waste streams including crustaceans, cephalopods, insects, and fungus in large scale annually.[1] It is the second most abundant biopolymer after cellulose and as such, constitutes the core component of the shell-biorefinery,[2] as a part of blue-biorefinery. Chitosan, as the deacetylated version of chitin, is a linear polysaccharide with amine functionality.[3] Chitosan features unique and attractive properties such as antimicrobial ability,[4, 5] biocompatibility,[6] and biodegradability,[7] applicable to many fields including biomedicine,[8] food,[9] agriculture,[10] cosmetics,[11] and water treatment.[12] Chitosan properties are largely dependent upon two features: its degree of deacetylation (DDA) and Mw.[13-15] Control over Mw is desirable as it can have considerable influence on the performance of the material in different applications. For instance, high Mw chitosan has a positive effect on water treatment by flocculation,[12] while, in aqueous solutions, viscosity increases sharply as a function of chitosan Mw,[16] so applications requiring aqueous processing usually favour lower Mw.[17] In another example, chitosan with Mw of 3000 Da showed the highest ability to inhibit pH decreases in dental plaque to reduce enamel surface damage, outperforming chitosan with Mw of 500 Da.[18] In food packaging, Liu and co-workers discovered that high Mw chitosan (HMwChs, 110 kDa) improved the mechanical properties, barrier properties, wettability and stability of packaging to extend the shelf life of strawberries in comparison to chitosan with lower Mw (50 kDa and 7 kDa).[19] Yet the control of chitosan Mw is challenging. Namely chitosan Mw depends on two factors: 1) the chitin source, as various biomass possesses different inherent chitin Mw and 2) deacetylation conditions, as alkaline conditions trigger concomitant deacetylation and glycosylic bond hydrolysis. Because it is not possible to independently control both reactions, deacetylation efficiency dominates the choice of reaction parameters and the resulting Mw chitosan is imposed by them. Thus, the development of methods towards controllable Mw reduction for chitosan is essential to afford chitosan of defined Mw. Table 1 compares approaches to chitin and chitosan hydrolysis. Controlled depolymerization of HMwChs is commonly performed in solution by acid hydrolysis,[20-22] oxidative depolymerization,[23, 24] or enzymatic degradation.[25] Most acid cleavage processes use hydrochloric, sulfuric, and phosphoric acid (Table 1, entries a-c),[26-28,29] while oxidation relies on H_2O_2 with a catalyst such as phosphotungstic acid (Table 1, entry d).[23] Enzymes are also powerful agents for Mw reduction, achieving LMwChs in the low Mw range on 20-40 kDa,[30] or even oligosaccharides (Table 1, entries e and f).[46]

Solid-state chemistry, especially mechanochemistry, has recently emerged as a way to significantly reduce or eliminate the amount of solvent in a chemical process.[31-33] Mechanochemistry utilises mechanical force to trigger chemical phenomena, and has been shown to allow a reduction in the amount of needed auxiliary substrate, chemical reaction acceleration,[34] and reduction energy requirement.[35] Besides reactions happening during mechanical treatment, the concept of aging has been introduced, in which reactions take place in the solid phase, after an initial mixing phase.[36] These solid-state methodologies are well suited in the context of biomass conversion, especially because such starting materials tend to be insoluble,[37] and have shown great promise in the context of chitin extraction, [38, 39] and conversion.[39, 40] Mechanochemistry has been used for the deacetylation of chitin into chitosan in a process pioneered by Yan and co-workers.[41] Chitin was ball-milled with solid NaOH to trigger both amide deacetylation and the nucleophilic attack of the chitin glycosidic bonds by hydroxide ions.[42] The Yan process afforded LMwChs with a tunable Mw of 1 – 13 kDa, with an excellent polydispersity index (PDI) at 1.1. Subsequently, our group introduced an aging phase to the process to afford high Mw chitosan.[43] While these processes afford chitosan in one pot, the precise control of the Mw was difficult because deacetylation cannot be decorrelated from hydrolysis.

Other methods have been explored to specifically depolymerize chitin using acid catalysed hydrolysis by ball milling, as demonstrated by Fukuoka and co-workers.[44, 45] During the process, chitin was milled extensively with mineral acid and a carbon-based solid catalyst in a planetary milling system to produce water soluble chitin-oligosaccharides with high selectivity. However, given the poor solubility of macromolecule chitin, the reaction mechanism of reducing chitin Mw, while still retaining its polymeric properties with the mechanochemical approach, was not investigated. With aging methods, the Giraud-Guille group used HCl vapours in the solid-state hydrolysis of chitosan, but the high polydispersity of the resulting chitosan hinders the applicability of the developed process (Table 1, entry g).[28]

Table 1. Summarization of the features of past works and present study on chitosan/chitin hydrolysis.

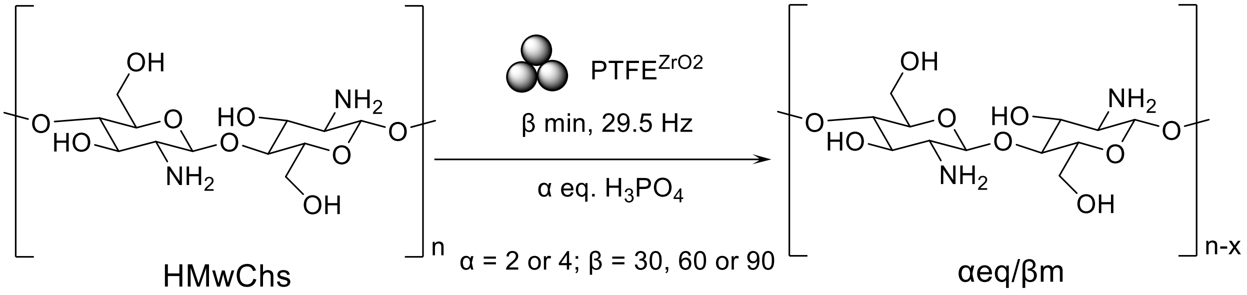
Entry	Active components/ method	Pros	Cons	Ref.
a	Chitosan, 2 M HCl/ solution state	Quick reduction, very low Mw	Reflux, large amount of acid used, low Mw control	20
b	Chitosan, 72 mM H_2SO_4 / solution state	Low acid concentration	Low yield (54%), low Mw control	26
c	Chitosan, 85% H_3PO_4 / solution state	Wide Mw range, high purity	Large amount of acid, low yield (49.3%)	21
d	Chitosan, 2% H_2O_2, 0.1% Phosphotungstic acid / solution state	Quick reaction, low H_2O_2 and acid concentration	Chitooligosaccharide only	23
e	Chitosan, No. 7-M chitosanase / solution state	Mild conditions (37˚C)	Chitooligosaccharide only	46
f	Chitin, H_2SO_4 / Mechanochemistry	Little amount of acid used	N-acetylated monomer only	45
g	Chitosan, HCl vapour / Aging	Mild conditions	Long reaction time High polydispersity	28
i	Chitosan, trifluoroacetic acid / solution state	Mild conditions	Large amount of acid used (5000 v/w%)	47
i	Colloidal chitin, 4M HNO_3 / solution state	Uniform product	Large amount of acid used, N-acetylated monomer only	48
j	Chitosan, 85% H_3PO_4 / Mechanochemistry and aging	High PMI Mild conditions Wide Mw range Excellent PDI	Long reaction time for the smallest Mw range	Present study

Herein we report a method combining mechanochemistry and aging for the conversion of HMwChs into LMwChs with Mw ranging from 858.7 to 19.5 kDa. This method leads to chitosan product with excellent PDI as low as 1.8. In particular, by varying our conditions, we can target Mw in the range of 20 to 200 kDa with PDI of 4 or under. We investigated the kinetics of this process during the mechanochemical and the aging steps to better understand the controlled chitosan Mw reduction. We investigated reaction parameters including milling time, aging time, and quantity of H_3PO_4. We also compared two sources of phosphoric acids, 85% H_3PO_4 and crystalline H_3PO_4 in order to gain insight on the role of water during the hydrolysis reaction. Finally, we demonstrate that our process features an improved process mass intensity (PMI) as compared to a comparable solution state method.

RESULTS AND DISCUSSION:

Mechanochemical hydrolysis of chitosan with a mixer mill

We first explored the mechanochemical hydrolysis of commercial HMwChs (Mw 310–375 kDa) with 85% H_3PO_4 (Scheme 1). A Retsch MM 400 mixer mill equipped with polytetrafluoroethylene (PTFE) SmartSnap grinding jars (15 mL) and one 7 mm zirconia ball in each jar was used. Reaction conditions include 2 or 4 equivalents of 85% H_3PO_4 (based on glycosidic bond) and milling time of 30, 60 or 90 min under 29.5 Hz. After set milling time, acid hydrolysis was stopped by triturating the resulting powders in deionised (DI) water. NaOH solution (1 M) was used to adjust the acidic aqueous solution to pH 12, where all excess H_3PO_4 and phosphates on the free amine groups of chitosan monomers were converted to trisodium phosphate salts. These salts, along with free NaOH, were removed by repeatedly washing with DI water,[49] leaving reduced Mw chitosan as a precipitate that was recovered by freeze-drying. Each chitosan sample produced in this experiment was abbreviated as αeq/βm in which α refers to the equivalents of acid used in milling and β refers to the milling time in minutes.



Scheme 1. Mechanochemical-based phosphoric acid hydrolysis of chitosan to *α*eq/*β*m. Typical experimental conditions: HMwChs (250 mg), α=2 or 4 eq. of H_3PO_4 (273 or 547 mg) are loaded into a PTFE jar with a 7 mm ZrO_2 ball and milled for β minutes (β = 30, 60 or 90)

The recovered chitosan was characterized by ^{13}C magic angle spinning nuclear magnetic resonance (NMR) to probe structural modification, quantitative ^{31}P NMR to check full removal of phosphate salts, and GPC in an acetic acid buffer consisting of 0.25 M acetic acid and 0.25 M sodium acetate. The original commercial HMwChs was measured to have an Mw value of 2686.9 kDa by GPC. We first tested the effect of ball milling for 30 to 90 min with 2 or 4 equivalents of 85% H_3PO_4 and measured the chitosan Mw (Figure 1, Table S2). The Mw of Sample 2eq/30m by GPC was 857.8 kDa, suggesting the method was successful in hydrolysing the chitosan. Longer milling time further reduced the Mw with values of 325.2 and 225.0 kDa for 2eq/60m and 2eq/90m, respectively. As the number of 85% H_3PO_4 equivalents was doubled to 4, a dramatic increase in hydrolysis efficiency was observed in the measured Mw with values of 116.8, 97.7, 77.3 kDa for 4eq/30m, 4eq/60m and 4eq/90m, respectively (Figure 1, Table S2). The highest degree of Mw reduction was achieved for 4eq/90m, in which the Mw was reduced by 77.4%. Interestingly the process also afforded reduced PDI. While the starting HMwChs featured an average PDI of 11.58, this value dropped to 9.7 for 2eq/30m and 2.5 for 4eq/30m. The developed process created a narrower distribution in Mw which is a desired feature of polymeric materials in application.[50-52] Solid state ^{13}C NMR spectra were acquired for all samples which showed that the molecular structure of chitosan was not altered after hydrolysis. Solution state ^{31}P NMR spectra were also acquired to confirm that the end products are pure and free of phosphate residues. All NMR spectra can be found in the Supplementary Information (SI).

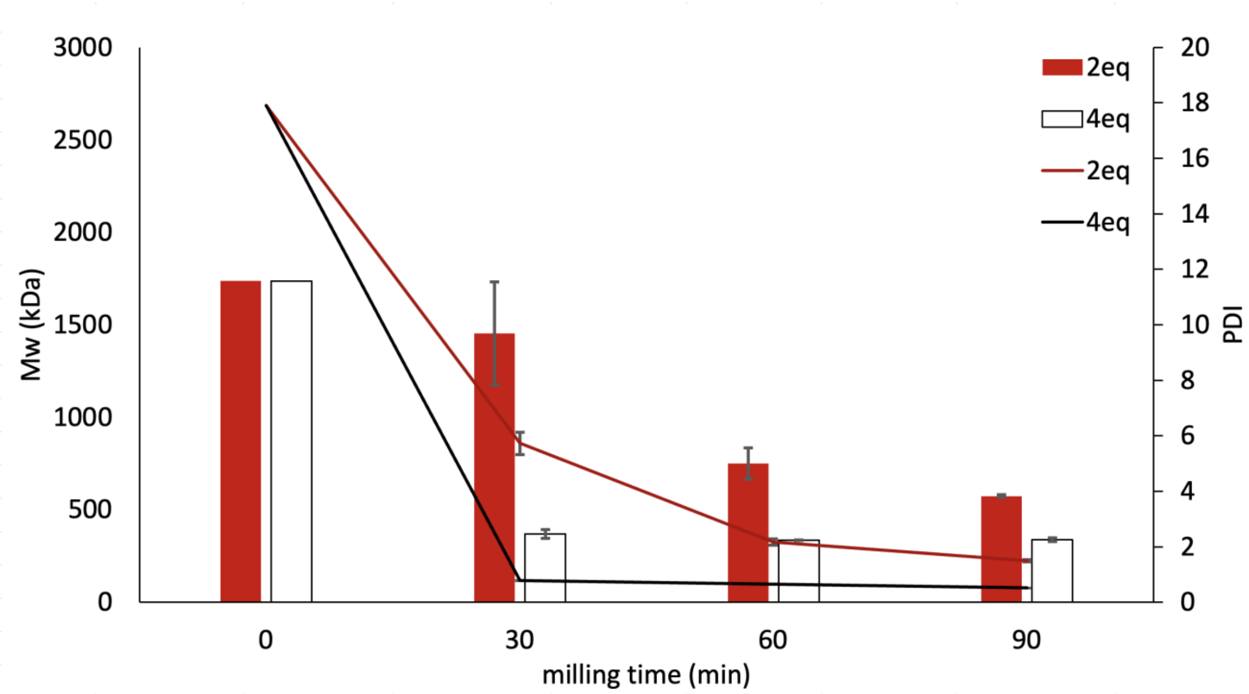


Figure 1. Effect of milling time and 85% H_3PO_4 equivalents on Mw of HMwChs milled in PTFE jar. (lines, left axis: Mw, red line 2 eq. and black line 4 eq.; Bars, right axis: PDI, red bar 2 eq. and black bar 4 eq.) Typical experimental conditions: HMwChs (250 mg), α=2 or 4 eq. of H_3PO_4 (273 or 547 mg) are loaded into a PTFE jar with a 7 mm ZrO_2 ball and milled for β minutes (β = 30, 60 or 90)

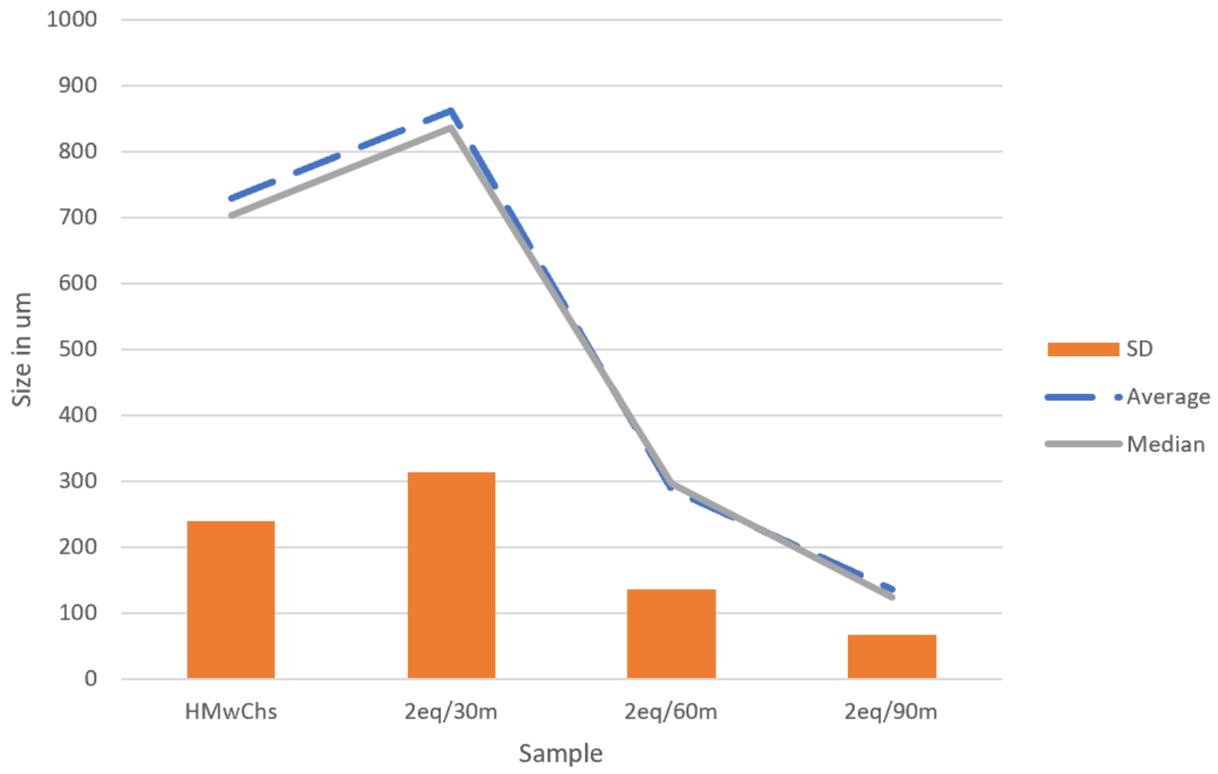


Figure 2. Effect of milling time with 2 equivalents of 85% H_3PO_4 on particle size distribution of HMwChs milled in PTFE jar, expressed in distribution standard deviation (orange bars), average size (dashed blue line) and median size (solid gray line). All samples were dispersed in isopropanol.

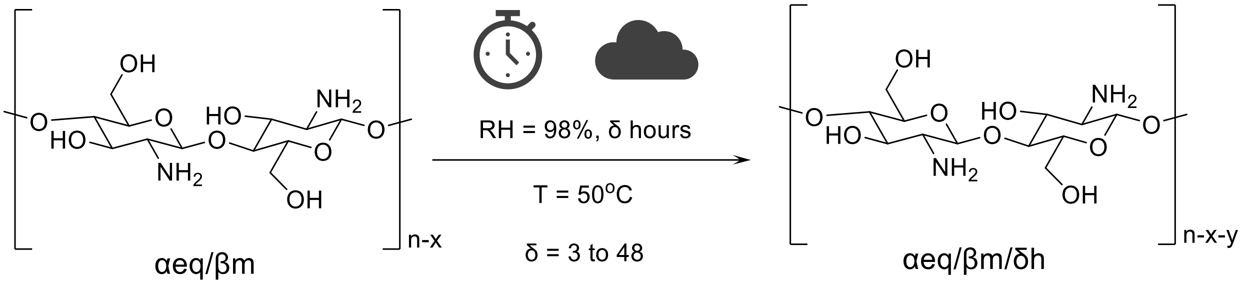
Comminution is an important, yet often overlooked, effect of mechanochemical treatment, so we turned to granulometry to study the effect grinding introduced by the mixer-mill on the particle size of chitosan. Figure 2 shows the change in particle size distribution as a function of milling time for starting material and samples treated with 2 equivalents of 85% H_3PO_4 for 30, 60, and 90 min, expressed in standard deviation, average size and median size. The starting material featured an average particle size of 729 μm with a standard deviation (SD) of 239 μm. After the first 30 min of milling, particles did increase in size, with 2eq/30m particles having an average particle size of 862 μm with a standard deviation of 314 μm. This increase in particle size and standard deviation is possibly caused by initial agglomeration of particles through binding of chitosan as a solid particle with 85% H_3PO_4 as a fluid. Further milling caused comminution, as 2eq/60m and 2eq/90m had an average particle size of 288 and 236 μm, respectively. and SD of 136 to 66.6 μm respectively. These results showed that milling beyond 60 min allowed a reduction in both particle size and size distribution from the starting material. For all samples, the median and average values of the measured distributions were similar and thus indicated fairly symmetrical distributions. The occurrence of comminution and agglomeration phenomena is intrinsic to the material being processed as a result of the mechanical properties.[53] The grinding of fragile particles was described in previous studies as a process of reduction of particle size first, and then agglomeration of the particles occurs, which was not the case that we observed on the grinding of chitosan with 85% H_3PO_3.[54] In contrary the trend observed on our system followed the pattern of grinding ductile materials, in which the two stages happen in opposite order.[55]

In another series of test, we mixer-milled HMwChs alone in a PTFE jar for 30 min and then exposed it to 2 equivalents of 85% H_3PO_4. This sample was then homogenized with a mortar and pestle to ensure some contact is established between the sample and reagent. In another trial, HMwChs and 2 equivalents of 85% H_3PO_4 were mixed with a mortar and pestle. The Mw of both samples was calculated over 2000 kDa with no statistical difference (2416.2 ± 271.5 with milling, 2040.0 ± 119.7 with mortar pestle). These result also did not suggest significant reduction in Mw from the starting material (2686.9 kDa) with these treatments. These tests demonstrate that H_3PO_4 is needed for hydrolysis to take place during the mixer milling part.

The lowest chitosan Mw value reported for this method was 77.3 kDa with 4eq/90m. Yet in high value applications such as pharmaceutics, the preferred Mw can be as low as 8 kDa for drug release applications.[56-58] We explored longer milling times, but found that while they afforded some improvement with 2 equivalents, they did not improve the 4 equivalents series. Additionally, they caused a significant rise in temperature, from room temperature (25.4°C) to 40.6°C after 90 min and 50.5°C at 180 min, which we thought would make the rationalization of mechanochemical vs. thermal effects difficult to disentangle beyond 90 min milling (see Discussion S1 in SI). We thus sought a method to further hydrolyse chitosan, and considered aging as a viable approach.[36, 43, 59, 60]

Hydrolysis of chitosan with combined mechanochemical and aging treatment

As this reaction proceeds through the hydrolytic cleavage of the chitosan glycosylic bond, water is consumed. Therefore, we explored aging under high humidity levels, and directly used the reaction samples after ball milling with 2 or 4 equivalents of 85% H_3PO_4 for 30, 60 or 90 min, to aging at 50 ℃ under 98% relative humidity (RH) for 0 to 48 h, as described in Scheme 2. We called the overall process, “Mechanochemical and Aging-based Phosphoric Acid Hydrolysis” (MABPAH). Each chitosan sample produced in this experiment was abbreviated as αeq/βm/δh in which α refers to the equivalents of acid used in milling; β refers to the milling time in min; and δ refers to the aging duration in hours.



Scheme 2. Aging of mechanochemically produced chitosan *α*eq/*β*m into further Mw reduced *α*eq/*β*m/δh. Typical experimental conditions: *α*eq/*β*m were aged at 50°C under 98% RH for *δ* h (*δ* = 3 to 48).

The Mw of samples after MABPAH were measured by GPC and presented in Figure 3. The most drastic Mw reduction observed for 2eq/30m/3h: the Mw dropped from 857.8 kDa for 2eq/30m to 297.9 kDa for 2eq/30min/3h, a 65.3% reduction assisted by 3 h of aging. In this example, with the lowest amount of H_3PO_4 added, the shortest milling time, and the shortest aging duration, the combination of mechanochemical milling and aging had a profound effect in chitosan depolymerization. Additional Mw reduction on 2eq/30min sample was observed with aging from 6 to 48 h; 2eq/30min/48h (77.3 kDa) was comparable to for the milled-only 4eq/90min (77.3 kDa). The assistance of 48 h aging was as effective as doubling amount of acid used and tripled milling time in terms of hydrolysis performance. Another interesting point is that aging seems to attenuate the effects for different milling times. With 2 equivalents, after 48 h of aging, samples achieved Mw of 72.2, 66.7 and 51.1 kDa for 30, 60 or 90 min milling, respectively. The significant gap of calculated Mw of 654.7 kDa between 2eq/30min and 2eq/90min converged to around 21.1 kDa through 48 h of aging on the respective samples 2eq/30min/48h and 2eq/90min/48h. As seen in Figure 3, Mw reduction was most effective over the first 6 h, with diminishing returns towards the total aging duration of 48 h. Importantly, this reaction condition, 2eq/90min/48h afforded excellent mass balance, with an overall yield of 92% (Table S1). This result, combined with the lowering of the chitosan PDI as the reaction progressing, is a clear indication that the hydrolysis reaction happens favourably internally rather than on chain end.

The effect of aging on extending the mixer-mill based acid catalysed chitosan hydrolysis reaction when the amount of acid was doubled to 4 equivalents was also studied. With the increased amount of H_3PO_4 the attenuation of the effects of milling times was even more profound. The Mw between 4eq/30min, 4eq/60min, and 4eq/90min converged after 48 h aging to around 20 kDa. Furthermore, Mw reduction was achieved more quickly at the higher acid content. The Mw of 4eq/30min/24h was 27.0 kDa, only slightly higher than that of 4eq/60min/24h (23.5 kDa) and 4eq/90min/24h (25.3 kDa). This can be attributed to the higher reaction kinetics facilitated by the doubled the amount of acid which enables chitosan hydrolysis, ultimately reaching a plateau around 20 kDa, which is comparable value to many other existing methods of LMwChs synthesis by hydrolysis.[20, 21, 30] The mass balance was however affected by the larger amount of reagent, as the yield was 52% for 4eq/30min/48h (Table S1).

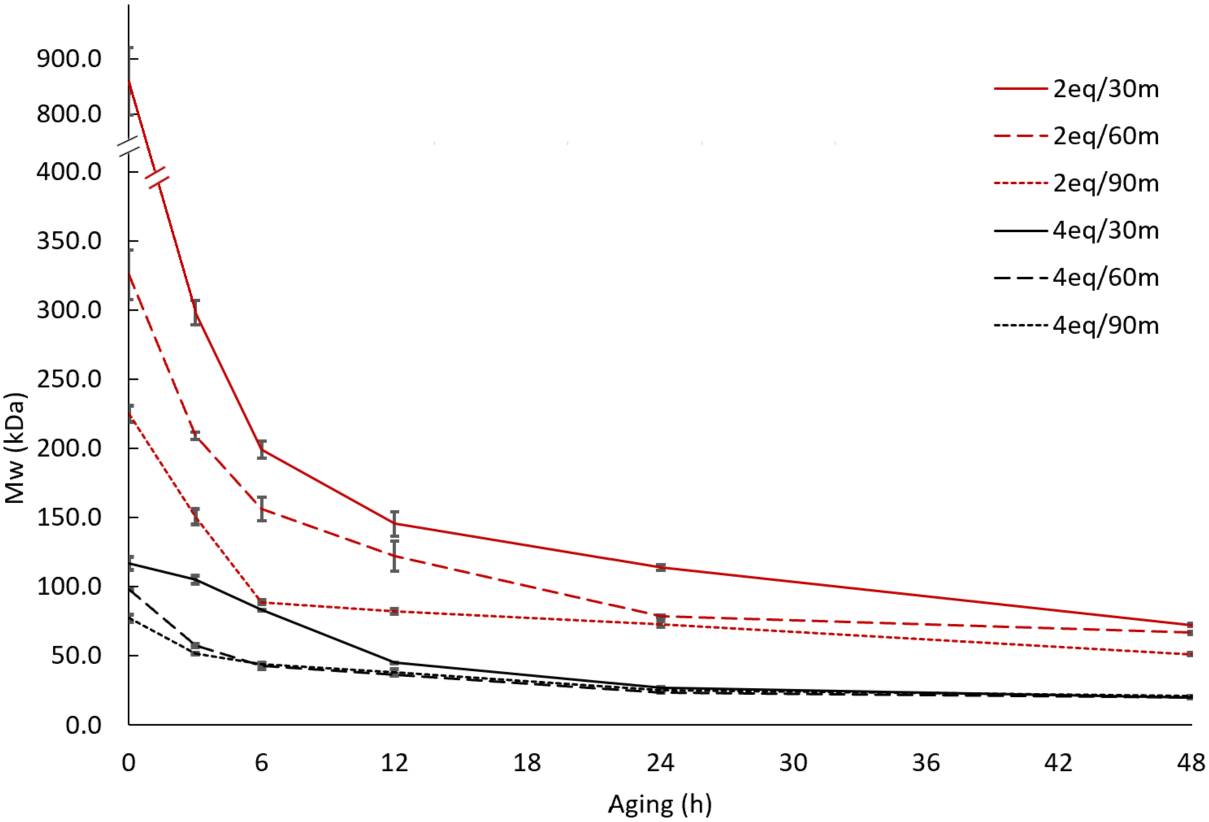


Figure 3. Effect of aging time on the Mw of HMwChs under MABPAH. Typical experimental conditions: *α*eq/*β*m were aged at 50°C under 98% RH for *δ* h (*δ* = 3 to 48).

Aging had a profound effect on the sample PDI as shown in Figure 4. After only 3 h of aging, the PDI of 2eq/30m dropped from 9.7 to 3.7 for 2eq/30min/3h. The measured PDI of the samples decreased with 48 h of aging and levelled off at about 2.0, regardless of the amount of acid used or the milling time in hydrolysing the chitosan.

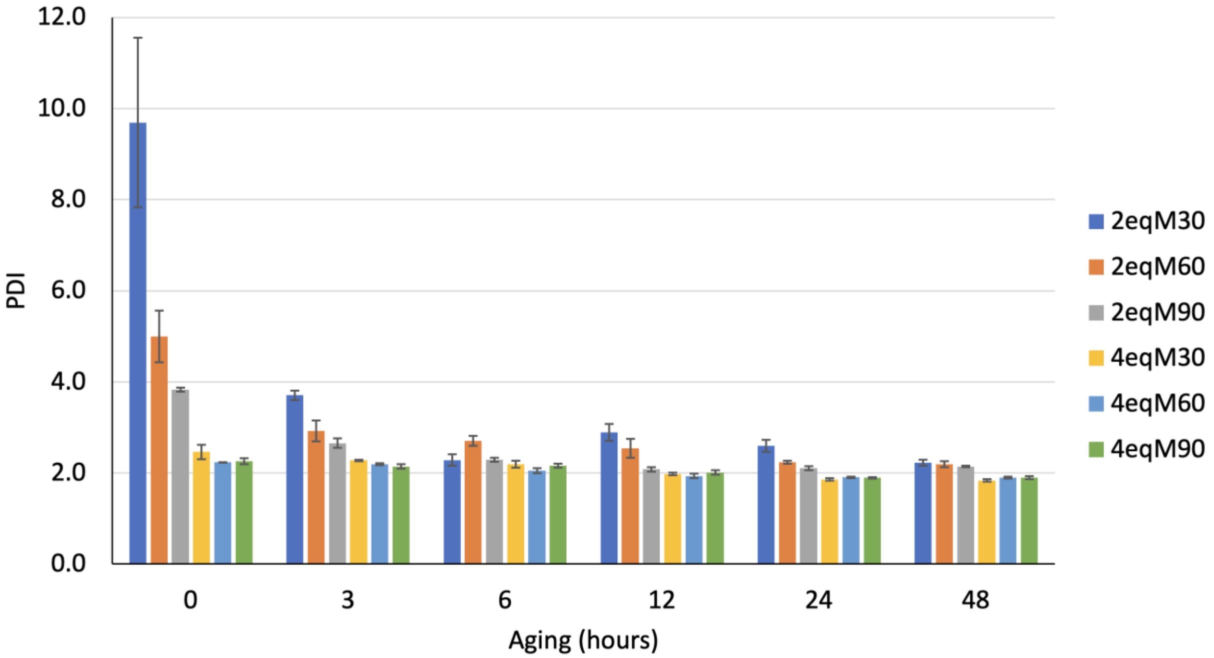


Figure 4. Effect of aging time on GPC calculated PDI of HMwChs under MABPAH. Typical experimental conditions: αeq/βm were aged at 50°C under 98% RH for δ h (δ = 3 to 48).

The role of water in the mechanochemical and aging steps in chitosan hydrolysis

When going from 2 equivalents (273 mg) of 85% H_3PO_4 to 4 (547 mg), the quantity of acid changes, the latter also contains 48 mg more H_2O. We sought to understand which component had the most effect in improving hydrolysis for the 4 equivalents H_3PO_4 reaction. Chitosan was thus milled with 2 equivalents of 85% H3PO$_4$ and an additional 48 mg H_2O for 30 min, followed by a 24 h aging under 98% RH. Under these conditions, the Mw of 2eq/30m with extra water was 2218.1 kDa (PDI 17.2), which means significantly less Mw reduction took place than with regular 2eq/30m (Mw = 858.7 kDa, PDI = 9.7). After 24 hours of aging, the Mw of the sample with water was reduced to 122.2 kDa (PDI 2.3), which was slightly higher than 2eq/30m/24h (Mw = 113.7 kDa, PDI = 2.6). The extra water slowed the reaction significantly during the milling stage, while during the aging stage, it seems to have a marginal detrimental effect. This is possibly due to the dilution of H_3PO_4, as the concentration of 85% H_3PO_4 dropped to 74% effectively with the addition of water. Another possible explanation is that the liquid assist grinding (LAG) conditions are altered and affect reaction progress, as the LAG coefficient η goes from 0.76 to 0.96. [33]

To confirm the effect of high humidity on aging, chitosan was milled with 2 and 4 equivalents of 85% H_3PO_4 for 30 min, then subjected to 24 h aging at the same temperature under static vacuum conditions with the presence of phosphorus pentoxide as drying aging for moisture removal. Under these dry aging conditions, the Mw of 2eq/30min/24h was 113.8 kDa (PDI 3.0), similar to samples aged for 12 h under 98% RH. In comparison, the Mw of 4eq/30min was 30.9 (PDI 2.2), which interestingly is also obtained from samples aged for 12 h at 98% RH. Although these numbers suggest that chitosan hydrolysis still occurs during dry aging, the reaction rate was significantly slower in dry conditions than in 98% RH, indicating high RH does play a key role in chitosan hydrolysis.

The influence of the form of acid

The mechanism of acid catalysed hydrolysis with the presence of water is well understood. In the H_3PO_4-catalysed chitosan hydrolysis reaction, other than the fraction of phosphate anions which form salts on the glucosamine free amine groups, the rest of acid catalytically activates the glycosidic oxygen, leading to the cleavage of the glycosidic linkage. With the addition of one H_2O molecule, the reaction results in two fragments of chitosan polymeric chain with the chain length shorter than the original chitosan polymer. Thus, water is essential for the reduction in chitosan Mw. We studied the role of water in the MABPAH process using crystalline, anhydrous H_3PO_4 in comparison with the 85% H_3PO_4 aqueous solution. All other parameters of the process were kept identical to allow for a direct comparison. With anhydrous H_3PO_4, the acid no longer carries water, as a reagent for the cleavage of the glycosidic linkage. During the initial mechanochemical step, the only source of water is chitosan itself. A thermogravimetric analysis (TGA) analysis revealed that the commercial HMwChs used in this reaction had a water content of 8.03%. Due to the absence of water from the acid added to the reaction. The reaction mixture of chitosan and 4 equivalents of crystalline H_3PO_4 did not turn in to a paste no matter the milling time. The reaction mixture did absorb moisture from the humid environment during aging and became a paste after 24 hr of aging.

Figure 5 compares the effects of using 85% (left) vs crystalline H_3PO_4 (right) on chitosan Mw. After the milling step (aging time = 0 h), all samples showed a lower Mw and better dispersity with crystalline H_3PO_4, as compared to the 85% solution. For instance for 2eq/30min, crystalline H_3PO_4 afforded a Mw of 408.0 kDa, with a PDI of 5.2, whereas we obtained 858.7 kDa (PDI of 9.7) with 85% H_3PO_4. With crystalline H_3PO_4, under conditions where water is restricted, milling likely leads to the formation of pockets of ultra-concentrated H_3PO_4, which can expedite the reaction. After the initial milling step, high humidity aging was performed (Figure 5, aging time >0), and both acid forms showed the reaction continued, to attain similar Mw after 48 h. Both acid forms do afford a similar kinetic of Mw reduction between aging time 3 and 48 h. This is consistent with the fact that the samples were then exposed to high humidity and could both rapidly adsorb water to further support Mw reduction. With 2 equivalents, the Mw rapidly converged to similar values rapidly, as after 6 h of aging, the Mw obtained, for crystalline resp. 85% H_3PO_4, were 191.5 and 198.9 kDa for 2eq/30min/6h, 146.2 resp. 156.2 kDa for 2eq/60min/6h and 118.5 resp. 88.8 kDa for 2eq/90min/6h (Table S2). With 4 equivalents, both sources of acid also led to similar values, but later in the aging time, around 12 h of aging.

Overall, these results suggest that mechanochemical grinding creates a homogeneous mixture of chitosan and H_3PO_4 and likely activates the hydrolysis reaction with mechanical impacts. Subsequently, aging at elevated temperature allows the hydrolysis reaction to continue under an ultra-high local concentration of acid. Water, from either the 85% H_3PO_4 solution, the native chitosan, and/or absorbed from the humid environment, drives the hydrolysis reaction catalyzed by local acidic protons in the chitosan-H_3PO_4 mixture and allows the reaction to progress within the reaction mixture. This mechanism is reminiscent of work previously reported by our group on chitin oxidation with ammonium persulfate in solid state, high humidity aging conditions.[40]

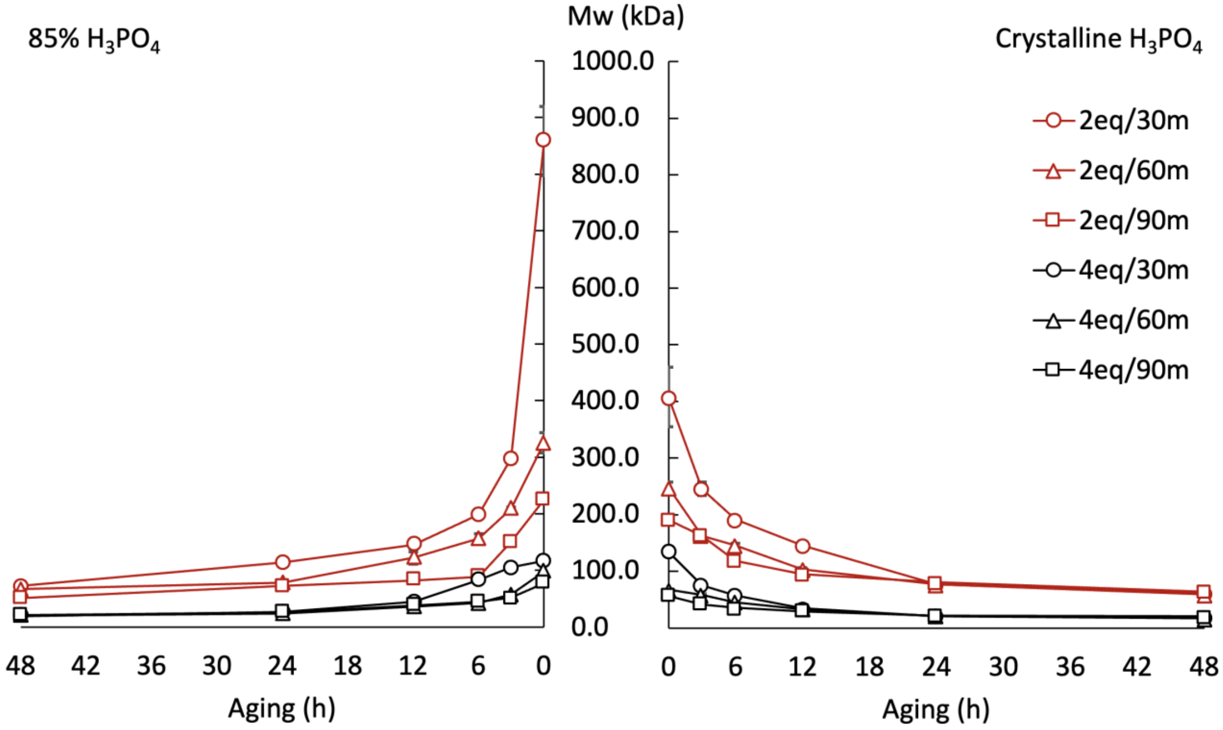


Figure 5. Comparison of Mw reduction of HMwChs by MABPAH using 85% or crystalline H_3PO_4 in PTFE jar, and aged in a humidity chamber at 50˚C and 98% RH.

Advantage of the method

We compared the MABPAH process for chitosan treated with 85% H_3PO_4 with a solution-based method of H_3PO_4-catalysed chitosan hydrolysis reported by Jia and Shen.[21] In their work, chitosan was mixed in a large quantity of 85% H_3PO_4 with constant stirring and heating at 60˚C, and the effect of reaction time and temperature on hydrolysis was investigated.[21] The MABPAH method eliminates the use of organic solvents, allowed the process to be done in a less complicated manner with the greener solvent water only in contrast to Jia and Shen's method.[61] MABPAH produces chitosan with precise control of Mw and narrow Mw distribution (PDI <2) which can be advantageous for obtaining specific Mw of chitosan for application development. For example, the optimum molecular weight for food packaging reported by Liu et al. was at 110 kDa.[19] With MABPAH, chitosan with a Mw of 135.0 kDa and PDI of 2.3 can be produced through 4 equivalents of H_3PO_4 and 30 min milling. On the other end, 4eq/90min/24h afforded Mw at 25.3 kDa with a PDI of 1.9. Within this range of chitosan's low Mw, conditions can be chosen to produce chitosan with more finely tuned Mw. For instance, 2eq/30min/48h afforded Mw at 61.1 kDa (PDI 2.1). Last but not least, all conditions mentioned above showed great reproducibility in triplicate, proving the liability of the Mw control achieved with present study.

We also wanted to compare the two methods from a green metrics perspective. The solution-based method reported by Jia and Shen served as a comparison.[21] In this method, 31 equivalents of H_3PO_4 were used, vs 2 or 4 equivalents for MABPAH. The solution-based method had a much higher calculated PMI of 6826, compared to 805 for MABPAH with 2 equivalents and 806 for the version with 4 equivalents. Additionally solvent use is much greater in the method of Jia and Shen at the workup level, although it is difficult to quantify because workup solvents quantities were not reported. One can say though that the Jia and Shen method relied on ethanol, likely in large quantity to precipitate chitosan out of the phosphoric acid solution and subsequently used in multiple washings, alongside water, for both ethylamine and NaOH treatments. For MABPAH, less than 0.5 L of water was used to work up 250 mg of chitosan. Finally, solutions already exist to recycle waste water from such processes, for instance with energy efficient non-thermal separation method such as reverse osmosis (RO) and forward osmosis (FO).[62] The only leftover waste after water separation will be a concentrated aqueous solution of trisodium phosphate, which can be used in other application such as cleaning agent,[63] or to be used in pH adjustment in water treatment.[64]

CONCLUSION: In this study, we present a solvent-free method to hydrolyse polydisperse HMwChs into LMwChs with narrow Mw distribution by a combination of mechanochemical ball milling with H_3PO_4 and aging in a humidity chamber. With ball milling, increasing molar equivalents of H_3PO_4 or increasing milling time promoted chitosan Mw reduction and lower PDI. By applying aging after the milling process, chitosan hydrolysis continued to occur, leading to even lower Mw. The net effect of these two processes can lead to controlled chitosan hydrolysis of up to 94.3% of the starting MW of the chitosan to 19.5 kDa and PDI of 1.8. The use of anhydrous, crystalline H_3PO_4 initially promotes greater chitosan Mw reduction compared to 85% H_3PO_4. However, the prolong aging under high humidity conditions normalizes the final Mw obtained.

The MABPAH method provides novelty in using soft mechanochemical ball milling to initiate hydrolysis of chitosan, followed by again, to produce LMwChs with low polydispersity. It provides a green alternative to conventional solution-based chemistry in reducing more than 85% of the H_3PO_4 required, no solvent use during reaction, no organic solvent in work-up and isolation of product, reduced energy consumption, and narrow Mw distribution. This method is simple with respect to the limited numbers of chemicals required and the use of non-sophisticated equipment for operation. In conclusion, MABPAH enables precise production of low Mw chitosan with minimal low cost for crustacean material valorisation, which can be used in value-added applications from biomedicine, food, cosmetics, water treatment, and etc.

EXPERIMENTAL:

Materials

HMwChs (310000-375000 Da as reported by company), deuterium oxide (99.9% D), and sodium acetate were purchased from Sigma-Aldrich Co. L.L.C. (St. Louis, MO, USA). 1 M NaOH solution was used to adjust pH and recover acid hydrolysed chitosan. Sodium hydroxide pellet, 85% O-H_3PO_4, anhydrous H_3PO_4, and acetic acid were purchased from Fisher Scientific L.L.C. (Ottawa, ON, Canada). A Shodex pullulan standard kit P-82 (mp 4400-788000) was purchased from Showa Denko K.K. (Japan).

Milling

In the following procedures, a Retsch MM 400 mixer mill was used, with polytetrafluoroethylene (PTFE) SmartSnap Jar (15 mL) manufactured by Form-Tech Scientific (Canada), equipped with 7 mm balls made of zirconia.

Controlled humidity chamber

A controlled humidity chamber was used for aging experiments. The RH level was set and tested as 98%. The chamber consisted of a 180 mL glass crystallizing dish, which was filled with 50 mL of saturated aqueous solutions of K_2SO_4 to access targeted RH. After milling, the grinding jar containing the solid-state reaction mixture was placed inside the chamber. The overall chamber could be heated by placing in a Fisher Scientific Isotemp oven.

Mechanochemical-based phosphoric acid hydrolysis experiments

In a typical experiment, 250 mg of HMwChs with 2 equivalents (273 mg) or 4 equivalents (547 mg) of H_3PO_4 to the glucosamine units were combined in the PTFE grinding jars, and milled for 30, 60 or 90 min with one 7 mm zirconia ball. Following milling, the reaction mixture was triturated in deionized (DI) water, worked up by adjusting to pH 11 with 1 M NaOH (335 mg for 2 equivalents, 670 mg for 4 equivalents) solution. The precipitate was washed repeatedly with DI water (160 mL) until neutral pH and freeze dried.

Mechanochemical and aging-based phosphoric acid hydrolysis experiments

In a typical experiment, chitosan was milled with H_3PO_4 as described in the previous step. After milling, the grinding jars containing the reaction mixture were transferred into the controlled humidity chamber and aged for 3 to 48 h at 50 ˚C. A brown caramel colour developed during the aging process overtime. The same work up and washing procedure was applied on the aged reaction mixture. Following milling and aging, the reaction mixture was triturated in DI water, worked up by adjusting to pH 11 with 1 M NaOH (335 mg for 2 equivalents, 670 mg for 4 equivalents) solution. The precipitate was washed repeatedly with DI water (160 mL) until neutral pH and freeze dried. Partially hydrolysed chitosan after drying had a pale off-white colour and a powdery look.

Analysis – characterization – equipment details and methods.

^{13}C magic angle spinning nuclear magnetic resonance (MAS-NMR). NMR spectra were recorded on a Varian VNMRS operating at 400 MHz for the solid-state ^{13}C acquisition using a 4 mm double-resonance Varian Chemagnetics T3 probe. A contact time of 1000 μs and a recycle delay of 3 s were used to acquire quantitative spectra. Five hundred scans were acquired of each sample for a total time of 1.5 h.

^{31}P NMR. NMR spectra were recorded on a Varian VNMRS spectrometer operating at 500 MHz for ^{1}H acquisitions with 0.3 M acetic acid D_2O solution as solvent. The amount of phosphate residue after washing was quantitatively analysed with a 48.5 mM H_3PO_4 D_2O sample as an indirect reference providing limit of detection of 0.061 mM, or 1.5% of phosphate formed on chitosan primary amie groups in present study.

GPC. GPC measurements were performed by Agilent Technologies 1260 Infinity II GPC/SEC System with refractive index, light scattering multi-detector system. A OHpak SB-805 HQ GPC column from Shodex with target pullulan M_w range of 100 – 1000 kDa was used with 0.25 M acetic acid/sodium acetate solution. Dried chitosan samples were dissolved in the same acetate buffer solution. After dissolution, samples were filtered through 0.20 μm Whatman syringe filter before injection. Concentration of injected sample was 0.1% w/w; the injection volume was 100 μL; the flow rate was 0.3 mL/min; temperature of the column and detectors was 40 ˚C. The molecular parameters were calculated by the Agilent GPC data analysis software using *K* and *a* values from the Mark – Houwink equation for chitosan of degree of acetylation in the range of 21 – 26%.[65] The column was calibrated with pullulan standard kit P-82 purchased from Shodex (mp 4400-788000).

Granulometry. Particle size distribution analysis was performed by Microtrac Flow Sync wet laser diffraction with particle size range from 0.020 to 2000 μm. Samples of chitosan (Refractive index = 1.70) were dispersed in isopropyl alcohol (RI = 1.38). The analysis was conducted at flow rate of 60% with 3 deaeration cycles.

TGA. TGA analysis was performed on a Discovery 5500 from TA Instruments under nitrogen with balance flow rate at 10 mL/min and sample flow rate at 25 mL/min. The temperature was raised from room temperature to 800˚C at ramp rate of 10˚C/min.

ASSOCIATED CONTENT

The supporting information document is available online and contains additional methods information, data on the comparison of H_3PO_4 source on Mw reduction of HMwChs, as well as representative ^{13}C NMR and ^{31}P NMR spectra and representative GPC trace.

AUTHOR INFORMATION

Corresponding Author

*Audrey Moores, E-mail: Audrey.moores@mcgill.ca

Present Addresses

GY, EL and AM: Centre in Green Chemistry and Catalysis, Department of Chemistry, McGill University, Montréal, Québec H3A 0B8, Canada

EL: Aquatic and Crop Resource Development Research Centre, National Research Council of Canada, Montréal, Québec H4P 2R2, Canada

Author Contributions

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ABBREVIATIONS

Mw, molecular weight; HMwChs, high molecular weight chitosan; GPC, gel permeation chromatography; LMwChs, low molecular weight chitosan; DDA, degree of deacetylation; PDI, polydispersity index; PMI, process mass intensity; PTFE, polytetrafluoroethylene; DI, deionised;

NMR, nuclear magnetic resonance; SI, Supplementary Information; RH, relative humidity; MABPAH, Mechanochemical and Aging-based Phosphoric Acid Hydrolysis; TGA, thermogravimetric analysis; RO, reverse osmosis; FO, forward osmosis

REFERENCES

1. Kerton, F. M.; Liu, Y.; Omari, K. W.; Hawboldt, K., Green chemistry and the ocean-based biorefinery. *Green Chem.* **2013,** *15* (4), 860-871.https://doi.org/10.1039/C3GC36994C
2. Vidal, J. L.; Jin, T.; Lam, E.; Kerton, F.; Moores, A., Blue is the new green: Valorization of crustacean waste. *Curr. Res. Green Sustainable Chem.* **2022,** *5*, 100330.https://doi.org/10.1016/j.crgsc.2022.100330
3. Kim, S. K., *Chitin, Chitosan, Oligosaccharides and Their Derivatives: Biological Activities and Applications*. CRC Press: Boca Raton, 2010; p 666.
4. Goy, R. C.; Britto, D. d.; Assis, O. B., A review of the antimicrobial activity of chitosan. *Polímeros* **2009,** *19*, 241-247.https://doi.org/10.1590/S0104-14282009000300013
5. Zheng, L.-Y.; Zhu, J.-F., Study on antimicrobial activity of chitosan with different molecular weights. *Carbohydr. Polym.* **2003,** *54* (4), 527-530.https://doi.org/10.1016/j.carbpol.2003.07.009
6. VandeVord, P. J.; Matthew, H. W.; DeSilva, S. P.; Mayton, L.; Wu, B.; Wooley, P. H., Evaluation of the biocompatibility of a chitosan scaffold in mice. *J. Biomed. Mater. Res., Part A* **2002,** *59* (3), 585-590.https://doi.org/10.1002/jbm.1270
7. Rinaudo, M., Main properties and current applications of some polysaccharides as biomaterials. *Polym. Int.* **2008,** *57* (3), 397-430.https://doi.org/10.1002/pi.2378
8. Wang, W.; Meng, Q.; Li, Q.; Liu, J.; Zhou, M.; Jin, Z.; Zhao, K., Chitosan derivatives and their application in biomedicine. *Int. J. Mol. Sci.* **2020,** *21* (2), 487.https://doi.org/10.3390/ijms21020487
9. No, H. K.; Meyers, S. P.; Prinyawiwatkul, W.; Xu, Z., Applications of Chitosan for Improvement of Quality and Shelf Life of Foods: A Review. *J. Food Sci.* **2007,** *72* (5), R87-R100.https://doi.org/10.1111/j.1750-3841.2007.00383.x
10. Malerba, M.; Cerana, R., Chitosan Effects on Plant Systems. *Int. J. Mol. Sci.* **2016,** *17* (7), 996.https://doi.org/10.3390/ijms17070996
11. Aranaz, I.; Acosta, N.; Civera, C.; Elorza, B.; Mingo, J.; Castro, C.; Gandía, M. D. L. L.; Heras Caballero, A., Cosmetics and cosmeceutical applications of chitin, chitosan and their derivatives. *Polymers* **2018,** *10* (2), 213.https://doi.org/10.3390/polym10020213
12. Yang, R.; Li, H.; Huang, M.; Yang, H.; Li, A., A review on chitosan-based flocculants and their applications in water treatment. *Water Res.* **2016,** *95*, 59-89.https://doi.org/10.1016/j.watres.2016.02.068
13. Chatelet, C.; Damour, O.; Domard, A., Influence of the degree of acetylation on some biological properties of chitosan films. *Biomaterials* **2001,** *22* (3), 261-268.https://doi.org/10.1016/S0142-9612(00)00183-6

14. Zhang, H.; Neau, S. H., In vitro degradation of chitosan by a commercial enzyme preparation: effect of molecular weight and degree of deacetylation. *Biomaterials* **2001,** *22* (12), 1653-1658.https://doi.org/10.1016/S0142-9612(00)00326-4

15. Huei, C. R.; Hwa, H.-D., Effect of molecular weight of chitosan with the same degree of deacetylation on the thermal, mechanical, and permeability properties of the prepared membrane. *Carbohydr. Polym.* **1996,** *29* (4), 353-358.https://doi.org/10.1016/S0144-8617(96)00007-0

16. Ogawa, K.; Yui, T.; Okuyama, K., Three D structures of chitosan. *Int. J. Biol. Macromol.* **2004,** *34* (1-2), 1-8.https://doi.org/10.1016/j.ijbiomac.2003.11.002

17. Wang, W.; Du, Y.; Qiu, Y.; Wang, X.; Hu, Y.; Yang, J.; Cai, J.; Kennedy, J. F., A new green technology for direct production of low molecular weight chitosan. *Carbohydr. Polym.* **2008,** *74* (1), 127-132.https://doi.org/10.1016/j.carbpol.2008.01.025

18. Shibasaki, K.; Sano, H.; Matsukubo, T.; Takaesu, Y., Effects of low molecular chitosan on pH changes in human dental plaque. *The Bulletin of Tokyo Dental College* **1994,** *35* (1), 33-39

19. Liu, Y.; Yuan, Y.; Duan, S.; Li, C.; Hu, B.; Liu, A.; Wu, D.; Cui, H.; Lin, L.; He, J.; Wu, W., Preparation and characterization of chitosan films with three kinds of molecular weight for food packaging. *Int. J. Biol. Macromol.* **2020,** *155*, 249-259.https://doi.org/10.1016/j.ijbiomac.2020.03.217

20. Aljbour, N. D.; Beg, M. D. H.; Gimbun, J., Acid Hydrolysis of Chitosan to Oligomers Using Hydrochloric Acid. *Chem. Eng. Technol.* **2019,** *42* (9), 1741-1746.https://doi.org/10.1002/ceat.201800527

21. Jia, Z.; Shen, D., Effect of reaction temperature and reaction time on the preparation of low-molecular-weight chitosan using phosphoric acid. *Carbohydr. Polym.* **2002,** *49* (4), 393-396.https://doi.org/10.1016/S0144-8617(02)00026-7

22. Il'ina, A. V.; Varlamov, V. P., Hydrolysis of Chitosan in Lactic Acid. *Appl. Biochem. Microbiol.* **2004,** *40* (3), 300-303.https://doi.org/10.1023/B:ABIM.0000025956.98250.30

23. Xia, Z.; Wu, S.; Chen, J., Preparation of water soluble chitosan by hydrolysis using hydrogen peroxide. *Int. J. Biol. Macromol.* **2013,** *59*, 242-245.https://doi.org/10.1016/j.ijbiomac.2013.04.034

24. de Farias, B. S.; Grundmann, D. D. R.; Rizzi, F. Z.; Martins, N. S. S.; Junior, T. R. S. A. C.; de Almeida Pinto, L. A., Production of low molecular weight chitosan by acid and oxidative pathways: Effect on physicochemical properties. *Food Res. Int.* **2019,** *123*, 88-94.https://doi.org/10.1016/j.foodres.2019.04.051

25. Muzzarelli, R. A. A.; Stanic, V.; Ramos, V., Enzymatic Depolymerization of Chitins and Chitosans. In *Carbohydrate Biotechnology Protocols*, Bucke, C., Ed. Humana Press: Totowa, NJ, 1999; pp 197-211.

26. Zamani, A.; Taherzadeh, M., Production of low molecular weight chitosan by hot dilute sulfuric acid. *BioResources* **2010,** *5* (3), 1554-1564

27. Hasegawa, M.; Isogai, A.; Onabe, F., Preparation of low-molecular-weight chitosan using phosphoric acid. *Carbohydr. Polym.* **1993,** *20* (4), 279-283.https://doi.org/10.1016/0144-8617(93)90100-I

28. Belamie, E.; Domard, A.; Giraud-Guille, M.-M., Study of the solid-state hydrolysis of chitosan in presence of HCl. *J. Polym. Sci., Part A: Polym. Chem.* **1997,** *35* (15), 3181-3191.https://doi.org/10.1002/(SICI)1099-0518(19971115)35:15<3181::AID-POLA11>3.0.CO;2-7

29. Cabrera, J. C.; Van Cutsem, P., Preparation of chitooligosaccharides with degree of polymerization higher than 6 by acid or enzymatic degradation of chitosan. *Biochem. Eng. J.* **2005,** *25* (2), 165-172.https://doi.org/10.1016/j.bej.2005.04.025

30. Kapadnis, G.; Dey, A.; Dandekar, P.; Jain, R., Effect of degree of deacetylation on solubility of low-molecular-weight chitosan produced via enzymatic breakdown of chitosan. *Polym. Int.* **2019,** *68* (6), 1054-1063.https://doi.org/10.1002/pi.5795

31. James, S. L.; Friščić, T., Mechanochemistry. *Chem. Soc. Rev.* **2013,** *42* (18), 7494-7496.https://doi.org/10.1039/C3CS90058D

32. Takacs, L., The historical development of mechanochemistry. *Chem. Soc. Rev.* **2013,** *42* (18), 7649-7659.https://doi.org/10.1039/C2CS35442J

33. Do, J.-L.; Friščić, T., Mechanochemistry: a force of synthesis. *ACS Cent. Sci.* **2017,** *3* (1), 13-19.https://doi.org/10.1021/acscentsci.6b00277

34. Lamour, S.; Pallmann, S.; Haas, M.; Trapp, O., Prebiotic sugar formation under nonaqueous conditions and mechanochemical acceleration. *Life* **2019,** *9* (2), 52.https://doi.org/10.3390/life9020052

35. Ardila‐Fierro, K. J.; Hernández, J. G., Sustainability assessment of mechanochemistry by using the twelve principles of green chemistry. *ChemSusChem* **2021,** *14* (10), 2145-2162.https://doi.org/10.3390/life9020052

36. Cliffe, M. J.; Mottillo, C.; Stein, R. S.; Bučar, D.-K.; Friščić, T., Accelerated aging: a low energy, solvent-free alternative to solvothermal and mechanochemical synthesis of metal–organic materials. *Chem. Sci.* **2012,** *3* (8), 2495-2500.https://doi.org/10.1039/C2SC20344H

37. Hajiali, F.; Jin, T.; Yang, G.; Santos, M.; Lam, E.; Moores, A., Mechanochemical Transformations of Biomass into Functional Materials. *ChemSusChem* **2022,** *15* (7), e202102535.https://doi.org/10.1002/cssc.202102535

38. Hajiali, F.; Vidal, J.; Jin, T.; de la Garza, L. C.; Santos, M.; Yang, G.; Moores, A., Extraction of Chitin from Green Crab Shells by Mechanochemistry and Aging. *ACS Sustainable Chem. Eng.* **2022,** *10* (34), 11348-11357.https://doi.org/10.1021/acssuschemeng.2c02966

39. Margoutidis, G.; Kerton, F. M., Biomass Processing via Mechanochemical Means. In *Biomass Valorization* [Online] Davide Ravelli, C. S., Ed. 2021; pp. 343-365. https://onlinelibrary.wiley.com/doi/abs/10.1002/9783527825028.ch12.

40. Jin, T.; Liu, T.; Hajiali, F.; Santos, M.; Liu, Y.; Kurdyla, D.; Régnier, S.; Hrapovic, S.; Lam, E.; Moores, A., High-Humidity Shaker Aging to Access Chitin and Cellulose Nanocrystals**. *Angew. Chem. Int. Ed.* **2022,** *61* (42), e202207206.https://doi.org/10.1002/anie.202207206

41. Chen, X.; Yang, H.; Zhong, Z.; Yan, N., Base-catalysed, one-step mechanochemical conversion of chitin and shrimp shells into low molecular weight chitosan. *Green Chem.* **2017,** *19* (12), 2783-2792.https://doi.org/10.1039/C7GC00089H

42. de Moura, C. M.; de Moura, J. M.; Soares, N. M.; de Almeida Pinto, L. A., Evaluation of molar weight and deacetylation degree of chitosan during chitin deacetylation reaction: used to produce biofilm. *Chemical Engineering and Processing: Process Intensification* **2011,** *50* (4), 351-355.https://doi.org/10.1016/j.cep.2011.03.003

43. Di Nardo, T.; Hadad, C.; Nguyen Van Nhien, A.; Moores, A., Synthesis of high molecular weight chitosan from chitin by mechanochemistry and aging. *Green Chem.* **2019,** *21* (12), 3276-3285.https://doi.org/10.1039/C9GC00304E

44. Kobayashi, H.; Suzuki, Y.; Sagawa, T.; Saito, M.; Fukuoka, A., Selective Synthesis of Oligosaccharides by Mechanochemical Hydrolysis of Chitin over a Carbon-Based Catalyst. *Angew. Chem. Int. Ed. 62* (3), e202214229.https://doi.org/10.1002/anie.202214229

45. Yabushita, M.; Kobayashi, H.; Kuroki, K.; Ito, S.; Fukuoka, A., Catalytic Depolymerization of Chitin with Retention of N-Acetyl Group. *ChemSusChem* **2015,** *8* (22), 3760-3763.https://doi.org/10.1002/cssc.201501224

46. Izume, M.; Nagae, S. i.; Kawagishi, H.; Mitsutomi, M.; Ohtakara, A., Action Pattern of Bacillus sp. No. 7-M Chitosanase on Partially N-Acetylated Chitosan. *Biosci., Biotechnol., Biochem.* **1992,** *56* (3), 448-453.https://doi.org/10.1271/bbb.56.448

47. Chandrasekharan, A.; Hwang, Y. J.; Seong, K.-Y.; Park, S.; Kim, S.; Yang, S. Y., Acid-Treated Water-Soluble Chitosan Suitable for Microneedle-Assisted Intracutaneous Drug Delivery. *Pharmaceutics* **2019,** *11* (5), 209.https://doi.org/10.3390/pharmaceutics11050209

48. Sri, W., STUDY OF COLLOIDAL CHITIN HYDROLYSIS TO PRODUCE THE N ACETYL GLUCOSAMINE FROM SHRIMP SHELL WASTE USING HYDROCHLORIC ACID AND NITRIC ACID. *KnE Life Sciences* **2015,** *1* (0), 77-84.https://doi.org/10.18502/kls.v1i0.89

49. Elvers, B., *Ullmann's encyclopedia of industrial chemistry*. Verlag Chemie Hoboken, NJ: 1991; Vol. 17.

50. Anwunobi, A.; Emeje, M., Recent applications of natural polymers in nanodrug delivery. *J. Nanomed. Nanotechnol.* **2011,** *4* (002), 1-6.http://dx.doi.org/10.4172/2157-7439.S4-002

51. Ouyang, X. P.; Zhang, P.; Tan, C. M.; Deng, Y. H.; Yang, D. J.; Qiu, X. Q., Isolation of lignosulfonate with low polydispersity index. *Chin. Chem. Lett.* **2010,** *21* (12), 1479-1481.https://doi.org/10.1016/j.cclet.2010.06.032

52. Whitfield, R.; Truong, N. P.; Messmer, D.; Parkatzidis, K.; Rolland, M.; Anastasaki, A., Tailoring polymer dispersity and shape of molecular weight distributions: methods and applications. *Chem. Sci.* **2019,** *10* (38), 8724-8734.https://doi.org/10.1039/C9SC03546J

53. Goodson, M.; Kraft, M.; Forrest, S.; Bridgwater, J. In *A multi-dimensional population balance model for agglomeration*, PARTEC 2004—International Congress for Particle Technology, Nuremberg, Germany, March 16-18, 2004; Nuremberg, Germany, 2004.

54. Opoczky, L., Fine grinding and agglomeration of silicates. *Powder Technol.* **1977,** *17* (1), 1-7.https://doi.org/10.1016/0032-5910(77)85037-7

55. Fadda, S.; Cincotti, A.; Concas, A.; Pisu, M.; Cao, G., Modelling breakage and reagglomeration during fine dry grinding in ball milling devices. *Powder Technol.* **2009,** *194* (3), 207-216.https://doi.org/10.1016/j.powtec.2009.04.009

56. Bruinsmann, F. A.; Pigana, S.; Aguirre, T.; Dadalt Souto, G.; Garrastazu Pereira, G.; Bianchera, A.; Tiozzo Fasiolo, L.; Colombo, G.; Marques, M.; Raffin Pohlmann, A.; Stanisçuaski Guterres, S.; Sonvico, F., Chitosan-Coated Nanoparticles: Effect of Chitosan Molecular Weight on Nasal Transmucosal Delivery. *Pharmaceutics* **2019,** *11* (2), 86.https://doi.org/10.3390/pharmaceutics11020086

57. Amoozgar, Z.; Park, J.; Lin, Q.; Yeo, Y., Low molecular-weight chitosan as a pH-sensitive stealth coating for tumor-specific drug delivery. *Mol. Pharmaceutics* **2012,** *9* (5), 1262-1270.https://doi.org/10.1021/mp2005615

58. Li, N.; Zhuang, C.; Wang, M.; Sun, X.; Nie, S.; Pan, W., Liposome coated with low molecular weight chitosan and its potential use in ocular drug delivery. *Int. J. Pharm.* **2009,** *379* (1), 131-138.https://doi.org/10.1016/j.ijpharm.2009.06.020

59. Mottillo, C.; Lu, Y.; Pham, M.-H.; Cliffe, M. J.; Do, T.-O.; Friščić, T., Mineral neogenesis as an inspiration for mild, solvent-free synthesis of bulk microporous metal–organic frameworks from metal (Zn, Co) oxides. *Green Chem.* **2013,** *15* (8), 2121-2131.https://doi.org/10.1039/C3GC40520F

60. Mottillo, C.; Friščić, T., Advances in solid-state transformations of coordination bonds: from the ball mill to the aging chamber. *Molecules* **2017,** *22* (1), 144.https://doi.org/10.3390/molecules22010144

61. Byrne, F. P.; Jin, S.; Paggiola, G.; Petchey, T. H.; Clark, J. H.; Farmer, T. J.; Hunt, A. J.; Robert McElroy, C.; Sherwood, J., Tools and techniques for solvent selection: green solvent selection guides. *Sustainable Chem. Processes* **2016,** *4* (1), 1-24.https://doi.org/10.1186/s40508-016-0051-z

62. Mazlan, N. M.; Peshev, D.; Livingston, A. G., Energy consumption for desalination — A comparison of forward osmosis with reverse osmosis, and the potential for perfect membranes. *Desalination* **2016,** *377*, 138-151.https://doi.org/10.1016/j.desal.2015.08.011

63. Agency, U. S. E. P., Sodium phosphate listing background document for the inorganic chemical listing determination. Agency, U. S. E. P., Ed. 2000; p 77.

64. Gilmour, R., *Phosphoric Acid: Purification, Uses, Technology, and Economics*. CRC Press: Boca Raton, 2013.

65. Kasaai, M. R.; Arul, J.; Charlet, G., Intrinsic viscosity–molecular weight relationship for chitosan. *J. Polym. Sci., Part B: Polym. Phys.* **2000,** *38* (19), 2591-2598.https://doi.org/10.1002/1099-0488(20001001)38:19

SYNOPSIS (Word Style “SN_Synopsis_TOC”).

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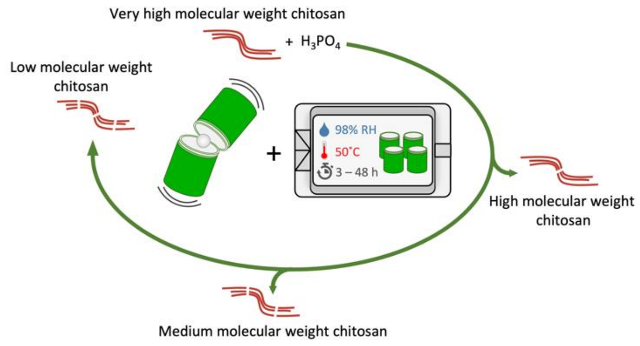

We developed a mechanochemical and aging-based method for the controlled depolymerization of chitosan affording high quality samples with defined molecular weight and low dispersity.