

Swelling by Hydrochloric Acid Partially Retains Cellulose-I Type Allomorphic Ultrastructure But Enhances Susceptibility toward Cellulase Hydrolysis Such as Highly Amorphous Cellulose

Shishir P. S. Chundawat^{1,*} and Umesh P. Agarwal²

¹Department of Chemical and Biochemical Engineering,
Rutgers-State University of New Jersey,
98 Brett Road, Piscataway, New Jersey 08854, United States

²Forest Products Laboratory, USDA Forest Service,
Madison, Wisconsin 53726, United States

*E-mail: shishir.chundawat@rutgers.edu.

Enzymatic conversion of cellulosic biomass into fermentable sugars such as glucose is a slow and catalytically inefficient process, largely due to limited accessibility of cellulose. Cellulose crystallinity can be reduced to increase substrate accessibility toward cellulolytic enzymes by either swelling or dissolving biomass in chemicals such as concentrated acids (e.g., phosphoric acid or H_3PO_4 , sulfuric acid or H_2SO_4) or ionic liquids. Phosphoric acid swollen cellulose (PASC) is one such highly digestible form of regenerated amorphous cellulose (RAC), enriched along with some cellulose-II allomorph that can be readily produced in the lab and is, therefore, the most widely reported form of amorphous cellulose in the literature. However, concentrated hydrochloric acid (HCl) can also be used to produce RAC completely free of inorganic esters, which is structurally more representative of native cellulosic substrates and, therefore, is a useful alternative model substrate for cellulolytic enzyme assays. Here, unlike previous reports, we found that concentrated HCl swells cellulose into a gel-like state at temperatures close to freezing (4 °C), while only partially hydrolyzing and dissolving a small fraction of cellulose as large celloextrins. Raman spectroscopy analysis suggests that cellulose-I transitions from its native allomorphic state to an intermediate swollen, gel-like state that retains some “memory” of the original starting structure, which facilitates its transition back into a cellulose-I-like state upon full solvent removal and lyophilization. Surprisingly, the cellulose regenerated from this HCl-treated cellulose gel-like state resulted in an amorphous PASC-like substrate before drying with comparable enzymatic digestibility.

Introduction

Concentrated acids, such as phosphoric, nitric, and sulfuric acid, have all been shown to swell and/or dissolve microcrystalline cellulose (1–3). These acids have long been known to act as efficient cellulose solvents that are also capable of hydrolysis of the solubilized polysaccharides at higher temperatures to produce high molecular weight celloextrins (2). The hydrolytic ability of concentrated acids can be minimized by decreasing the cellulose dissolution temperature, which can be optimized based on the cellulose source/properties and type of acid. Acid-based solvents can produce a highly decrystallized and amorphous cellulose-enriched substrate that can be readily hydrolyzed by enzymes into fermentable sugars. One major advantage of using concentrated acids as pretreatment solvents is that these processes are typically feedstock agnostic, and can work effectively on a wide range of lignocellulosic feedstocks. Cellulose solvent and organic solvent-based lignocellulose fractionation (COSLIF) process was developed in the mid-2000s to fractionate lignocellulose using concentrated phosphoric acid and an organic solvent (e.g., acetone or ethanol) to decrystallize and fractionate biomass under modest reaction conditions (4). Several biofuel companies, such as BlueFire Renewables and Virdia, employed concentrated acid-based biomass pretreatment and saccharification processes in the early 21st century. While these technologies have not yet been readily commercialized, concentrated acid-based solvents are still used in research labs to generate amorphous cellulose-based substrates that are more amenable to high-throughput enzyme assays or screening. Also, since these acid-based solvent systems are more readily available, compared to some exotic solvent, such as ionic liquids, these solvent systems can and have been used to study the mechanism of cellulose dissolution to produce fully molecular solutions of cellulose.

As highlighted by various research groups over the last 150 years (5), cellulose swelling and dissolution are distinct but closely related processes that often vary in the severity of the dissolution process parameters (e.g., time, temperature, mixing rate, moisture content) and/or cellulose physicochemical properties (e.g., degree of polymerization, crystallinity index). Cellulose swelling is often thought to retain the original underlying parallel-chains structure of native crystalline cellulose-I, while undergoing a significant increase of the cellulose crystallite volume due to the uptake of the swelling agent between individual fibril chains. Concentrated bases, such as anhydrous liquid ammonia, have been reported to readily swell (but not dissolve) cellulose into an intermediate crystalline structure called *ammonia-cellulose-I complex* (6–9). This intermediate complex finally results in a distinct allomorph, called *cellulose-III*, upon complete removal of liquid ammonia under anhydrous conditions. Cellulose-III is a metastable allomorph of cellulose with a $P2_1$ monoclinic unit cell with parallel packed cellulose chains, but with hydroxymethyl groups in *gauche-trans* (GT) conformation unlike the *trans-gauche* (TG) conformation of cellulose-I. Cellulose-III can be further converted back into a partially decrystallized cellulose-I-like state upon boiling in hot water/air/glycerol (10–13). On the other hand, cellulose-II is the dominant cellulose allomorphic structure formed, which consists of a two-chain $P2_1$ monoclinic unit cell where cellulose chains are stacked with opposite polarity, also called an “antiparallel structure,” which results from either the treatment of cellulose-I by alkali solution or during cellulose recrystallization or regeneration from a homogenous cellulose solution. Cellulose-II has been shown to be produced during regeneration of celloextrins and cellulose dissolved in concentrated acid solutions (14, 15) or other solvent systems (e.g., LiCl-DMAc (16), NaOH (17), 1-butyl-3-methylimidazolium chloride) (18, 19). For most of the last century, scientists believed that cellulose-I could not be regenerated from a fully molecular cellulose solution and would only exist in its native cellulose-I metastable form during biosynthesis, where chain polymerization and crystallization would occur simultaneously to give

rise to the parallel-chains-based self-assembled microfibril structure (20–22). This paradigm was reinforced by the observation that fully solubilized cellodextrins crystallize into precipitates with unit cell structures similar to cellulose-II (23, 24). However, Atalla and co-workers suggested the possibility of regenerating crystalline cellulose-I (and/or cellulose-IV-like structures in some cases) upon precipitation of cellulose from an 85% phosphoric acid solution using glycerol as an antisolvent agent at 170 °C (25). However, Wada and co-workers have recently called into question the validity of the distinct cellulose-IV polymorphic state versus a more likely laterally disordered cellulose-I structure instead (13). Nevertheless, this suggests that regeneration of cellulose into its native cellulose-I-like lattice structure from a fully homogenous solution or solvent swollen states has several open questions that still need to be addressed.

Walseth was the first to develop a protocol for swelling cellulose in concentrated phosphoric acid to produce regenerated amorphous cellulose (RAC) suitable for well-defined cellulase activity assays (26). Zhang later performed a systematic analysis to study the effect of phosphoric acid concentration on cellulose swelling (3). Studies using phosphoric acid and various ionic liquids have shown that full cellulose dissolution is a two-step transition that takes place from a two-phase system to a one-phase system when the native crystalline structure of cellulose is fully decrystallized and solvated to dissolve individual molecular cellulose polymer chains (3, 27). In the case of microcrystalline cellulose (e.g., Avicel PH-101) dissolution using phosphoric acid, there seems to be a gradual transition between the swelling process and the full dissolution process at acid concentrations greater than ~80% w/w (which is also a function of cellulose concentration and cellulosic substrate properties) (3). Ice-cold concentrated phosphoric acid (80–85% w/w) has been shown to produce a fully homogeneous solution of cellulose. Furthermore, precipitation of cellulose from such homogeneous solutions using cold or room temperature-based antisolvents (e.g., water, ethanol, or acetone) invariably leads to the production of RAC precipitates that are enriched in mostly amorphous cellulose with a cellulose-II-like structure (19). Further lyophilizing of the precipitated PASC or RAC has shown partial hornification or recrystallization of the cellulose, but the dried precipitate is still mostly enriched in cellulose-II and amorphous cellulose based on X-ray diffraction (XRD), Raman spectroscopy, and NMR-based structural characterization of the recrystallized materials (3, 16, 28). Regeneration or recrystallization of cellulose-II from a homogenous molecular solution of cellulose has been justified from a thermodynamic point of view, suggesting that cellulose-II likely has a lower free-energy state than other allomorphic states of cellulose (29, 30). However, this is still a hotly debated field since the cellulose allomorphic state has been shown to be closely dependent on the exact cellulose regeneration conditions, solvent type, and the original starting material properties.

Lastly, understanding the relationship between the cellulose ultrastructure and enzyme activity is also an active area of study, with several unanswered questions still plaguing the research community. Establishing consensus is critical to developing effective pretreatments and engineered enzyme cocktails that can facilitate the cost-effective conversion of cellulosic biomass into fuels and chemicals (31). A decrease in intrasheet and intrachain cellulose polysaccharide hydrogen bonds during pretreatment that results in the formation of amorphous cellulose (32), cellulose-II (33, 34), or cellulose-III (35–37) is invariably associated with some degree of decrystallization that is thought to directly impact enzyme accessibility/activity. The relationship between cellulose crystallinity of various cellulose allomorphs and their enzymatic digestibility has been closely examined for both cellulose-II and cellulose-III in the last decade (34, 36). The disruption of the hydrogen bond network in cellulose-III was correlated with an increased number of solvent-exposed cellulose chain hydrogen bonds with water by ~50% versus native cellulose-I (36). This was accompanied by

enhanced cellulase saccharification rates by up to fivefold for cellulose-III versus cellulose-I, depending on cellulose source and cellulase family. The enhancement in apparent cellulase activity was attributed to the “amorphous-like” nature of the cellulose-III fibril surface that facilitated easier glucan chain extraction into the enzyme active site; however, there was no significant enhancement in overall accessible surface area for cellulose-III that is seen for highly amorphous substrates (e.g., PASC). This explains why substrates such as PASC are still more easily digestible than any known cellulose allomorphs studied to date. However, subtle alterations within the cellulose allomorphic ultrastructure have recently been shown to impact cellulase binding, which could enhance activity by reducing nonproductive enzyme binding (37). Furthermore, it is also well-known that PASC has a significant fraction of phosphate esters formed during cellulose dissolution in phosphoric acid. Such chemical modifications of PASC could chelate metal ions and possibly have a deleterious impact on the activity of certain classes of cellulolytic enzymes. This is particularly relevant in light of recent discoveries made regarding lytic polysaccharide monooxygenases (LPMOs), which are dependent upon the availability of divalent cations such as Cu^{2+} within the LPMO active site for driving catalytic activity (38). This further highlights the need to develop alternative pretreatment strategies in the lab to generate highly amorphous cellulose of varying degrees of crystallinity without chemical modification that can be used for characterizing and screening diverse cellulolytic enzymes.

Here, we follow up on a previous paper published by Hsu and Penner that reported a protocol to prepare RAC (or high-molecular weight cellooligosaccharides) using concentrated HCl (1). One of the curious findings reported in this study was the need to use ultra-low freezing temperatures (-25 to -30 °C) to fully dissolve cellulose into solution followed by partial hydrolysis of the cellulose into cellooligosaccharides at room temperature (25 °C) for varying periods of time (0–60 min) prior to regeneration of cellulose using water as antisolvent to produce a highly amorphous cellulose along with cellulose-II. Instead, we were originally interested in developing a modified cellulose dissolution process to generate amorphous cellulose using ice-cold (0–4 °C) concentrated reagent grade HCl, which is similar to the PASC preparation methods reported earlier in its ease to set up and perform (26). However, to our surprise, we noticed that the cellulose became swollen using ice-cold concentrated HCl and resulted in the formation of two distinct phases: denser gel-like phase and lighter liquid-phase. Cellulose regenerated from these two HCl swollen or dissolved cellulose phases was found to have distinct cellulose allomorphic states, with varying degrees of susceptibility toward hydrolytic cellulases. Surprisingly, the cellulose regenerated from this HCl-treated cellulose gel-like state resulted in an amorphous PASC-like substrate, that has comparable enzymatic digestibility to PASC before air-drying or lyophilization. However, after drying the HCl-based cellulose reverted to an enzymatically recalcitrant highly crystalline cellulose-I-like allomorphic state, while wet PASC reverted to more readily digestible dry amorphous cellulose state enriched in some cellulose-II. We provide evidence of our experimental findings, Raman spectra for the pretreated celluloses, and speculate on the possible mechanism for the origin of distinct cellulose allomorphic states regenerated from the concentrated HCl-based cellulose–solvent solution phases.

Materials and Methods

Cellulosic Materials

Microcrystalline cellulose, Avicel PH-101, was procured from Sigma-Aldrich (St. Louis, MO) and is reported as cellulose-I in this study. Cellulose-I was used as is for all experiments and had a total original moisture content of under 4–5% (total weight basis). Concentrated reagent grade

HCl (36.5–37.5% w/w) and sodium hydroxide were available from Fisher Scientific. Cellulose-II hydrate was prepared by mercerization of 0.25 g of Avicel (cellulose-I) in 25 mL of 5 N- NaOH solution for 1 h with intermittent mixing at room temperature, followed by extensive washing with water until neutral pH, as reported by Wada (34). Cellulose-II hydrate substrate was lyophilized to produce cellulose-II. RAC or phosphoric acid swollen cellulose (PASC) from Avicel was prepared using 83% w/w phosphoric acid (at 4 °C for 1 h) based on a previously published protocol (3, 36). PASC samples were either freeze-dried (lyophilized) prior to characterization or used as is without drying. The cellulose crystallinity index (CrI) for some dry (e.g., cellulose-I) or lyophilized samples was estimated based on the XRD or Raman spectroscopy-based methods as reported previously (36, 39).

Cellulose Swelling Using Ice-Cold Concentrated HCl

Cellulose-I (Avicel PH-101) was swollen using ice-cold concentrated reagent grade HCl on ice at ~4 °C for 1 h (see Figure 1A for a general schematic overview of the entire dissolution and cellulose recovery process). Briefly, 200 mg of dry microcrystalline cellulose-I is added into a 50 mL Falcon tube. Next, while swirling the tube, 10 mL of concentrated ice-cold HCl solution (~36.5–37.5 wt %) is added to the tube. To ensure complete saturation and swelling of the cellulose, a glass rod was used to vigorously mix the slurry solution to facilitate the breakup of any cellulose clumps to facilitate swelling/dissolution. The tube was left on ice for at least 1 h with intermittent mixing to fully swell and dissolve the cellulose. A translucent cellulose gel-like viscous layer was clearly seen at the bottom of the tube with no visible Avicel particles and with a lower viscosity second phase of liquid seen at top of the gel-like layer. The top layer was easily pipetted off into a separate tube (after centrifugation of the Falcon tubes for 30 min at 4 °C) to isolate the bottom gel-like layer for further analysis. To precipitate and regenerate cellulose as whole HCl acid swollen cellulose (HASC) from the entire solution, continue to mix the two phases well before adding cold water and proceed to recover cellulose as discussed below. Or, to precipitate and regenerate the cellulose specifically from either the top supernatant liquid layer (HASC-SN) and bottom gel-like pellet layer (HASC-P) phases, add 40 mL of ice-cold water in 10 mL increments to separate tubes containing the supernatant or gel pellet phases. Mix thoroughly in between each addition in order to regenerate cellulose out of concentrated acid solution as visible white solid precipitates. Centrifuge the tubes containing the precipitated cellulose for 5 min at 4200 rpm to pellet the regenerated cellulose and discard the supernatant after checking pH. Wash the cellulose with water and repeat the process until the supernatant has a pH close to neutral. If needed, add NaOH solution to neutralize the solution pH prior to extensive washing with DI water to remove all salts formed. The precipitated cellulose-water slurry can then be either air-dried overnight in a fume hood or lyophilized after slow-freezing to generate dry samples for further spectroscopic analysis, as detailed later in the study. Neutral pH washed HCl-swollen cellulose can also be stored in a slurry form at 4 °C for 1 week as is or up to 2 months if sodium azide (0.05% w/v) is added to prevent any microbial growth.

Enzymatic Hydrolysis

The enzymatic hydrolysis procedure was based on a modified NREL LAP-009 protocol. Briefly, all samples were hydrolyzed in a 0.05 M citrate buffer (pH 4.5) at 0.4% glucan loading (at 1 or < 5 mL total reaction volume) with the necessary commercial cellulases (Celluclast or C.Tec2) and

β -glucosidase (Novo 188 supplemented with Celluclast only), procured from suitable commercial sources. Celluclast (56 ± 3 mg/mL; Sigma-Aldrich), C. Tec2 (86 ± 1 mg/mL; Novozymes), and Novozyme 188 (26 ± 3 mg/mL; Sigma-Aldrich) total protein concentration after desalting was based on Bradford assay method using bovine serum albumin (BSA) as standards (40). Necessary amount of cellulose was delivered to each reaction vial, either in wet slurry or dry powder form as reported previously (41). Enzymatic hydrolysis experiments were conducted using either a low, medium, or high-total cellulase enzymes loadings, as explained below. Low enzyme loading experiments were carried out either using 1.5 mg C.Tec2 cellulase/g dry biomass (DBM) equiv or 1.5 mg Celluclast cellulase/g DBM equiv supplemented with Novo 188 (1.5 mg enzyme/g DBM). Medium enzyme loading experiments were carried out either using 5 mg C.Tec2 cellulase/g DBM equiv or 5 mg Celluclast cellulase/g DBM equiv supplemented with Novo 188 (5 mg enzyme/g DBM). High enzyme loading experiments were carried out either using 15 mg C.Tec2 cellulase/g DBM equiv or 15 mg Celluclast cellulase/g DBM equiv supplemented with Novo 188 (15 mg enzyme/g DBM). Sodium azide was added to prevent any microbial growth (0.01% w/v final concentration). All samples were incubated at 50 °C without shaking. The hydrolyzed samples were analyzed for total reducing sugar concentrations (using glucose as standard) at predetermined time periods (i.e., 4 or 24 h) using the standard dinitrosalicylic acid (DNS) colorimetric assay or glucose hexokinase-based methods as reported earlier (42). All experiments were carried out in at least duplicates. Error bars reported in figures represent 1 standard deviation from mean values for replicates.

Total Cellulose-Reducing Ends Estimation

The number average degree of polymerization (DP) for cellulose polymers is directly correlated to the total number of cellulose-reducing ends per unit mass of cellulose (43). Therefore, the total number of cellulose-reducing ends per unit mass of cellulose was estimated using a modified DNS-based colorimetric assay as originally reported elsewhere (37). Briefly, 1.15 mg of cellulose (dry weight basis) was added along with 0.1 mL DI water and mixed with 0.1 mL DNS stock reagent in PCR tubes. The slurry was well-mixed and incubated at 95 °C for 10 min on a thermal cycler prior to rapid cool down to 4 °C. The absorbance of the resulting cellulose-free SN was measured at 540 nm. The total reducing sugar equivalents concentrations (using glucose equivalents as standard) were determined to estimate total cellulose-reducing chain ends per unit mass of cellulose to predict relative cellulose DP for different substrates.

Raman Spectroscopy

A MultiRam FT-Raman spectrometer (Bruker) was used to collect Raman spectra for all cellulose samples. The FT-Raman spectrometer was equipped with a 1064 nm, 1000 mW Nd:YAG laser. For Raman analysis, cellulose pellets were first prepared from either lyophilized or air-dried samples prior to analysis. In most cases, spectra with high signal-to-noise ratios were obtained using a 660 mW laser power setting and collecting over 1024 scans per sample. Bruker's proprietary OPUS version 7.2 software was used to process all collected spectral data, which involved selection of a spectral region, background correction, and if needed, normalization of spectra typically at 1096 cm^{-1} . Background correction was performed using a 64-point "rubber band option" available in OPUS software. The spectra were then converted to ASCII format and exported to Microsoft Excel for

plotting and analysis. The absolute CrI, based on the relative Raman spectral intensity at 380 versus 1096 cm^{-1} , was determined using a previously published method and $\text{CrI}_{\text{Raman}}$ formula (39, 44).

Results and Discussion

Here, we revisit the original ultra-low freezing temperature ($-30\text{ }^{\circ}\text{C}$) process proposed by Hsu and Penner (1) for the preparation of RAC using concentrated HCl to better understand the role of HCl swelling temperature on cellulose dissolution, crystalline ultrastructure of recovered cellulose after dissolution, and its subsequent impact on enzymatic activity. We used a very-low cellulose concentration ($<0.5\%$ w/v) to avoid possible issues related to anisotropy and its impact on the cellulose dissolution process. As shown in Figure 1A, ice-cold concentrated HCl was first used to dissolve microcrystalline cellulose-I, which can be readily used to produce a highly amorphous form of cellulose, called HASC, upon precipitation of the acid-solubilized cellulose in cold water. If the cellulose–HCl solution was allowed to phase separate (i.e., via gravity or centrifugal separation), two distinct phases after cellulose dissolution can be noticed: a slightly viscous and denser gel-like, clear layer can be seen at the bottom of the Falcon tube; and a less dense transparent liquid-phase layer can be seen at the top of the gel-like layer. As defined in the Materials and Methods section above, cellulose regenerated specifically from the *top* supernatant liquid layer and *bottom* gel-like pellet layer phases are called HASC-SN and HASC-P, respectively. These two phases can be readily isolated via centrifugation following decanting/pipetting, and cellulose can be precipitated out separately from the supernatant (SN) and gel-like pellet (P) phases using cold water to produce the regenerated HASC-SN and HASC-P cellulosic substrates, respectively.

Interestingly, we found that ice-cold concentrated HCl solvent seems to mostly swell microcrystalline cellulose into a gel-like state at temperatures close to freezing ($4\text{ }^{\circ}\text{C}$). However, this gel-like layer formation and swelling of cellulose were not seen if the concentrated HCl solution was contacted with the cellulose at ambient temperatures. These findings are consistent with previous work that reported issues with the dissolution of cellulose using HCl at ambient temperatures, even after prolonged incubation and extensive mixing (1). It is likely that the dissolution of cellulose in concentrated HCl solvent is a thermodynamically-limited and not a kinetically-limited process as has been also reported for other cellulose–solvent systems (45). This could likely explain why certain lower temperature regimes play a critical role in finely modulating the dominant conformational and entropic forces driving cellulose–solvent interactions and, hence, driving overall solubilization efficiency. It is likely that this low- versus high-temperature dependence of cellulose swelling behavior can be explained by the combination of enthalpic versus entropic forces at play that facilitate disruption of noncovalent bonds within cellulose by HCl (vs selective partitioning of HCl toward water) that ultimately results in cellulose swelling or dissolution. Similar explanations have been provided by others in the literature regarding the role of temperature in cellulose dissolution for other solvent systems (45). In the case of concentrated HCl, however, as well as H_3PO_4 (15), temperature also plays a critical role in impacting the rate of acid hydrolysis of cellulose into lower molecular weight polymers that are possibly more readily solubilized into solution. Considering that HCl is a stronger acid than H_3PO_4 , it is interesting to note that only a small fraction ($<1\%$ w/w of original material) of the original cellulose was hydrolyzed into soluble sugars for either acids after incubation at $4\text{ }^{\circ}\text{C}$ (Figure 1B). However, a reducing sugar assay for the insoluble cellulose was also performed to check the total number of accessible reducing sugar chain ends created due to acid hydrolysis as detailed below.

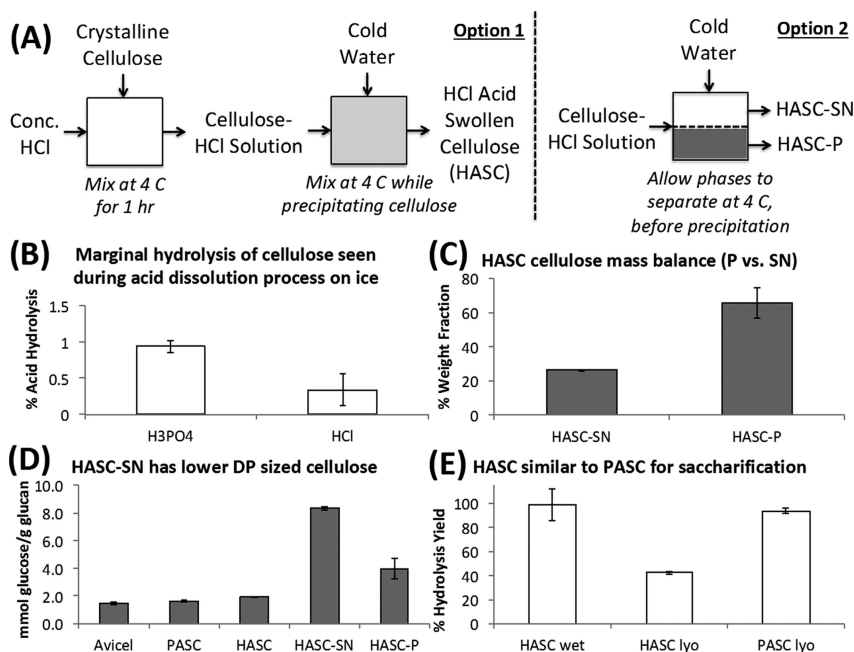


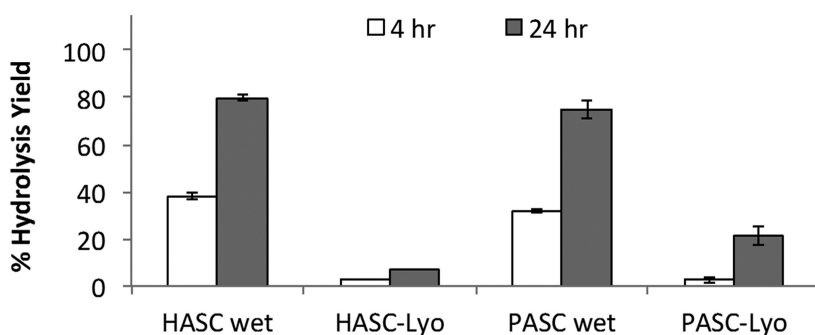
Figure 1. (A) Schematic for preparation of an amorphous cellulose substrate HASC. Top supernatant and gel-like bottom pellet layers can be also recovered separately to generate HASC-SN and HASC-P celluloses (Option 1 vs 2). (B) Mass loss due to acid hydrolysis of cellulose during the dissolution process in concentrated HCl or phosphoric acid is marginal at 4 °C. (C) Over two-thirds of the cellulose recovered after precipitation from concentrated HCl process was found to be in the gel-like HASC-P phase. (D) HASC-SN recovered cellulose has a lower DP than other insoluble cellulosic materials, based on total reducing sugars availability per unit mass substrate. (E) Never-dried wet HASC is readily hydrolyzed by commercial cellulase cocktails; however, lyophilization significantly lowered HASC digestibility compared to lyophilized PASC. Cellulose hydrolysis was conducted using a high enzyme loading of 15 mg/g glucan of Celluclast and Novo 188, each at 50 °C for 22 h.

Two distinct phases were seen upon dissolution on ice: a denser P-phase and a lighter SN-phase that could be readily separated, if desired. These distinct phases eventually produce the HASC-P, and HASC-SN regenerated cellulose after separately recovering the cellulose from the two phases using cold water, respectively. Interestingly, the majority of the cellulose recovered was found in the pellet phase (~70 wt % of original starting material) with a smaller fraction fully solubilized in the lighter supernatant phase (~30 wt % of original starting material). A DNS-based colorimetric assay was used to quantify the total number of accessible reducing sugar chain ends associated with the insoluble cellulose phases and to make comparisons between various substrates. Cellulose recovered from the SN-phase was found to have 8.3 mmol glucose equiv chain ends available per gram of HASC-SN compared to 3.9 mmol glucose equiv chain ends available per gram of HASC-P (Figure 1D). This suggests that the SN-phase solubilized cellulose has a lower DP than the P-phase associated material, likely due to acid hydrolysis of some fraction of the accessible cellulose chains during the dissolution process. Hsu and Penner (1) showed that the final DP of cellulose recovered after the process was directly dependent on the total hydrolysis time (0–60 min) at room temperature prior to the recovery of acid-solubilized cellulose. They showed that the recovered cellulose had a DP that dropped between 1.1 to 3.4-fold, compared to the starting material depending on the hydrolysis time (1). The total number of chain ends created due to acid hydrolysis was higher for HASC versus

PASC, compared to the original starting material in our study. Phosphoric and HCl resulted in a 10% and 30%, respectively, increase in the total accessible reducing sugar chain ends, compared to native cellulose-I. These results are consistent with previously reported work that showed a more significant drop in the number of average DP of cellulose for HASC versus PASC (1). Our work also confirms that concentrated HCl produces amorphous cellulose with a slightly lower DP compared to concentrated H_3PO_4 at comparable solubilization conditions (i.e., swelling at 4 °C for 1 h).

We also found that the cellulose regenerated from the concentrated ice-cold HCl solution using cold water as the antisolvent was easily susceptible to enzymatic hydrolysis such as PASC (Figure 1E). Never-dried wet HASC was found to be readily digested into soluble sugars using a commercial cellulase cocktail. For results reported in Figure 1E, cellulose hydrolysis was conducted using a high enzyme loading of 15 mg/g glucan at 50 °C for 22 h. However, surprisingly, if the HASC sample was lyophilized prior to hydrolysis, the dried substrate had a significantly lower digestibility compared to the lyophilized PASC. Interestingly, the lyophilized/air-dried HASC material also had a spongier, aerogel-type morphology that was clearly distinct compared to the starting powdery microcrystalline cellulose-I material. This led us to more closely investigate the enzymatic digestibility of overall combined HASC and separate HASC-SN and HASC-P-phase-based regenerated celluloses either with or without drying.

(A) Lyophilized HASC is very recalcitrant to enzymes



(B) HASC-P slightly more recalcitrant than HASC-SN

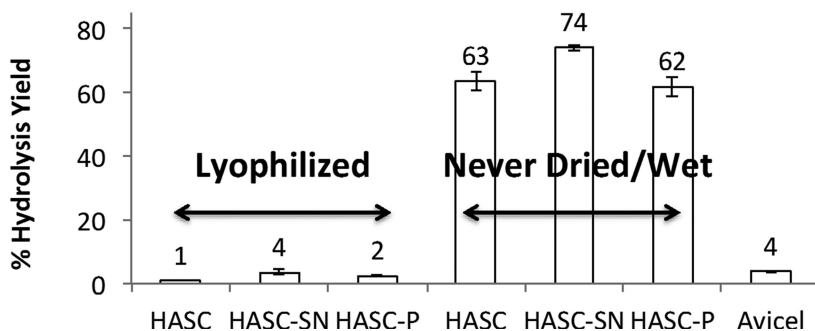


Figure 2. (A) Lyophilization of HASC reduced cellulase enzymatic hydrolysis rate to a greater extent compared to lyophilized PASC. (B) HASC-SN was found to be marginally less recalcitrant than HASC-P, with or without lyophilization. The relative rate of enzymatic hydrolysis of lyophilized HASC was always comparable to original cellulose-I (or Avicel). In all cases, cellulose hydrolysis was conducted using a low enzyme loading of 1.5 mg/g glucan of *C. Tec2* at 50 °C for 24 h.

HASC was found to have a comparable or marginally higher rate of enzymatic hydrolysis than PASC if either substrate was never dried (Figure 2A). For results reported in Figure 2A, cellulose hydrolysis was conducted using a low enzyme loading of 1.5 mg/g glucan at 50 °C for 4 and 24 h. As expected, lyophilization of HASC and PASC resulted in dried substrates that had a significant 14.5-fold and 12.5-fold drop in enzymatic hydrolysis rates after 4 h, respectively. The lyophilized HASC sample was still largely undigested (~5% hydrolysis yield) even after 24 h incubation; however, the lyophilized PASC was close to 20–25% hydrolyzed. These results clearly show that lyophilized HASC is more recalcitrant to cellulase hydrolysis compared to lyophilized PASC. It was unclear if the amorphous cellulose recovered after lyophilization from either the SN or P fractions was responsible for the increased recalcitrance toward cellulases. Therefore, we carried out enzymatic hydrolysis for the HASC-SN and -P recovered celluloses either with or without lyophilization (Figure 2B). Our results clearly showed that the never-dried HASC-SN substrate was less recalcitrant to cellulase hydrolysis compared to the never-dried HASC-P substrate. Surprisingly, lyophilization of HASC produced a substrate that had similar or marginally increased recalcitrance compared to the original native cellulose-I (i.e., Avicel PH-101) to hydrolytic cellulases. Prolonged incubation time with cellulases, even at a medium enzyme loading with the lyophilized substrates, also showed the similar general observations reported here (data not shown). Furthermore, air-drying of HASC instead of lyophilization also produces similar relative hydrolysis trends reported here (data not shown). While lyophilization and air-drying of amorphous cellulose are well-known in the literature to cause a significant retardation in the overall rate of enzymatic hydrolysis (1, 46), it was rather surprising that the lyophilized HASC-P substrate was as recalcitrant as the native cellulose-I original material. More importantly, lyophilized PASC was significantly more digestible than either the lyophilized HASC or native cellulose-I, as reported in this study and others (1, 47). This suggests that while never-dried HASC and PASC may show similar rates of enzymatic hydrolysis due to the overall improved substrate accessibility compared to native cellulose-I, both forms of amorphous cellulose are likely not equivalent to each other at the ultrastructural level.

While XRD is often reported in the literature to estimate CrI of cellulose, it is challenging to use this method alone to conclusively determine the various allomorphs of cellulose formed during regeneration of cellulose. For example, it has been shown to be challenging to use XRD peak positions alone to distinguish cellulose-I-like allomorph from cellulose-IV formed during regeneration of cellulose from solution (16). However, as shown by Atalla and Agarwal, Raman spectra of various cellulose allomorphs, such as cellulose-I, -II, -III, and -IV, can be readily distinguished (25, 44). Previous peak region assignments of the vibrational spectrum of cellulose have been described in terms of multiple group motions, since the traditional normal-coordinate peak analyses are not straightforward for cellulose (44, 48). Normal-coordinate calculations for model cellodextrins (e.g., cellotetraose) has shown that the frequency distribution below 700 cm⁻¹ is sensitive to dihedral angles at the glycosidic linkages. Therefore, the region of 250–550 cm⁻¹ for cellulose has predominant motions attributed to skeletal-bending modes involving C–C–C, C–O–C, O–C–C, and O–C–O internal bond coordinates. Similarly, the region of 550–750 cm⁻¹ for cellulose has been shown to correspond to mostly out-of-plane bending modes involving C–C–C, C–O–C, O–C–O, C–C–O, and O–H internal bond coordinates. Furthermore, the cluster of peaks around 900 cm⁻¹ is shown to involve bending of H–C–C and H–C–O bonds, localized at C-6 atoms of the hydroxymethyl group. The region of 950–1200 cm⁻¹ for cellulose was shown to correspond to mostly stretching motions involving C–C and C–O internal bond coordinates. The region of 1200–1500 cm⁻¹ for cellulose was shown to correspond to mostly bending

motions involving H–C–C, H–C–O, H–C–H, and C–O–H internal bond coordinates. The region of 1400–1500 cm^{-1} for cellulose has been shown to be particularly sensitive to the CH_2 scissor bending modes that are sensitive to the TG (1480 cm^{-1}) and GT (1460 cm^{-1}) conformations of the hydroxymethyl group (44, 49). Furthermore, Agarwal has now developed and validated a simple Raman spectroscopy-based method to estimate cellulose CrI using the relative peak intensity of the 380 cm^{-1} to the 1096 cm^{-1} wavenumber relative peak intensities (39). The method has been shown to produce CrI for diverse cellulosic samples that are highly correlated to the traditional analytical methods used to estimate CrI. Therefore, we analyzed all samples using Raman spectroscopy alone to simultaneously estimate relative CrI and determine the type of cellulose allomorph formed. For comparison purposes, Raman spectra of cellulose-I (Avicel), lyophilized cellulose-II, and lyophilized PASC sample are shown in Figure 3. Raman spectrum of lyophilized whole HASC contained spectral band features that were representative of cellulose-I (clear peaks or shoulder intensity increased at 350, 380, 438, 913, 999, 1180, 1294, and 1480 cm^{-1}), cellulose-II (clear peaks or shoulder intensity increased at 355, 421, 580, 899, 1267, and 1463 cm^{-1}), and amorphous cellulose (features at 355, 899, and 1463 cm^{-1}) based on peak assignments reported previously (39, 44).

The HASC spectrum was similar to PASC, but clearly with some minor differences that showed overlapping bands seen in cellulose-I as well. For example, compared to PASC, HASC contained lower amounts of cellulose-II (based on relative intensity at 355 cm^{-1}) and amorphous cellulose (spectra compared after normalization on 1096 cm^{-1}). Several band intensities for HASC at 350–355, 375–380, 580, 899, 913, 999, 1267, and 1463 cm^{-1} were shifted by a few wavenumbers toward bands also seen in cellulose-I and/or were also lower in relative intensity compared to PASC as well. The CrI based on the Raman spectral intensity at 380 versus 1096 cm^{-1} was calculated using previously published formula for cellulose-I, cellulose-II, HASC, and PASC to be 81%, 59%, 73%, and 69%, respectively. Clearly, lyophilized HASC is more crystalline than lyophilized PASC, based on this simple quantitative analysis method as well.

Raman spectra for HASC-P and HASC-SN phases recovered cellulose after lyophilization were collected as well and are shown in Figure 4. The spectra of air-dried and lyophilized samples were very similar; therefore, only the lyophilized sample spectra are shown in Figure 4. For comparison purposes, the spectrum of native cellulose-I (Avicel) is also included here. Clearly, the Raman spectrum of the SN layer recovered HASC-SN lyophilized sample was similar to cellulose-II spectrum shown in Figure 3. However, the P layer recovered HASC-P lyophilized sample produced spectral features that closely resemble native cellulose-I, although there were clearly some minor differences. For example, the HASC-P compared to native cellulose-I (after normalization at 1096 cm^{-1}) had: (1) lower ratio of 380–350 cm^{-1} band intensities; (2) smaller peaks at 580 and 1267 cm^{-1} ; and (3) higher peak intensity at 900 cm^{-1} . Moreover, the peak usually seen in cellulose-I at 1480 cm^{-1} was broader and shifted toward 1463 cm^{-1} in the spectrum of HASC-P. The CrI based on the Raman spectral intensity at 380 versus 1096 cm^{-1} was calculated using previously published formulas for cellulose-I, HASC-P, and HASC-SN to be 81%, 78%, and 62%, respectively. Clearly, lyophilized HASC-P is significantly more crystalline than lyophilized HASC-SN, and has nearly comparable CrI to native cellulose-I based on this simple quantitative analysis method.

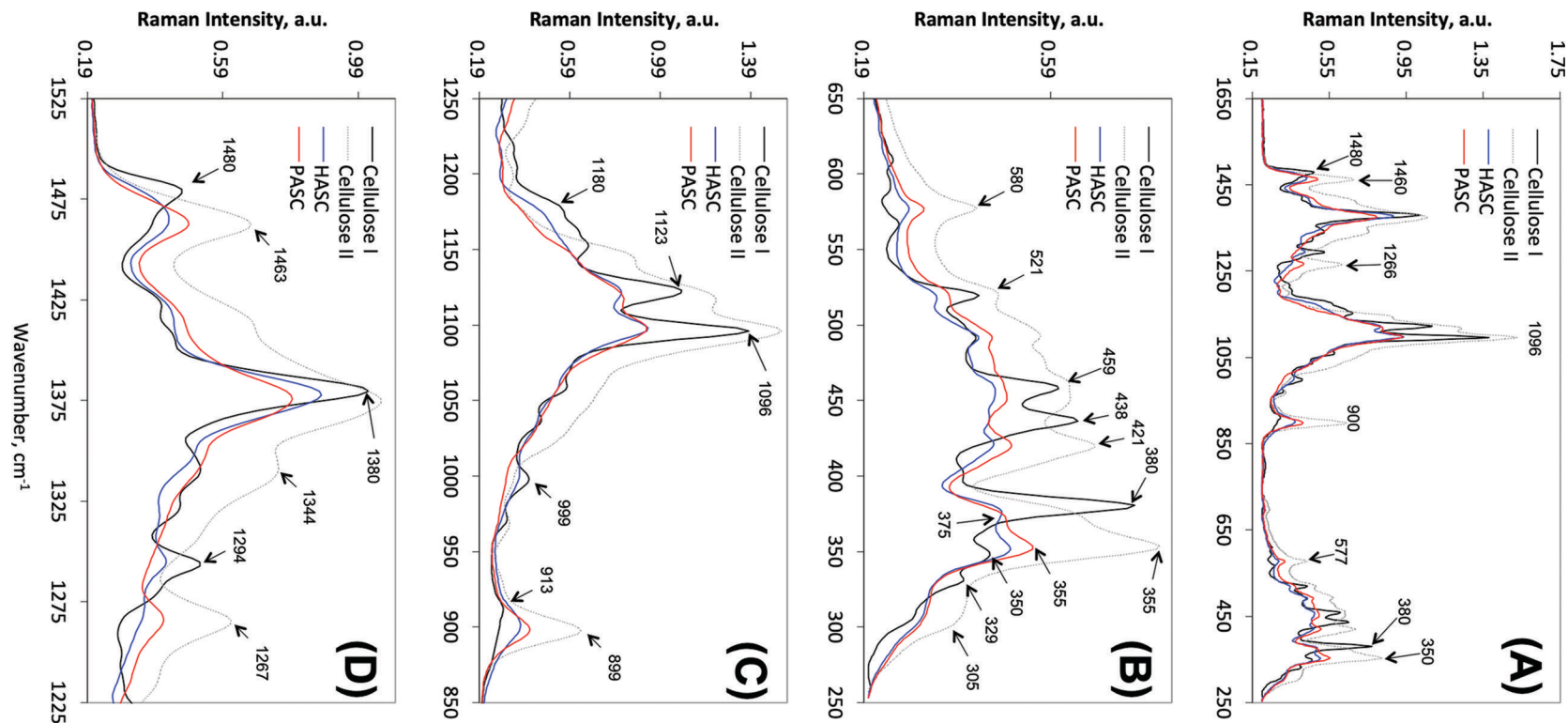


Figure 3. Impact of concentrated HCl treatment on cellulose structure was studied using Raman spectroscopy (A). Zoomed in spectral regions for low (B), medium (C), and high frequency (D) regions are shown, highlighting key spectral features for clarity. Overlaid Raman spectra of native dry Avicel cellulose-I (solid black line), dried cellulose-II (dotted gray line), dried HASC (solid blue line), and dried PASC (solid red line) are shown. Raman spectra for HASC and PASC alone have been normalized at 1096 cm⁻¹ for the sake of comparison.

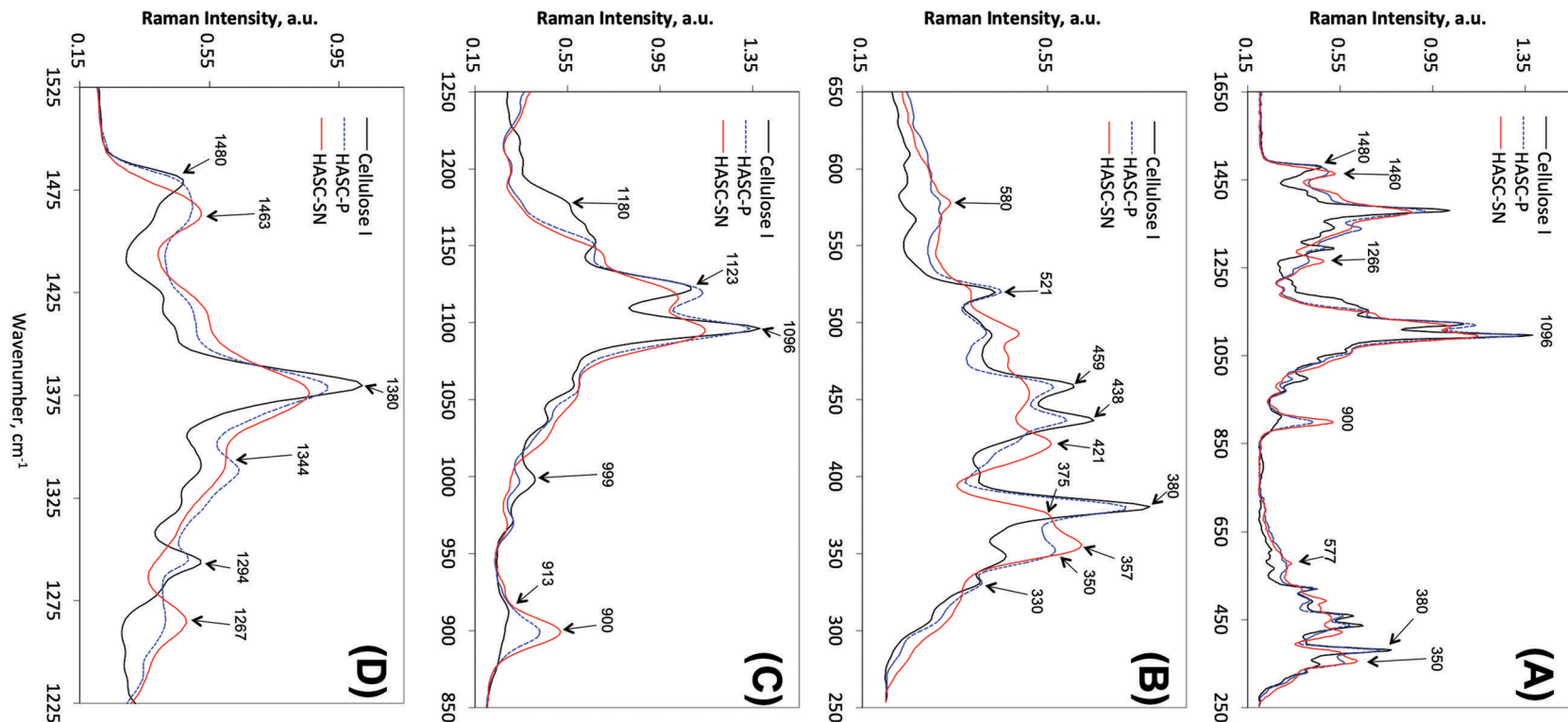


Figure 4. Raman spectra for lyophilized HASC-P (dotted blue line) and lyophilized HASC-SN (solid red line) celluloses are shown here (A). Zoomed in spectral regions for low (B), medium (C), and high-frequency (D) regions are shown, highlighting key spectral features for clarity. Note that upon lyophilization of the recovered cellulose samples, a similar spectrum to the air-dried samples was also obtained (data not shown). Overlaid Raman spectra of native Avicel cellulose-I (solid black line) is shown for comparison to HASC-P phase recovered cellulose sample spectra. Raman spectra for HASC-P and HASC-SN alone have been normalized at 1096 cm^{-1} for the sake of comparison.

All reported Raman spectra characteristics clearly implied that the lyophilized state of HASC-P was undoubtedly enriched in a cellulose-I-like allomorphic state, with possibly some degree of cellulose-II and/or amorphous cellulose-like faint background signals as well. Regeneration of cellulose-I from a fully molecular solution of dissolved cellulose has been rarely reported in the literature, partly due to the extreme sensitivity of the regenerated cellulose lattice structure to the exact regeneration environment and the starting cellulose material (15, 16, 25). Nevertheless, it has been shown by Atalla and co-workers that cellulose-I-like allomorphic state of cellulose can be regenerated upon precipitation of dissolved cellulose from a concentrated phosphoric acid solution into a hot glycerol solvent only after a defined period of incubation time (15). Recently, Wan and co-workers have also shown that a high crystallinity, aerogel type cellulose-I-like material can be regenerated from LiCl·DMAc solution driven by a thermally-induced sol-gel mechanism under certain swelling conditions alone (16). However, to date, it has never been demonstrated that it is possible to regenerate a cellulose-I-like allomorph from a concentrated HCl dissolved or swollen cellulose intermediate state. Hsu and Penner reported only the regeneration of a highly amorphous and cellulose-II-like substrate upon regeneration of cellulose from a concentrated HCl solution (1). It is unclear how exactly concentrated HCl is able to swell cellulose at nonfreezing temperatures to produce a gel-like state; and how it then produces a highly amorphous form of cellulose upon regeneration, which is readily accessible to hydrolytic cellulases such as PASC. While the mechanism of cellulose solubilization and recovery from concentrated HCl as a function of temperature and substrate properties is still far from being fully understood, it is clear that not all forms of amorphous cellulose are created equally. Obviously, the removal of water from a fully hydrated state of PASC versus HASC during lyophilization or air-drying results in cellulosic substrates with distinct cellulose ultrastructures and, therefore, varying accessibility/reactivity toward cellulases.

Hsu and Penner showed that concentrated HCl could fully dissolve cellulose only upon temperature reduction close to -30 °C to produce fully RAC and cellulose-II upon precipitation with water at room temperature (1). They also mentioned that microcrystalline cellulose must be properly dispersed/mixed in the concentrated HCl solution at room temperature, followed by immediate cooling of the solution in a -37 °C freezer or solid CO₂-ethanol bath to achieve complete cellulose dissolution starting at approximately -25 °C. In our study, we found that it more convenient to swell/dissolve cellulose at temperatures close to 0–4 °C in concentrated HCl and then recover regenerated cellulose at low temperatures to minimize acid-catalyzed cellulose hydrolysis. Furthermore, it is unclear if the high cellulose concentration employed in the previously reported work (i.e., 8 g cellulose dissolved in 100 mL solvent) could have had a deleterious impact on the optimum conditions reported to achieve full dissolution. Cellulose concentrations under 1% (w/v) are often necessary to achieve fully isotropic solutions, as also seen in the case of other reported cellulose solvents, including H₃PO₄ (50–52). Therefore, it would be prudent to use lower starting concentrations for such studies unless a detailed cellulose-HCl-water ternary phase diagram becomes available in the near future for different DP-based cellulose materials of varying starting crystallinity index. Hsu and Penner did not use Raman spectroscopy to characterize or report the detailed cellulose allomorph type produced for cellulose regenerated after annealing the frozen cellulose-HCl solution at 25 °C prior to cellulose regeneration. Nevertheless, their results clearly suggest that there is some degree of recrystallization taking place to a form of regenerated cellulose that is less digestible with increasing time of acid hydrolysis at 25 °C despite a lowering of cellulose DP [see Table 1 in Hsu and Penner (1)].

Currently, we can only speculate to the molecular origins of the cellulose-I-like allomorphic state of regenerated HASC, the temperature-dependent mechanism, and the role of cellulose hydration in driving this complex process. For example, it is possible that incomplete cellulose-I dissolution could have given rise to the cellulose-I-like allomorph seen upon regeneration and lyophilization. However, this seems unlikely for several reasons. Firstly, we did not see a clear cellulose-I signal during Raman spectroscopy of the never-dried wet HASC samples (data not shown). Secondly, the cellulose–acid solution was mostly clear and no undissolved Avicel particles were visually observed. This clearly changed when cold water was added, which resulted in the formation of a milky white precipitate of regenerated cellulose. Thirdly, the morphology of the regenerated HASC was similar to PASC with a very-low-bulk density and uniformly suspended in water, unlike native cellulose-I that is much denser in appearance and readily settles in water. Fourthly, only when the regenerated HASC was lyophilized was a clear Raman spectra for cellulose-I, cellulose-II, and amorphous cellulose seen in the various phase fractions representative of the overall material balance. Lastly, the morphology of the lyophilized regenerated HASC material was spongier, much like an aerogel, and very distinct from the native cellulose-I powder/fibrillar morphology. Another possible explanation is the reversible swelling of native cellulose-I in concentrated HCl at 4 °C and the formation of multimorphic “memory”, retaining nucleated cellulose aggregates in the gel-like phase that is structurally distinct from the starting material. This gel-like state of cellulose–solvent has been reported for other systems and could be a highly swollen state of individual parallel cellulose chains, which are not fully solvated by HCl–H₂O into a truly molecular solution of cellulose at intermediate temperatures. This is likely due to the high degree of polymerization of cellulose that could possibly limit individual cellulose chain solubilization. This could explain why the supernatant phase was found to be enriched in fully solvated chains of cellulose, but with a lower DP compared to the gel-like phases and starting cellulose material. What is really fascinating is that the highly swollen cellulose ultrastructure with parallel chains orientation seems to be mostly recovered even after precipitation and regeneration with water. This could explain why a cellulose-I-like allomorphic structure is seen to be formed after controlled removal of water from the regenerated HASC, either by lyophilization or air-drying. Atalla and co-workers have also suggested that a minor fraction of polymeric crystalline cellulose aggregates is likely present as well-defined multimorphic cellulose in the cellulose–solvent solution that controls the final composition of allomorphic states seen within regenerated cellulose (15). However, in our case, we only found regeneration of a cellulose-I-like allomorphic state when water was fully removed from the hydrated HASC sample, but not hydrated PASC sample. It would be critical to characterize the transition structure of cellulose in solution with solvents (such as cold concentrated HCl) prior to cellulose precipitation, as well as to characterize the transition during lyophilization of hydrated cellulose, to fully understand the possible role of minor nucleating multimorphic cellulose allomorphs, which could control the final allomorphic state of cellulose, but often escape detection using conventional cellulose structure bulk analytical techniques (such as XRD, solid-state NMR, optical microscopy, and Raman spectroscopy).

Conclusions

The mechanism for how concentrated H₃PO₄ acts as an effective solvent to dissolve, and ultimately produce highly amorphous cellulose and/or distinct cellulose allomorphs, such as cellulose-I or cellulose-II under well-defined processing conditions, has been somewhat explored; however, mechanistic understanding of how other acids, such as concentrated HCl, swell or dissolve cellulose is still poorly understood. Here, we showed that using a low, but nonfreezing swelling temperature (0–4 °C), and reagent-grade concentrated HCl to dissolve native microcrystalline

cellulose-I, ultimately result in the formation of a two-phase cellulose–solvent system: a less dense supernatant phase and a denser gel-like phase. The cellulose samples regenerated after precipitation from the gel-like and liquid-phases using cold water were characterized by Raman spectroscopy and also were subjected to enzymatic hydrolysis using synergistic cellulase enzymes. We found that cellulose regenerated from the gel-like state retained ultrastructural features that resembled native crystalline cellulose-I allomorph upon either lyophilization or air-drying but had a slightly spongier aerogel type morphology compared to the original Avicel material. While the cellulose regenerated from the liquid-phase retained features of highly amorphous cellulose along with features typically seen with crystalline cellulose-II. This HASC recovered after the treatment was subjected to enzymatic hydrolysis at varying cellulase loadings, either with or without HASC lyophilization. Surprisingly, cellulose regenerated from either the gel-like or soluble supernatant state resulted in a highly amorphous substrate comparable to PASC in relative ease of enzymatic digestibility. However, lyophilization or air-drying of this RAC resulted in the formation of a cellulose-I-like allomorph with lowered enzymatic digestibility that was comparable or marginally lower than the original native cellulose-I substrate. Obviously, more work is needed to better understand the actual mechanism at work regarding cellulose dissolution/recovery using concentrated HCl and the regeneration of cellulose-I allomorphic state upon lyophilization of HASC. Nevertheless, we now have a simple, validated method for preparation of highly amorphous cellulose using HCl (or HASC) at nonfreezing temperatures that is comparable in simplicity to the widely used PASC preparation protocol.

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