

**INHIBITING ICE RECRYSTALLIZATION BY
NANOCELLULOSES: EFFECTS OF SURFACE CHARGE
DENSITY AND FIBRIL LENGTH, AND APPLICATIONS
IN CELL CRYOPRESERVATION**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

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August 2020**

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DEDICATION

*This dissertation is dedicated to my lifelong treasure of destiny Weilong Liang
In the vastness of space and immensity of time, it is my joy to meet you and spend the rest of my
life with you*

*This dissertation is dedicated to my little son Knox, whose innocent smile brings me a lot
happiness but without whom this dissertation could have been completed one month earlier*

This dissertation is also dedicated to my parents for their love and support

ACKNOWLEDGEMENTS

First, I would like to thank my major advisor, Dr. Tao Wu, for his wonderful supervision and support during the past three years. His enthusiasm and focus on research inspired me greatly. Dr. Wu not only shared his knowledge about research but also his own experience about life and work with me, and this sharing will benefit my future development. I would like to thank my co-advisor Dr. Qixin Zhong. I am especially impressed by his carefulness and creativity in research. Each time I had difficulties with my experiments, Dr. Zhong always gave me illuminating suggestions. I truly appreciate the guidance and encouragement he has provided to me for three years.

I would also to thank my committee members Dr. Bin Zhao, Dr. Siqun Wang, and Dr. Scott Lenaghan. Dr. Zhao, it was a very wise choice for me to have organic polymer chemistry, and I will remember your encouragement. Dr. Wang, thank you for providing switchgrass and pulp for my experiments. Dr. Lenaghan, thank you for your insightful suggestions on my research and for your help with the cryopreservation of soybean protoplasts. I thank Dr. Dia for allowing me to use the cell lab. I also thank Dr. Philipus Pangloli for his kind technical and administrative supports in experiments.

I would like to thank my lab mates: Ying Zhao, Min Li, Wenhui Qi, Josué, and Safoura. I would like to thank Nan Li, Shan Hong, Anyi Wang, Jennifer Vuia-Riser, Melody, Andrea, Yang Lin, Francisco, Sarah, Manuel, and all the staff in the food science department. Thank you for your helps. It is my honor to meet all of you in UTIA.

Finally, I would like to thank our dear friends at Wallace Memorial Baptist Church. You made us not feel lonely in Knoxville. Your warm and kind helps always remind us of Dr. Bill Wallace, who devoted his life in my wife's hometown Wuzhou. Thank you!

ABSTRACT

Ice recrystallization (IR) is the change in size, number, and morphology of ice crystals during storage and thawing, which causes cell death in cryopreservation. Recent studies link a facial amphiphilic structure to the IR inhibition (IRI) activity in antifreeze (glyco) proteins (AF(G)Ps) and mimics. Recent studies also found that nanocelluloses are amphiphilic. We hypothesized that nanocelluloses are IRI active and can be used to improve the cell viability in cryopreservation.

The first part of this research explored the IRI activity in nanocelluloses. At mg/mL level, Cellulose nanocrystals (CNCs) and 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils (TEMPO-CNFs) inhibited IR in 0.01 M NaCl without thermal hysteresis or dynamic ice shaping activity. The IRI activity of nanocelluloses was not affected by the quenching temperature but decreased with the increase of annealing temperature.

The second part investigated the relationship between surface charge density (SCD) and the IRI activity in nanocelluloses. Nanocelluloses with similar fibril lengths but varied SCDs were synthesized, characterized, and compared for IRI activity. Reducing SCD promotes the IRI activity until fibril aggregation occurred.

The third part studied the effect of fibril length on the IRI activity of nanocelluloses. TEMPO-CNFs with similar SCDs and fibril widths but with different fibril lengths were prepared, characterized, and compared for IRI activity. Longer nanocelluloses are more IRI active. The IRI activity was not correlated with the viscosity or gelling properties of nanocellulose dispersions.

The fourth part examined the potential application of nanocelluloses in cryopreservation. A novel type of nanocelluloses - electrosterically stabilized cellulose nanocrystals (ECNCs) were synthesized. ECNCs remained dispersed and IRI-active at high ionic strengths. ECNCs improved the post-thaw viability of HCT-116 colorectal cancer cells in slow/fast freezing-slow thawing

protocols in the presence of 1-20% dimethyl sulfoxide (DMSO), as well as in slow/fast freezing-fast thawing protocols at reduced DMSO concentrations. The effectiveness in cryoprotection did not match the IRI activity in ECNCs, polyethylene glycol, and polyvinyl alcohol; and in ECNCs with different surface charge densities.

Nanocelluloses are inexpensive, biocompatible, and sustainable materials, this dissertation has demonstrated their IRI activity, which have great potentials for frozen foods and cryopreservation applications.

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Chapter 1. Introduction and literature review

My primary contributions to this chapter include visualization and Writing - Original Draft.

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1.1 Introduction

Ice recrystallization (IR) is the change in the size, number, and shape of ice crystals during freezing and frozen storage (Adapa, Schmidt, Jeon, Herald, & Flores, 2000; Cook & Hartel, 2010). There are three main IR processes: iso-mass, accretion, and migratory recrystallization (Cook & Hartel, 2010). The overall effect of IR is the reduction of free energy at the ice/water interface. Thus, IR is a thermodynamically driven process that is difficult to control. IR can cause mechanical damage to cells, tissues, and organs during cryopreservation (Pegg, 2010; Takamatsu & Zawlodzka, 2006), and it also negatively impacts the quality of frozen foods (Adapa et al., 2000).

In nature, some polar fishes, insects, and plants have evolved the ability to inhibit IR by producing antifreeze proteins (AFPs) or antifreeze glycoproteins (AFGPs) (Voets, 2017). Although AF(G)Ps are effective IR inhibitors, the high cost associated with their isolation and synthesis limits their large-scale application. Thus, many studies have explored the use of inexpensive IR inhibitors, including low-molecular-weight compounds, polymers, and nanomaterials, as AF(G)Ps mimics (Balcerzak, Capicciotti, Briard, & Ben, 2014; Biggs et al., 2017; He, Liu, & Wang, 2018). To design novel materials for ice recrystallization inhibition (IRI), an understanding of the structure and IRI activity relationship in AF(G)Ps is crucial. Recently, a key structural feature responsible for the IRI activity of AF(G)Ps has been associated with a facial amphiphilicity (Biggs et al., 2017; Tachibana et al., 2004). Several facially amphiphilic materials with IRI activity have been identified (Drori et al.,

2016; Mitchell & Gibson, 2015). A few IRI-active facially amphiphilic materials have also been synthesized (Graham et al., 2017; Graham, Fayter, Houston, Evans, & Gibson, 2018).

Nanocelluloses are cellulose fibrils with lateral dimensions at the nanoscale and lengths of up to hundreds of nanometers or several micrometers (Klemm et al., 2011). Nanocelluloses can be isolated from various sources by mechanical treatment, acid hydrolysis, or chemical oxidation (Habibi, Lucia, & Rojas, 2010; Isogai, Saito, & Fukuzumi, 2011; Klemm et al., 2011). Nanocelluloses are emerging as a renewable and biocompatible material for many potential applications (Gomez et al., 2016; Markstedt et al., 2015). The crystalline structure of nanocelluloses demonstrates amphiphilicity; it includes planes rich in CH groups that are hydrophobic and planes rich in hydroxyl groups that are hydrophilic (Bruehl, Tavares, Carreau, & Heuzey, 2019; Yamane et al., 2006). The emulsion stabilization ability of nanocelluloses and molecular simulation studies provide evidence for the amphiphilicity (Biermann, Hadicke, Koltzenburg, & Muller-Plathe, 2001; Kalashnikova, Bizot, Cathala, & Capron, 2012). Based on the association between amphiphilicity and IRI activity, we hypothesized that nanocelluloses have IRI activity. We further hypothesized that IRI-active nanocelluloses have a cryoprotective effect because of the reduced damage resulting from IRI in cryopreservation. The goals of this dissertation are to explore the IRI activity of nanocelluloses, understand the structure-IRI activity relationships, and investigate their potential application in cryopreservation. This chapter reviews current knowledge about IR, IR inhibitors, the structure-IRI activity relationships

of IR inhibitors, nanocelluloses, and cryopreservation.

1.2 Ice and ice recrystallization

Understanding the structure of ice is essential in discussing the IR process and how AF(G)Ps interact with ice. Hexagonal ice (I_h) ice is the most common crystalline structure of ice formed under atmospheric pressure (Owston & Lonsdale, 1948; Pauling, 1935). IR is a thermodynamically driven process that occurs in several forms. This section reviews the fundamentals of I_h ice and IR.

1.2.1 Hexagonal ice

Ice with a variety of crystalline structures, and even amorphous ice lacking a crystal structure, can be formed depending on the temperature and pressure conditions (Benoit, Bernasconi, Focher, & Parrinello, 1996; Hemley, Chen, & Mao, 1989; Svishchev & Kusalik, 1994). I_h is the common form of ice that is formed by freezing water at atmospheric pressure. The crystalline structure of I_h was proposed by Pauling in 1935 and later confirmed by a neutron diffraction study (Pauling, 1935; Peterson & Levy, 1957). Each I_h crystal has two basal planes, six prism planes, six secondary prism planes, and six pyramidal planes (Vrielink, Aloï, Olijve, & Voets, 2016) (**Figure 1-1**). In I_h , each water molecule is associated with four neighboring water molecules through four hydrogen bonds in a tetrahedral arrangement (Pauling, 1935). I_h has been widely used as a model in studies of the interactions between ice and IR inhibitors (Naullage, Lupi, & Molinero, 2017; Naullage, Qiu, & Molinero, 2018; Weng, Stott, & Toner, 2017).

1.2.2 Ice recrystallization

Due to the high surface free energy of water molecules at the ice/water interface, there is a tendency to minimize the area of the ice/water interface (Cook & Hartel, 2010). This process leads to IR. As shown in **Figure 1-2A-C**, there are three main forms of IR: iso-mass, accretion, and migratory recrystallization (Adapa et al., 2000; Cook & Hartel, 2010). Iso-mass recrystallization refers to smoothing the surfaces of irregular ice crystals without a change in the mass of the ice (Cook & Hartel, 2010). Accretion recrystallization refers to the tendency of adjacent crystals to merge into a single large crystal (Cook & Hartel, 2010). Migratory recrystallization refers to the general propensity of larger crystals to grow at the expense of smaller ones (Cook & Hartel, 2010). Smaller ice crystals are less stable than larger ones because their surface area is larger relative to their mass. Therefore, it is thermodynamically favorable for water molecules on smaller ice crystals to melt, diffuse, and deposit on larger ones. Ostwald ripening is a unique form of migratory recrystallization that occurs at a constant temperature (Cook & Hartel, 2010). At the initial stage of recrystallization, accretion is dominant due to the presence of a large number of small ice crystals that are in close contact. In the later stage, with the reduction in the number of ice crystals and the increase of crystal size, Ostwald ripening becomes a dominant recrystallization process (Sutton, Lips, Piccirillo, & Sztchlo, 1996). The main result of IR is an increase in the size of individual ice crystals and a reduction in their number (**Figure 1-2D, E**).

In the absence of IR inhibitors, the diffusion rate of water in the liquid phase between ice crystals determines the IR rate when Ostwald ripening becomes dominant (Cook & Hartel, 2010). The Lifshitz Slyozov Wagner (LSW) theory (Lifshitz & Slyozov, 1961; Wagner, 1961) models the kinetics of IR for separated ice crystals. The mean crystal radius $r(t)$ at time t follows Eq (1) (Budke, Heggemann, Koch, Sewald, & Koop, 2009):

$$r^3(t) = r_0^3 + k_d t \quad (1)$$

where r_0 is the mean ice crystal radius at $t = 0$ and k_d is the rate constant of recrystallization. The k_d is dependent on the ice volume fraction $Q = V_{ice}/(V_{ice} + V_{liquid})$, where V_{ice} and V_{liquid} are the ice volume and the liquid volume, respectively. The relationship between k_d and Q follows Eq (2) (Budke et al., 2009):

$$k_d(Q) = k_{d0} \frac{\alpha^3}{1 - PQ^{1/3}} \quad (2)$$

where k_{d0} is the apparent rate constant at $Q = 0$; it is obtained by fitting Eq (2) with different Q values. P is a temperature-dependent scaling factor, $\alpha = r/r_c$, in which r is the mean crystal radius and r_c is the critical radius of a crystal that neither grows nor melts. α can be approximated by $\alpha = 1 - mQ$ and $m = 0.15$ with $Q \leq 0.4$ (Budke et al., 2009).

1.3 Antifreeze activity of AF(G)Ps

AF(G)Ps were first discovered in polar fishes (DeVries & Wohlschlag, 1969) and have since been found in a wide range of organisms living in environments with subzero temperatures (Gilbert, Davies, & Laybourn-Parry, 2005; Graether et al., 2000; Sidebottom

et al., 2000). AF(G)Ps have thermal hysteresis (TH), IRI, and dynamic ice shaping (DIS) activities resulting from the adsorption (binding) of AF(G)Ps on the ice surface (Voets, 2017). The exact ice-binding motifs of AF(G)Ps are currently a subject of debate. Recently, facial amphiphilicity has emerged as the key structural motif for the ice-binding of AF(G)Ps (Nutt & Smith, 2008; Tachibana et al., 2004).

1.3.1 Thermal hysteresis activity

The first antifreeze activity of AF(G)Ps to be discovered was the TH activity: an ability to depress the freezing point of a solution relative to its melting point (DeVries & Wohlschlag, 1969). For small molecules such as salts, the freezing point depression (ΔT) depends linearly on the solute osmolality and is calculated according to Eq (3):

$$\Delta T = K_F \times b \times i \quad (3)$$

where K_F is the cryoscopic constant, b is the molality, and i is the Van't Hoff factor. On a molar basis, AF(G)Ps depress the freezing point 200-300 times more efficiently than the values estimated from Eq (3) (Devries, 1971), indicating that the depression is not colligative. A later study proposed an adsorption-inhibition mechanism to explain the TH activity of AF(G)Ps (Raymond & Devries, 1977). The thermodynamic aspects of the adsorption-inhibition mechanism are described below (Kristiansen & Zachariassen, 2005; Raymond & Devries, 1977).

At the melting point T_m , ice and water phases are at equilibrium. From a thermodynamic viewpoint, their free energies per mol is equal, and their free energy

difference ΔG_v is 0 (Kristiansen & Zachariassen, 2005):

$$\Delta G_v = \Delta H_v - T_m \Delta S = 0; \Delta S = \frac{\Delta H}{T_m} \quad (4)$$

where ΔH_v and ΔS are the melting enthalpy and entropy, respectively, of ice (both are negative). At temperatures below the melting point (T_m), the free energy of the ice phase is lower than that of water, and this difference increases with decreasing temperature. The ΔG_v at a temperature T (K) below T_m is expressed by Eq (5) (Kristiansen & Zachariassen, 2005):

$$\Delta G_v = \Delta H - T \Delta S = \Delta H - \frac{T \Delta H}{T_m} = \Delta H \left(\frac{T_m - T}{T_m} \right) = \frac{\Delta H \Delta T}{T_m} \quad (5)$$

The growth of ice across a curved water/ice interface must overcome the Laplace pressure that on a spherical ice crystal with radius r , follows the Laplace Eq (6):

$$\Delta P = \frac{2\gamma}{r} \quad (6)$$

where γ is the interfacial tension at the ice/water interface. At the critical radius r_c , ice is at equilibrium with its surrounding water: $\Delta G_v = \Delta P$ (Glicksman, 2010). By combining (5) and (6), the critical radius r_c of the convex ice interface can be obtained:

$$r_c = \frac{2\gamma T_m}{\Delta H \Delta T} \quad (7)$$

If $r < r_c$, the system can decrease its free energy through the melting of ice, and ice growth is inhibited. If $r > r_c$, the system can decrease its free energy by growing ice. As shown in **Figure 1-3A**, ice only grows in the area between two successive AF(G)Ps binding sites separated by a distance d , forming an ice “cap” with an r_c and a semi-center angle θ :

$$r_c = \frac{d}{2\sin\theta} \quad (8)$$

Combining (7) and (8), the ΔT follows:

$$\Delta T = \frac{4\gamma T_m \sin \theta}{\Delta H d} \quad (9)$$

The surface coverage density (D) of AF(G)P on a binding surface follows Eq (10):

$$D = K[AF(G)Ps] \quad (10)$$

where K is the adsorption constant and $[AF(G)Ps]$ is the concentration of AF(G)P in the solution. The distance between binding sites, d , is calculated using Eq (11):

$$d = \frac{1}{\sqrt{K[AF(G)Ps]}} \quad (11)$$

Combining (9) and (11), ΔT follows Eq (12):

$$\Delta T = \frac{4\gamma \sin \theta \sqrt{K[AF(G)Ps]}}{\Delta H} \quad (12)$$

From Eq (12), at a given $[AF(G)Ps]$, when $\theta = 0.5 \pi$ and $r = 0.5 d$, the maximum ΔT can be reached. With a further decrease in temperature, burst growth of ice crystals will occur. This model predicts that the TH of AF(G)Ps is proportional to the root square of the molar/mass concentration, and this prediction has been supported by experiments (Kristiansen & Zachariassen, 2005; Raymond & Devries, 1977). The TH activity is commonly measured by the nanoliter osmometry method. Briefly, approximately 10 nL of a sample solution is injected into an oil droplet and subjected to nucleation at -30°C to -40°C inside a cryostage. The temperature of the cryostage is then increased to melt excessive ice crystals until a single small ice crystal is obtained, and the temperature is recorded as $T_{melting}$ (Braslavsky & Drori, 2013). The single ice crystal is incubated at $T_{melting}$ for a short time to allow adsorption of AF(G)Ps on its surface. The temperature

is then decreased, and the temperature at which burst growth of ice crystals occurs is recorded as $T_{freezing}$. The difference between $T_{melting}$ and $T_{freezing}$ is TH. Sonocrystallization technique has also been used in TH measurements (Gaede-Koehler, Kreider, Canfield, Kleemeier, & Grunwald, 2012).

1.3.2 Ice recrystallization inhibition activity

The IRI activity of AF(G)Ps was reported by Knight and coworkers (Knight, Devries, & Oolman, 1984; Knight, Hallett, & Devries, 1988). AF(G)Ps are highly effective IR inhibitors and display potent IRI activity in 45 wt.% sucrose at concentrations of 10^{-3} - 10^{-1} mg/mL (Budke et al., 2014). Assuming irreversible ice binding, the growth rate of ice (k_{ice}) for a spherical ice crystal is linearly proportional to the free surface area not covered by AF(G)Ps (Geng et al., 2017; Kowalczyk et al., 2012):

$$k_{ice}(\alpha) = k_{ice-0}(1 - \alpha) \quad (13)$$

where k_{ice-0} is the ice growth rate in the absence of AF(G)Ps and α is the surface coverage of AF(G)Ps. The adsorption rate of AF(G)Ps at time t follows (Geng et al., 2017; Kowalczyk et al., 2012):

$$\frac{d\alpha}{dt} = k_{ad}C_{AF(G)Ps}(1 - \alpha); \quad t = 0, \quad \alpha = 0 \quad (14)$$

where k_{ad} is the adsorption rate constant of AF(G)Ps and $C_{AF(G)Ps}$ is the concentration of AF(G)Ps. Assuming that $C_{AF(G)Ps}$ is constant during the formation of a single crystal, Eq (14) can be solved to yield the AF(G)Ps coverage as a function of time t :

$$\alpha(t) = 1 - \exp(-k_{ad}C_{AF(G)Ps}t) \quad (15)$$

Combining (13) and (15), the k_{ice} follows:

$$k_{ice}(t) = k_{ice-0} \exp(-k_{ad} C_{AF(G)Ps} t) \quad (16)$$

At a given time t , the growth rate (k_{ice}) of ice crystals is proportional to $\exp(-k_{ad} C_{AF(G)Ps} t)$.

Several methods have been developed for determining IRI activity. The “splat assay” is the method most widely used in the field (Knight et al., 1988). Samples are usually dispersed in salt solutions such as NaCl solution or PBS. A glass slide is prechilled on a polished metal block surrounded by dry ice (-78°C) in a foam box for at least 30 min. A drop of sample dispersion (ca. 10 μL) is then dropped from 1-2 m onto the surface of a prechilled glass slide; a thin ice wafer of small ice crystals is formed immediately. The glass slide is then transferred to a cryostage and annealed at -8°C or -6°C for 30-60 min. The chamber housing the cryostage is purged with N_2 to prevent moisture condensation during annealing. The resulting ice wafers are imaged by polarized optical microscopy, and the images are analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The mean largest grain size (MLGS) of the ten largest crystals or the mean grain area (MGA) of all ice crystals in each image is usually obtained. The MGLS or MGA values of the samples are compared to those of dispersion medium (blank control), yielding values that are expressed as %MLGS or %MGA. A rough method of comparing %MGLS and MGA% is $\%MGLS = \sqrt{\%MGA}$. Poly(ethylene glycol) (PEG) is typically used as a negative control in IRI measurements (Biggs et al., 2019).

Another commonly used method for IRI determination is the sandwich assay (Biggs et al., 2017). This assay is widely used with samples that show less colloidal stability in salt solution. The samples are dispersed in 18-49% sucrose solution and sealed between two glass slides using silicon grease to prevent moisture evaporation (Budke & Koop, 2006; Drori et al., 2016; Leiter, Ludwig, & Gaukel, 2017). The samples are then immersed in liquid N₂ for nucleation and incubated at a subzero temperature to observe ice recrystallization. Recently, high-throughput IRI screening methods using superhydrophobic-coated sapphire slides (Graham, Agrawal, Oleschuk, & Davies, 2018), glass capillaries (Tomczak, Marshall, Gilbert, & Davies, 2003), and gold nanoparticle probes (Mitchell, Congdon, Rodger, & Gibson, 2015) have also been developed.

It is noteworthy that the presence of solutes such as salts (in the splat assay) and sucrose (in the sandwich assay) is essential to exclude false positive results (Knight, Wen, & Laursen, 1995). If pure water is used as a dispersion medium, IRI-inactive particles trapped in ice grain boundaries can, due to the insufficient amount of unfrozen water, act as physical barriers that prevent water diffusion, leading to false positive results. The presence of solutes promotes the formation of liquid channels between ice crystals, and these channels can facilitate migratory recrystallization and eliminate the effect of nonspecific ice binding (Knight et al., 1995). A minimum NaCl concentration of 0.1 mg/mL is required to exclude false positive results in the splat assay (Knight et al., 1995). The unfrozen water fraction is proportional to the concentrations of solutes (Pongsawatmanit

& Miyawaki, 1993). The unfrozen water fraction in the sandwich assay is much higher than that in the splat assay due to the higher sucrose concentrations used in the sandwich assay (Biggs et al., 2017). This indicates that the concentration of IRI-active material in the sandwich assay is lower than that in the splat assay. Therefore, the IRI activity obtained using the two types of assays cannot be directly compared.

1.3.3 Dynamic ice shaping activity

DIS refers to the anisotropic ice growth that occurs due to the adsorption of AF(G)Ps on a single ice crystal plane or multiple ice crystal planes (Voets, 2017). The growth of ice crystal planes to which AF(G)Ps are bound is inhibited, and ice only grows at unbound planes, resulting in hexagonal, lemon-shaped or hexagonal bipyramidal forms (Geng et al., 2017; Graham, Liou, Walker, & Davies, 1997). The DIS property has been widely used as an indicator of ice adsorption of IR inhibitors (G. Bai et al., 2017; Bai, Gao, & Wang, 2017; Mizrahy, Bar-Dolev, Guy, & Braslavsky, 2013). The procedures used to analyze ice morphology are similar to TH measurements but emphasize determination of the morphology of a single ice crystal after growth. The morphology of a growing ice crystal is recorded by a high-speed camera. Anisotropic ice growth indicates the adsorption of IR inhibitors and DIS activity, while isotropic ice growth yielding ice crystals with a spherical shape indicates lack of adsorption and lack of DIS activity (Braslavsky & Drori, 2013). Different AF(G)Ps bind to different ice planes, and several AF(G)Ps can bind to multiple ice planes (Olijve et al., 2016; Rahman et al., 2019) (**Figure 1-4A**). The binding of AF(G)Ps

to crystal planes is commonly analyzed by the ice-etching method in which an ice crystal is grown from a dilute AF(G)Ps solution to produce a large ice hemisphere (**Figure 1-4B**) (Knight, Cheng, & Devries, 1991). Accumulation of AF(G)Ps results in visible etched regions that give clear spatial information on the binding planes (**Figure 1-4C**). The use of fluorescently labeled AF(G)Ps is preferred in this experiment because it enhances visualization of the etched regions (Rahman et al., 2019).

Although it has been commonly believed that TH, IRI and DIS all result from the absorption of AF(G)Ps, these phenomena are not strongly correlated. For example, *Tm*AFPs is TH hyperactive but shows relatively low IRI activity (Biggs et al., 2017). Conversely, AFGPs and winter flounder type I-AFPs are moderately TH active but have high IRI activity (Biggs et al., 2017). Some AF(G)Ps mimics show strong IRI activity but no DIS activity (Balcerzak et al., 2014; Biggs et al., 2017).

1.3.4 The adsorption-inhibition mechanism

TH, DIS, and IRI activities have been explained by an adsorption-inhibition mechanism (Raymond & Devries, 1977). Two models have been proposed to describe the adsorption of AF(G)Ps on ice. In the step-pinning model (**Figure 1-3B**), regions in which AF(G)Ps are adsorbed develop paths of growing steps in which the steps percolate between adsorption sites and form curvatures (Raymond & Devries, 1977). The elevated curvature increases the solid-liquid interfacial energy and depresses the freezing point, thereby inhibiting the further advancing of steps. The step-pinning model predicts that the TH

activity depends on the square root of the AF(G)Ps concentration (Raymond & Devries, 1977), a prediction that is confirmed by experimental results (Kristiansen & Zachariassen, 2005; Raymond & Devries, 1977). In the mattress model (**Figure 1-3C**), AF(G)Ps inhibit ice growth in the direction normal to the adsorption plane (Knight et al., 1991). AF(G)Ps are not required to be absorbed on exactly the same ice lattice plane to function in the mattress model, consistent with the adsorption of AF(G)Ps on atomically rough curved surfaces (Knight et al., 1991; Shtukenberg, Ward, & Kahr, 2017).

1.3.5 Structures of AF(G)Ps

Four classes of AFPs (types I, II, III, and IV) have been identified in various species of polar fishes in earlier studies (**Figure 1-5**) (Deng, Andrews, & Laursen, 1997; Vrielink et al., 2016). Additional AFPs have recently been found in insects (*Tm*AFPs and *Ri*AFPs), grass (*Lp*IBPs), and bacteria (*Ti*AFPs) (Dolev, Braslavsky, & Davies, 2016). AFPs vary in amino acid composition, molecular weight (M_w) (3.3-34 kDa), and secondary structure (Capicciotti, Doshi, & Ben, 2013; Dolev et al., 2016; Raymond, Radding, & DeVries, 1977). Type I AFPs are alanine-rich with M_w of 3.3-5.0 kDa, and they adopt an amphipathic α -helical conformation in solution (Sicheri & Yang, 1995). Type II AFPs are cysteine-rich with M_w of 14-24 kDa (Liu et al., 2007). Type II AFPs present a globular conformation consisting of two α -helices, two β -sheets, and some loops and turns. Five disulfide bridges between cysteine residues have been found in Type II AFPs (Sönnichsen, Sykes, & Davies, 1995). No particular amino acids dominate in the amino acid compositions of Type III

AFPs (Davies & Hew, 1990; Yamashita et al., 2003). Type III AFPs are 6-7 kDa globular proteins containing several short, irregular β sheets and one α -helical turn (Jia, DeLuca, Chao, & Davies, 1996; Frank D Sönnichsen, Carl I DeLuca, Peter L Davies, & Brian D Sykes, 1996). Type IV AFPs have a high content of glutamine and a M_w of 12.3 kDa; they consist of four amphiphilic α -helical regions that are folded into a four-helix bundle (Deng et al., 1997; Deng & Laursen, 1998).

Eight fractions of AFGPs, 1-8, have been identified and named according to their M_w , with a smaller number indicating a larger M_w (DeVries, Komatsu, & Feeney, 1970). AFGPs 1-5 feature a tripeptide repeat of (alanine-alanine-threonine)_n in their backbones and glycosylated threonine residues containing the disaccharide β -D-galactosyl-(1-3)- α -N-acetyl-D-galactosamine (DeVries et al., 1970; DeVries, Vandenheede, & Feeney, 1971). The M_w of AFGPs 1-5 ranges from 10.5 to 33.0 kDa (DeVries et al., 1970). AFGPs 6-8 differ from AFGPs 1-5 in amino acid sequence; proline replaces some alanine residues starting from the first alanine in the third (alanine-alanine-threonine) repeat (Lin, Duman, & DeVries, 1972; Morris et al., 1978). Specifically, AFGPs in fraction 8 consist of three different glycopeptides that have the same chain length but show minor variations in amino acid sequence (Morris et al., 1978). Among AFGPs, AFGPs-8 have the smallest M_w , 2.6 kDa (Knight, Driggers, & DeVries, 1993). AFGPs are proposed to adopt a left-handed helix similar to the polyproline II-type helix; the formation of this helix is independent of M_w (Lane, Hays, Crowe, Crowe, & Feeney, 1998; Nguyen, Colvin, Yeh, Feeney, & Fink, 2002;

Tachibana et al., 2004). The classification and representative structural properties of AF(G)Ps 1-5 are shown in **Figure 1-5**.

1.3.6 Structural motifs of AF(G)Ps essential for ice binding

1.3.6.1 Hydrophilic groups

Although the adsorption-inhibition mechanism has been well accepted in academia, the essential structural motif required for ice binding is not fully understood. Early works focused on the role of hydrogen bonding between AF(G)Ps and ice (Chou, 1992; Knight et al., 1993). The hydrogen bonding theory was based on the matching of the spacing between the polar groups of AF(G)Ps and that of water molecules in the ice lattice (DeVries, 1984). The distance between the Asp and Thr residues in the AFPs of some polar fishes matches the distance between adjacent oxygens in the basal and prism planes of I_h (DeVries, 1984). Chou et al. reported that flounder AFPs bind to ice in a zipper-like fashion because the spacing between the four threonine residues in the AFP helix matches well with the spacing of water molecules along the $[01\bar{1}2]$ direction in the $(20\bar{2}1)$ ice lattice plane (Chou, 1992). However, the lifetime of hydrogen bonds (in the order of ps magnitude) in the liquid phase is too short (Bandyopadhyay, Chakraborty, & Bagchi, 2005; Meister et al., 2013) to explain the irreversible AF(G)Ps binding. Knight et al. proposed that two hydroxyl groups of each disaccharide moiety in AFGPs 7-8 were embedded in the ice and became less mobile due to the formation of six stable hydrogen bonds with less mobile water molecules in the ice (Knight et al., 1993). The importance of the hydroxyl groups in the disaccharide moieties

of AFGPs has been confirmed by experiments in which the disaccharide moieties were removed or the hydroxyl groups of the disaccharide moieties were modified, leading to the elimination of the TH activity of AFGPs (Shier, Lin, & DeVries, 1972; Tachibana et al., 2004). More recently, the role of the hydroxyl groups in the ice-binding faces of AFPs has been revealed by molecular dynamic simulations (MDS). The hydroxyl groups in the ice-binding face of *Tm*AFPs anchor a layer of ice-like clathrate water through direct hydrogen bonding, thereby positioning and slowing the dynamics of water at the trough of the binding site (Hudait, Qiu, Odendahl, & Molinero, 2019; Midya & Bandyopadhyay, 2018). It has been proposed that hydrophilic groups in the non-ice binding face are also crucial for antifreeze activity. The hydrophilic groups in the non-ice binding face protect AF(G)Ps from being overgrown by disruption of the water structure around the rest of the protein (Liu et al., 2016; Nutt & Smith, 2008; Zanetti-Polzi, Biswas, Del Galdo, Barone, & Daidone, 2019).

1.3.6.2 Hydrophobic groups

The first study to address the importance of hydrophobic groups in ice binding was that of Sönnichsen and coworkers; it was based on the solution conformation of Type III AFPs (F. D. Sönnichsen, C. I. DeLuca, P. L. Davies, & B. D. Sykes, 1996). The hypothesis was further supported by two later studies using type I AFP mutants (Chao et al., 1997; Haymet, Ward, & Harding, 1999). Mutants in which Thr had been replaced by hydroxyl-group-rich Ser or by Gly lost ice-binding capacity and therefore TH activity (Chao et al.,

1997; Haymet et al., 1999). Similarly, replacing the γ -methyl groups of Thr in the backbone of AFGPs with hydrogens eliminates TH activity (Tachibana et al., 2004). In contrast, type I AFP mutants in which Thr is replaced by hydrophobic Val or Ala retain TH activity (Chao et al., 1997; Haymet et al., 1999) but adsorb to distinct ice planes (Haymet et al., 1999). Recent studies suggest that the role of the hydrophobic regions of AF(G)Ps in ice binding may be to organize the ice-like clathrate water by hydrophobic interactions. Molecular dynamic simulations (MDS) have suggested that the entropy increment caused by the desolvation of ice-like clathrate water around hydrophobic groups promotes the incorporation of AF(G)Ps into the grooves and cavities of certain ice planes (Mochizuki & Molinero, 2018; Nada & Furukawa, 2008). Other MDS studies have shown that the ice-like clathrate water layer around an ice-binding face recognizes and anchors AFPs on ice surfaces by hydrogen bonding (Chakraborty & Jana, 2018; Garnham, Campbell, & Davies, 2011; Nutt & Smith, 2008). However, it has also been reported that ice recognition of AFPs does not rely on the preordering of water and that water at the ice-binding face of AFPs in solution does not display an anchored clathrate-like order (Hudait et al., 2018).

1.3.6.3 Importance of facial amphiphilicity in the antifreeze activity of AF(G)Ps

It was recently proposed that the facially amphiphilic nature of AF(G)Ps, which show a segregated display of hydrophobic and hydrophilic planes, is the key structural motif of these proteins. The ice-binding face of AF(G)Ps is hydrophobic due to the presence of methyl groups (Liu et al., 2016; Tachibana et al., 2004). The non-ice binding face, which

consists of disaccharide moieties and charged groups, is hydrophilic (Liu et al., 2016; Tachibana et al., 2004). Both the hydrophobic and the hydrophilic faces are essential for the antifreeze activity of AF(G)Ps (Midya & Bandyopadhyay, 2018; Nutt & Smith, 2008).

1.4 Inhibition of ice recrystallization by other materials

Although AF(G)Ps are quite effective IR inhibitors, their large-scale application is limited by their high cost (10,000 dollars per gram) (Protein, 2020), which is associated with their isolation and synthesis. Therefore, it is of great interest to explore inexpensive AF(G)P mimics.

1.4.1 Ions and low-molecular-weight compounds

Ions are the smallest substances that demonstrate IRI activity (S Wu et al., 2017). The IRI activity of anions follows the order of $\text{HPO}_4^{2-} > \text{SO}_4^{2-} > \text{F}^- > \text{AC}^- > \text{SCN}^- > \text{ClO}_4^- > \text{Br}^- > \text{Cl}^- > \text{NO}_3^- > \text{I}^-$ (S Wu et al., 2017). The IRI activity of cations follows the order of $\text{NH}_4^+ > \text{Mg}^{2+} > \text{Ca}^{2+} > \text{Li}^+ > \text{TMA}^+ > \text{Na}^+ > \text{Gua}^+ > \text{K}^+ > \text{Cs}^+$. MDS studies have revealed that ions with high charge densities have greater ion-water interaction energy than water-water interaction energy in the bulk ice. Therefore, ions with high charge densities cause great disruption to the local ice structure and are more difficult to be incorporated into ice; this means that they can inhibit ice recrystallization effectively at the ice/water interface. Ions with lower charge densities are more likely to be incorporated into ice, leading to weak IRI (S Wu et al., 2017). This ion-specific ice recrystallization has been used to fabricate porous 2D materials with controllable pore sizes (S. Wu et al., 2017).

Mono/disaccharides have moderate IRI activity without observable TH or DIS activity (Tam, Ferreira, Czechura, Chaytor, & Ben, 2008). The IRI activity of mono/disaccharides correlates with their hydration index, which is the number of tightly bound water molecules divided by the molar volume of carbohydrates (Tam et al., 2008). When ice is in an aqueous solution, the interface between the ice and the bulk water is not an abrupt transition. A semiordered layer named the quasi-liquid layer exists between the highly ordered ice lattice and the disordered bulk water phase surrounding ice crystals (Hayward & Haymet, 2001; Sasaki, Zepeda, Nakatsubo, Yokomine, & Furukawa, 2012). Monosaccharides/disaccharides are proposed to inhibit IR at the bulk water between quasi-liquid layer of two adjacent ice crystals by disrupting the preordering of water and making water transfer to ordered ice crystals less energetically favorable (Tam et al., 2008). Mono/disaccharides with larger hydration indices cause greater disruption of bulk water ordering and therefore have stronger IRI activity (Balcerzak et al., 2014). The hydration of carbohydrate moieties also affects the IRI activity of C-linked AFGP analogues. Analogues linked to highly hydrated galactose show the strongest IRI activity, followed by glucose-, mannose-, and talose-linked ones (Czechura, Tam, Dimitrijevic, Murphy, & Ben, 2008). Carbohydrate hydration is also important for the IRI of carbohydrate-based low-molecular-weight surfactants. For surfactants containing the same hydrophobic moiety, those with a more hydrated galactose moiety are more IRI active than those with a less hydrated glucose moiety (Capicciotti et al., 2012).

Some amphiphilic low-molecular-weight compounds are also IRI active. Potent IRI activity in specially designed low-molecular-weight surfactants has been reported (Abraham et al., 2015; Adam et al., 2016; Balcerzak, Febbraro, & Ben, 2013), and both hydrophilic and hydrophobic moieties are important for the IRI of these compounds (Balcerzak et al., 2013; Capicciotti et al., 2012). Common low-molecular-weight surfactants have different effects on IR (Fan et al., 2020). For nonionic 2-(tridecyloxy) ethanol and zwitterionic OA-12, mean ice grain size increases as the concentration of surfactant is increased. For the 6 ionic surfactants studied, mean ice grain size tends first to increase and to reach a maximum at 5 μM ; it then decreases with further increases in the concentration of the surfactant (Fan et al., 2020). Addition of surfactants can lower the melting point of ice, thereby decreasing the stability of smaller ice crystals. Therefore, the number of small ice crystals is reduced at relatively low surfactant concentrations, leading to an increase in the mean ice grain size (Fan et al., 2020). In contrast, at relatively high surfactant concentrations, recrystallization of crystals is inhibited by the counterions that are concentrated at the ice-water interface and in the bulk water between two adjacent ice crystals (Fan et al., 2020). As a result, mean ice grain size decreases. Amphiphilic metallohelicenes without hydroxyl groups inhibit ice recrystallization at just 20 μM (Mitchell et al., 2017), indicating that amphiphilicity is the key structural motif for IRI and that hydroxyl groups are not essential.

1.4.2 Synthetic polymers

Poly(vinyl alcohol) (PVA) is the most IRI-active synthetic polymer discovered to date; it can effectively inhibit IR at sub-mg/mL concentrations (Congdon, Notman, & Gibson, 2013). The IRI mechanism of PVA is still not fully understood. MDS studies have shown that PVA binds to prismatic ice planes through hydrogen bonds (Naullage et al., 2017; Naullage & Molinero, 2020; Naullage et al., 2018). PVA can form local hydrophobic regions, indicating that a potential role of hydrophobic interactions cannot be excluded (Deller et al., 2013). However, inclusion of hydrophobic acetate groups above 10 mol % in PVA side chains leads to a reduction in IRI activity (Congdon et al., 2013). More recently, PVA has been found to exist as colloidal particles rather than as flexible polymer chains in the unfrozen water fraction between ice crystals (Ishibe et al., 2019). However, the role of this colloidal structure in IRI is not clear. The reported TH of PVA (weight-average M_w 13-23 kDa) is only 0.034°C at 40.0 mg/mL (Inada & Lu, 2004). The percentage of ice-absorbed PVA is only slightly higher than that of IRI-inactive PEG and much lower than that of type III AFPs (Burkey, Riley, Wang, Hatridge, & Lynd, 2018).

Inspired by the association between facial amphiphilicity and the antifreeze activity of AF(G)Ps, amphiphilic polymers have been synthesized for IRI. Polyproline has been found to adopt an amphiphilic polyproline II helix structure in aqueous solutions. Polyproline displays moderate IRI activity without DIS activity (Graham et al., 2017). Glycopolymers featuring segregated hydrophilic and hydrophobic faces have been shown to have potent

IRI activity without DIS activity (B. Graham et al., 2018). The IRI activity of glycopolymers was reduced when a hydrophilic oxo ether was added on the glycan-opposing face, but the lost activity was restored after incorporation of a hydrophobic dimethylfulvene residue (B. Graham et al., 2018). However, simply incorporating hydrophobic acetate groups into PVP fails to enhance its IRI activity, indicating that hydrophobicity alone is not sufficient to activate IRI (Stubbs, Congdon, & Gibson, 2019).

1.4.3 Natural biopolymers

Some polysaccharides have been shown to be IRI active. κ -carrageenan is a potent IR inhibitor that can effectively inhibit IR in 49% sucrose solution at 1.0 mg/mL (Leiter, Ludwig, et al., 2017). Gelation of κ -carrageenan induced by metal ions reduces the IRI (Leiter, Emmer, & Gaukel, 2018). Both κ -carrageenan and ι -carrageenan have been shown to inhibit IR and to modify ice morphology in 49% sucrose solution, but they have no observable TH (Kiran-Yildirim & Gaukel, 2020). Locust bean gum and guar gum have been used in ice creams to inhibit IR (Adapa et al., 2000; Sutton & Wilcox, 1998), but their IRI activity has not been measured by the splat assay or the sandwich assay. The IRI mechanism of polysaccharides has been proposed to be associated with increased viscosity and gelation (Adapa et al., 2000; Regand & Goff, 2002).

IRI activity of some non-AF(G)Ps proteins and protein hydrolysates has been reported. The IRI activity of C-type lectins can be activated by Ca^{2+} ions (Mitchell & Gibson, 2015). Peptides obtained from gelatin hydrolysis demonstrate moderate IRI activity at 40 mg/mL

in 23% sucrose solution (Wang, Agyare, & Damodaran, 2009; Wang & Damodaran, 2009), but their IRI activity in salt solutions has not been tested by the splat assay.

1.4.4 Self-assembled materials and nanomaterials

Three low-molecular-weight molecules can self-assemble into fibril-like structures and inhibit IR by the adsorption-inhibition mechanism. Safranin O is a synthetic dye that has been found to possess IRI activity comparable to that of highly evolved AFGPs (Drori et al., 2016). Safranin O molecules self-assemble into amphiphilic fibril-like stacks and bind reversibly to ice, resulting in IRI, TH and DIS activity (Drori et al., 2016). Zirconium acetate and zirconium acetate hydroxide show pH-dependent IRI, TH, and DIS activity (Mizrahy et al., 2013). In aqueous solutions, they are proposed to form polymers in which hydrophobic acetate groups are exposed to ice and only hydroxyl groups are exposed to water (Mizrahy et al., 2013).

The IRI activity of nanomaterials has been explored in recent years. Graphene oxide (GO), the most IRI-active nanomaterial identified to date, effectively inhibits IR at 0.1 mg/mL (Geng et al., 2017). GO binds to the basal planes of ice crystals via hydrogen bonds and demonstrates both TH and DIS activity (Geng et al., 2017). Oxidized quasi-carbon nitride quantum dots (OQCNs) have been shown to exhibit TH, DIS, and IRI activity by binding to ice (G. Bai et al., 2017). The proposed mechanism of ice binding is the formation of hydrogen bonds between neighboring tertiary nitrogen atoms on OQCNs and repeating oxygen atoms along the c-axis on the primary prism plane of the ice lattice (G. Bai et al.,

2017). TH and DIS activity of sulfur-doped OQCNs have been reported (G. Y. Bai et al., 2017). Recently, IRI activity of PVA and antifreeze peptides decorated with gold nanoparticles has been reported (Lee, Lee, Lim, Ahn, & Lee, 2019; Stubbs, Wilkins, Fayter, Walker, & Gibson, 2019). Metal-organic frameworks are periodic porous materials that are typically self-assembled through metal nodes and organic linkers (Zhu et al., 2019). A zirconium-based metal-organic framework has been shown to inhibit IR by adsorption to primary ice planes via hydrogen bonds (Zhu et al., 2019).

1.5 Mw and surface charge dependence of IRI

AF(G)Ps demonstrate molecular weight dependence of their TH and IRI activity (Mueller, McKown, Corotto, Hague, & Warren, 1991; Tachibana et al., 2004; Wilkinson et al., 2012). AF(G)Ps must possess a minimum M_w to be active, and activity initially increases with increasing M_w and then reaches a plateau (Mueller et al., 1991; Tachibana et al., 2004; Wilkinson et al., 2012). Assembly to dimers and multimers can enhance the activity of AF(G)Ps (Bouvet, Lorello, & Ben, 2006; Can & Holland, 2011; Stevens, Drori, Zalis, Braslavsky, & Davies, 2015). M_w -dependent IRI activity is also observed in PVA and κ -carrageenan. The presence of a longer PVA chain (higher DP and higher M_w) results in stronger IRI activity (Congdon et al., 2013; Inada & Lu, 2003). PVA with a DP of 10 does not possess IRI activity, but the compound becomes very active when the DP increases to 20 (Congdon et al., 2013). The IRI activity of inactive, low-DP PVA can be activated by assembly of the compound into supramolecular star polymers linked by Fe^{3+} (Phillips,

Congdon, & Gibson, 2016). Hydrolysis and dialysis have been found to reduce the M_w and therefore the IRI activity of κ -carrageenan (Leiter, Mailander, Wefers, Bunzel, & Gaukel, 2017).

Charge density-dependent IRI activity is observed in synthetic polymers and GO. Typically, charged synthetic polymers are not IRI active (Gibson, Barker, Spain, Albertin, & Cameron, 2009). Poly(acrylic acid), poly(2-aminoethyl methacrylate), poly(L-glutamate), and poly(lysine) have been found to have weak or no IRI activity (Gibson et al., 2009). Vinyl-based poly(ampholytes) display moderate IRI activity only when they possess a 1:1 ratio of cationic to anionic groups (Mitchell, Lilliman, Spain, & Gibson, 2014). GO demonstrates strong IRI activity, but fully carboxyl-functionalized GO (GCOOH) becomes inactive because GCOOH can form more hydrogen bonds with water than with ice, resulting in weak ice-binding affinity (Geng et al., 2017).

1.6 Cellulose and nanocelluloses

Cellulose is one of the most abundant natural polymers, with an annual biosynthesis yield of more than 7.5×10^{10} tons (Habibi et al., 2010). Cellulose is a homopolymer of anhydro-D-glucose units linked by β -1,4-glycosidic bonds. Each glucose unit is corkscrewed 180° with respect to its neighbor, and the repeat segment is known as cellobiose, which is the monomer unit of cellulose (Habibi et al., 2010) (**Figure 1-6A**). In nature, cellulose exists as fibrils assembled from individual cellulose chains via van der Waals forces, stacking interactions, and intra/intermolecular hydrogen bonds (Habibi et al.,

2010; Parthasarathi et al., 2011). The role of hydrophobic interactions in the assembly of cellulose fibrils has recently been recognized (Lindman et al., 2017; Medronho, Romano, Miguel, Stigsson, & Lindman, 2012). The hydrophobic interaction originates from the -CH groups along the axial direction of anhydro-glucose rings (Medronho et al., 2015; Yamane et al., 2006). The importance of hydrophobic interactions in the assembly of cellulose is supported by the finding that the self-association of cellulose chains is inhibited by the presence of certain amphiphilic cosolutes (Medronho et al., 2015; Medronho et al., 2012). Typically, approximately 36 individual cellulose chains assemble into a larger unit known as an elementary fibril, which further assembles into fibrils and larger fibers (**Figure 1-6B**) (Habibi et al., 2010). Cellulose fibrils have both crystalline regions and amorphous regions (Le Gars, Douard, Belgacem, & Bras, 2019) (**Figure 1-6C**). Nanocelluloses refer to nanostructured cellulose fibrils isolated by different methods. Nanocelluloses vary in fibril length, fibril width, and structure and density of surface charge depending on the source, production method, and post-production modification process (Habibi et al., 2010; Kalashnikova et al., 2012; Klemm et al., 2011). Due to their nontoxicity, biocompatibility and renewability, nanocellulose-based materials are emerging as green and sustainable solutions to challenges in the 21st century (Golmohammadi, Morales-Narváez, Naghdi, & Merkoçi, 2017; Gomez et al., 2016; Klemm et al., 2011; Salas, Nypelo, Rodriguez-Abreu, Carrillo, & Rojas, 2014)

1.6.1 Types of nanocelluloses and production methods

Many nanocelluloses have been reported, and cellulose nanofibrils (CNFs), cellulose nanocrystals (CNCs), and 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils (TEMPO-CNFs) have been intensively studied.

CNFs are long, flexible, branched fibrils that exhibit aggregation (Klemm et al., 2011). CNFs are produced by forcing suspensions of wood-based cellulose fibers through mechanical devices such as high-pressure homogenizers that result in delamination of the fibers and liberation of the fibrils (Klemm et al., 2011). Other mechanical treatments such as high-intensity ultrasound have also been used (Pappas, Tarantilis, Daliani, Mavromoustakos, & Polissiou, 2002). CNFs are barely charged and cannot be dispersed well in water (Klemm et al., 2011).

TEMPO-CNFs are cellulose nanofibrils with several nanometers in width and several hundred nanometers in length (Zhou, Saito, Bergstrom, & Isogai, 2018). TEMPO-CNFs are prepared by TEMPO-catalyzed oxidation in the presence of NaBr at pH 10.0, and NaClO is commonly used as the oxidant (**Figure 1-7A**) (Isogai et al., 2011). An alternative method is to use a combination of NaClO and NaClO₂ as oxidants with the pH controlled at 4.8 (Shinoda, Saito, Okita, & Isogai, 2012). TEMPO-CNFs are negatively charged because carboxylate groups are formed by oxidation at C-6 (Isogai et al., 2011). However, a small number of aldehyde groups remain after oxidation (Okita, Saito, & Isogai, 2010). Further oxidation by NaClO₂ or reduction by NaBH₄ is required to convert the aldehydes

to carboxylate groups or hydroxyl groups (Shinoda et al., 2012; Takaichi, Saito, Tanaka, & Isogai, 2014). TEMPO-CNFs exist as fibril aggregates after TEMPO-mediated oxidation (Saito, Nishiyama, Putaux, Vignon, & Isogai, 2006). Mechanical treatments such as homogenization and sonication are required to nanofibrillate TEMPO-CNFs (Fukuzumi, Tanaka, Saito, & Isogai, 2014; Saito et al., 2009). TEMPO-CNFs have good dispersibility in water but gradually aggregate with increases in ionic strength due to the charge screening effect (Fukuzumi et al., 2014).

CNCs are rodlike nanocrystals with high crystallinity (Dong, Revol, & Gray, 1998). CNCs are produced by the use of acid to preferentially hydrolyze the amorphous regions; with this treatment, crystalline regions with a higher resistance to acid attack are left. Sulfuric acid (64 wt.%) is commonly used, and the duration of hydrolysis depends on the hydrolysis temperature (Jiang & Hsieh, 2015; P. Lu & Y. L. Hsieh, 2012; Reiner & Rudie, 2013). The mass ratio of sulfuric acid to cellulose used in hydrolysis is 8.75-10:1 (Dong et al., 1998; Lu & Hsieh, 2010; P. Lu & Y.-L. Hsieh, 2012). Hydrolysis is quenched by 10-fold dilution in cold water, and acid is removed by centrifugation and dialysis in the laboratory or by ultrafiltration in industry (P. Lu & Y. L. Hsieh, 2012; Reiner & Rudie, 2013). During hydrolysis, sulfuric acid reacts with the hydroxyl groups of CNCs to form sulfate ester groups ($-\text{OSO}_3^-$) on the CNC surface (**Figure 1-7B**) (Beck, Méthot, & Bouchard, 2014). Phosphoric, hydrobromic, and hydrochloric acids are also used to produce CNCs (Araki, Wada, Kuga, & Okano, 1998; Camarero Espinosa, Kuhnt, Foster,

& Weder, 2013; Sadeghifar, Filpponen, Clarke, Brougham, & Argyropoulos, 2011). The electrostatic repulsion provided by the sulfate groups results in good dispersibility of CNCs in water, but the charge screening effect at an increased ionic strength can also causes aggregation of CNCs (Leguy et al., 2018).

To address the problem of aggregation, novel sterically stabilized dialdehyde/dicarboxylate hairy CNCs are prepared by chemical oxidation (**Figure 1-7C**) (Kim, Kuga, Wada, Okano, & Kondo, 2000; Yang, Alam, & Van de Ven, 2013; Yang, Chen, & Van de Ven, 2015). Hairy CNCs are rodlike fibrils with cellulose chains protruding at both ends (Sheikhi & Van de Ven, 2017). The cellulose chains protruding at both ends of the fibrils provide steric interactions, resulting in good dispersibility of the fibrils under conditions of high ionic strength (Safari, Sheikhi, & Van de Ven, 2014; Yang et al., 2013). In NaIO_4 oxidation, C-2 and C-3 hydroxyls are oxidized to aldehyde groups and anhydroglucose rings are opened, forming dialdehyde hairy CNCs; after removal of NaIO_4 by repeated washing and filtering, the dispersion is heated at 80°C for several hours to obtain water-dispersible dialdehyde hairy CNCs (Kim, Wada, & Kuga, 2004). Propanol is added to precipitate the dialdehyde hairy CNCs (Yang et al., 2015). The dispersity of the precipitated dialdehyde hairy CNCs in water is poor due to the presence of interfibrillar hemiacetal linkages (Munster et al., 2017; Takaichi et al., 2014). The dispersibility of dialdehyde hairy CNCs can be promoted by NaBH_4 reduction (Leguy et al., 2018). Dialdehyde hairy CNCs are not stable in water and degrade with time possibly by β -

elimination (Munster et al., 2017; Yang et al., 2015). Dicarboxylate hairy CNCs are obtained after NaClO_2 oxidation of dialdehyde groups are also referred to electrosterically stabilized nanocrystals (ECNCs) (Yang et al., 2013). The density of carboxylate groups in these CNCs is approximately 6 mmol/g, significantly higher than that of conventional CNCs and TEMPO-CNFs (Yang et al., 2013).

1.6.2 Structural parameters of nanocelluloses and characterization

Surface charge density (SCD), fibril length (degree of polymerization), and fibril width are three most important structural parameters of nanocelluloses. The SCD of CNCs is determined as the sulfate group density (Beck et al., 2014), whereas the SCD of TEMPO-CNFs and ECNCs is estimated as that of carboxylate group (Isogai et al., 2011; Yang et al., 2013). The SCD of nanocelluloses is normally measured by conductometric titration. Sulfate groups require protonation by a strong acid cation exchange resin before titration (Beck et al., 2014). Carboxylate groups can be protonated by lowering the pH to 2.5 (Saito & Isogai, 2004). To calculate the SCD, the protonated nanocellulose dispersions are titrated against NaOH. The SCD of CNCs can also be estimated based on the sulfur content determined by inductively coupled plasma atomic emission spectroscopy or energy-dispersive X-ray spectroscopy (Beck et al., 2014; P. Lu & Y. L. Hsieh, 2012). The sulfate group density of CNCs can be modulated by 2.5 N HCl or 5 N trifluoroacetic acid hydrolysis without significantly changing fibril length or DP (Kalashnikova et al., 2012). However, long duration of HCl hydrolysis (10 h) reduces fibril length and affects the

crystalline structure of the fibrils (Kalashnikova et al., 2012).

The fibril length of nanocelluloses is commonly measured by atomic force microscopy (AFM) or transmission electron microscopy (TEM) (Kalashnikova et al., 2012; Shinoda et al., 2012; Zhou et al., 2018). The number average fibril length of nanocelluloses is calculated as (Zhou et al., 2018):

$$L_n = \frac{\sum_{i=1}^n N_i L_i}{\sum_{i=1}^n N_i} \quad (17)$$

where N_i is the number of fibrils with length L_i . If the lengths of nanocellulose are polydisperse, the length-weight average can emphasize the presence of long fibrils (Zhou et al., 2018):

$$L_l = \frac{\sum_{i=1}^n N_i L_i^2}{\sum_{i=1}^n N_i L_i} \quad (18)$$

where N_i is the number of fibrils with length L_i . The DP is correlated with the fibril length of nanocelluloses (Shinoda et al., 2012). The DP of nanocelluloses is commonly measured by the viscosity method. Nanocelluloses are dissolved in 0.5 M cupriethylenediamine (CED), and the relative viscosity (η_{rel}) of CED-containing nanocelluloses compared to 0.5 M CED is measured using a Ubbelohde viscometer (a capillary with a diameter of 0.84 mm) at 25 °C (Saito & Isogai, 2004). The product of intrinsic viscosity ($[\eta]$) and nanocellulose concentration (C) can be obtained from a predetermined table in the ISO 5351:2010 standard according to the obtained η_{rel} value (ISO, 2010). DP is calculated according to Eq (19) (Smith, Bampton, & Alexander, 1963):

$$DP = 1.75 \times [\eta] \quad (19)$$

Notably, the remaining aldehyde groups on TEMPO-CNFs should be reduced to hydroxyls before dissolving the material in CED, as if this is not done, the measured DP value will be lower than the actual value (Takaichi et al., 2014). The DP of nanocelluloses can also be determined as the ratio of glucosyl monomer concentration determined by the phenol-sulfuric acid method to the reducing end concentration determined by a modified 2, 2'-bicinchoninate (BCA) method, which works for CNCs but not for TEMPO-CNFs with extra reducing aldehyde groups (Zhang & Lynd, 2005). The fibril length of TEMPO-CNFs can be decreased by treatment with high-intensity ultrasound (Arcari et al., 2019; Zhou et al., 2018).

The width of nanocelluloses can be measured from TEM images (Kalashnikova et al., 2012). Fibril width determined by AFM is not accurate because it depends on the morphology of the AFM tip rather than on the width of the fibril (Arcari et al., 2019). However, the width can also be obtained as the fibril height in AFM images, assuming that the width and height are equal (Arcari et al., 2019; Zhou et al., 2018).

1.6.3 Amphiphilicity of celluloses

Celluloses are generally regarded as hydrophilic materials because of the presence of a rich array of hydroxyl, carboxylate, and sulfate groups on their surfaces. The amphiphilicity of celluloses has only gradually been recognized. An early MDS study showed that both hydrophilic and lipophilic areas exist on the surfaces of cellulose crystals (Biermann et al., 2001). The lipophilicity is attributed to the presence of hydrophobic atoms

such as ether oxygen atoms (O4 and O5) and nonpolar carbon atoms (C1, C4, and C5) (Biermann et al., 2001). Later MDS studies have revealed that celluloses interact with GO and furosemide through hydrophobic interactions (Alqus, Eichhorn, & Bryce, 2015; Lombardo et al., 2018). Lindman and coworkers proposed that hydrophobic interactions among hydrophobic -CH groups play important roles in the (in)solubility of celluloses in different media (Glasser et al., 2012; Lindman, Karlström, & Stigsson, 2010; Lindman et al., 2017; Medronho et al., 2012). One piece of evidence for this is that the association of cellulose chains is inhibited and the dissolution of cellulose is promoted by amphiphilic solutes such as betaine derivatives and PEG (Medronho et al., 2015; Yan & Gao, 2008). Further studies have assigned the hydrophilic surfaces of celluloses to the staggered crystallographic (110) and (1 $\bar{1}$ 0) crystalline planes rich in hydroxyl groups and the hydrophobic surface to the flat face of the crystallographic (200) plane mainly containing CH groups (**Figure 1-8A**) (Bruel et al., 2019; Ding & Himmel, 2006; Yamane et al., 2006).

Nanocelluloses can stabilize emulsions through the Pickering mechanism (**Figure 1-8B**), providing solid evidence for their amphiphilic nature (Jimenez Saelices & Capron, 2018; Kalashnikova, Bizot, Cathala, & Capron, 2011). It has been proposed that nanocelluloses expose hydrophobic (200) planes to the oil phase (**Figure 1-8C**) (Kalashnikova et al., 2012). The ability of CNCs to stabilize emulsions is affected by SCD and by fibril length. Capron and coworkers found that CNCs with a SCD higher than 0.03 e/nm² had no emulsion stabilization ability unless NaCl or KCl was added to screen the

sulfate groups (Kalashnikova et al., 2012). Similarly, TEMPO-CNFs stabilize emulsions more effectively when their carboxylate groups are protonated (Jia et al., 2016). Higher surface coverage of nanocelluloses has been observed with short fibrils, while interconnected networks of low covered droplets have been found when longer nanocelluloses are used (Kalashnikova, Bizot, Bertoncini, Cathala, & Capron, 2013).

1.6.4 Applications of nanocelluloses

Nanocelluloses have no oral toxicity (DeLoid et al., 2019), dermal toxicity (O'Connor, Berry, & Goguen, 2014), pulmonary toxicity (O'Connor et al., 2014), or cytotoxicity (Alexandrescu, Syverud, Gatti, & Chinga-Carrasco, 2013; Souza et al., 2018). Nanocelluloses have a wide variety of applications in the field of biomedicine. Nanocellulose-based hydrogels are good materials for tissue engineering (Rodriguez, Renneckar, & Gatenholm, 2011). Nanocellulose-based hydrogels, aerogels, and emulsions have been widely used as delivery systems to enhance the bioavailability of drugs and other bioactive compounds (Ngwabebhoh, Erdagi, & Yildiz, 2018; Supramaniam, Adnan, Kaus, & Bushra, 2018; Valo et al., 2013). Nanocelluloses can reduce fat adsorption both *in vitro* and *in vivo* (DeLoid et al., 2018). In addition to various applications in biomedicine, nanocelluloses have many potential applications in the food industry (Gomez et al., 2016). Nanocelluloses have been used as emulsifiers in mayonnaise and sausage products because of their amphiphilic nature (Choublab & Winuprasith, 2018; Qi et al., 2020). Nanocelluloses are good stabilizers for ice cream; they can reduce the melting rate of ice

creams (Velásquez-Cock et al., 2019). Nanocelluloses are functional food ingredients as dietary fibers (Gomez et al., 2016). Due to their nontoxicity and biodegradability, nanocelluloses have emerged as novel coating and packaging materials for foods (Azeredo, Rosa, & Mattoso, 2017).

1.7 Cryopreservation

1.7.1 Lethal factors in cryopreservation

For long-term storage, cells, tissues, and organs must be cryopreserved at subzero temperatures, typically at -80°C or in liquid N₂ (Elliott, Wang, & Fuller, 2017; Polge, Smith, & Parkes, 1949). However, prior to the discovery of cryoprotectants, applications of cryopreservation were limited by cryoinjury, which especially occurs during freezing and thawing (P. Mazur, 1965; Mazur, Leibo, & Chu, 1972). Osmotic pressure imbalance and intra/extracellular IR are two major lethal factors associated with cryoinjury (Mazur et al., 1972). During freezing, solutes such as the salts in the culture medium are excluded from the ice crystals, leading to an increase in the solute concentration in the unfrozen phase. This creates an imbalance in the osmotic pressure across cell membranes, leading to dehydration of the cells (Mazur, 1970). Cell dehydration can create high mechanical stress that results in cell membrane leakage and cell death (Wolfe & Bryant, 1999). During freezing, ice crystals form inside cells, and these small ice crystals can grow into large crystals via an intracellular ice recrystallization process during storage and thawing (Mazur et al., 1972; Seki & Mazur, 2011). Intracellular ice recrystallization causes structural

damages to the cytoplasm and cell membranes (Acker & McGann, 2001; Bank, 1973; P Mazur, 1965; Mazur et al., 1972), especially at slow thawing rates that facilitate ice recrystallization (Seki & Mazur, 2008). Extracellular ice recrystallization also causes mechanical damage to cells and organs in cryopreservation (Fujikawa & Miura, 1986; Pegg, 2010; Takamatsu & Zawlodzka, 2006). These two lethal factors indicate that an optimal freezing rate is required for each cell to achieve the highest post-thaw cell viability (Mazur et al., 1972). If cells are frozen more slowly than the optimal rate, they will be killed by long-term exposure to mechanical stress caused by dehydration. If cells are frozen more rapidly than the optimal rate, they will be killed by the formation of intracellular ice and its subsequent recrystallization on thawing. This hypothesis, which is known as the two-factor hypothesis, was proposed by Professor Peter Mazur of the University of Tennessee (Mazur et al., 1972). A fast thawing rate is normally used with most cell types to achieve high viability (Baboo et al., 2019; El-Naggar et al., 2006; Loretz, Li, Flye, & Wilson, 1989; Mazur & Seki, 2011).

1.7.2 Conventional cryoprotectants

The serendipitous discovery of the efficacy of glycerol in the cryopreservation of spermatozoa by Polge and coworkers opened the door to modern cryobiology (Polge et al., 1949). Cryoprotectants are classified as penetrating or nonpenetrating types based on the permeability of cell membranes to these compounds. Penetrating cryoprotectants include glycerol, dimethyl sulfoxide (DMSO), 1,2-propanediol, ethylene glycol, and propylene

glycol (Elliott et al., 2017; Polge et al., 1949), all of which can cross cell membranes and enter cells. DMSO is the most effective of these and is widely used in all types of cells (Elliott et al., 2017). Several mechanisms of action of penetrating cryoprotectants have been suggested. Lovelock proposed that penetrating cryoprotectants act by reducing the concentration of electrolytes in the unfrozen fraction (salt buffering) (Lovelock, 1954). In addition, cells can achieve vitrification in the presence of penetrating cryoprotectants, which reduce the cryoinjuries associated with ice (Elliott et al., 2017). DMSO can interact with cell membranes and facilitate membrane fusion, thereby enabling cells to withstand stress during cryopreservation by reducing the barrier to molecular transport and assisting in pore formation (Notman, Noro, O'Malley, & Anwar, 2006). Although penetrating cryoprotectants are quite effective, some of them are toxic and should be removed after thawing (Best, 2015).

Nonpenetrating cryoprotectants do not cross the cell membrane, and they act outside the cell. Nonpenetrating cryoprotectants mainly include small carbohydrates and (bio)polymers. Sugars are widely used in the cryopreservation of marine organisms and cells (Rodrigues et al., 2008; Tsai, Chong, Meng, & Lin, 2018). Sugars are proposed to interact with the polar head groups of the lipid bilayer via hydrogen bonds, thereby maintaining suitable spacing between the lipids and stabilizing membranes (Leekumjorn & Sum, 2008). In addition, vitrified sugars mechanically hinder the occurrence of conformational changes in dehydrated lipid bilayers, leading to a reduction in lateral

pressure (Koster, Lei, Anderson, Martin, & Bryant, 2000; Wolfe & Bryant, 1999). Typical polymeric cryoprotectants include hydroxyethyl starch (HES), PEG, PVP, and proteins (Elliott et al., 2017; Grondin et al., 2013). Poly(ampholytes) are emerging as novel polymeric cryoprotectants (Bailey et al., 2019; Matsumura & Hyon, 2009; Stubbs, Bailey, Murray, & Gibson, 2020). It has been demonstrated that the balance of negatively and positively charged groups in poly(ampholytes) is crucial to their high protective activity (Bailey et al., 2019). The mechanism through which polymeric cryoprotectants act is not fully understood but has been suggested to involve membrane stabilization by hydrophobic interactions between cell membranes and polymers (Rajan, Hayashi, Nagashima, & Matsumura, 2016) and the facilitation of vitrification (Takahashi, Hirsh, Erbe, & Williams, 1988). Because nonpenetrating cryoprotectants alone cannot provide high post-thaw cell viability, they are normally used together with penetrating co-cryoprotectants (Bailey et al., 2019; Deller, Pessin, Vatish, Mitchell, & Gibson, 2016; Tomas, Bailey, Hasan, & Gibson, 2019).

1.7.3 Applications of IRI-active materials as novel cryoprotectants

IRI-active materials are emerging as novel cryoprotectants due to their ability to inhibit extracellular ice recrystallization. Early work revealed that low concentrations (5-150 µg/mL) of AFPs enhanced the survival of red blood cells cryopreserved in hydroxyethyl starch solutions (Carpenter & Hansen, 1992). However, high concentrations of AFPs (e.g., 1.54 mg/mL) reduced cell viability, an effect that has been ascribed to their DIS activity:

ice crystals grow to needle-like shapes in the presence of AFPs, and the needle-like crystals pierce cell membranes and kill cells (Carpenter & Hansen, 1992). Carbohydrate-based surfactants have been shown to enhance the post-thaw viability of red blood cells cryopreserved in reduced concentrations of glycerol (Briard et al., 2016; Capicciotti et al., 2015). Combined with PEG, DMSO, and proline, IRI-active polymers such as PVA and polyproline can improve the viability of microorganisms, cell monolayers, and cell suspensions subjected to cryopreservation (Deller, Vatish, Mitchell, & Gibson, 2014, 2015; Graham et al., 2017; Hasan, Fayter, & Gibson, 2018). Nanomaterials such as GO, quantum dots, and zirconium-based MOF have been shown to protect sperm and red blood cells during cryopreservation, an effect that has been proposed to be associated with their IRI activity (G. Bai et al., 2017; Geng et al., 2017; Zhu et al., 2019).

More recently, IRI-active materials have been delivered into cells to inhibit intracellular IR and thereby protect cells (Poisson, Acker, Briard, Meyer, & Ben, 2019; Tomas et al., 2019). Glucoside-based small-molecule IR inhibitors have been shown to penetrate human umbilical vein endothelial cells and control the rate and extent of intracellular IR over a broad range of subzero temperatures, and the cryoprotective effect of these inhibitors was observed in red blood cells (Poisson et al., 2019). Type III AFPs have been delivered into A549 cells by cell-penetrating peptides, resulting in the improved cell viability compared to cells cryoprotected by DMSO alone (Tomas et al., 2019). However, type III AFPs are less effective when used in cryopreservation of the same cells

in suspension form, indicating that the cryopreservation protocol is also crucial (Tomas et al., 2019).

1.8 Research hypothesis of the dissertation research

The overall objective of the dissertation is to explore the potential of nanocelluloses as IR inhibitors and cryoprotectants.

The first hypothesis of this dissertation is that nanocelluloses may have IRI activities because of their amphiphilic nature. This hypothesis is based on the association between amphiphilicity and IRI activity in AF(G)Ps and mimics and the amphiphilic nature of nanocelluloses (Capron, Rojas, & Bordes, 2017; Tachibana et al., 2004). To test this hypothesis, the IRI activity of CNFs, CNCs, and TEMPO-CNFs was evaluated by the splat assay, as described in the second chapter. The effects of ionic strength, quenching temperature, and annealing temperature on IRI activity were also evaluated.

The second hypothesis is that the IRI activity of nanocelluloses is affected by the SCD and fibril length. This hypothesis is based on the charge and molecular weight dependence of IRI activity in AF(G)Ps and mimics and also on the influence of SCD and fibril length on the emulsion stabilization activity of nanocelluloses (Congdon et al., 2013; Geng et al., 2017; Gibson et al., 2009; Kalashnikova et al., 2013; Kalashnikova et al., 2012; Tachibana et al., 2004). In chapter three, the SCD of nanocelluloses was modulated by a mild acid hydrolysis without significant alteration of the fibril length. The IRI activity of nanocelluloses with various SCDs was measured. The relationships between IRI and the

surface charge density of nanocelluloses were discussed. In chapter -four, the fibril lengths of TEMPO-CNFs were modulated by a high-intensity ultrasound treatment. The IRI activity of TEMPO-CNFs with various fibril lengths was measured. The relationship between fibril length and the IRI activity of TEMPO-CNFs was discussed. The knowledge obtained from the work in chapters 3 and 4 will be useful in the production of nanocelluloses with enhanced IRI activity and will contribute to a better understanding of IRI mechanisms.

The third hypothesis is that nanocelluloses may have a cryoprotective effect. IR inhibitors have emerged as novel cryoprotectants. Conventional nanocelluloses cannot form stable colloidal dispersions and lose IRI activity at high ionic strengths. In chapter 5, a novel type of nanocellulose, ECNCs, was synthesized to obtain good dispersibility and IRI activity in cell culture media and subsequently tested as a cryoprotectant for HCT-116 colorectal cancer cells. The relationship between the IRI activity of ECNCs and their cryoprotective effects was investigated.

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Appendix

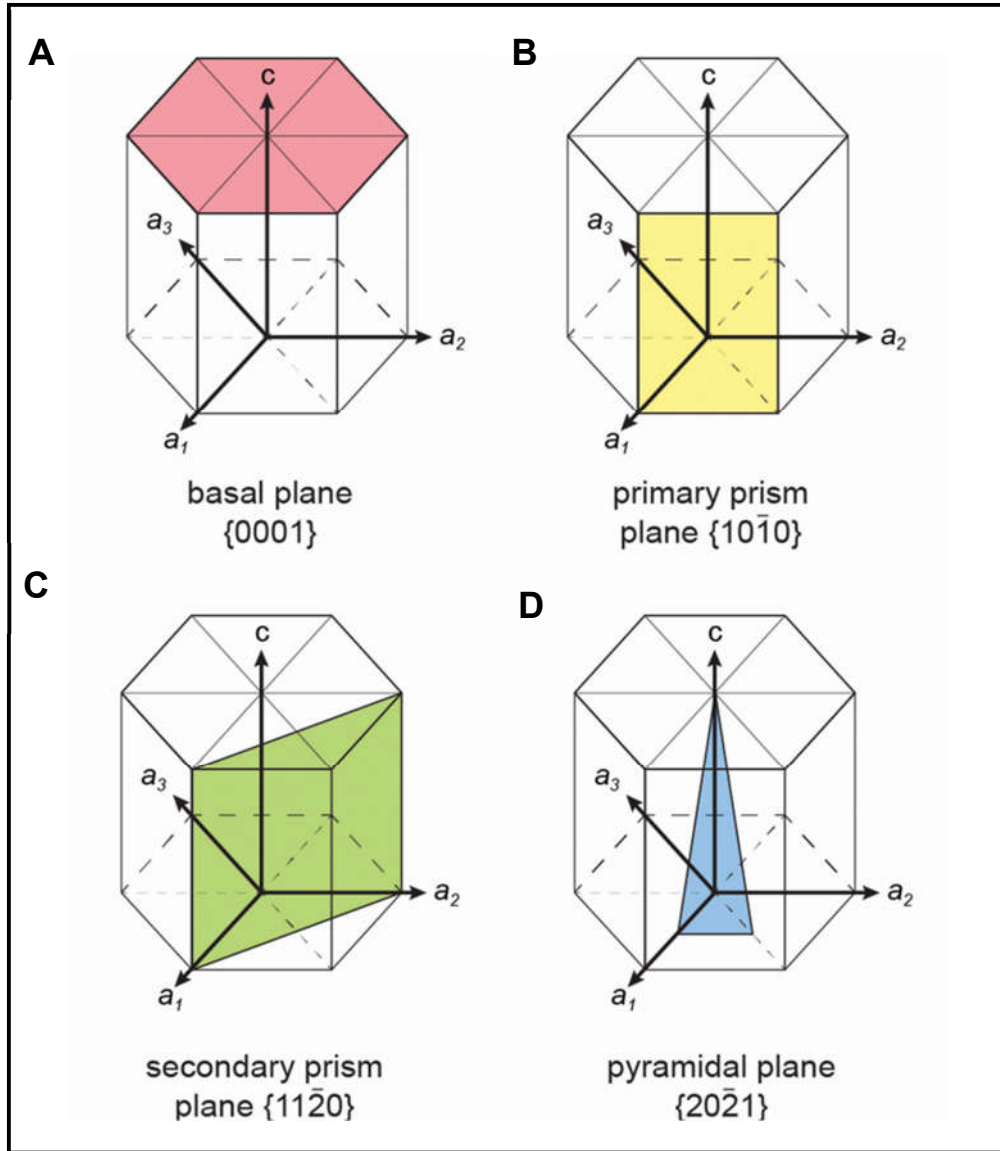


Figure 1-1. (A) The basal plane, (B) primary prism plane, (C) secondary prism plane, and (D) pyramidal plane of hexagonal ice crystals. Adapted from (Vrielink et al., 2016) with permission. Copyright (2016), American Vacuum Society.

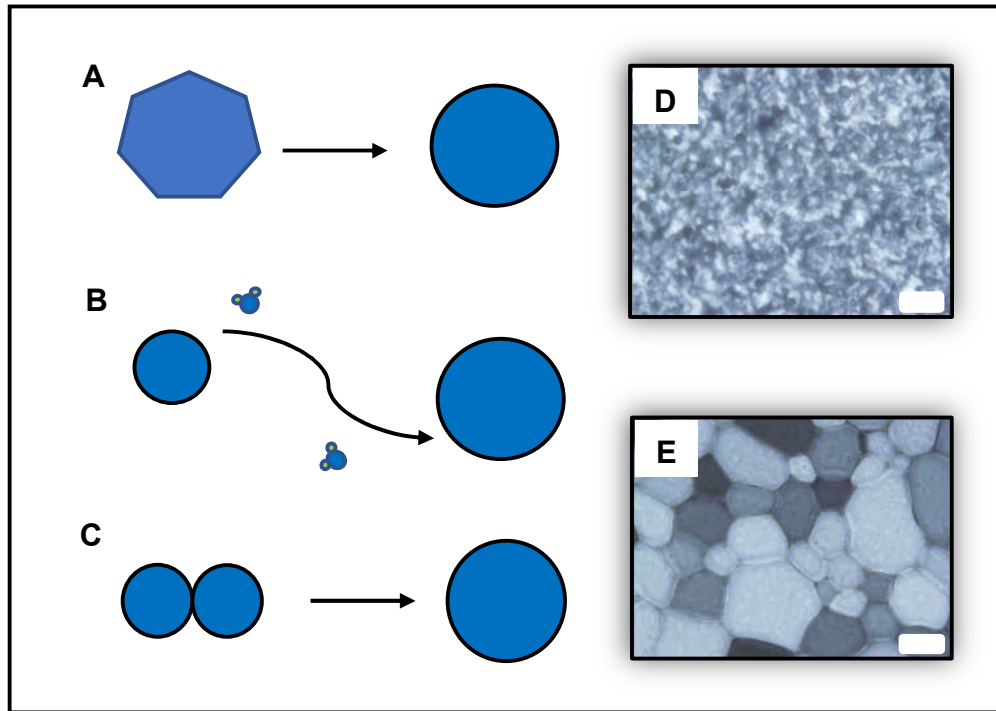


Figure 1-2. (A) Iso-mass recrystallization; (B) migratory/Ostwald ripening recrystallization; and (C) accretion recrystallization. Morphology of ice crystals in PBS before (E) and after (F) ice recrystallization. Scale bar = 50 μm .

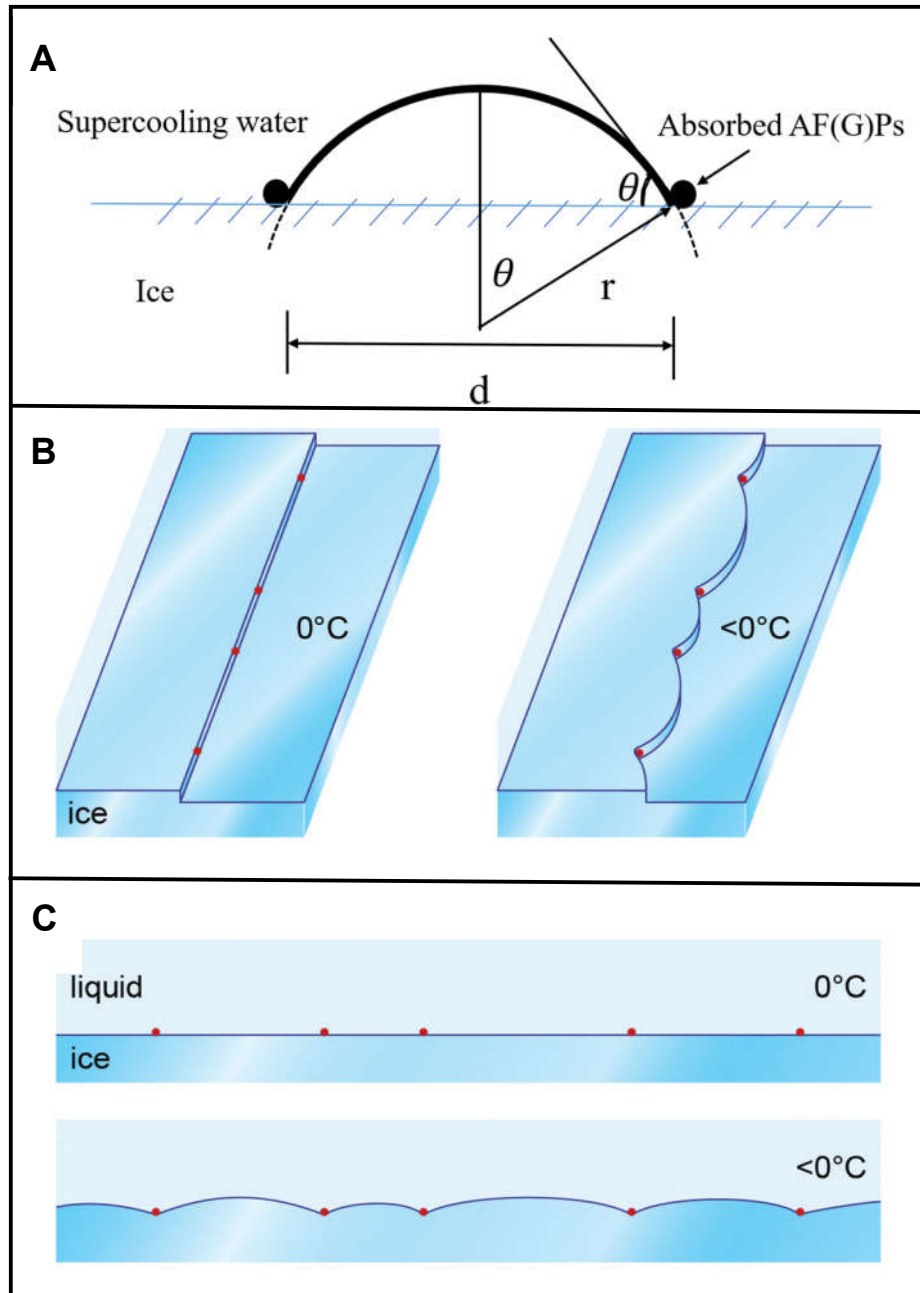


Figure 1-3. Adsorption of AF(G)Ps on ice. (A) Spherical ice “cap” growth in the region between adjacent adsorbed antifreeze proteins. (B) Step-pinning and (C) mattress model for adsorption of AF(G)Ps on ice. Adapted from (Vrieling et al., 2016) with permission. Copyright (2016), American Vacuum Society.

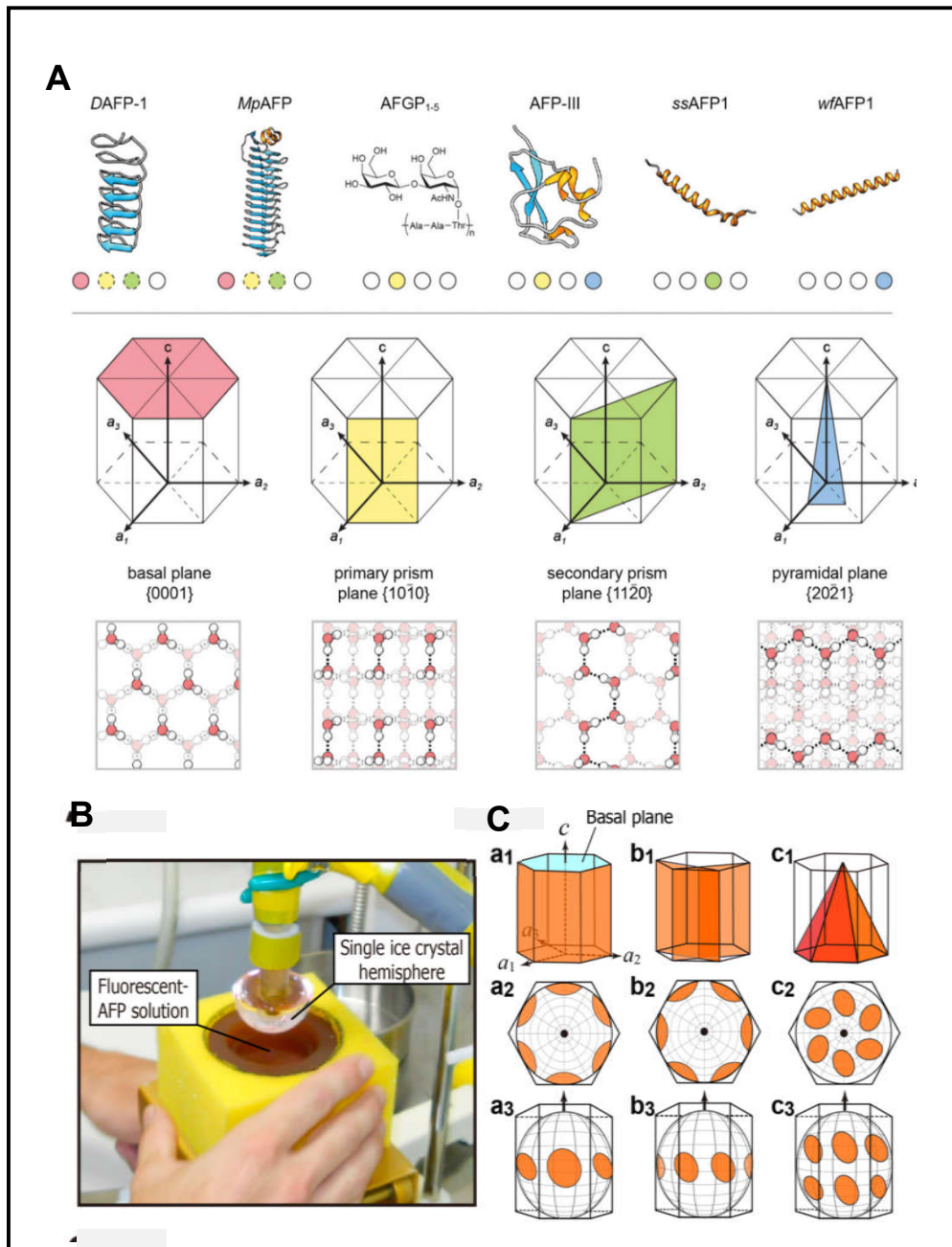


Figure 1-4. (A) Ice-binding planes of different AFPs. (B) The crystal hemisphere formed in the ice-etching method. Adapted from (Olijve et al., 2016) with permission. (C) The binding planes and corresponding etched regions (Rahman et al., 2019).

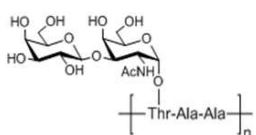
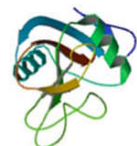
Characteristic	AFGP 1-5	Type I AFP	Type II AFP	Type III AFP	Type IV AFP
Mass (kDa)	10.5-33	3.3 – 4.5	14 – 24	6.5	12
Key Properties	AAT repeat; disaccharide	Alanine-rich α -helix	Disulfide bonded	β -sandwich	Alanine rich; helical bundle
Representative Structure					
Natural Source	Antarctic Notothenioids; northern cods	Right-eyed flounders; sculpins	Sea raven; smelt; herring	Ocean pout; wolfish; eel pout	Longhorn sculpin

Figure 1-5. Classification and structural properties of antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs). Adapted from (Capicciotti et al., 2013).

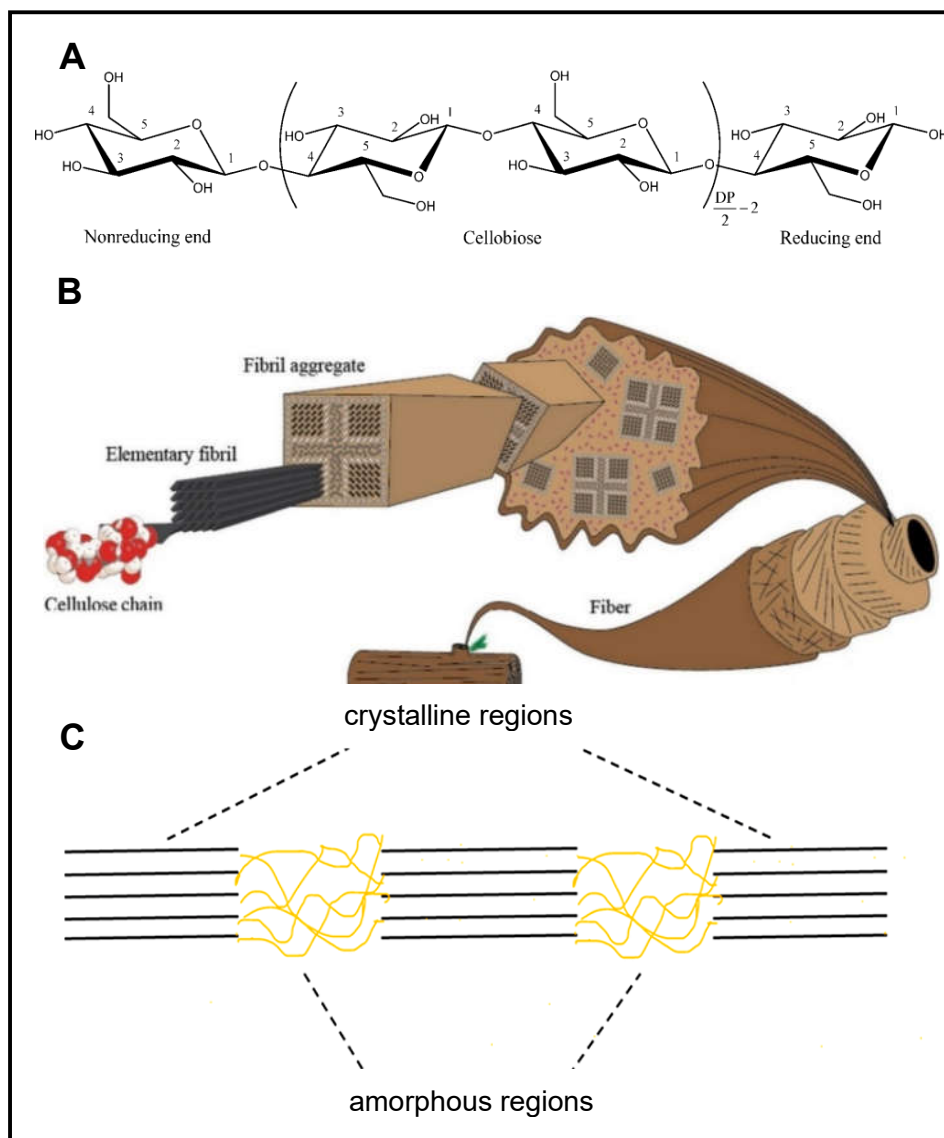


Figure 1-6. Structure of cellulose. (A) Single cellulose chain. Adapted from (Habibi et al., 2010) with permission. Copyright (2010) American Chemical Society. (B) Cellulose chains, elementary fibrils, fibril aggregates, and fibers. Adapted from (Capron et al., 2017) with permission. Copyright (2017), Elsevier. (C) Crystalline and amorphous regions of cellulose. Adapted from (Rajinipriya, Nagalakshmaiah, Robert, & Elkoun, 2018) with permission. Copyright (2018) American Chemical Society.

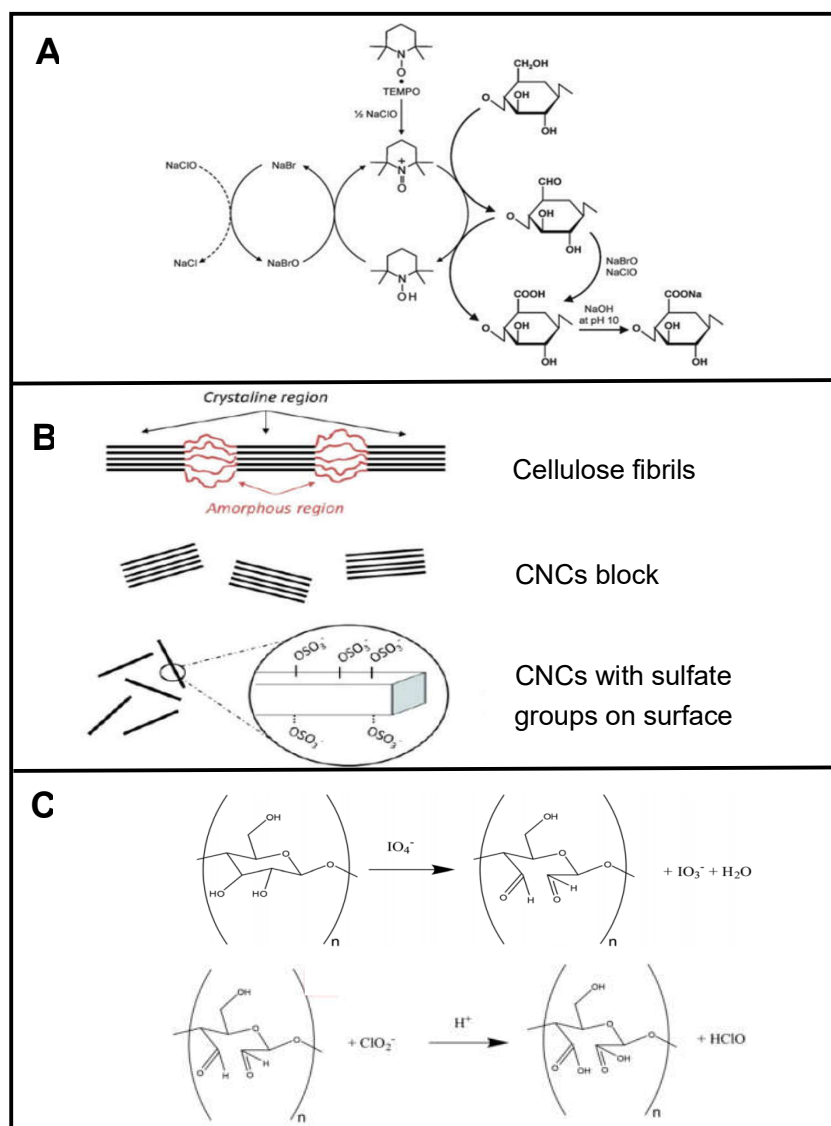


Figure 1-7. Common methods of production of nanocelluloses. (A) TEMPO-mediated oxidation of celluloses. Adapted from (Isogai et al., 2011) with permission. Copyright (2018) Royal Society of Chemistry. (B) Sulfuric acid hydrolysis of cellulose to produce CNCs. Adapted from (Le Gars et al., 2019). (C) Preparation of uncharged/charged hairy CNCs by NaIO_4 and NaClO_2 oxidation. Adapted from (Van de Ven & Sheikhi, 2016) with permission. Copyright (2016), Royal Society of Chemistry

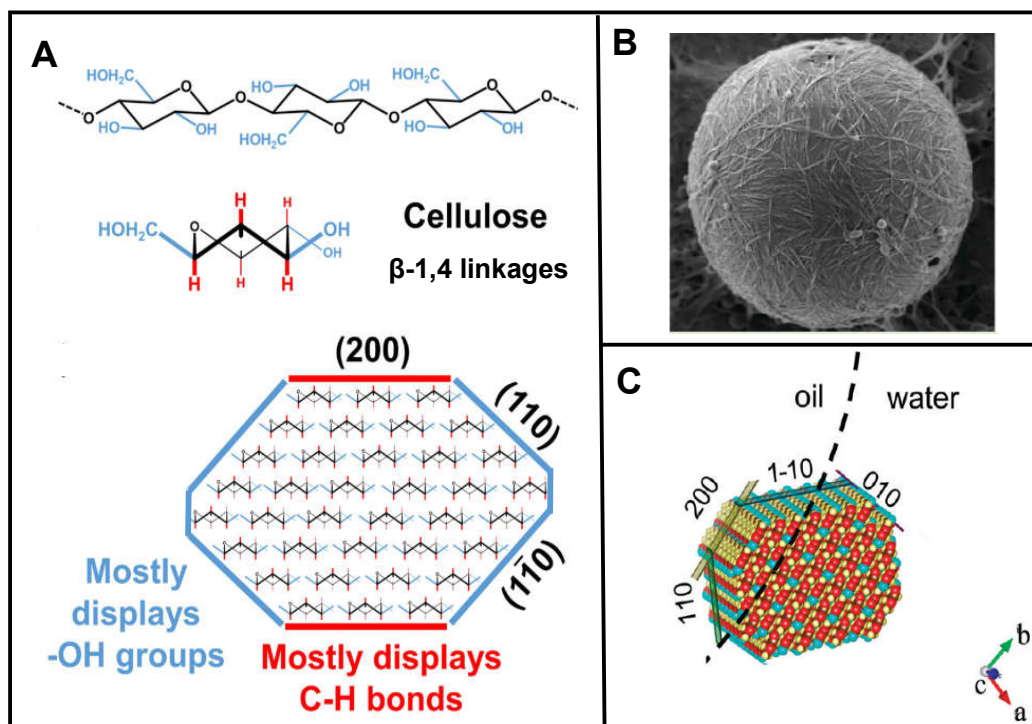


Figure 1-8. Amphiphilicity of nanocelluloses. (A) Crystalline structure of nanocelluloses. Adapted from (Bruehl et al., 2019) with permission. Copyright (2019) Elsevier. (B) Adsorption of CNCs on an oil phase. Adapted from (Kalashnikova et al., 2012) with permission. Copyright (2012) American Chemical Society. (C) Exposure of hydrophobic (200) plane in CNCs to the oil phase. Adapted from (Kalashnikova et al., 2012) with permission. Copyright (2012), American Chemical Society.

Chapter 2. Determination of ice recrystallization inhibition activity of nanocelluloses by the splat assay

A version of this chapter was originally published by Teng Li, Ying Zhao, Qixin Zhong, and Tao Wu:

Li, T, Zhao, Y, Zhong, Q, and Wu, T. (2019). Inhibiting ice recrystallization by nanocelluloses. *Biomacromolecules*, 20(4), 1667-1674.

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Author contribution statement of the published version:

Teng Li: Methodology, Investigation, Validation, Visualization, Writing - Original Draft,

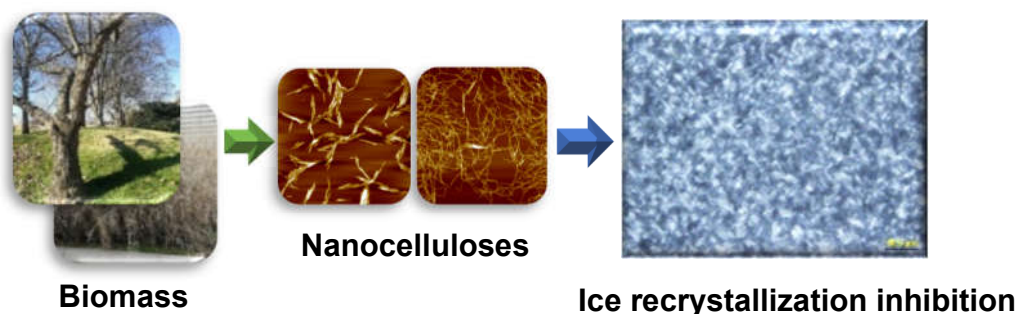
Yin Zhao: Investigation

Qixin Zhong: Resources, Funding acquisition, Writing - Review & Editing, Supervision

Tao Wu: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition

2.1 Abstract

Biocompatible materials with ice recrystallization inhibition (IRI) activity have potential applications in several fields. Emerging studies have associated the IRI activity of antifreeze proteins/glycoproteins and several mimics of synthetic materials with a facially amphipathic structure. Nanocelluloses are a new family of renewable materials that demonstrate amphiphilicity. Herein the IRI activity of cellulose nanocrystals (CNCs) and 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils (TEMPO-CNFs) is reported. In 0.01 M NaCl, ice recrystallization was effectively inhibited by 5.0 mg/mL CNCs or 2.0 mg/mL TEMPO-CNFs. In phosphate-buffered saline, observable IRI activity was found with 30.0 mg/mL CNCs. IRI assays in sucrose solutions showed that the decreased IRI activity of nanocelluloses in saline was caused by the aggregation of nanocelluloses due to charge screening. Neither thermal hysteresis nor dynamic ice shaping activity was observed in nanocelluloses. These findings may lead to the use of nanocelluloses as novel ice recrystallization inhibitors.



Keywords: Nanocelluloses, Ice recrystallization inhibition, Amphiphilicity

2.2 Introduction

Inhibiting ice recrystallization is crucial to the development of high-quality frozen foods (Soukoulis & Fisk, 2016). It was also believed that ice recrystallization inhibition was important to cryopreservation (Balcerzak, Capicciotti, Briard, & Ben, 2014; Biggs et al., 2017). Potential application of ice recrystallization inhibitors as kinetic hydrate inhibitors was also explored in the petroleum industry (Ke & Kelland, 2016). Recently, inhibiting ice recrystallization was utilized in preparing porous materials with controlled pore size (S. Wu et al., 2017). Nature has evolved a variety of antifreeze proteins (AFPs) and glycoproteins (AFGPs) that enable the survival of life in sub-zero environments (Balcerzak et al., 2014; Voets, 2017), and these proteins are featured by their thermal hysteresis (TH), ice recrystallization inhibition (IRI), and dynamic ice shaping (DIS) activities. However, use of AF(G)Ps as ice recrystallization inhibitor is limited by their high production cost. Thus, exploring synthetic materials that mimic the AF(G)Ps activity has attracted wide interest.

A typical structure of AFGPs features a disaccharide moiety attached to a repeated tripeptide. Early studies in this field attempted to elucidate the role of carbohydrates in IRI (Balcerzak et al., 2014). A number of glycopeptides with potent IRI activity but without TH or DIS activity were synthesized (Czechura, Tam, Dimitrijevic, Murphy, & Ben, 2008; Liu & Ben, 2005). A highly hydrated galactose residue is essential for the strong IRI activity of these glycopeptides (Czechura et al., 2008). Further studies proposed that IRI

activity of low-molecular-weight carbohydrates is correlated with the degree of hydration of carbohydrates (Tam, Ferreira, Czechura, Chaytor, & Ben, 2008). Later studies examined the role of hydrophobic groups in the binding of AF(G)Ps on ice. A molecular simulation study demonstrated that AF(G)Ps bind to ice via hydrophobic groups (Mochizuki & Molinero, 2018). Another molecular simulation study showed both polar and nonpolar groups contribute to the binding of AFPs on ice (Midya & Bandyopadhyay, 2018). Inspired by the presence of hydrophobic patches in AF(G)Ps, O-aryl-glycosides bearing a hydrophobic acryl group and carbohydrate-based surfactants with strong IRI activity were synthesized (Balcerzak, Febbraro, & Ben, 2013; Capicciotti et al., 2012; Capicciotti et al., 2016).

Extensive studies have attempted to understand the IRI activity in synthetic polymers (Biggs et al., 2017). Poly(vinyl alcohol) (PVA) is a water-soluble synthetic polymer with strong IRI activity but without appreciable TH activity (Biggs et al., 2017). Inspired by the hydroxyl-rich nature of PVA, the IRI activity of various polyols rich in hydroxyl groups was examined. However, most polyols examined demonstrate only weak IRI activity (Deller et al., 2013). A hydrophobic dye inclusion assay highlights the capability of PVA to possess hydrophobic domain (Mitchell, Lilliman, Spain, & Gibson, 2014). However, random incorporation of hydrophobic groups on PVA polymer chain reduces the IRI activity, indicating that a proper orientation of hydrophobic groups is crucial to its activity (Congdon, Notman, & Gibson, 2013). This occurrence is also shown in polyampholytes,

where hydrophobic domains are essential for the IRI activity, although increasing hydrophobicity alone does not necessarily lead to increased activity (Stubbs, Lipecki, & Gibson, 2017).

Recently, a facial amphiphilicity with segregated hydrophilic and hydrophobic groups was proposed to be the key motif for the TH activity of AF(G)Ps (Tachibana et al., 2004). This facial amphiphilicity is also shared by several other IRI active materials. Nisin, an antimicrobial peptide, exhibits a pH switchable amphiphilicity and IRI activity (Mitchell & Gibson, 2015). Zirconium acetate and zirconium acetate hydroxide demonstrate strong IRI activity by presenting hydrophobic acetate groups on one side of polymer chain and hydroxyl groups on the other (Mizrahy, Bar-Dolev, Guy, & Braslavsky, 2013). Graphene oxide (GO) is found to have potent IRI activity, and one of the most notable properties of GO is its amphiphilicity (Geng et al., 2017; Kim et al., 2010). Safranin O is a synthetic dye that demonstrates comparable IRI activity to that of AFGPs by self-assembling into an amphiphilic fiber (Drori et al., 2016). Amphiphilic metallohelices inhibit the ice growth at just 20 μ M (Mitchell et al., 2017). Recently, glycopolymers with facial amphiphilicity were successfully synthesized to enable strong IRI activity (Graham, Fayter, Houston, Evans, & Gibson, 2018).

Cellulose is the most abundant renewable biopolymer in existence, and natural cellulose is present as highly crystalline nanofibrils separated by amorphous regions (Habibi, Lucia, & Rojas, 2010). These nanofibrils can be isolated by mechanical treatments,

acid hydrolysis, or chemical oxidation to produce nanocelluloses in various forms, such as cellulose nanofibrils (CNFs), cellulose nanocrystals (CNCs), or 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils (TEMPO-CNFs) (Klemm et al., 2011). Nanocellulose can be also produced from bacteria (*Gluconacetobacter*) (Klemm et al., 2011). As a result of their biocompatibility, biodegradability, and low toxicity (Lin & Dufresne, 2014), nanocelluloses are attractive for applications in biomedicine and food (Gomez et al., 2016; Lin & Dufresne, 2014). Historically, cellulose has not been considered as an amphiphilic polymer, but recent studies demonstrated the importance of hydrophobic interactions in its physicochemical properties, suggesting its amphiphilic nature (Lindman et al., 2017; Medronho, Romano, Miguel, Stigsson, & Lindman, 2012). This amphiphilicity resides on the crystal surface of celluloses (Biermann, Hadicke, Koltzenburg, & Muller-Plathe, 2001), and has been experimentally demonstrated by the oil/water interfacial absorption and the excellent emulsion stabilization ability of nanocelluloses (Kalashnikova, Bizot, Cathala, & Capron, 2011, 2012).

Based on the association of facial amphiphilicity with IRI and the amphiphilic structure of nanocelluloses, we hypothesize that nanocelluloses may be IRI active materials. In this paper, the IRI activity of several nanocelluloses was assayed in salt and sucrose solutions by the standard “splat” assay and results are reported here.

2.3 Materials and methods

2.3.1 Materials

CNFs manufactured by the Process Development Center at the University of Maine (Orono, ME), CNCs, TEMPO-CNFs manufactured by the USDA Forest Products Laboratory were purchased from the Process Development Center of the University of Maine. The CNFs in the form of slurry (3.0 wt.%) were made from natural lignocellulosic fibers by grinding with an ultrafine grinder. The CNCs in the form of slurry (10.4 wt.%) were produced by sulfuric acid hydrolysis, followed by dilution, setting, and concentration by membrane filtration. The sulfate content of CNCs was 0.36 mmol of $-\text{OSO}_3\text{Na}$ / g dry mass according to the product label. The TEMPO-CNFs in the form of slurry (1.1 wt.%) were produced by the TEMPO-catalyzed hypochlorite oxidation at pH 10.0. The carboxylate content of TEMPO-CNFs was 1.4 mmol $-\text{COONa}$ / g dry mass according to the manufacturer. Polyethylene glycol (PEG) with number average molecular weight of 6,000 and polyvinyl alcohol (PVA) (88% hydrolyzed) with weight average molecular weight of 88,000 were purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). De-ionized (D.I.) water was used in all experiments unless specified.

2.3.2 Physicochemical characterization of nanocelluloses

2.3.2.1 Cellulose content in nanocellulose

The cellulose contents of CNFs and CNCs were determined as the content of glucose. The glucose content was determined by anthrone-sulfuric acid method with slight modification (Viles & Silverman, 1949). The anthrone-sulfuric acid reagent was prepared by dissolving 0.25 g anthrone into 250 mL 84.5 wt.% sulfuric acid. Around 35 mg lyophilized nanocellulose sample was dispersed in 100 mL water by sonication (VCX- 750, Sonics and Materials., Newton, CT) at 60% pressure amplitude for 90 s. Glucose standard solutions at concentrations from 0 to 412 µg/mL were used to make the calibration curve. A 4.5 mL anthrone- sulfuric acid reagent was added into a 0.5 mL nanocellulose dispersion or glucose standard solution in a 15 mL centrifuge tube, mixed and chilled in an ice bath. The mixtures were then incubated at 95°C for 10 min and cooled to room temperature in an ice bath before recording the absorbance at 620 nm with an Evolution 201 UV-visible spectrophotometer (Thermo Fisher Scientific., Waltham, MA). The cellulose contents of CNFs and CNCs were calculated using Eq 1:

$$\text{Cellulose content} = \frac{m_{\text{glucose}}}{m_{\text{CNF or CNC}}} \times 100\% \quad (1)$$

where m_{glucose} and $m_{\text{CNF or CNC}}$ are the mass of glucose determined by anthrone-sulfuric acid method and the mass of CNFs or CNCs, respectively.

The cellulose content of TEMPO-CNFs was determined as the content of glucose and glucuronic acid (oxidation product of glucose) (Kintner & Vanburen, 1982). The

glucuronic acid content in TEMPO-CNFs was determined by the meta-hydroxydiphenyl method. Reagent A was prepared by dissolving 0.478 g sodium tetraborate in 100 mL sulfuric acid (98 wt.%). Reagent B was prepared by dissolving 0.150 g meta-hydroxydiphenyl into 100 mL 5.0 mg/mL NaOH solution. Around 20 mg lyophilized nanocellulose sample was dispersed in 100 mL water by sonication at 60% pressure amplitude for 90 s. Glucuronic acid standard solutions at concentrations from 0 to 80 µg/mL were used to make the calibration curve. A 6.0 mL reagent A was added into 1.0 mL nanocellulose dispersion or glucuronic acid standard solution in a 15.0 mL centrifuge tube, mixed, and incubated in a boiling water bath for 5 min, and immediately cooled in an ice bath to room temperature. Then 100 µL reagent B was added, mixed, and incubated at room temperature for 20 min. The absorbance of the mixture was measured at 520 nm. The glucose content was determined by the anthrone-sulfuric acid assay described above. Preliminary results showed that the determination of glucuronic acid content by the meta-hydroxydiphenyl method was not interfered with the glucose from TEMPO-CNFs, and the determination of glucose by the anthrone-sulfuric acid method was not interfered with the glucuronic acid from TEMPO-CNFs. The cellulose content of TEMPO-CNFs was calculated using Eq 2:

$$Cellulose\ content = \frac{m_1 + m_2}{m_{TEMPO-CNF}} \times 100\% \quad (2)$$

where m_1 is the mass of glucose determined by the anthrone-sulfuric acid method and m_2 is the mass of glucuronic acid content determined by the meta-hydroxydiphenyl

method.

2.3.2.2 Determination of crystallinity index (CI) by X- Ray Diffraction (XRD)

The CI of lyophilized nanocelluloses was determined with a Panalytical Empyrean X-ray diffractometer with a Ni-filtered Cu K α radiation at 40 kV and 40 mA (Malvern Panalytical Ltd., Malvern, UK). Scans were conducted with scattering angle (2 θ) from 4 to 60° for each scan at a scan rate of 6.7°/min by step of 0.025°. The XRD spectra of three nanocelluloses are shown in **Figure 2-1**. The CI (%) was calculated according to the method of Segal et al. (L. Segal, 1959):

$$CI (\%) = \frac{I_{max} - I_{am}}{I_{max}} \times 100\% \quad (3)$$

where I_{max} is the maximum intensity (in arbitrary units) of the principle peak at 2 θ between 22° and 25° and I_{am} is the intensity of the amorphous peak at 2 θ = 18°.

2.3.2.3 Determination of Z-average hydrodynamic diameter and ζ -potential of nanocellulose

Nanocelluloses were dispersed in 0.01-0.05 M NaCl to a concentration of 0.5 mg/mL. The pH of all the samples was adjusted to 6.0. The Z-average hydrodynamic diameter was determined with a Malvern dynamic light scattering system (Nano ZS, Malvern Instruments., UK) using a scattering angle of 173°, refractive index of 1.470, and adsorption of 0.001. An autocorrelator was used to analyze the time-dependent fluctuations of scattered intensity to obtain the autocorrelation function of the signal. The intensity weighted diffusion coefficient (C) that describes the speed of Brownian motion of a particle

was obtained from the autocorrelation function. The intensity weighted hydrodynamic diameter (D_h) was calculated according to the Stokes-Einstein equation (Edward, 1970):

$$D_h = \frac{K_B T}{3\pi\eta C} \quad (4)$$

where K_B is the Boltzmann constant, T is the temperature in Kelvin and η is the viscosity of water (0.8872 cP at 25°C). The Z-average hydrodynamic diameter was calculated as the harmonic mean of D_h . ζ - potential was obtained by measuring the electrophoretic mobility (U_E) of charged particles according to the Henry equation (Henry, 1931):

$$\zeta = \frac{3\eta U_E}{2\varepsilon F(ka)} \quad (5)$$

where ε is the dielectric constant of water (78.36 F/m at 25°C), η is the viscosity of water (0.8872 cP at 25°C). Smoluchowski model is used in calculation with an $F(ka)$ value of 1.5. All measurements were conducted at $25 \pm 0.1^\circ\text{C}$ with equilibrium time of 3 min before each measurement.

2.3.2.4 Determination of carboxylate group and sulfate group content by conductivity titration

Nanocelluloses were dispersed in water at a concentration of 3.0 mg/mL and dialyzed against excessive water for 3 days in dialysis tubing with a molecular weight cut-off of 6,000-8,000 Da (Thermo Fisher Scientific, Waltham, MA). To fully protonate the carboxyl groups on the surface of CNFs and TEMPO-CNFs, 1.0 N HCl was added into the dispersions of CNFs and TEMPO-CNFs to adjust the pH to 2.5 and stirred for 10 min (Saito & Isogai, 2004). Next, protonated CNFs and TEMPO-CNFs dispersions (0.3 g CNFs or

TEMPO-CNFs) were titrated with 0.03 M NaOH solution. The conductivity during titration was monitored by an YSI 3200 conductivity meter with a 3256 cell (YSI Inc, Yellow Springs, OH). The carboxylate content of the sample was determined from the conductometric titration curves, as shown in **Figure 2-2A**. The carboxylate content (mmol/g) was calculated according to the Eq (6):

$$\text{Carboxylate content (mmol/g)} = \frac{0.03 \times (v_2 - v_1)}{m} \quad (6)$$

where V_1 and V_2 are the NaOH volume used at the first and second equivalent point, respectively, and m is the mass of CNFs or TEMPO-CNFs (0.3 g). The CNCs dispersion after dialysis was protonated by the Dowex Marathon C hydrogen form strong acid cation exchange resin (Dow Chemical, Midland, MI) using a batch method (Beck, Méthot, & Bouchard, 2014). The resin was repeatedly washed with water until a constant pH was reached and dried by a filter cloth. Resin (12 g resin / g CNCs) was added into 3.0 mg/mL CNCs dispersion and stirred on a magnetic stirrer for 180 min. The resin was changed every 30 min by filtration. Then 100 mL protonated CNCs dispersion (total 0.3 g CNC in dispersion) was titrated with 0.01 M NaOH solution. The conductivity was monitored by the same conductivity meter described above. The sulfate group content of the sample was calculated from the conductometric titration curve, as shown in **Figure 2-2B**. The sulfate group content (mmol/g) was calculated according to the following Eq (7):

$$\text{Sulfate group content (mmol/g)} = \frac{0.01 \times V_1}{m_{\text{CNC}}} \quad (7)$$

where V_1 is the NaOH volume at the first equivalent point and m_{CNC} is the mass of CNCs.

2.3.2.5 Determination of degree of polymerization (DP) by intrinsic viscosity

Prior to the DP measurement, the C6- aldehydes and C2/C3 ketones in TEMPO-CNFs were reduced to alcoholic hydroxyls by a literature method (Shinoda, Saito, Okita, & Isogai, 2012). A 0.5 g TEMPO-CNFs was dispersed in 95 mL water and reacted with 0.5 g NaBH₄ at room temperature and a constant pH of 9.0, which was monitored continuously and adjusted by adding 0.5 M NaHCO₃. After 48 h reaction, the TEMPO-CNFs were washed with water and lyophilized. For DP determination, a 40 mg nanocellulose sample was dissolved in 20 mL 0.5 M cupriethylenediamine hydroxide (CED) (Shinoda et al., 2012). The relative viscosities (η_{rel}) of nanocellulose CED solutions were measured using an AVS 370 Ubbelohde viscometer (0.84 mm capillary tube) in a $25 \pm 0.1^\circ\text{C}$ water bath (SI Analytics, Weilheim, Germany). The η_{rel} was calculated as $\eta_{rel} = t_s/t_0$, where t_s and t_0 are efflux time of nanocellulose CED solutions and 0.5 M CED. The product of intrinsic viscosity and nanocellulose concentration ($[\eta] \times C$) was taken from a predetermined table in the ISO 5351:2010 standard according to the obtained η_{rel} value (ISO, 2010). DP was calculated according to the Mark-Houwink-Sakurada equation (Smith, Bampton, & Alexander, 1963):

$$DP = 1.75 \times [\eta] \quad (8)$$

2.3.3 Morphological analysis of nanocelluloses

Morphological analysis of nanocelluloses was conducted using atomic force microscopy (AFM). Nanocelluloses were dispersed in 0.01 M NaCl solution (pH 6.0) at a

concentration of 1.0 mg/mL. A freshly peeled mica sheet was taped on an AFM sample mounting disk and rinsed with 1.0 mL water at 2000 rpm for 40 s in a P6700 spin coater (Specialty Coating Systems Inc, Indianapolis, IN), followed by coating the mica surface with 30 μ L of the nanocellulose dispersions at 4000 rpm for 60 s. The samples were dried at room temperature for 2 h before collecting AFM (model Multimode VIII, Bruker Corp, Santa Barbara, CA) images using a ScanAsyst-AIR probe in the ScanAsyst-Air mode. The scanning area was 5.0 μ m $\times$ 5.0 μ m and the scanning rate was 1.0 Hz. All AFM images were flattened by the Nanoscope Analysis software (Bruker Corp, Santa Barbara, CA). Individual fibrils without aggregation were used to estimate the heights of nanocelluloses (Figure 2-3).

2.3.4 Ice recrystallization inhibition assay

IRI activity of nanocelluloses was determined using a standard “splat” assay (Knight, Hallett, & Devries, 1988). PEG and PVA was used as a negative control and a positive control, respectively. Nanocelluloses, PEG and PVA were dispersed in 0.01 M NaCl (pH 6.0), PBS (pH 7.4) and sucrose solutions (pH 6.0) at different concentrations. Microscope glass slides (7.5 cm $\times$ 2.5 cm $\times$ 1.0 mm, Thermo Fisher Scientific, Waltham, MA) were placed on the surface of a metal block (9.5 cm $\times$ 7.5 cm $\times$ 7.5 cm, Reacti-Therm, Thermo Fisher Scientific, Waltham, MA) and pre-chilled with dry ice kept in a foam box for at least 30 min. Then, around 10 μ L of sample was dropped from a 1.40 m height above a pre-chilled microscope glass slide using a 3 mL syringe (Becton & Dickinson, Franklin Lakes,

NJ). Upon hitting the chilled microscope glass slide, an ice wafer with a diameter of around 10 mm and a thickness of around 10 μm was formed instantaneously. The microscope glass slide was immediately transferred to another metal block with temperature controlled at -8°C by a 50:50 (v/v) water-glycerol bath (Isotemp 3016D, Thermo Fisher Scientific, Waltham, MA). The sample was covered with a plastic lid of 16 mL culture tubes (VWR International, Radnor, PA), sealed by silicone oil to avoid the condensation of moisture on the surface of ice wafers, and annealed for 30 min. After annealing, the microscope glass slide was immediately loaded into an HCS 302 cryostage (Instec Instruments, Boulder, CO) with temperature set at -8.0°C under the purge of N_2 . Optical images were obtained by a polarized light microscope (BX51, Olympus, Tokyo, Japan) with a built-in digital camera (DP 70, Olympus, Tokyo, Japan) under a $400\times$ magnification. The grain areas of the ten largest ice crystals in the view field were measured using the ImageJ software (National Institutes of Health, Bethesda, MD). For each sample, 5 images from at least 2 independent wafers (total 50 ice crystals) were used to calculate the mean grain area (MGA). The percentage mean grain area (% MGA) was obtained by dividing the MGA of a sample to that of background medium at same assay condition.

2.3.5 Ice growth kinetics during annealing

CNCs and TEMPO-CNFs dispersions in 0.01 M NaCl (pH 6.0) were prepared at concentrations of 1.0-5.0 mg/mL and 0.5-2.0 mg/mL, respectively. CNCs dispersions in PBS (pH 7.4) were also prepared at final concentrations of 10.0-50.0 mg/mL. A 0.01 M

NaCl or PBS was used as a blank control. After obtaining an ice wafer of each sample, the microscope glass slides were transferred to the cryostage and incubated at -8°C under the purge of N₂. Images were collected every 5 min from 0 to 30 min. The MGA of sample was compared to that of 0.01 M NaCl or PBS annealed at the -8°C cryostage for 30 min to obtain % MGA.

2.3.6 Effects of quenching and annealing temperature on IRI activity

CNCs and TEMPO-CNFs dispersions in 0.01 M NaCl (pH 6.0) were prepared at concentrations of 5.0 mg/mL and 2.0 mg/mL, respectively. CNCs dispersion in PBS (pH 7.4) was also prepared at a concentration of 50.0 mg/mL. For quenching temperature, the sample was dropped onto a microscope glass slide pre-chilled within the cryostage with cover removed and temperatures set at -80, -70, -60, and -50°C. The MGA of sample was compared to that of 0.01 M NaCl or PBS quenched at the same temperature to obtain % MGA. For annealing temperature, after obtaining the ice wafer, the microscope glass slide was loaded into the cryostage with the temperature set at -18, -10, -8, -6, -4, and -2°C and annealed for 30 min under the purge of N₂. The MGA of samples was compared to that of 0.01 M NaCl or PBS annealed at the same annealing temperature to obtain % MGA.

2.3.7 Thermal hysteresis and ice morphology analysis

The analysis of ice morphology and thermal hysteresis of CNCs and TEMPO-CNFs was conducted using a literature method after modification (Drori et al., 2016). A droplet of immersion oil was placed on a concave microscope glass slide, followed by injection of

0.1 μL sample into the immersion oil using a 701NPT5 10 μL syringe (Hamilton, Reno, Nevada), and covered with a coverslip. The glass slide was then loaded into the cryostage and ice nucleation was initiated at -40°C . After ice nucleation, the temperature of the cryostage was increased at a rate of $5^{\circ}\text{C}/\text{min}$ to -2°C , and further increased at a rate of $0.05^{\circ}\text{C}/\text{min}$ to melt most ice until a single ice crystal (with a diameter of around $50\text{ }\mu\text{m}$) was obtained. A 3 mL syringe (Becton & Dickinson, Franklin Lakes, NJ) was used to introduce air to the surface of the coverslip to accelerate the melting of unwanted ice without causing significant temperature fluctuation within the cryostage. After obtaining a single ice crystal, the temperature was decreased at a rate of $0.05^{\circ}\text{C}/\text{min}$ to a certain temperature until the size of ice crystals remained unchanged (usually $20\text{ }\mu\text{m}$). This temperature was recorded as T_0 . The ice crystal was incubated at T_0 for 30 s. Further decrease of temperature at a rate of $0.05^{\circ}\text{C}/\text{min}$ was applied until a burst growth of ice crystals was observed at another temperature, recorded as T_1 . Thermal hysteresis was calculated as $T_0 - T_1$. The same experimental design was also used to analyze the ice morphology. Further decrease of temperature from T_0 caused the growth of the single crystal, and the ice morphology was analyzed before and after growth.

2.4 Calculation of unfrozen water fraction in NaCl and sucrose solutions

When annealed at temperature above eutectic points, salt and sucrose exist in unfrozen water phase. The unfrozen water fraction can be calculated using the following Eq (Pongsawatmanit & Miyawaki, 1993):

$$\frac{C_{solute}}{C_{solute}+C_{unfrozen\ water}} = C_{solute\ in\ unfrozen\ water} \quad (9)$$

Where C_{solute} is the mass concentration of solute, $C_{unfrozen\ water}$ is the mass concentration of unfrozen water and $C_{solute\ in\ unfrozen\ water}$ is the mass concentration of solute in unfrozen water phase. $C_{solute\ in\ unfrozen\ water}$ only depends on the annealing temperature and can be obtained from phase diagram data in literatures. For example, $C_{solute\ in\ unfrozen\ water}$ of sucrose solution at -8°C is 52.0 wt.%(Pongsawatmanit & Miyawaki, 1993). $C_{solute\ in\ unfrozen\ water}$ of NaCl solution at -18, -10, -8, -6, -4 and -2°C are 21.5, 14.4, 12.0, 9.3, 6.8 and 3.9 wt.%, respectively (Farnam, Bentz, Sakulich, Flynn, & Weiss, 2014). For 0.21 M sucrose, C_{solute} is 7.19 wt.% and $C_{solute\ in\ unfrozen\ water}$ is 52% at -8°C, $C_{unfrozen\ water}$ can be calculated as below:

$$\frac{0.0719}{0.0719+C_{unfrozen\ water}} = 0.52 \quad (10)$$

$C_{unfrozen\ water}$ of 0.21 M sucrose is calculated to be 0.066 (6.6%) at -8°C. For PBS, all the salts are considered as NaCl to simplify calculation, and C_{solute} is 0.9 wt.%. $C_{solute\ in\ unfrozen\ water}$ of NaCl is 12.0 wt.% at -8°C. $C_{unfrozen\ water}$ can be calculated as below:

$$\frac{0.009}{0.009+C_{unfrozen\ water}} = 0.12 \quad (11)$$

$C_{unfrozen\ water}$ of PBS is calculated to be 0.066 (6.6%) at -8°C.

2.5 Results and discussion

2.5.1 Physicochemical and morphological properties of nanocelluloses

The morphology of nanocelluloses imaged by AFM is shown in **Figure 2-3** and **Figure 2-4**. CNFs were long fibrils with severe aggregation. CNCs were rodlike crystals with lateral aggregation. TEMPO-CNFs were fibrils with heights of 2.5-4.0 nm, and lengths of up to several hundred nanometers. The cellulose content, crystallinity index and other physicochemical properties of nanocelluloses are presented in **Table 2-1**. CNFs had a low ζ -potential and a low carboxylate content, which explained the significant aggregation of CNFs. Both CNCs and TEMPO-CNFs had high ζ -potential values, but the carboxylate content in TEMPO-CNFs was higher than the sulfate content in CNCs. This difference might explain the little aggregation in TEMPO-CNFs, but lateral aggregation in CNCs.

2.5.2 IRI activity of nanocelluloses in 0.01 M NaCl and PBS

The representative polarized light microscopical images of ice wafers of blank controls (0.01 M NaCl and PBS), negative control (PEG) and nanocelluloses are shown in **Figure 2-5**. Larger ice crystals were observed in blank controls and negative control, and smaller ice crystals were observed in nanocelluloses, which indicated that nanocelluloses tested were IRI active. In 0.01 M NaCl, where a false-positive result can be effectively excluded (Balcerzak et al., 2013; Knight, Wen, & Laursen, 1995), CNFs demonstrated a weak IRI activity at 5.0 mg/mL; CNCs and TEMPO-CNFs demonstrated observable IRI activity at

5.0 mg/mL and 2.0 mg/mL, respectively. In PBS, observable IRI activity was found with 30.0 mg/mL CNCs. The IRI activity of CNFs and TEMPO-CNFs at higher concentrations in PBS was not determined with the “splat” assay, due to the high viscosity of samples. The limited IRI activity of CNFs might be caused by their aggregation, which was evidenced by their larger Z-average hydrodynamic diameter (**Table 2-1**). This aggregation decreased the number of nanocelluloses available to inhibit ice recrystallization. By the same mechanism, CNCs showed weaker IRI activity than TEMPO-CNFs because of the lateral aggregation of CNCs.

The IRI activity of nanocelluloses is more clearly seen in **Fig 2-6**, where the % MGA of nanocelluloses and PEG are plotted against the polymer concentrations. A smaller value of % MGA indicates stronger IRI activity. Nanocelluloses were more effective than PEG at all concentration levels in both 0.01 M NaCl and PBS, especially in 0.01 M NaCl (**Fig 2-6A**). Due to the high viscosity of CNFs and TEMPO-CNFs samples in PBS, only CNCs were determined for IRI activity in PBS. A 10-folds increase in CNCs concentration was necessary to achieve observable ice recrystallization inhibition in PBS. As it was discussed later, this decrease of IRI activity was mainly caused by the aggregation of CNCs at high ionic strength due to charge screening. As shown in **Figure 2-6B**, nanocelluloses were less active than PVA on a mass basis. One possible reason is that nanocelluloses are assembled by tens to hundreds of cellulose molecules (Habibi et al., 2010), and they have higher molecular mass than PVA. This is the reason that PVA is less than AF(G)Ps on a mass

basis (Biggs et al., 2017). While nanocelluloses were 10-folds less active than PVA in 0.01 M NaCl, they were 100-fold less active than PVA in PBS. If the first 10-folds was caused by the higher molecular mass of nanocelluloses than PVA, another 10-folds decrease of IRI activity in PBS might be caused by aggregation of nanocelluloses in high ionic strength condition. Nevertheless, compared to PVA, the IRI activities of CNCs and TEMPO-CNFs were low. The IRI activity of TEMPO-CNFs in 0.01 M NaCl was highly sensitive to the change of nanocellulose concentration: from slightly active at 1.0 mg/mL to highly active at 2.0 mg/mL. Similar property was also found with high-molecular-weight PVA (Gibson, Barker, Spain, Albertin, & Cameron, 2009). The IRI activity of CNCs in 0.01 M NaCl was also sensitive to the change of concentration, but not as sensitive as TEMPO-CNFs.

2.5.3 Effects of ionic strength on IRI activity of nanocelluloses

With the increase of ionic strength from 0.01 M NaCl to PBS, the electrostatic repulsive interactions between nanocelluloses were screened, which led to the aggregation of nanocelluloses and decreased the number of nanocelluloses available to inhibit ice recrystallization. Meanwhile, the fraction of unfrozen water was increased, which decreased the concentration of nanocelluloses in solutions. Both factors can lead to the decreased IRI activity. To identify which factor was mainly responsible for the decreased IRI activity at high ionic strength condition, a series of “splat” assays were conducted in 0.01-0.05 M NaCl and 0.0135-0.0675 M sucrose solutions. These NaCl and sucrose solutions were formulated to have similar unfrozen water fractions at corresponding

concentrations. As it is shown for in **Figure 2-7A**, increase of salt concentration led to increase of sample turbidity and particle size of CNCs. Consequently, a decrease of IRI activity was observed (**Figure 2-7B**). When the splat assays were conducted in sucrose solutions, the sample turbidity and particle size remained unchanged with the increase of sucrose concentration. Consequently, the IRI activity of CNCs remained unchanged despite the increase of unfrozen water fraction. It indicated that the decrease of IRI activity of CNCs in high ionic strength was mainly caused by the fibril aggregation. Future work can be conducted to enhance the salt tolerance of nanocelluloses by surface modification and reduce their concentration necessary in applications at high ionic strength conditions (Araki, Wada, & Kuga, 2001; Azzam, Heux, Putaux, & Jean, 2010). Nevertheless, nanocelluloses may be directly used as ice recrystallization inhibitors in low ionic strength conditions, such as in frozen desserts and preparing porous materials (Soukoulis & Fisk, 2016; S. Wu et al., 2017).

2.5.4 IRI activity of nanocelluloses in 0.21 M sucrose

To compare the IRI activity of nanocelluloses with other materials without aggregation of fibrils, IRI assays of nanocelluloses were conducted in 0.21 M sucrose solution, which has a similar unfrozen water fraction of 6.6% to that of PBS at -8°C (Pongsawatmanit & Miyawaki, 1993). Both CNCs and TEMPO-CNFs demonstrated stronger IRI activity than PEG at the same mass concentration (**Figure 2-8**). The % MGA of CNCs was 31.4 % and 15.7% at 5.0 and 10.0 mg/mL, respectively. The % MGA of TEMPO-CNFs was 52.3%

and 34.5% at 5.0 and 10.0 mg/mL, respectively. It was obvious that CNCs and TEMPO-CNFs were more IRI active than most polyols (Gibson et al., 2009; Laezza et al., 2017). Compared to other non-AF(G)Ps IRI active materials reported (**Table 2-2**), CNCs and TEMPO-CNFs demonstrated weaker IRI activity than GO and safranin O on a mass basis (Drori et al., 2016; Geng et al., 2017), but comparable or superior to nylon-3 polymers, polyproline, oxidized quasi-carbon nitride quantum dots, nisin, lectins concanavalin A (Bai et al., 2017; Graham et al., 2017; MacDonald, Cornejo, & Gellman, 2017; Mitchell & Gibson, 2015). Without fibrils aggregation, nanocelluloses demonstrated observable IRI activity.

2.5.5 Ice growth kinetics

The size of ice crystals is affected by both nucleation and recrystallization processes (S Wu et al., 2017). To differentiate the influences of these processes, polarized light microscopy images of polycrystalline ice crystals immediately after quenching were compared. As shown in **Figure 2-9**, the size of ice crystals did not vary appreciably between blank controls and samples, and among the three types of nanocelluloses. Monitoring the recrystallization of ice crystals during the first 30 min offered more insights on the effect of nucleation (**Fig 2-10**). In blank controls of 0.01 M NaCl and PBS, ice crystals grew continuously during the first 30 min, whereas, in the presence of nanocelluloses, the growth of ice crystals leveled off within 30 min. When CNCs concentrations were higher than 20.0 mg/mL, the initial sizes of ice crystals were larger

than those in PBS control, which indicated that the IRI activity of CNCs was underestimated by calculating % MGA relative to that of PBS because the ice crystals were not grown at CNCs concentrations higher than 30.0 mg/mL.

2.5.6 Effects of quenching and annealing temperatures on IRI activity

Monitoring the effects of quenching and annealing temperatures on the growth of ice crystals can also be used to differentiate the role of nucleation and ice recrystallization (S Wu et al., 2017). Quenching temperature had little effect on the size of ice crystals (**Figure 2-11 A, B**), which confirmed that smaller ice crystals observed in the presence of nanocelluloses was resulted from the IRI. The IRI activity of CNCs and TEMPO-CNFs did not change significantly from annealing temperature of -18 to -6°C but decreased appreciably at -4°C and -2°C (**Figure 2-11 C, D**). After calculation, it was known that the unfrozen water fraction was doubled at -4°C and tripled at -2°C (**Figure 2-12**), but these increases were not the main reason of decreased IRI activity because nanocelluloses still demonstrated IRI activity in sucrose solutions with similar or even higher unfrozen water content at -8°C (**Figure 2-7B**). Therefore, the decreased IRI activity at higher annealing temperatures might be caused by the increase in ice recrystallization rate (S Wu et al., 2017).

2.5.6 TH and DIS analysis

Neither TH (**Table 2-1**) nor DIS (**Figure 2-13**) activity was observed with nanocelluloses. Nevertheless, the absence of correlation between IRI and TH/DIS was also

observed in AFGPs, synthetic polymers and small molecular weight compounds (Capicciotti et al., 2012; Graham et al., 2018; Olijve et al., 2016; Wilkinson et al., 2018).

It should be point out that no observable TH and DIS activity does not mean nanocelluloses do not bind to ice. TH and DIS were conducted at a temperatures close to zero, where nanocelluloses lost all IRI activity.

2.6 Conclusion

In contradiction to many polyols that demonstrate only weak IRI activity, this study shows that CNCs and TEMPO-CNFs have observable IRI activity without TH or DIS activity. This is the first report of IRI activity found in a non-AF(G)P biopolymer from biomass. Even though the amphiphilicity of nanocelluloses has been confirmed in colloidal science, evidence to associate the IRI activity of nanocelluloses with their amphiphilicity is still missing and requires further study. This situation, however, does not decrease the potential applications of nanocellulose as ice recrystallization inhibitors in many fields, due to the biocompatibility, low toxicity, low-cost of nanocelluloses, and the observable IRI activity reported here. Combined with the numerous approaches available to modify the surface of nanocelluloses, i.e., enhanced IRI activity or improved salt tolerance may be expected, which opens a tremendous opportunity for the utilization of nanocelluloses.

2.7 Acknowledgements

We thank Dr. Douglas Hayes from the Department of Biosystems Engineering and Soil Science on the conductometric titration experiment. We thank Professor Matthew I. Gibson from the University of Warwick for his suggestions on how to conduct the “splat” assay.

2.8 Abbreviations used in this chapter

CNF, cellulose nanofibrils; CNCs, cellulose nanocrystals; TEMPO-CNFs, 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils; IRI, ice recrystallization inhibition; PEG, polyethylene glycol; PVA, polyvinyl alcohol; (%) MGA, (percentage) mean grain area; AFPs, antifreeze proteins; AFGPs, antifreeze glycoproteins; AFM, atomic force microscopy; TH, thermal hysteresis; DIS, dynamic ice shaping; GO, graphene oxide.

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Appendix

Table 2-1. Physicochemical properties of nanocelluloses used in IRI evaluation (Data are mean $\pm$ standard deviation of triplicate determinations).

Parameters	CNFs	CNCs	TEMPO-CNFs
Cellulose (%) ^A	90.2 $\pm$ 0.6	95.1 $\pm$ 0.2	93.6 $\pm$ 0.3
Crystallinity index (CI)	86.7 $\pm$ 1.0	91.1 $\pm$ 1.1	74.7 $\pm$ 0.6
Zeta potential (mV)	-11.5 $\pm$ 0.9	-31.5 $\pm$ 1.4	-33.3 $\pm$ 0.3
Sulfate/Carboxylate content (mmol/g dry mass) ^B	0.10 $\pm$ 0.00	0.40 $\pm$ 0.00	1.47 $\pm$ 0.02
Degree of polymerization (DP)	1211 $\pm$ 8	134 $\pm$ 0	196 $\pm$ 0
Z- average hydrodynamic diameter (nm)	5099.0 $\pm$ 12.8	85.1 $\pm$ 1.4	167.3 $\pm$ 1.2
TH	Not observed (NA)	NA	NA

^A: The cellulose (%) is calculated based on the glucose content for CNFs and CNCs or the content of glucose and its oxidation product, glucuronic acid, for TEMPO-CNFs.

^B: Sulfate group for CNCs and carboxylate group for CNFs and TEMPO-CNFs.

Table 2-2. IRI activity of ice recrystallization inhibitors on a mass basis.

Ice recrystallization inhibitors	Concentration (mg/mL)	Assay media	Annealing condition	% MGA
Safranine O	1.47	40% sucrose	-6°C for 120 min	< 10 (Drori et al., 2016)
Graphene oxide	0.1, 5.0	0.5 mg/mL NaCl	-6°C for 30 min	20, 10 (Geng et al., 2017)
Nylon- 3 polymers	6.0	PBS	-8°C for 30 min	14-75 (MacDonald et al., 2017)
Oxidized Quasi-Carbon Nitride Quantum Dots (180- 3)	5.0, 10.0	PBS	-6°C for 30 min	22, 15 (Bai et al., 2017)
Nisin	5.0, 10.0	Acetate buffer (pH 5)	-8°C for 30 min	25, 25 (Mitchell & Gibson, 2015)
Lectins concanavalin A	5.0	HEPEs buffer with 5 mg/mL Ca ²⁺	-8 °C for 30 min	36 (Mitchell & Gibson, 2015)
Oxidized Quasi-Carbon Nitride Quantum Dots (200- 6)	5.0, 10.0	PBS	-6°C for 30 min	42, 22 (Bai et al., 2017)
Dextran	10.0	PBS	-8°C for 30 min	65 (Laezza et al., 2017)
Polyproline (DP 20)	10.0, 20.0	PBS	-8°C for 30 min	80, 49 (Graham et al., 2017)

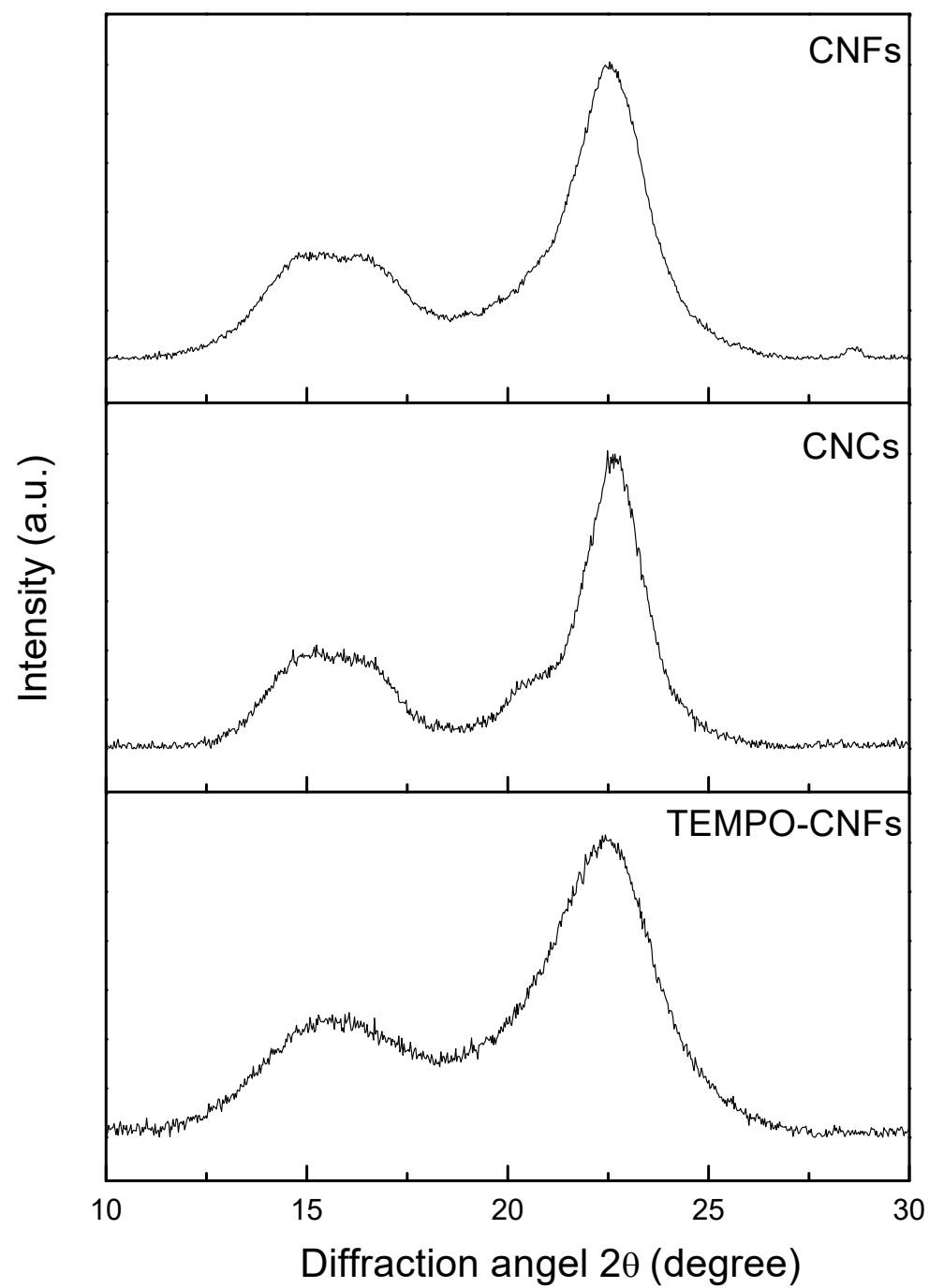


Figure 2-1. XRD spectra of CNFs, CNCs, and TEMPO-CNFs.

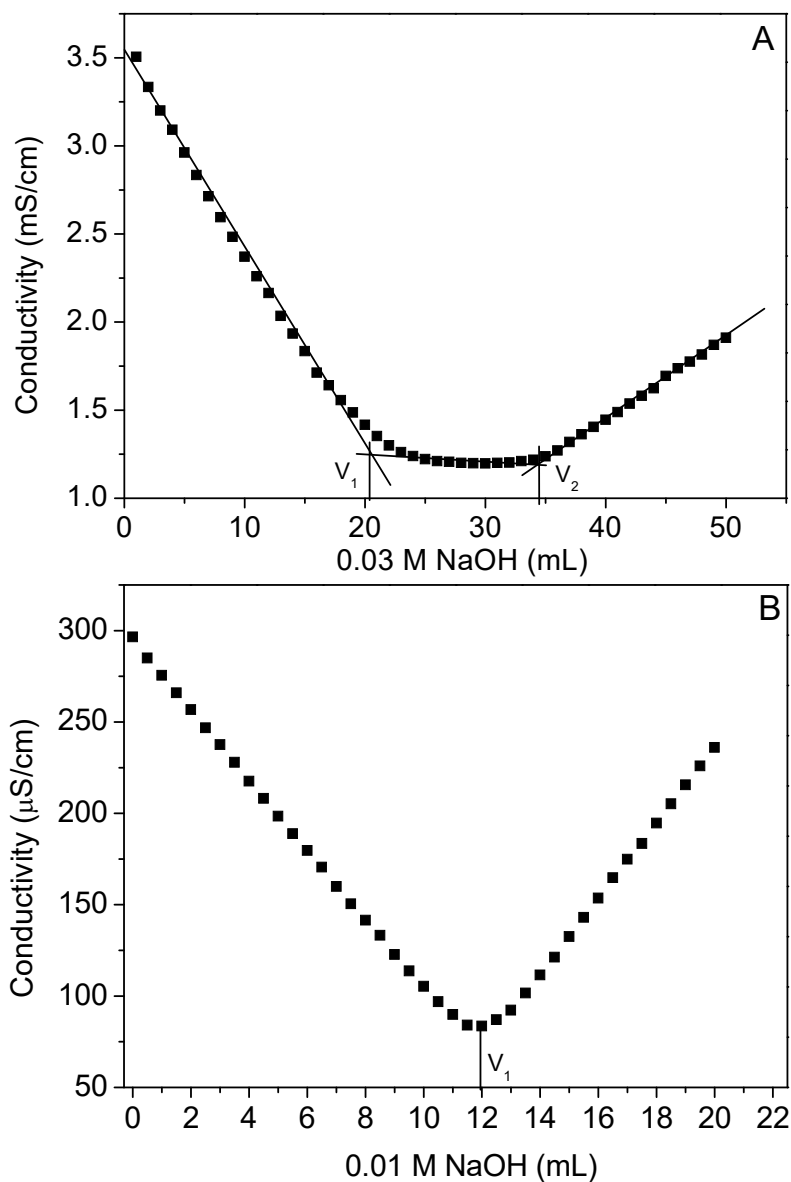


Figure 2-2. Typical conductometric titration curves of nanocelluloses: (A) protonated CNFs and TEMPO-CNFs dispersions (0.3 g CNFs or TEMPO-CNFs) titrated with 0.03 M NaOH; (B) protonated CNCs dispersion (0.3 g CNCs) titrated with 0.01 M NaOH.

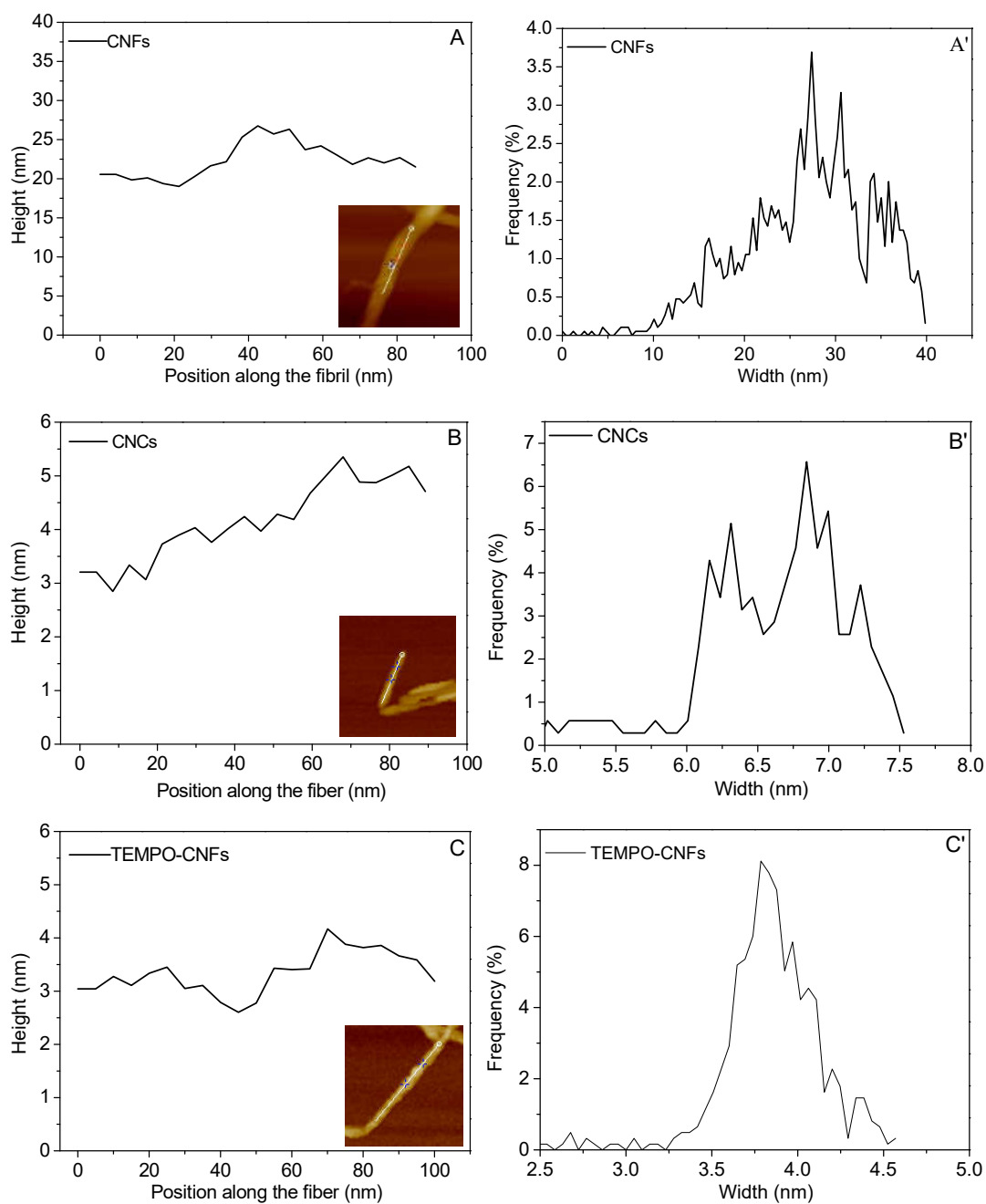


Figure 2-3. Height and width analysis of nanocelluloses by AFM: height distribution of (A) CNFs, (B) CNCs, and (C) TEMPO-CNFs; width distribution of (A') CNFs, (B') CNCs, and (C') TEMPO-CNFs.

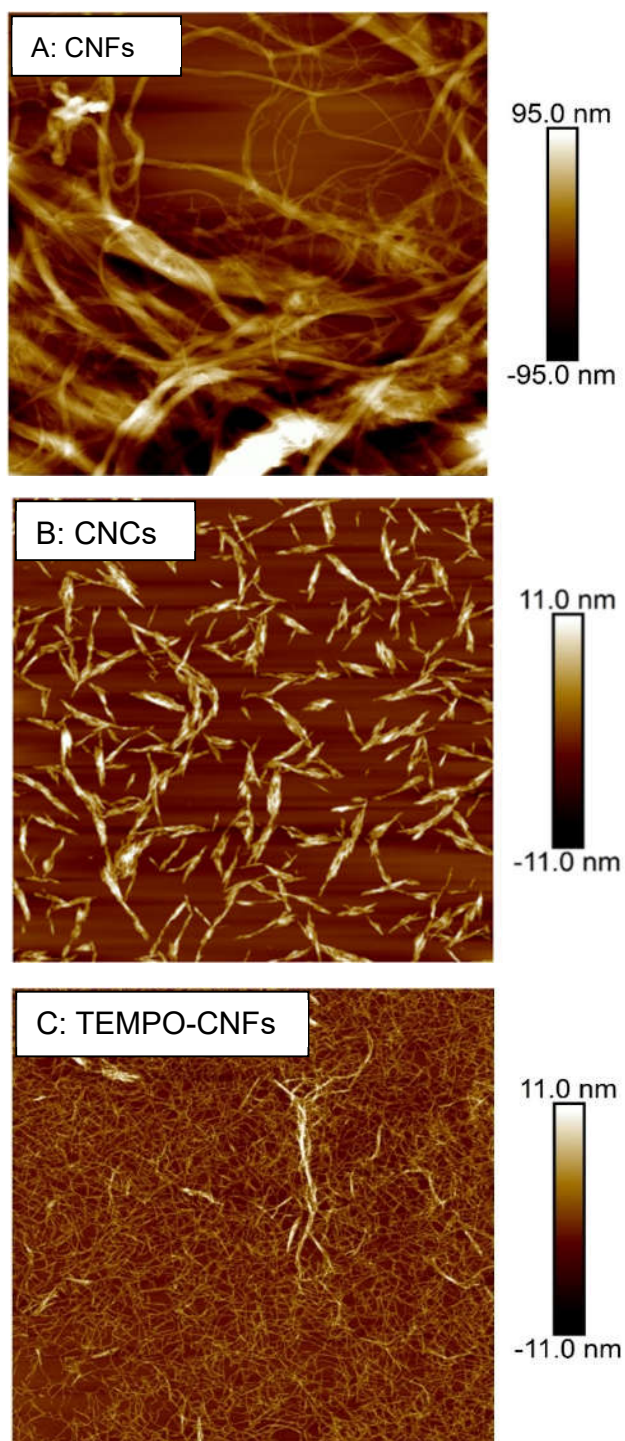


Figure 2-4. Atomic force microscopic images of (A) CNFs, (B) CNCs, and (C) TEMPO-CNFs. The size of images is $5\ \mu\text{m} \times 5\ \mu\text{m}$.

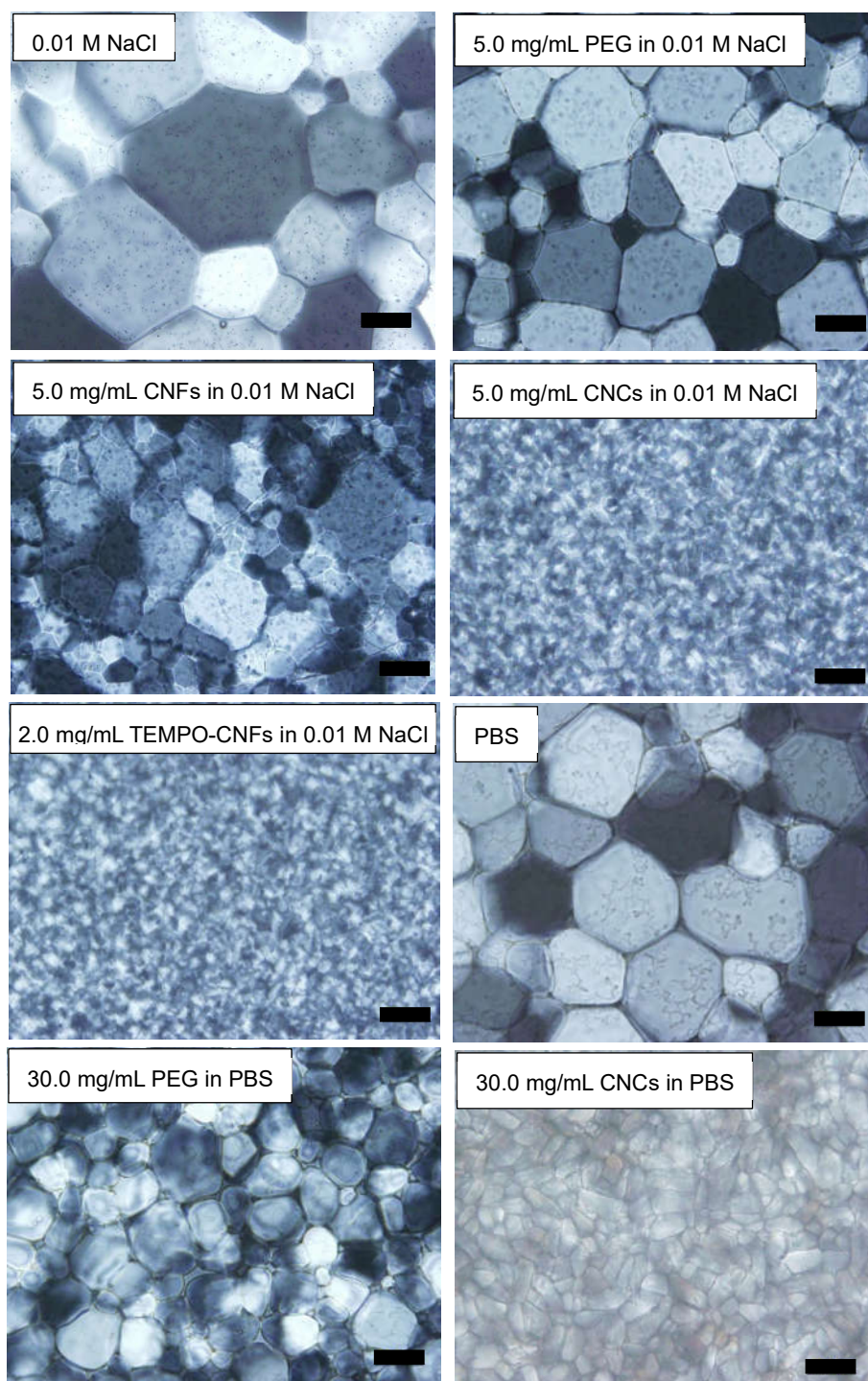


Figure 2-5. Representative polarized light microscopy images of ice wafers after 30 min annealing at -8°C . Scale bar = $50\text{ }\mu\text{m}$

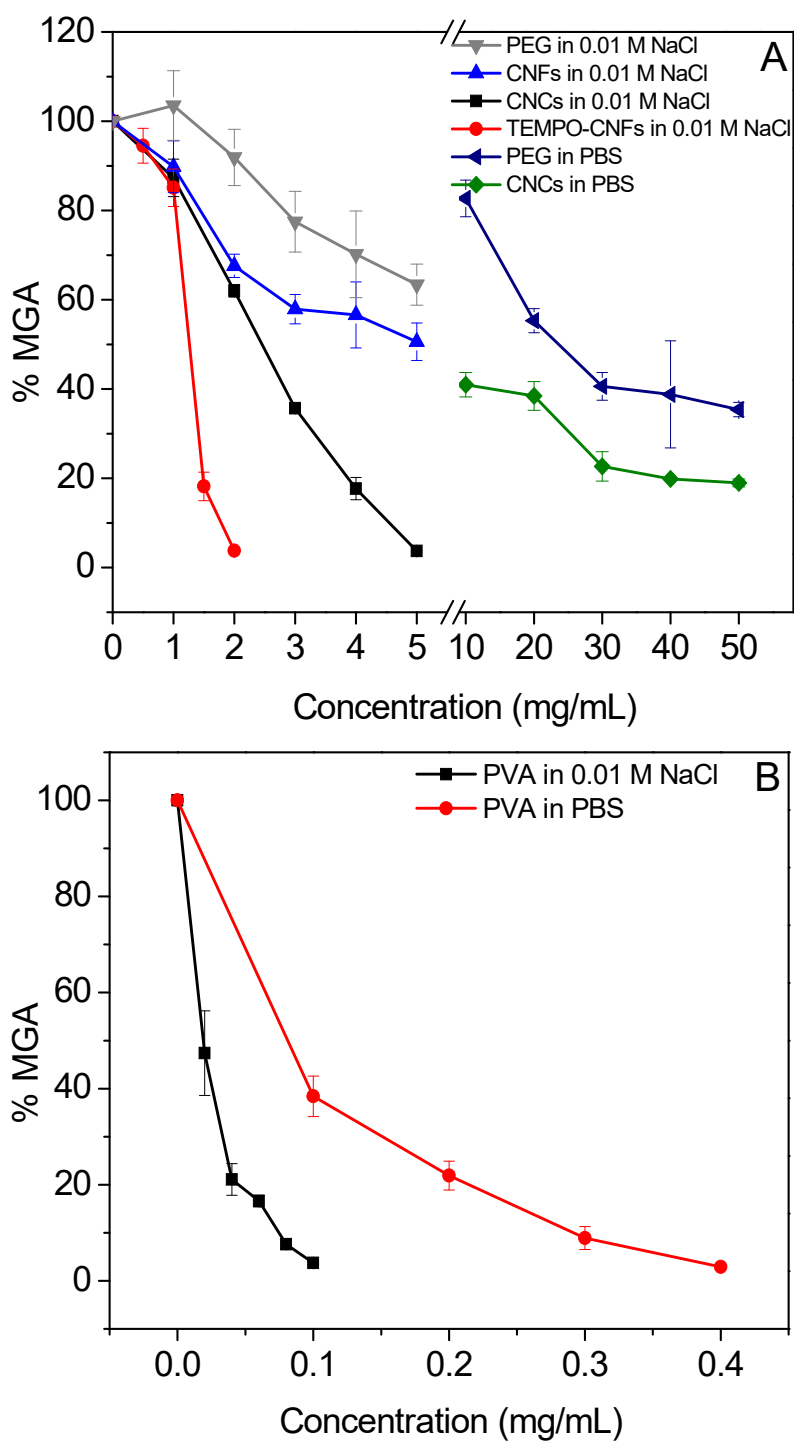


Figure 2-6. The IRI activity of PEG, PVA, CNFs, CNCs, and TEMPO-CNFs in percentages of mean grain area (% MGA).

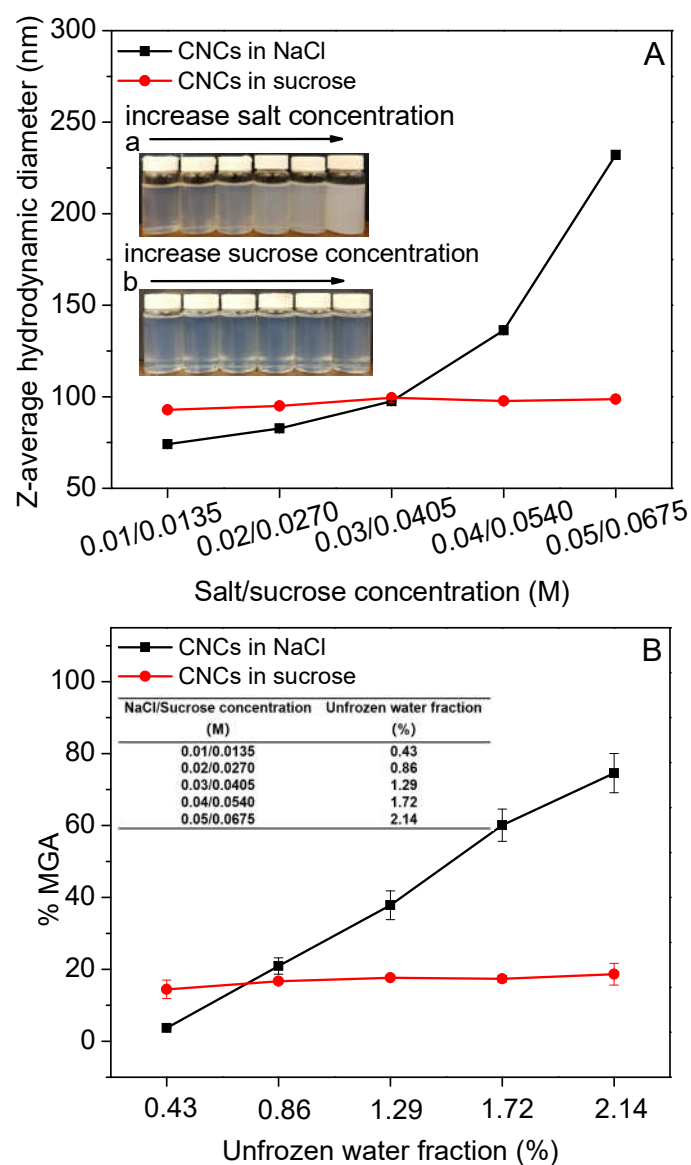


Figure 2-7. (A) Z-average hydrodynamic diameter of 0.5 mg/mL CNCs in 0.01-0.05 M NaCl and 0.0135-0.0675 M sucrose solutions. The inset figures are the dispersion appearance of CNCs. (B) % MGA of 5.0 mg/mL CNCs in 0.01-0.05 M NaCl and 0.0135-0.0675 M sucrose. % MGA was obtained by dividing the MGA of sample to that of NaCl or sucrose solutions at the same solute concentration. The inset table shows the unfrozen water fraction of NaCl and sucrose solutions at -8°C.

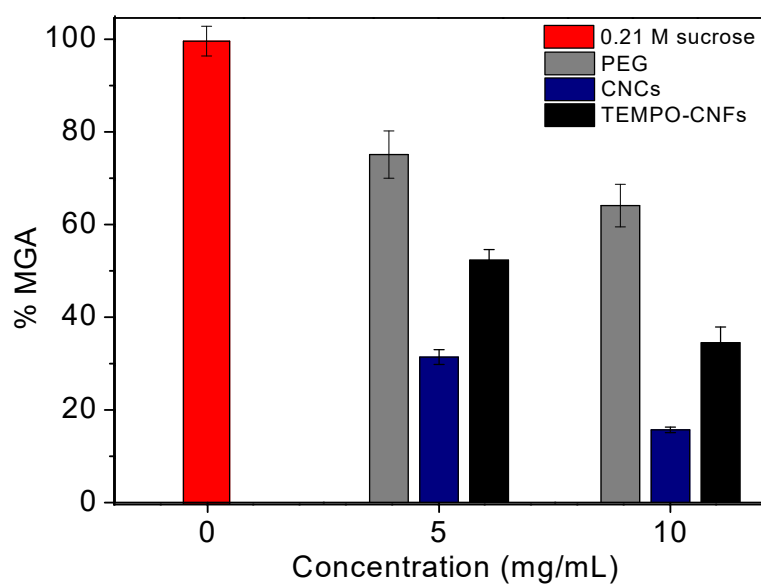
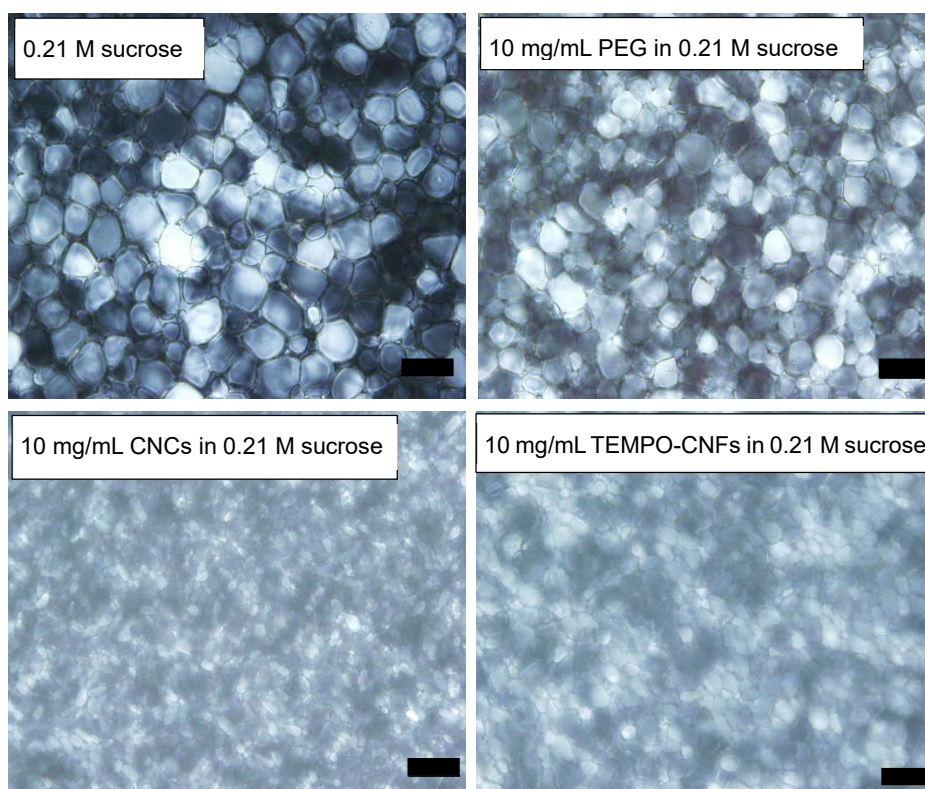


Figure 2-8. Polarized light microscopy images of ice wafers of 0.21 M sucrose containing PEG and nanocelluloses after 30 min annealing at -8°C . Scale bar = 50 μm ; % MGA was calculated by dividing the MGA of sample to that of 0.21 M sucrose.

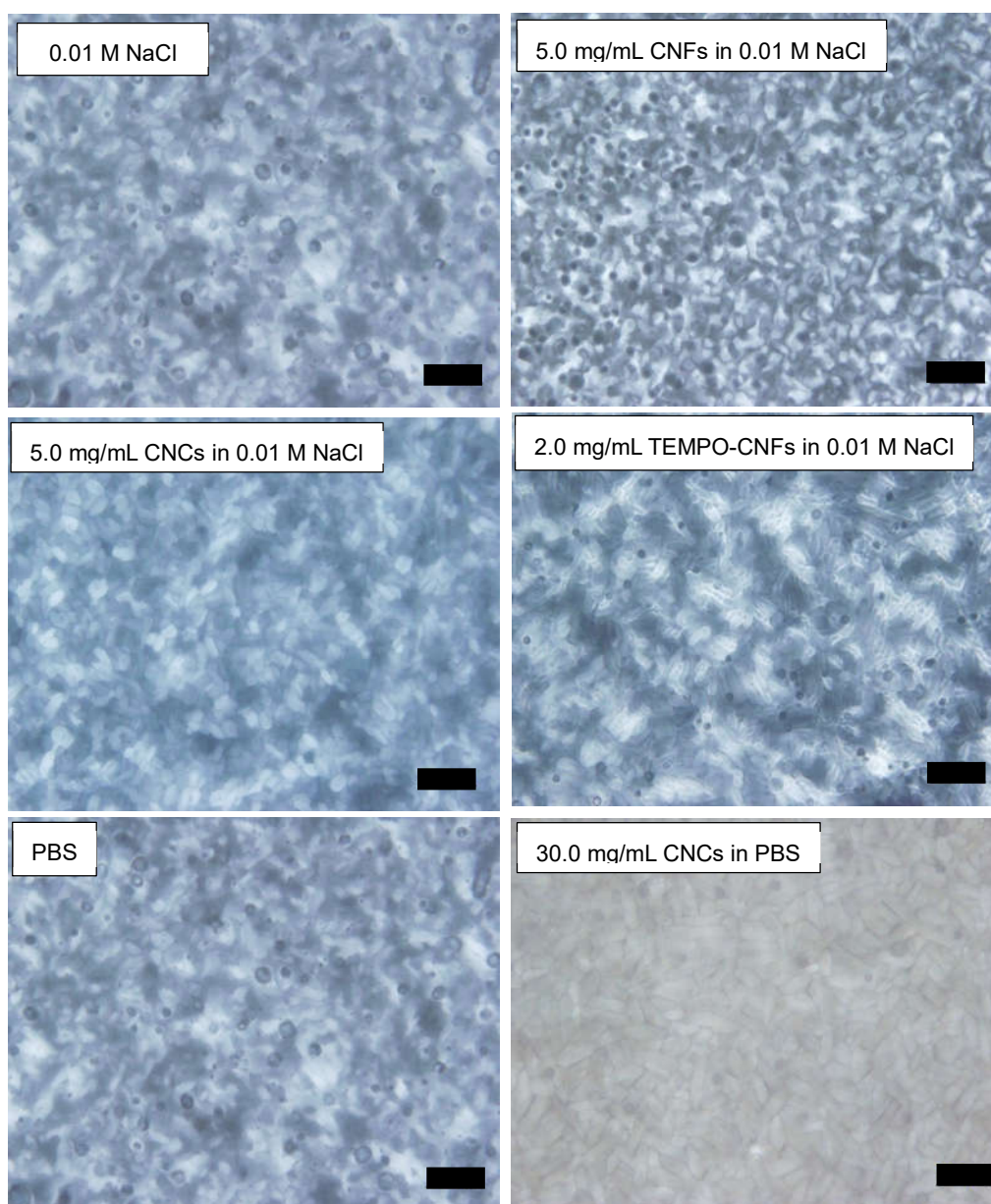


Figure 2-9. Polarized light microscopy images of ice wafers formed immediately after quenching. Scale bar = 50 μm .

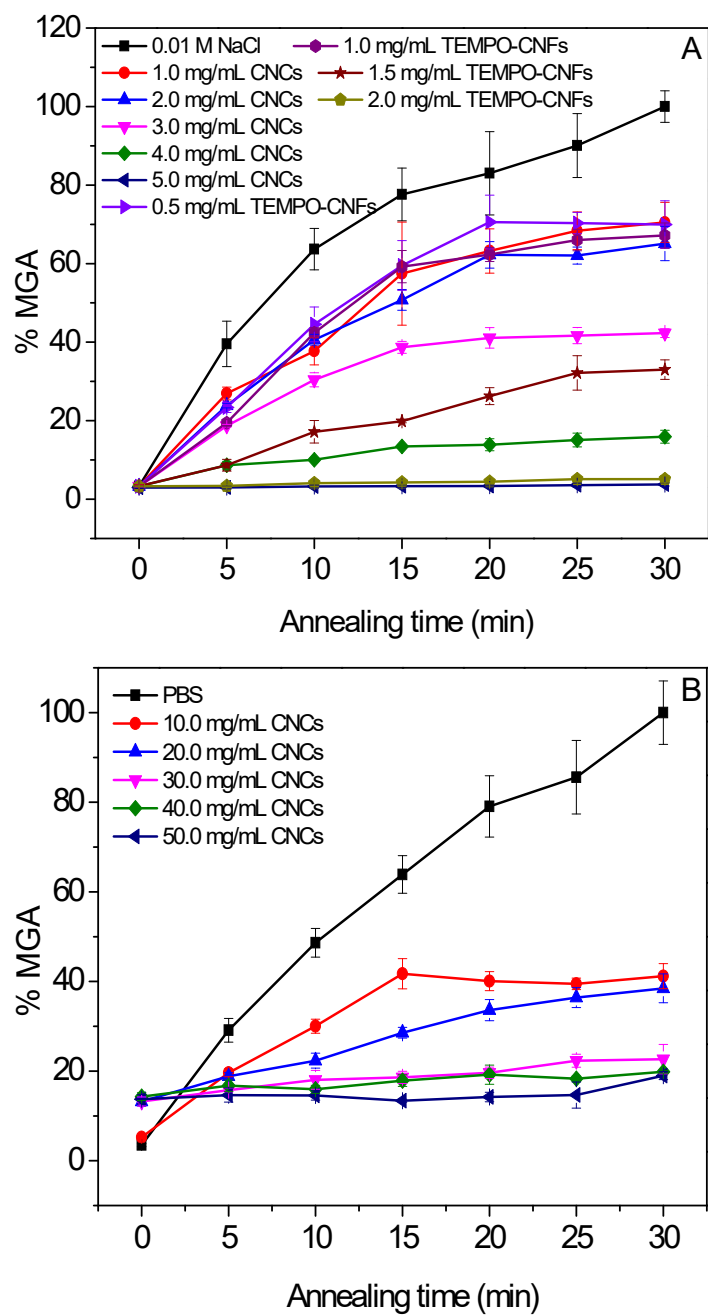


Figure 2-10. Ice growth kinetics of (A) CNCs and TEMPO-CNFs in 0.01 M NaCl and (B) CNCs in PBS annealed at -8°C . % MGA was calculated by dividing the MGA of sample at different annealing time to that of 0.01 M NaCl and PBS annealed at -8°C for 30 min.

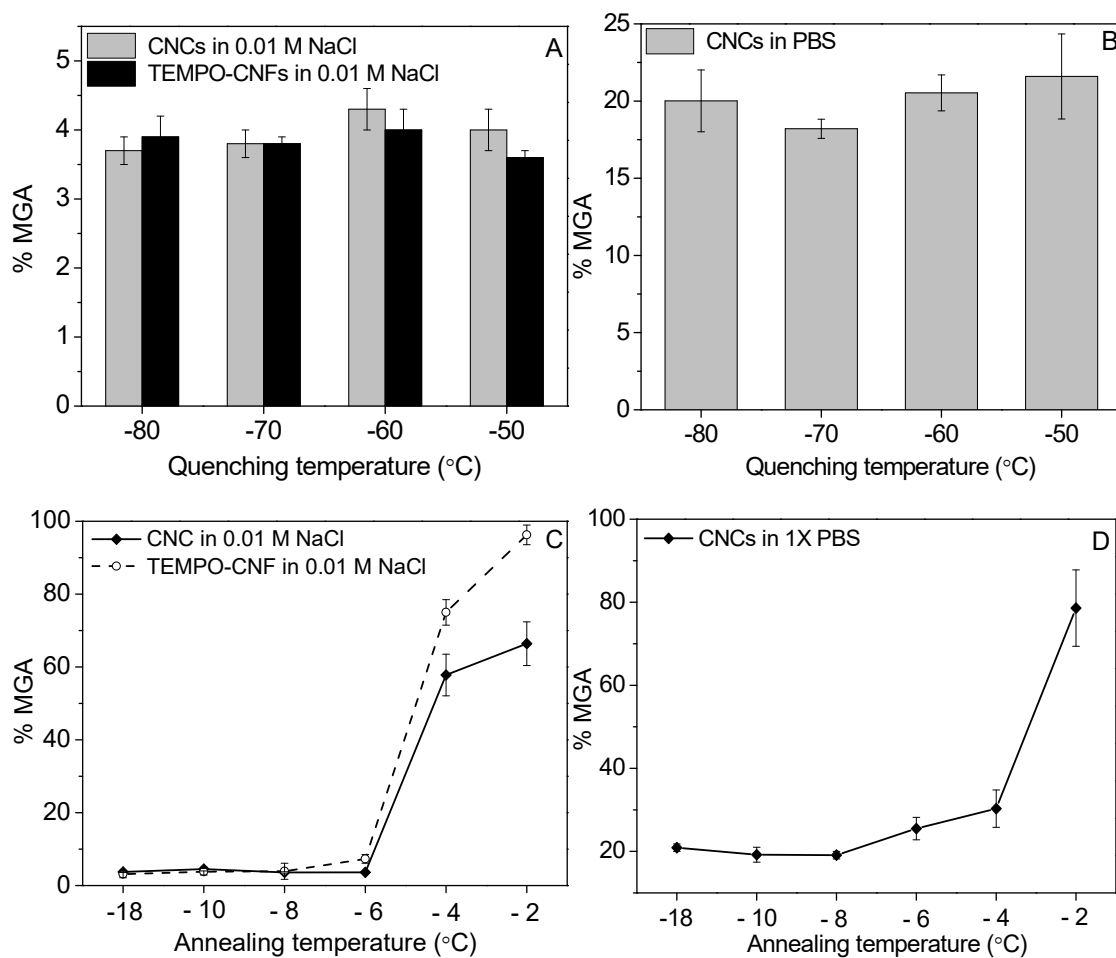


Figure 2-11. Effects of (A, B) quenching temperature and (C, D) annealing temperature on the IRI activity of nanocelluloses. The concentration of CNCs and TEMPO-CNFs in 0.01 M NaCl was 5.0 mg/mL and 2.0 mg/mL, respectively. The concentration of CNCs was 50.0 mg/mL in PBS. % MGA was calculated by dividing the MGA of samples to that of 0.01 M NaCl and PBS quenched or annealed at the same temperature.

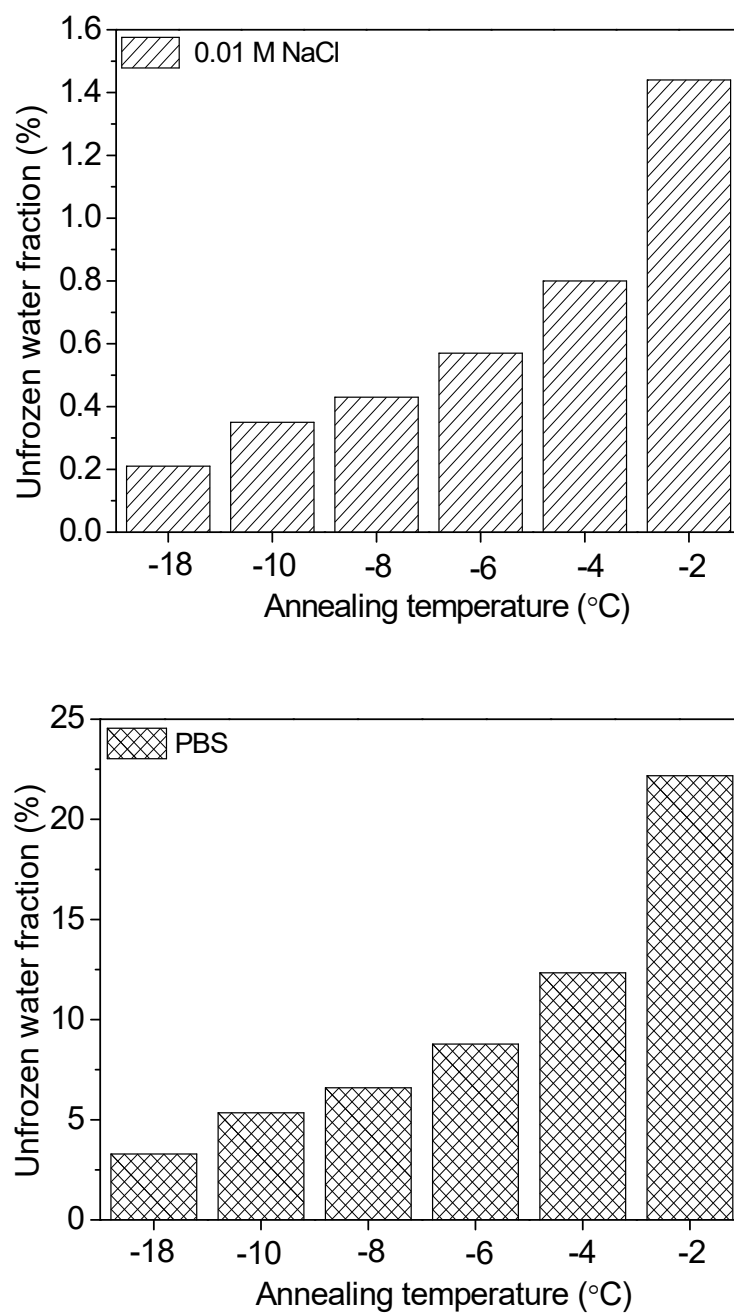


Figure 2-12. Unfrozen water fraction (%) of 0.01 M NaCl and PBS at different annealing temperatures.

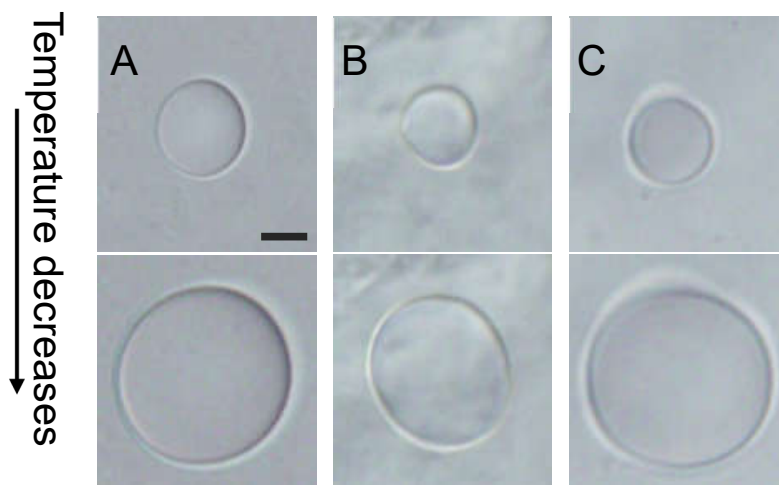


Figure 2-13. Optical microscopy images of ice morphology: (A) 0.01 M NaCl solution; (B) 25.0 mg/mL CNCs in 0.01 M NaCl; and (C) 2.0 mg/mL TEMPO-CNFs in 0.01 M NaCl. Ice crystals in the bottom row were grown from those in the top row. Scale bar = 10 μm .

Chapter 3. Effect of surface charge density on the ice recrystallization inhibition activity of nanocelluloses

A version of this chapter was originally published by Teng Li, Qixin Zhong, Bin Zhao, Scott Lenaghan, Siqun Wang, Tao Wu.

Li, T., Zhong, Q., Zhao, B., Lenaghan, S., Wang, S., & Wu, T. (2020). Effect of Surface Charge Density on the Ice Recrystallization Inhibition Activity of Nanocelluloses. *Carbohydrate Polymers*, 115863.

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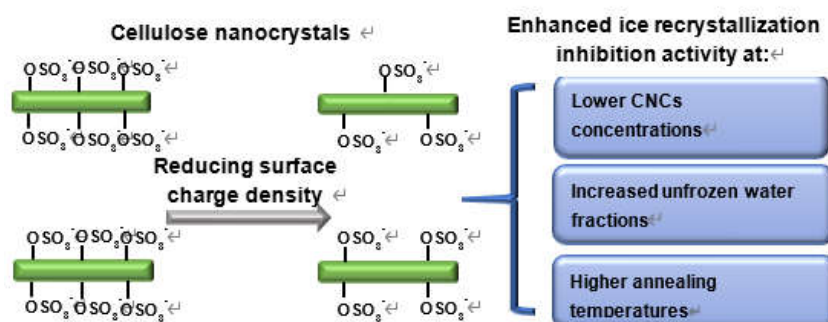
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3.1 Abstract

Recently nanocelluloses have been found to possess ice recrystallization inhibition (IRI) activity, which have several potential applications. The present study focuses on the relationship between the surface charge density (SCD) of nanocelluloses and IRI activity. Cellulose nanocrystals (CNCs) and 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils (TEMPO-CNFs) with similar degrees of polymerization (DP) or fibril lengths but with different SCDs were prepared and characterized for IRI activity. When the SCD of CNCs was progressively reduced, an initial increase of IRI activity was observed, followed by a decrease due to fibril aggregation. CNCs with a low SCD became IRI active at increased unfrozen water fractions and higher annealing temperatures. TEMPO-CNFs with a low SCD also had higher IRI activity. Additionally, lowering pH to protonate the carboxylate groups of TEMPO-CNFs enhanced the IRI activity. These research findings are important in producing nanocelluloses with enhanced IRI activity and understanding their structure-activity relationship.



KEYWORDS: Nanocellulose; Ice recrystallization inhibition; Surface charge density; Amphiphilicity.

3.2 Introduction

Ice recrystallization is a ubiquitous thermodynamically-driven process that adversely affects cryopreserved cells and foods (Adapa, Schmidt, Jeon, Herald, & Flores, 2000; Balcerzak, Capicciotti, Briard, & Ben, 2014). Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) are accumulated by a variety of fishes, insects, and plants to enable their survival in subzero environments (Voets, 2017). These proteins feature thermal hysteresis (TH) activity in lowering the freezing point of water, dynamic ice shaping (DIS) activity in altering ice crystal morphology, and ice recrystallization inhibition (IRI) activity via the adsorption-inhibition mechanism (Raymond & Devries, 1977; Voets, 2017). However, the application of AF(G)Ps to industrial processes are limited by high costs associated with extraction and purification, or synthesis. Thus, various low molecular weight compounds, synthetic polymers, and nanomaterials have been explored for IRI activity (Biggs et al., 2017; Capicciotti et al., 2012; He, Liu, & Wang, 2018).

A successful design and synthesis of AF(G)P mimics relies on a thorough understanding of their ice-binding motifs. Despite a consensus that AF(G)Ps bind to a certain or multiple ice planes (Knight, Cheng, & Devries, 1991), the structural diversity among AF(G)Ps makes it difficult to predict the essential features of ice-binding motifs. Early studies have focused on the role of hydrogen bonds in ice binding (Chou, 1992; Knight, Driggers, & Devries, 1993). However, a later study using different mutants of type I-AFPs has revealed the importance of hydrophobic groups (Haymet, Ward, & Harding,

1999). Further modeling studies have proposed that hydrophobic groups of AF(G)Ps are incorporated into grooves and cavities of certain ice planes (Mochizuki & Molinero, 2018; Nada & Furukawa, 2008), which is entropically driven by desolvation of water molecules around hydrophobic groups (Mochizuki & Molinero, 2018). Recent studies have associated ice binding of AFPs with a layer of ice-like clathrate water around an ice binding face that recognizes and anchors AFPs on ice surfaces (Chakraborty & Jana, 2018; Garnham, Campbell, & Davies, 2011; Nutt & Smith, 2008).

Poly(vinyl alcohol) (PVA) is a potent IRI active polymer that functions at a sub-mg/mL concentration (Congdon, Notman, & Gibson, 2013). However, PVA bears few structural similarities with AF(G)Ps. Molecular dynamic simulation studies have shown that PVA binds to ice via hydrogen bonds formed between hydroxyl groups of PVA and water molecules of ice (Naullage, Lupi, & Molinero, 2017; Weng, Stott, & Toner, 2017). PVA has been found to also possess local hydrophobic domains (Deller et al., 2013), suggesting that the potential role of hydrophobicity in its IRI activity cannot be excluded. Small molecular weight compounds including mono/disaccharides and carbohydrate-based surfactants are IRI active (Capicciotti et al., 2012; Tam, Ferreira, Czechura, Chaytor, & Ben, 2008). The IRI activity of mono/disaccharides is proposed to be positively correlated with their hydration indexes, which are the number of tightly bound water molecules divided by the molar volume of carbohydrates (Tam et al., 2008). Mono/disaccharides are hypothesized to be concentrated at the bulk water between quasi-liquid layers of ice

crystals and disrupt the ordering of bulk water molecules, making the transfer of water molecules to ordered ice crystals less energetically favorable (Tam et al., 2008). Mono/disaccharides with a larger hydration index cause a greater disruption of the bulk water ordering and have stronger IRI activity (Tam et al., 2008). Recently, Ben et al. have demonstrated that long alkyl chains are necessary for the potent IRI activity of lysine-based surfactants, indicating the importance of hydrophobic moieties for the IRI activity of small amphiphilic molecules (Balcerzak, Febbraro, & Ben, 2013). Ions are the smallest substances that influence ice recrystallization (S. Wu et al., 2017). Ions with higher charge densities are less likely to be incorporated into ice crystals, thus effectively inhibiting ice growth at ice/water interface (S. Wu et al., 2017).

Several IRI active materials were found to have facial amphiphilic structures (Drori et al., 2016; Geng et al., 2017; Mitchell & Gibson, 2015), and a few materials with strong IRI activity were designed based on an amphiphilic structure (Graham, Fayter, Houston, Evans, & Gibson, 2018; Mitchell et al., 2017). Nanocelluloses are cellulose fibrils with widths between 3 and 50 nm and lengths from 50 to several 1000 nm (Peng, Dhar, Liu, & Tam, 2011). They can be produced by mechanical treatments, chemical oxidation, or acid hydrolysis from a variety of sources (Peng et al., 2011). As an emerging biomass-based material, nanocelluloses can be used in forms of colloidal dispersions, hydrogels, and aerogels in various of fields (De France, Hoare, & Cranston, 2017; Gomez et al., 2016). The amphiphilic nature of nanocelluloses has been recognized (Biermann, Hadicke,

Koltzenburg, & Muller-Plathe, 2001; Lindman et al., 2017; Medronho, Romano, Miguel, Stigsson, & Lindman, 2012). Based on the observed relationship between amphiphilicity and IRI activity, we hypothesized that nanocelluloses would have IRI activity, which was confirmed in our previous study (Li, Zhao, Zhong, & Wu, 2019). However, the IRI activity of nanocelluloses is low and their structure-activity relationship remains to be elucidated.

Surface charge density (SCD) is one of the most important structural parameters of nanocelluloses as SCD modulates the repulsive electrostatic interaction and influences the colloidal stability and self-assembly of nanocelluloses (Jiang & Hsieh, 2016; Wu, Li, Fu, Li, & Wang, 2017). It has been shown that the emulsion stabilization ability of nanocelluloses is affected by their SCD (Kalashnikova, Bizot, Cathala, & Capron, 2012). Given the strong possibility that emulsion stabilization and IRI activity are associated with amphiphilicity, we hypothesized that SCD plays an important role in the IRI activity of nanocelluloses. In this work, we modified the SCDs on cellulose nanocrystals (CNCs) with similar DPs and on 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils (TEMPO-CNFs) with similar fibril lengths and studied their IRI activity before and after SCD modification. This research is important in producing biomass-derived nanocelluloses with enhanced IRI activity and understanding the structure and IRI activity relationship of nanocelluloses.

3.3 Materials and method

3.3.1 Materials.

Commercial CNCs (10.4 wt.%) and cellulose nanofibrils (CNFs) (3.0 wt.%) in the slurry form were purchased from the Process Development Center of the University of Maine (Orono, ME) and were used as received. The CNCs and CNFs were produced by concentrated sulfuric acid hydrolysis and mechanical grinding, respectively. The morphology and physicochemical properties of CNCs and CNFs were reported in our previous study (Li et al., 2019). Other chemicals were purchased from Fisher Scientific (Pittsburgh, PA). De-ionized (D.I.) water was used throughout the experiment.

3.3.2 De-sulfation of CNCs by HCl hydrolysis

The CNCs with different charge densities were prepared by a mild HCl hydrolysis to remove surface sulfate groups (Kalashnikova et al., 2012). Commercial CNCs (designated as CNCs-0) were dispersed in water at a concentration of 20.0 mg/mL and sonicated (VCX-750, Sonics and Materials, Newton, CT) at 60% pressure amplitude for 60 s. Seventy-five mL CNCs-0 dispersion was mixed with 75 mL 5.0 N HCl and evenly distributed into six 50 mL Erlenmeyer flasks, which were sealed and transferred into a shaking water bath. Acid hydrolysis of CNCs was conducted at a temperature of $99.0 \pm 0.1^{\circ}\text{C}$ and a shaking speed of 120 rpm for 10, 20, 40, and 60 min. The obtained CNCs were designated as CNCs-X, with X being the hydrolysis time in min. After hydrolysis, the reaction mixtures were cooled to room temperature in an ice-water bath and centrifuged at 26,000 g for 15 min

using a Sorvall RC5B-PLUS centrifuge (Thermo Scientific, Waltham, MA) with an SS 34 centrifuge rotor. After centrifugation, the pellets were collected and redispersed in 150 mL water. The centrifugation and redispersion steps were repeated until the supernatant started to become turbid. Then the CNCs pellets were dispersed in 20 mL water and dialyzed at 4°C against excessive water to neutral pH in a dialysis tubing with a molecular weight cut-off of 6,000-8,000 Da (Thermo Fisher Scientific, Waltham, MA).

3.3.3 Preparation of TEMPO-CNFs with different surface charge densities

TEMPO-CNFs with varied SCDs were prepared by a TEMPO-catalyzed oxidation method using different amounts of NaClO (Shinoda, Saito, Okita, & Isogai, 2012). An illustrative scheme for TMEPO-CNFs preparation is shown in **Figure 3-1**. NaBr (0.50 g) and TEMPO (0.08 g) were dissolved in 338 mL water and mixed with 167.0 g of a slurry containing 5.0 g dry CNFs on a magnetic stirrer for 5 min. Then NaClO was added at levels of 10.0 and 6.0 mmol/g dry CNFs to prepare TEMPO-CNFs-10 and TEMPO-CNFs-6, respectively. The mixtures were stirred at 22.0°C and the pH was maintained at 9.8-10.2 by adding 0.5 M NaOH until no decrease of pH was observed. After oxidation, a white fibril-like aggregate of TEMPO-CNFs was collected by centrifugation at 4°C and 10,000 g for 10 min. The pellet was re-dispersed in 250 mL water and dialyzed at 4°C against excessive water to neutral pH in a dialysis tubing with a molecular weight cut-off of 6,000-8,000 Da (Thermo Fisher Scientific, Waltham, MA). TEMPO-CNFs-10 and TEMPO-CNFs-6 were dispersed in water or 0.223 M sucrose at a concentration of 5.0 mg/mL in a

100 mL beaker. To prepare TEMPO-CNFs-10 and TEMPO-CNFs-6 with similar fibril lengths, TEMPO-CNFs-10 and TEMPO-CNFs-6 dispersions were sonicated at 60% pressure amplitude for 10 and 35 min in a pulse mode (59 sec on and 59 sec off), respectively. During sonication, the beaker was kept in an ice-water bath to avoid excessive heating. After sonication, TEMPO-CNFs dispersions became optically transparent. The metal particles formed from the cavitation-induced corrosion of ultrasound probe were removed by centrifugation at 4°C for 10 min (10,000 g).

3.3.4 Surface charge density determined by conductometric titration

SCD was determined by conductometric titration and calculated as the sulfate group density for CNCs and carboxylate group density for TEMPO-CNFs. The sulfate groups of CNCs were protonated with the Dowex Marathon C hydrogen form strong acid cation exchange resin (Dow Chemical, Midland, MI) by a batch method (Beck, Méthot, & Bouchard, 2014). Prewashed resin (12 g resin/g CNCs) was added into CNC dispersion and stirred on a magnetic stirrer. Resin was changed every 30 min for 6 times by filtering with filter cloth (Millipore, Burlington, MA). Then 100 mL of the protonated dispersion containing 0.1 g CNCs was titrated with 0.01 M NaOH. The conductivity was monitored using a YSI 3200 conductometric meter with a 3256 cell (YSI Incorporated, Yellow Springs, OH). The sulfate group density was determined from the titration curves as shown in **Figure 3-2A** and calculated using Eq (1).

$$\text{Sulfate group density } \left(\frac{\text{mmol}}{\text{g}} \right) = \frac{0.01 \times V_1}{m_{\text{cnc}}} \quad (1)$$

Where V_1 is the volume of 0.01 M NaOH solution (mL) at the equivalent point and m_{cnc} is the mass of CNCs (0.1 g). The TEMPO-CNF dispersion (50 mL, 1.0 mg/mL) was adjusted to pH 2.5 with 1.0 N HCl and stirred for 10 min to protonate carboxylate groups (Saito & Isogai, 2004). Then TEMPO-CNF dispersions were titrated with 0.01 M NaOH. The conductivity was monitored using the above conductometric meter. The carboxylate group density of the sample was determined from the titration curves as shown in **Figure 3-2B** and calculated using equation (2):

$$\text{Carboxylate group density } \left(\frac{\text{mmol}}{\text{g}} \right) = \frac{0.01 \times (V_2 - V_1)}{m_{\text{TEMPO-CNFs}}} \quad (2)$$

Where V_1 and V_2 are the volume of NaOH at the first and second equivalent points, respectively, and $m_{\text{TEMPO-CNFs}}$ is the mass of TEMPO-CNFs (0.05 g).

3.3.5 Hydrodynamic size and ζ -potential of nanocelluloses

CNCs and TEMPO-CNFs were dispersed in 0.01 N NaCl or 0.223 M sucrose to a concentration of 0.5 mg/mL. The Z-average hydrodynamic diameter and ζ -potential were determined by a Malvern dynamic light scattering system (Nano ZS, Malvern Instruments, UK) according to referenced methods (Wilkins et al., 2019). Three replications were conducted for each sample.

3.3.6 Reduction of C₆-aldehydes to hydroxyls in TEMPO-CNFs.

During the preparation of TEMPO-CNFs, a small number of C₆-aldehydes are introduced on the surface by the TEMPO-catalyzed oxidation, which would cause an underestimation of DP measurement (Shinoda et al., 2012). Before DP determination, the

C₆-aldehydes of TEMPO-CNFs were reduced to hydroxyls by NaBH₄ according to a literature method (Takaichi, Saito, Tanaka, & Isogai, 2014). Briefly, 0.15 g of TEMPO-CNFs were dispersed in 30.0 mL of 0.3 M NaHCO₃, and 0.15 g of NaBH₄ was added. The reaction was conducted at room temperature for 48 h under constant stirring. TEMPO-CNFs were collected as gel-like precipitate by centrifugation at 10,000 g for 15 min at 4°C, redispersed in 30 mL water, dialyzed against excessive water at 4°C for 3 days, and lyophilized before DP measurement.

3.3.7 Degree of polymerization (DP) of nanocelluloses

The nanocelluloses were dissolved in 0.5 M cupriethylenediamine hydroxide (CED) to a concentration of 2.0 mg/mL (Shinoda et al., 2012). The relative viscosities (η_{rel}) of nanocelluloses CED solutions were measured using an AVS 370 Ubbelohde viscometer with a 0.84 mm capillary tube in a 25 ± 0.1°C water bath (SI Analytics, Weilheim, Germany). The efflux time of nanocelluloses-CED solutions and 0.5 M CED (blank) were recorded. The η_{rel} was calculated as the following equation:

$$\eta_{rel} = t_s / t_0 \quad (3)$$

where t_s and t_0 were efflux time of nanocelluloses-CED solutions and 0.5 M CED, respectively. The product of intrinsic viscosity and nanocelluloses concentration ($[\eta] \times C$) was taken from a predetermined table in the ISO 5351:2010 standard according to the obtained η_{rel} value (ISO, 2010). DP was calculated according to the Mark–Houwink–Sakurada equation (Smith, Bampton, & Alexander, 1963):

$$DP = 1.75 \times [\eta] \quad (4)$$

Mean and standard deviation were reported from 3 replications.

3.3.8 Morphological analysis of CNCs and TEMPO-CNFs by atomic force microscopy (AFM)

The morphologies of nanocelluloses were analyzed by AFM (model Multimode VIII, Bruker Corp, Santa Barbara, CA). CNCs (1.0 mg/mL) and TEMPO-CNFs (0.05 mg/mL) were dispersed in water or 0.01 M NaCl. Nanocellulose dispersions were spin-coated on a fresh mica surface, dried and scanned by AFM (Li et al., 2019). The fibril lengths of TEMPO-CNFs were measured using the ImageJ software (National Institutes of Health, Bethesda, MD) with at least 150 individual fibrils.

3.3.9 IRI activity of CNCs and TEMPO-CNFs in 0.01 M NaCl

IRI activity of CNCs and TEMPO-CNFs in 0.01 M NaCl (pH 6.0) was determined by the “splat” assay (Knight, Hallett, & Devries, 1988). Briefly, a microscope glass slide was pre-chilled on the surface of a metal block, which was surrounded by dry ice in a foam box. A thin ice wafer was obtained by dropping ca.10 μ L dispersion from a 1.4 m height onto the pre-chilled microscope glass slide using a syringe. Then the microscope glass slide was incubated in an HCS 302 cryostage (Instec Instruments, Boulder, CO) held at -8.0°C under the purge of N₂ for 30 min. For each ice wafer, three 400x magnification images in random locations were obtained using a polarized light microscope (BX51, Olympus, Tokyo, Japan) with a built-in digital camera (DP 70, Olympus, Tokyo, Japan). Ten largest ice crystals in

each image were measured for the single largest length in any axis. The average of the single largest length was calculated from two images from each ice wafers. The mean largest grain size (MLGS) was calculated from three independent ice wafers. The %MLGS was obtained by dividing MLGS of a sample to that of 0.01 M NaCl.

3.3.10 The pH effect on IRI activity of TEMPO-CNFs-10

The 0.223M sucrose solution was used in the splat assay to maintain a relatively constant unfrozen water fraction at 7.1% when the pH was varied (Li et al., 2019). The unfrozen water fraction of sucrose solution at -8.0°C was calculated from the sucrose-water phase diagrams (**Table 1**) (Li et al., 2019; Pongsawatmanit & Miyawaki, 1993). To protonate the carboxylate groups, TEMPO-CNFs-10 were dispersed in 0.223 M sucrose solution at a concentration of 5.0 mg/mL and the pH was adjusted to 3.5. The IRI activity of nanocelluloses was determined by the splat assay described above. The %MLGS was obtained by dividing MLGS of a sample to that of 0.223 M sucrose at the same pH value.

3.3.11 Interfacial tension measurements

The interfacial tension was measured using a Krüss FM40 shape analysis system (Hamburg, Germany). Dodecane was passed 3 times through an alumina column and distilled 3 times to remove interfacial active impurities. Water was also distilled in an all-glass apparatus three times. Before sample measurements, the surface tension of water in air and interfacial tension of dodecane in water were measured and compared to standard values in database to make sure that the instrument worked well. One mL dodecane was

added to a cuvette of 12.5 (L) mm $\times$ 12.5 (W) mm $\times$ 45 (H) mm (BrandTech., Wertheim, Germany). A pendant drop of 30 μ L water or CNCs (5.0 mg/mL in water) dispersion was formed on the end of a poly(tetrafluoroethylene) needle (diameter 1.068 mm), which was immersed in the dodecane phase. The data was collected every 10 min from 0 to 60 min.

3.3.12 Pyrene assay

Pyrene assay was conducted based on a reported method (Amiji, 1995). Briefly, pyrene was dissolved in methanol and mixed with CNCs solutions (1.0 mg/mL in 0.01 M NaCl, pH 6.0) to give a final concentration of 2.0 μ M. Samples were incubated in dark for 2 h. The pyrene probe was excited at 343 nm and the emission spectrum was collected in the range of 360-500 nm using a Perkin Elmer LS 55 Fluorescence spectrophotometer (Waltham, MA). The intensity of peak I₁ (372 nm) and peak I₃ (384 nm) was recorded and the ratio of I₁/I₃ was calculated. A smaller I₁/I₃ value indicates higher hydrophobicity. Mean and standard deviation were reported from 3 replications for interfacial tension measurement and pyrene assay.

3.3.13 Aggregation behavior of CNCs with different SCDs

The aggregation behavior of CNCs was analyzed by measuring the decrease of light transmittance with the increase of CNCs concentration (Fukuzumi, Tanaka, Saito, & Isogai, 2014). CNCs were dispersed in 0.01 M NaCl at concentrations from 0.01 mg/mL to 5.0 mg/mL. Transmittance of CNCs dispersions at 550 nm was measured using an Evolution 201 UV-visible spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

3.3.14 IRI activity of CNCs at increased unfrozen water fractions

Unfrozen water fraction at -8°C is proportional to the concentration of solutes in dispersion mediums. Sucrose was used to modulate unfrozen water fraction due to the good colloidal stability of CNCs at high sucrose concentrations. CNCs-0 and CNCs-20 were dispersed in aqueous solution containing 7.2-40.0 wt.% sucrose at a concentration of 10.0 mg/mL (pH 6.0). The unfrozen water fraction in different sucrose solutions at -8°C was calculated according to a literature method (**Table 3-1**) (Li et al., 2019). The IRI activity of CNCs in 7.2 to 20.0 wt.% sucrose solution was determined by the splat assay with an annealing time of 60 min, and the %MLGS was obtained by dividing MLGS of a sample to that of a sucrose solution at the same sucrose concentration. IRI activity of CNCs in 40.0 wt.% sucrose solution was determined by a sandwich method with modification (Budke & Koop, 2006). A 5.0 µL CNCs dispersion or sucrose solution was deposited on the surface of a microscope glass slide, covered by a cover slide, and sealed with silicone grease. Thereafter, samples were subjected to fast freezing by immersing in liquid nitrogen for 10 sec, transferred into the cryostage, and annealed at -8°C for 60 min under the purge of N₂ before taking images.

3.3.15 IRI activity of CNCs at higher annealing temperatures

CNCs-0 and CNCs-20 were dispersed in 0.01 M NaCl at a concentration of 5.0 mg/mL (pH 6.0). Then the IRI activity of CNCs was determined by the splat assay with annealing

temperatures at -8, -6, -4, and -2°C. The %MLGS was obtained by dividing of MLGS of a sample to that of 0.01 M NaCl annealed at the same temperature.

3.4 Results and discussion.

3.4.1 Physicochemical and morphological properties of nanocelluloses

The charge density of sulfate group for CNCs-0 was 0.40 ± 0.00 mmol/g (**Figure 3-3A**). Compared to values from 0.06 to 0.41 mmol/g reported in the literature (Lin & Dufresne, 2014), the CNCs used in this study had a high SCD. HCl hydrolysis effectively cleaved the sulfate groups from the surface of CNCs. With an increase in hydrolysis time, a decreased charge density was observed (**Figure 3-3A**), which was also confirmed by the decrease of ζ -potential (**Table 3-2**). Consequently, an increase in Z-average hydrodynamic diameter (**Table 3-2**) and dispersion turbidity (**Inset in Figure 3-3A**) was observed. TEMPO-CNFs oxidized by 10.0 and 6.0 mmol NaClO per gram of dry CNFs had a carboxylate group density of 1.70 ± 0.00 and 1.20 ± 0.00 mmol/g, respectively (**Figure 3-3B**), which agreed with their corresponding ζ -potential values (**Table 3-2**). Both TEMPO-CNFs-10 and TEMPO-CNFs-6 were dispersed well in 0.01 M NaCl with a clear appearance (**Inset in Figure 3-3B**).

The morphology of CNCs and TEMPO-CNFs was characterized by AFM. With the increase of acid hydrolysis time, a greater extent of lateral aggregation of CNCs was observed (**Figure 3-4A-E**). The extent of aggregation was higher in 0.01 M NaCl due to the charge screening effect (**Figure 3-4A'-E'**). Large fibril aggregates were also directly

observed in CNCs-40 and CNCs-60 dispersions under an optical microscope (**Figure 3-5**). The DP of CNCs-0 was 140 ± 0 , which was unchanged after 10 min acid hydrolysis but decreased slightly to 133 ± 0 and 132 ± 0 after 20 min and 40 min hydrolysis (**Table 3-2**). This indicated that the fibril length of CNCs was not significantly affected by the acid hydrolysis, which was in agreement with a previous study (Kalashnikova et al., 2012). The morphology of TEMPO-CNFs is shown in **Figure 3-6**. Individual fibrils without severe aggregation were observed for both TEMPO-CNFs-10 and TEMPO-CNFs-6 in water or in 0.01 M NaCl. As shown in **Figure 3-6C, D**, the fibril length distributions of TEMPO-CNFs-10 and TEMPO-CNFs-6 were similar, which agreed with their similar DPs and Z-average hydrodynamic diameters (**Table 3-2**). The heights of TEMPO-CNFs-10 and TEMPO-CNFs-6 from AFM were between 2 nm to 3 nm and were also similar (**Figure 3-7**). In short, CNCs with similar DPs and TEMPO-CNFs with similar fibril lengths but with different SCDs were obtained.

3.4.2 IRI activity of nanocelluloses with varied SCD

The representative polarized light microscopy images of ice wafers are shown in **Figure 3-8**. A smaller size of ice crystals indicates stronger IRI activity. For 3.0 mg/mL CNCs, the increase of acid hydrolysis time resulting in an increase of IRI activity at CNCs-10. After reaching the maximum at CNCs-20, the IRI activity began to decrease with CNCs-40 and became weaker with CNCs-60 (**Figure 3-8B-F**). The Z-average hydrodynamic diameters (**Table 3-2**) demonstrated the severe fibril aggregation of CNCs-

40 and CNCs-60, which led to a decreased number of fibrils available for IRI. As for TEMPO-CNFs, TEMPO-CNFs-6 with a lower carboxylate group density demonstrated stronger IRI activity than TEMPO-CNFs-10 (**Figure 3-8G, H**). The relationship between the concentration and IRI activity of nanocelluloses is plotted in **Figure 3-9**. A smaller value of %MLGS indicates stronger IRI activity. As shown in **Figure 3-9A**, for CNCs-0, the concentration required to obtain a %MLGS < 20% was 5.0 mg/mL. However, the concentrations required to obtain similar % MLGS were 4.0 and 3.0 mg/mL for CNCs-10 and CNCs-20, respectively. Similar results were found for TEMPO-CNFs, where 2.0 mg/mL of TEMPO-CNFs-10 but only 1.5 mg/mL of TEMPO-CNFs-6 was required to obtain %MLGS < 30% (**Figure 3-9B**). These results indicate that lowering the SCD of nanocelluloses can reduce the concentration needed for IRI.

Another experiment was conducted to observe the effect of SCD by decreasing the pH to protonate the carboxylate groups of TEMPO-CNFs-10. By decreasing the pH from 6.0 to 3.5, more carboxylate groups were protonated and an increase of IRI activity was observed (**Figure 3-10A**), despite that the Z-average hydrodynamic diameter was increased due to the decrease of ζ -potential magnitude (**Figure 3-10B**). The IRI activity of PVA in different media decreased in the order: 0.1 M NaOH > 0.1 M NaCl > 0.1 M HCl. Poly(ethylene oxide) and poly(glycerol) were IRI active in 0.1 M NaOH but inactive in 0.1 M NaCl (Burkey, Riley, Wang, Hatridge, & Lynd, 2018). The IRI activity of CNCs-0 in different media also followed the same order: 0.1 M NaOH > 0.1 M NaCl > 0.1 M HCl

(**Figure 3-11**). The mechanism is unclear and may be related to the change of hydrogen bonding environment or surface characteristics of the ice in different media (Burkey, Riley, Wang, Hatridge, & Lynd, 2018). However, the enhancement of IRI activity for TEMPO-CNFs from pH 6.0 to 3.5 was not caused by the change of hydrogen bonding environment or ice surface characteristics because the IRI activity of CNCs-0 was not changed under the same experimental conditions (**Figure 3-12**). A similar observation was made with a cationic peptide (nisin), which became IRI active with a decrease of pH from 7.4 to 5.0 and was explained by a change in conformation due to the protonation of histidine 27 and 31 (Mitchell & Gibson, 2015).

The influence of charged side groups on the IRI activity has been discussed in several polymers (Gibson, Barker, Spain, Albertin, & Cameron, 2009). Neutral polymers do not necessarily have IRI activity: that is, strong IRI activity was found with PVA and poly(L-hydroxyproline), but not with poly(ethylene glycol) (Gibson et al., 2009). However, charged polymers including poly(acrylic acid), poly(2-aminoethyl methacrylate), poly(L-glutamate), and poly(lysine) were found to have weak or no IRI activity (Gibson et al., 2009). Similarly, graphene oxide demonstrated strong IRI activity, but fully carboxyl-functionalized graphene displayed weak IRI activity (Geng et al., 2017). It seems that the IRI activity is weakened by the presence of charged side groups. However, some of these polymers have different backbones and relative conformations, which may contribute to their differences in IRI activity (Gibson et al., 2009). By using the same nanocelluloses

with varied SCDs, the contribution from polymer backbone and conformation can be ruled out. Therefore, our study provides new evidence that IRI is negatively affected by the presence of charged groups. This is in contrast to the case of ions, where ions with higher charge densities have higher IRI activity (S. Wu et al., 2017).

3.4.3 Hydrophobicity of nanocellulose with varied SCD

The hydrophobicity of CNCs at different SCDs were evaluated by the interfacial tension (IFT) measurement, pyrene assay, and aggregation behavior analysis (**Figure 3-13**). The measured IFTs decreased with the increase of measurement time and reached equilibrium at 40 min for most samples (**Figure 3-13A**). CNCs-60 reached equilibrium at 60 min, indicating a slower diffusion rate and a larger size of CNCs-60, which agreed with its size data in **Table 3-2**. All CNCs can reduce the IFT. However, significant IFT reduction was observed only for CNCs-60, which indicated CNCs-60 had the highest hydrophobicity. The results were slightly different in pyrene assay, where a smaller I_1/I_3 value indicates a more hydrophobic microenvironment to trap pyrene (**Figure 3-13B**). Compared to CNCs-0, a decrease of I_1/I_3 value was observed for all CNCs after HCl hydrolysis. The I_1/I_3 values of CNCs-40 and CNCs-60 were higher than that of CNCs-20, which might be explained by the aggregation of CNCs-40 and CNCs-60 that reduced the hydrophobic microenvironment to trap pyrene. While IFT measurement showed that CNCs-60 had the highest hydrophobicity, pyrene assay indicated that CNCs-20 had the highest hydrophobicity. In IFT measurement, the aggregates of CNCs-60 may be restructured and

spread at the dodecane/water interface, which is similar to the conformation changes of proteins after adsorption at interfaces (Roach, Farrar, & Perry, 2005), and thus can effectively reduce the IFT. However, in pyrene assay, there is no interface for CNCs-60 to spread. Therefore, CNCs-60 had lower hydrophobicity than CNCs-20. The hydrophobicity of CNCs was further studied by analyzing the aggregation behavior through a transmittance study, where a decrease of transmittance indicates the formation of aggregates (Fukuzumi et al., 2014). As shown in **Figure 3-13C**, with the increase of HCl hydrolysis time from 0 to 60 min, the decrease of dispersion transmittance started at lower concentrations of CNCs, which can be explained by an increase of hydrophobicity with an increase of hydrolysis time. Although giving different results of the highest hydrophobicity, the IFT measurement, pyrene assay and aggregation behavior analysis demonstrated a trend that the hydrophobicity of CNCs increases with the decrease of SCD. The increase of hydrophobicity with decrease of SCD also can be deduced from the hydrophile-lipophile-balance (HLB) group number of chemical groups. The HLB group numbers for ionized sulfate groups ($-\text{OSO}_3\text{Na}$) and carboxylate groups ($-\text{COONa}$) are 38.7 and 19.1, respectively, whereas they are 1.9 and 2.1 for hydroxyl groups and protonated carboxylate groups, respectively (Davies, 1957). These results indicate that the IRI activity of nanocelluloses is favored by an increase of surface hydrophobicity. The effect of SCD on IRI activity was similar to its effect on emulsion stabilization activity of nanocelluloses. While nanocelluloses with lower sulfate/carboxylate group densities can stabilize

emulsions more effectively (Jia et al., 2016; Kalashnikova et al., 2012), highly charged CNCs cannot stabilize emulsions unless NaCl or KCl is added to screen charges (Kalashnikova et al., 2012). Graphene oxide also stabilizes emulsions more effectively when the carboxylate groups are protonated at pH 2.0 (Kim et al., 2010). Previous studies either identified amphiphilic structures in IRI active materials or synthesized IRI active materials based on amphiphilic structures (Graham et al., 2018; Mitchell et al., 2017; Mitchell & Gibson, 2015; Mizrahy, Bar-Dolev, Guy, & Braslavsky, 2013). Our study provides new evidence supporting the hypothesis that IRI activity is associated with amphiphilic structures. Furthermore, because the SCD of nanocelluloses can be manipulated by varying the production conditions and post-processing (Kalashnikova et al., 2012; Lin & Dufresne, 2014; Shinoda et al., 2012), our results provide useful information in the production of nanocelluloses with enhanced IRI activity.

3.4.4 IRI activity of nanocelluloses with a low SCD at higher unfrozen water contents and annealing temperatures

Enhancement of IRI activity by reducing SCD is beneficial to the practical applications of nanocelluloses. Both CNCs-0 and CNCs-20 demonstrated IRI activity in 7.2 wt.% sucrose solution which contains a similar unfrozen water fraction (6.6%) to PBS. However, CNCs-0 gradually lost IRI activity with increased unfrozen water fractions and became IRI inactive in 15.0 wt.% sucrose solution. In contrast, the IRI activity of CNCs-20 was almost unchanged in solutions with a sucrose concentration increased from 7.2 to 20.0 wt.%

(**Figure 3-14A-%MLGS and Figure 3-15-size of ice crystals**). In 40.0 wt.% sucrose solution, the size of ice crystals in CNCs-0 was similar to that in the blank control, whereas the size of ice crystals in CNCs-20 was much smaller (**Figure 3-14B-D**). Therefore, CNCs-20 even demonstrated observable IRI activity in 40.0 wt.% sucrose solution. The IRI activity of CNCs-0 and CNCs-20 at higher annealing temperatures are shown in **Figure 3-14E**. CNCs-0 lost most of their IRI activity at -4°C and became completely inactive at -2°C, which was in line with our previous study (Li, Zhao, Zhong, & Wu, 2019). In contrast, the IRI activity of CNCs-20 remained unchanged at -4°C and slightly decreased at -2°C. These results indicate that CNCs with a low SCD are more suitable for applications at higher unfrozen water fractions and higher annealing temperatures. TEMPO-CNFs-6/10 were not IRI active at increased unfrozen water fractions or high annealing temperatures (data not shown). The enhancement by decreasing SCD was probably not enough to ensure IRI activity of TEMPO-CNFs at increased unfrozen water fractions or high annealing temperatures.

3.5 Conclusions

Nanocelluloses are biomass derived IRI active materials, which may provide a green and sustainable solution in combating ice recrystallization. The influence of SCD on the IRI activity of nanocelluloses is studied in this paper. CNCs with varied SCDs were prepared by varying the duration of a HCl hydrolysis, which cleaved the surface sulfate groups of CNCs without causing fibril fragmentation. TEMPO-CNFs with varied SCDs

were prepared by varying the amount of NaClO used in the TEMPO-catalyzed oxidation. The fibril lengths of TEMPO-CNFs were controlled by a sonication treatment. Physicochemical and morphological analyses confirmed that the prepared nanocelluloses had similar DPs or fibril lengths but varied SCDs. Reducing the SCD of CNCs without causing severe fibril aggregation led to an enhanced IRI activity. CNCs with a high SCD displayed a little IRI activity in 15-20% sucrose solutions or annealed at -4°C and -2°C. In contrast, CNCs with a low SCD showed observable IRI activity under these conditions. TEMPO-CNFs with a low carboxylate group density demonstrated stronger IRI activity. Lowering pH to protonate the carboxylate groups of TEMPO-CNFs also enhanced the IRI activity. The surface charge effect indicates that the IRI activity of nanocelluloses is associated with their amphiphilicity and higher hydrophobicity leads to an increase of IRI activity until fibril aggregation occurs. Although the exact IRI mechanism of nanocelluloses remains unanswered, the important role of SCD on IRI is identified in the present study, which is useful in the production of nanocelluloses with enhanced IRI activity.

3.6 Acknowledgements

We thank Dr. Douglas Hayes from the Department of Biosystems Engineering and Soil Science on the conductometric titration experiment. We thank Dr. Edward Wright from the Department of Biochemistry & Cellular and Molecular Biology for the use of the

fluorescence spectrometer. We thank Caleb A. Bohannon from the Department of Chemistry for purification of dodecane and water.

3.7 Abbreviations used in this chapter

CNF, cellulose nanofibrils; CNCs, cellulose nanocrystals; TEMPO-CNFs, 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils; SCD, surface charge density; IRI, ice recrystallization inhibition; PVA, polyvinyl alcohol; (%) MLGS, (percentage) mean largest grain size; AFPs, antifreeze proteins; AFGPs, antifreeze glycoproteins; AFM, atomic force microscopy; HLB, hydrophile-lipophile-balance.

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Appendix

Table 3-1. Unfrozen water fractions at different sucrose concentrations for IRI activity assay at -8°C.

Media	Unfrozen water fractions (%)
0.223 M	7.1
7.2 wt. % sucrose	6.6
10.0 wt. % sucrose	9.2
15.0 wt. % sucrose	13.8
20.0 wt. % sucrose	18.5
40.0 wt. % sucrose	36.9

Table 3-2. The DP, Z-average hydrodynamic diameter, and ζ -potential of nanocelluloses.

Values are represented as mean $\pm$ standard deviation from three replicates.

Nanocelluloses	DP	Z-average hydrodynamic diameter in 0.01 M NaCl (nm)	ζ -potential in 0.01M NaCl (mV)
CNCs-0	140 $\pm$ 0	86 $\pm$ 1	-31.0 $\pm$ 0.2
CNCs-10	140 $\pm$ 0	112 $\pm$ 1	-26.1 $\pm$ 1.0
CNCs-20	133 $\pm$ 0	683 $\pm$ 60	-20.0 $\pm$ 1.6
CNCs-40	132 $\pm$ 0	1754 $\pm$ 138	-7.9 $\pm$ 0.1
CNCs-60	132 $\pm$ 0	7952 $\pm$ 857	-5.1 $\pm$ 0.1
TEMPO-CNFs-10	216 $\pm$ 0	253 $\pm$ 20	-37.5 $\pm$ 0.6
TEMPO-CNFs-6	223 $\pm$ 0	255 $\pm$ 14	-34.8 $\pm$ 0.8

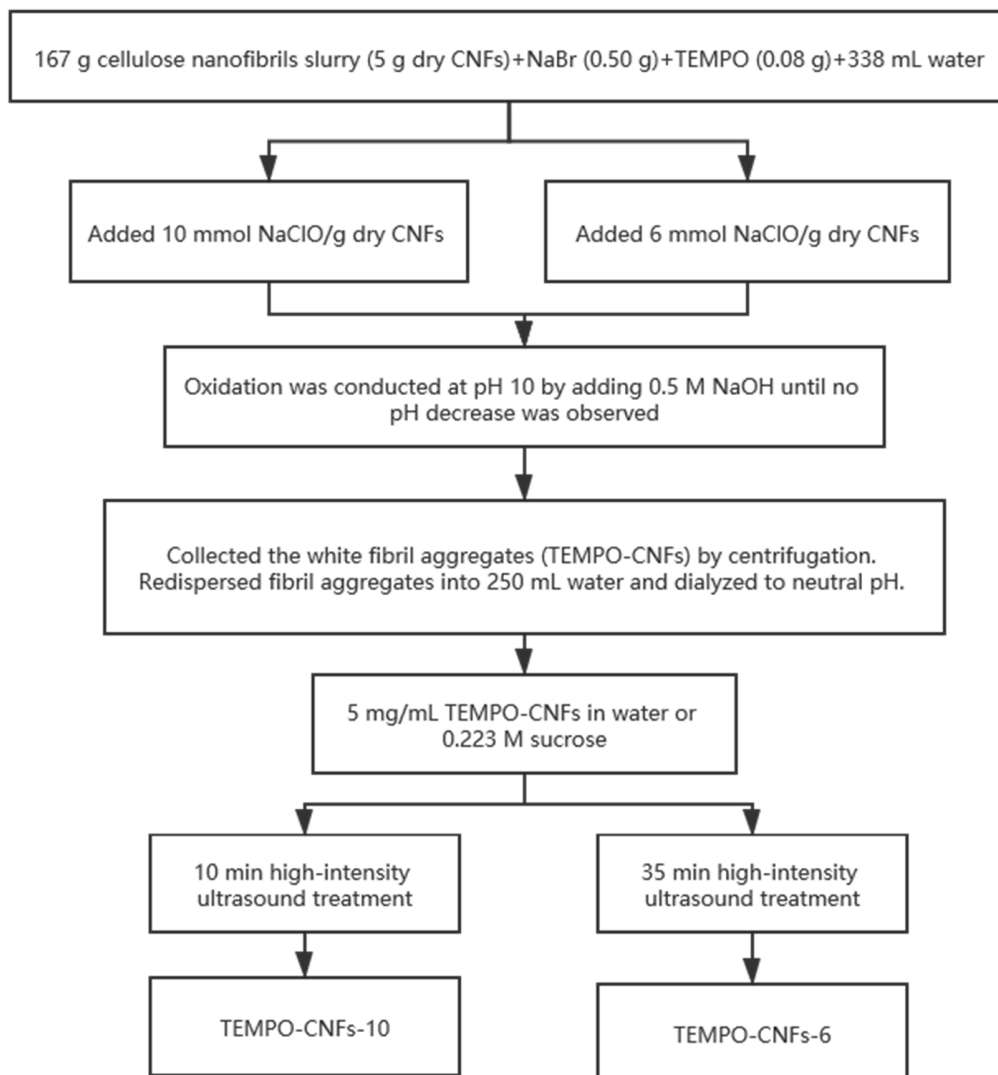


Figure 3-1. The illustrative scheme for the preparation of TMEPO-CNFs-6/10.

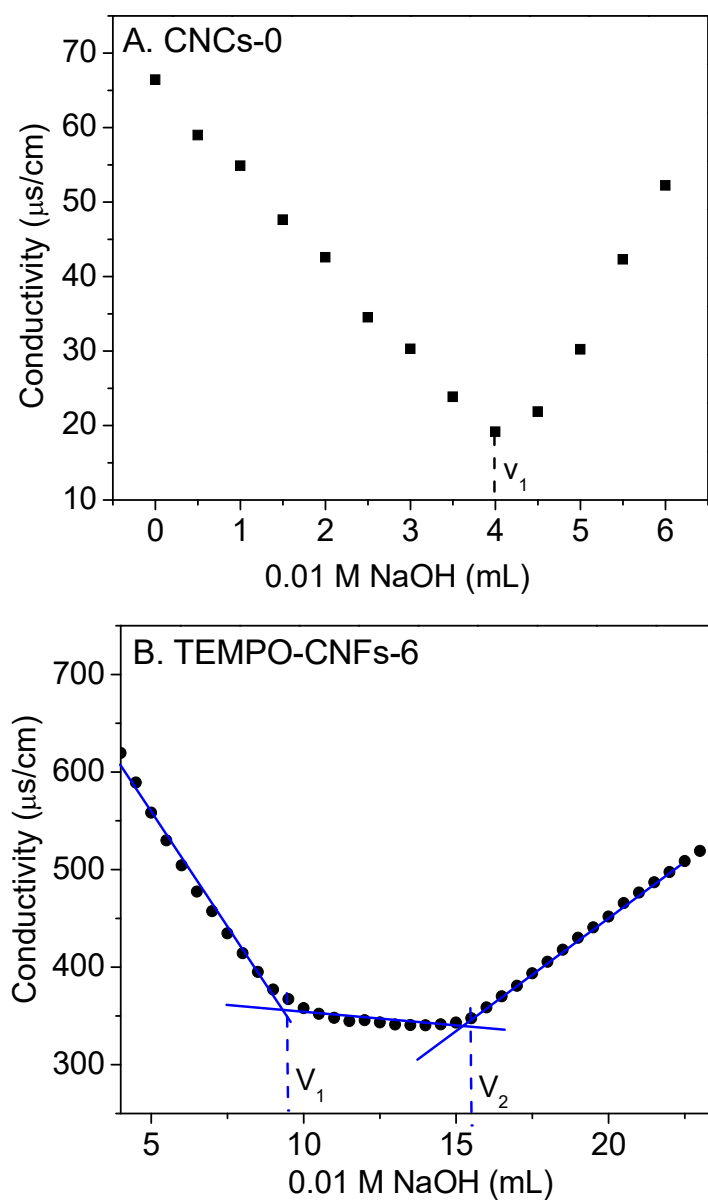


Figure 3-2. Conductometric titration curves of (A) CNCs-0 (100 mL, 1.0 mg/mL) and (B) TEMPO-CNFs-6 (50 mL, 1.0 mg/mL)

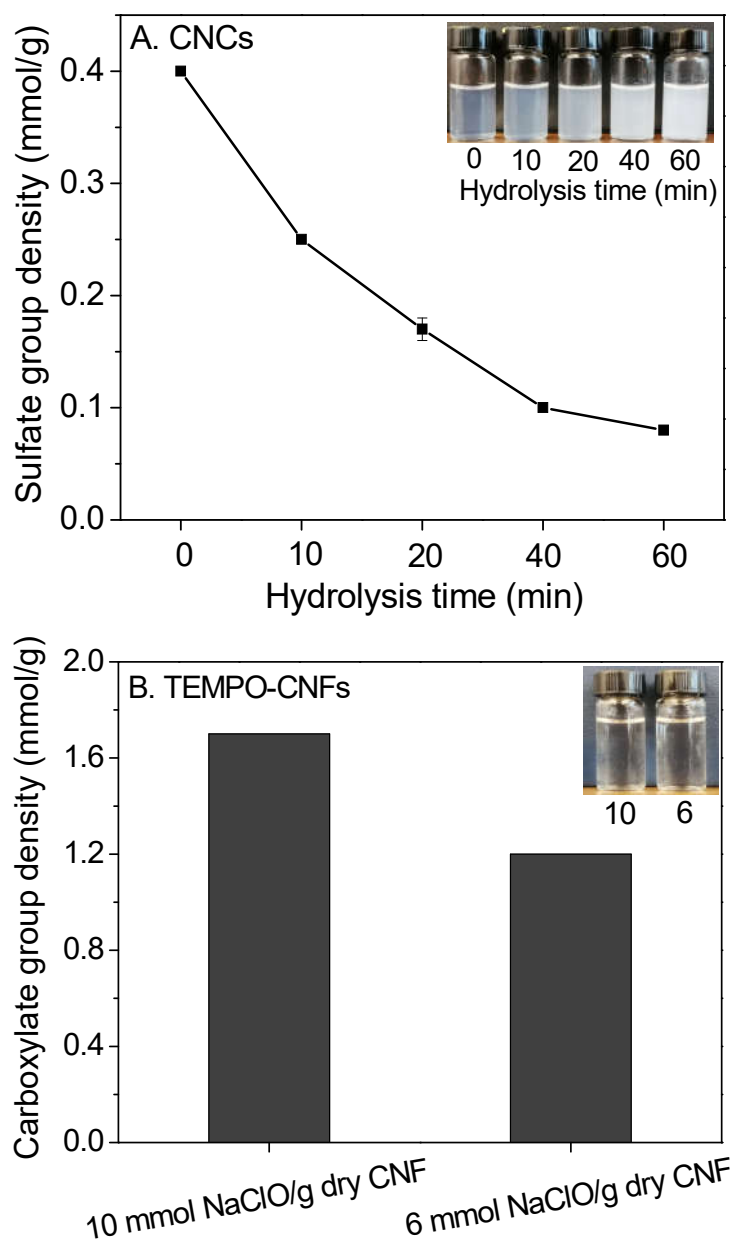


Figure 3-3. (A) Sulfate group densities of CNCs obtained with different HCl hydrolysis time. The inset in A shows 5.0 mg/mL CNCs with different hydrolysis time dispersed in 0.01 M NaCl. (B) Carboxylate group densities of TEMPO-CNFs-10 and TEMPO-CNFs-6. The inset in B shows 2.0 mg/mL TEMPO-CNFs-10 (left) and TEMPO-CNFs-6 (right) dispersed in 0.01 M NaCl.

CNCs in DI water

CNCs in 0.01 M NaCl

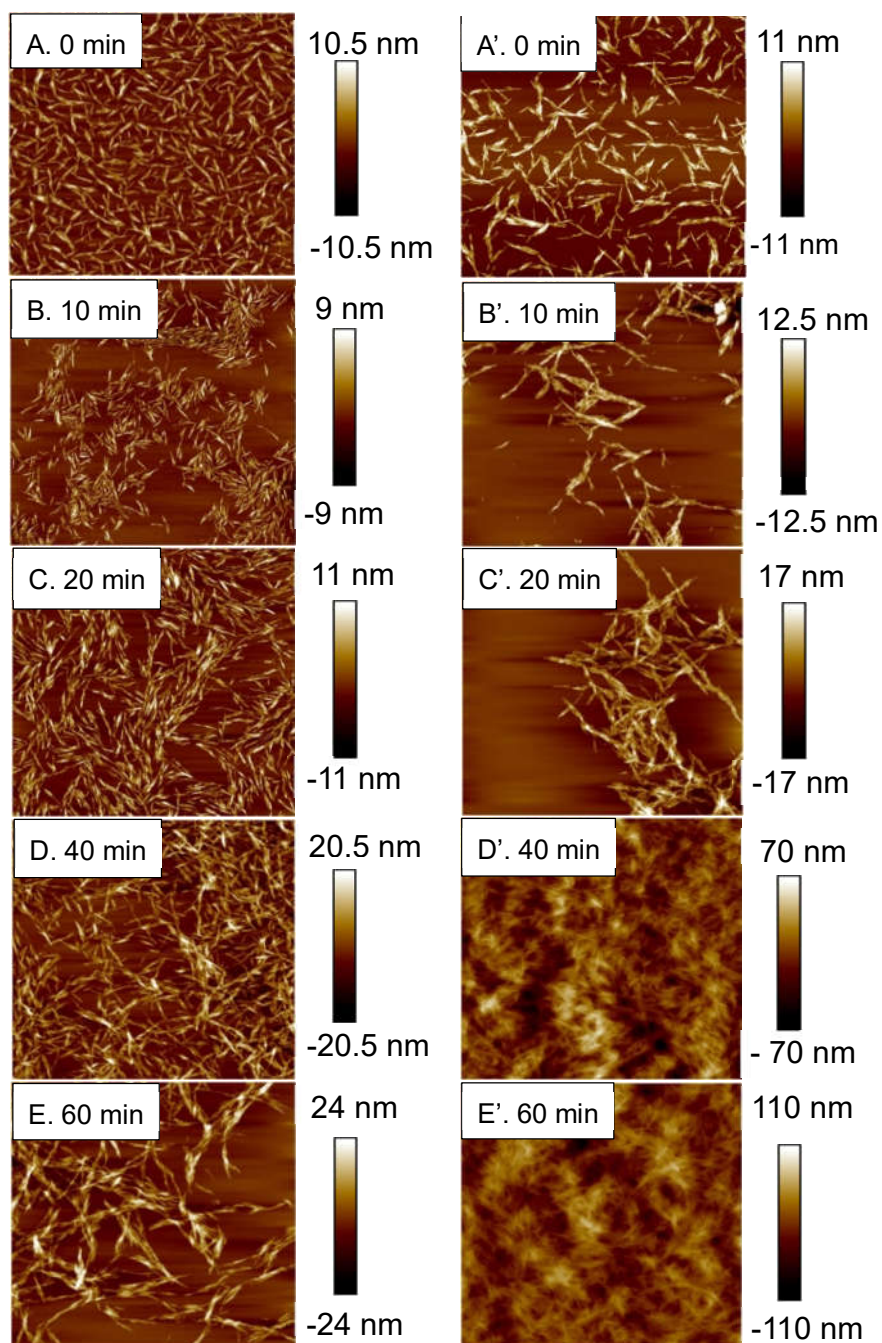


Figure 3-4. Atomic force microscopy images of CNCs after HCl hydrolysis for 0-60 min.

(A-E) CNCs (1.0 mg/mL) were dispersed in DI water or (A'- E') 0.01 M NaCl. The image dimension is 5 μm $\times$ 5 μm

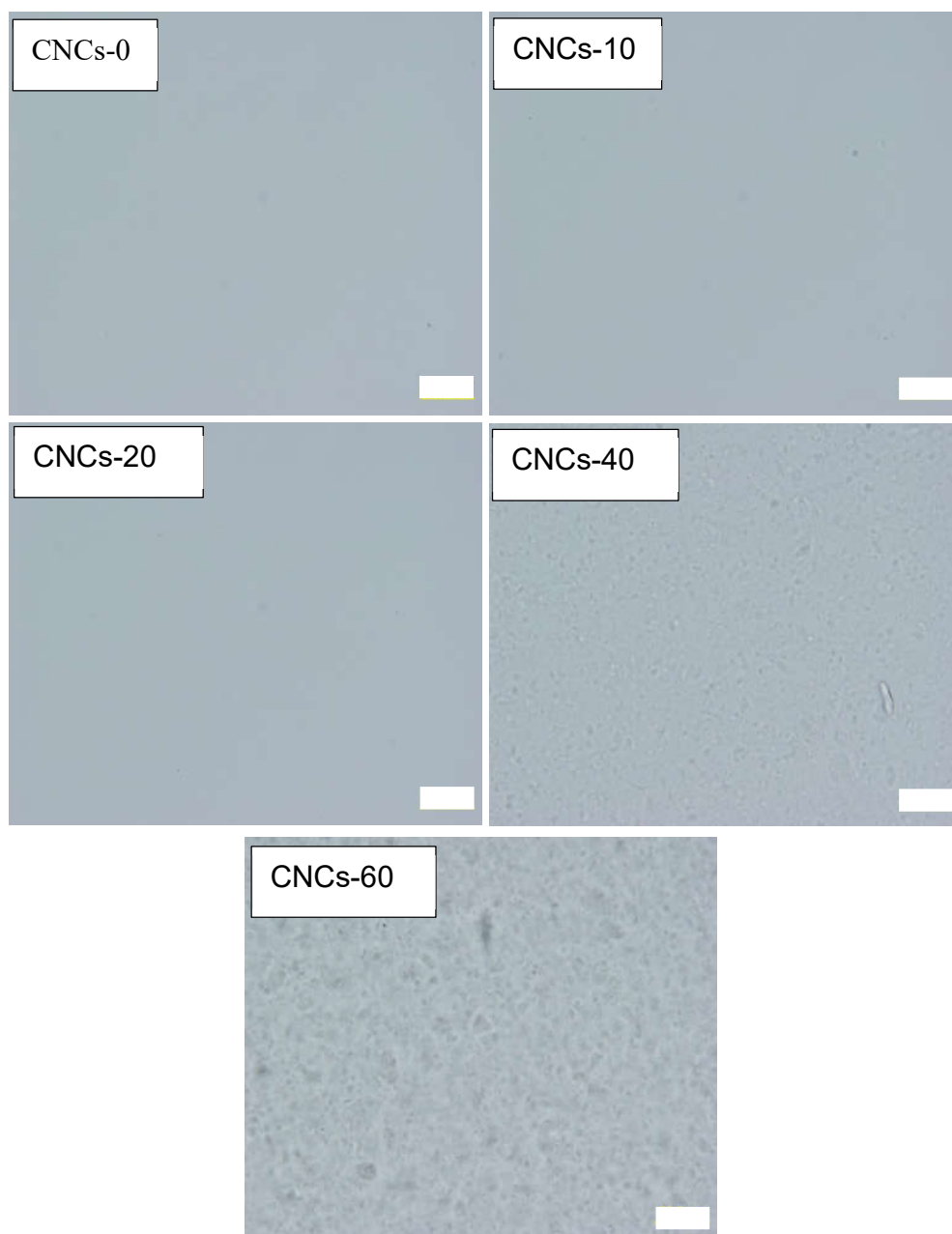


Figure 3-5. Optical microscope images of CNCs dispersions after HCl hydrolysis for 0-60 min. CNCs were dispersed in 0.01 M NaCl at 10.0 mg/mL. Scale bar = 50 μ m.

TEMPO-CNFs in DI water

TEMPO-CNFs in 0.01 M NaCl

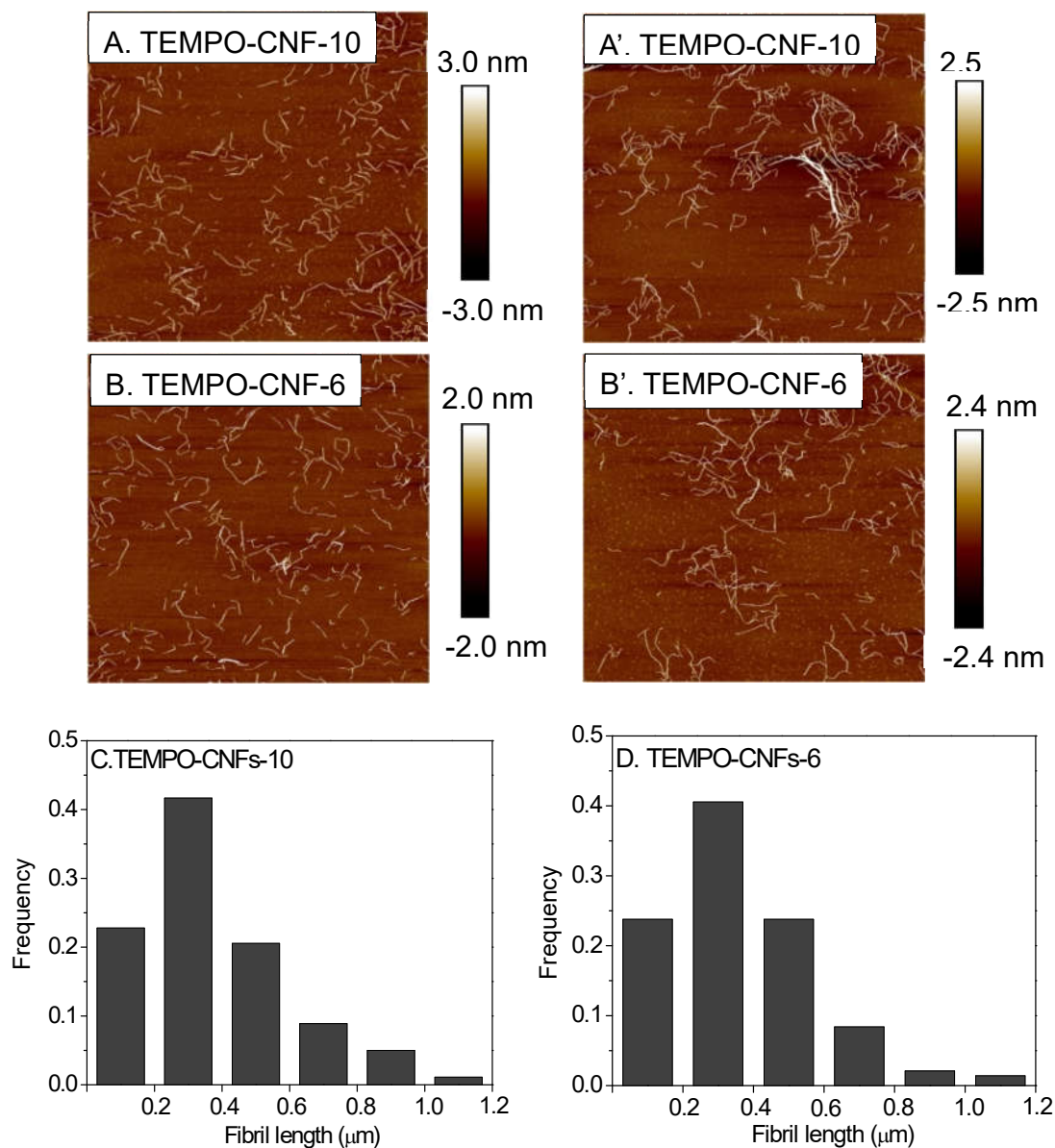


Figure 3-6. Atomic force microscopy images of TEMPO-CNFs-10 and TEMPO-CNFs-6.

The image dimension is $5 \mu\text{m} \times 5 \mu\text{m}$. TEMPO-CNFs (0.05 mg/mL) were dispersed in DI water (A and B) or 0.01 M NaCl (A' and B'). Fibril length distribution analysis of (C) TEMPO-CNFs-10 and (D) TEMPO-CNFs-6.

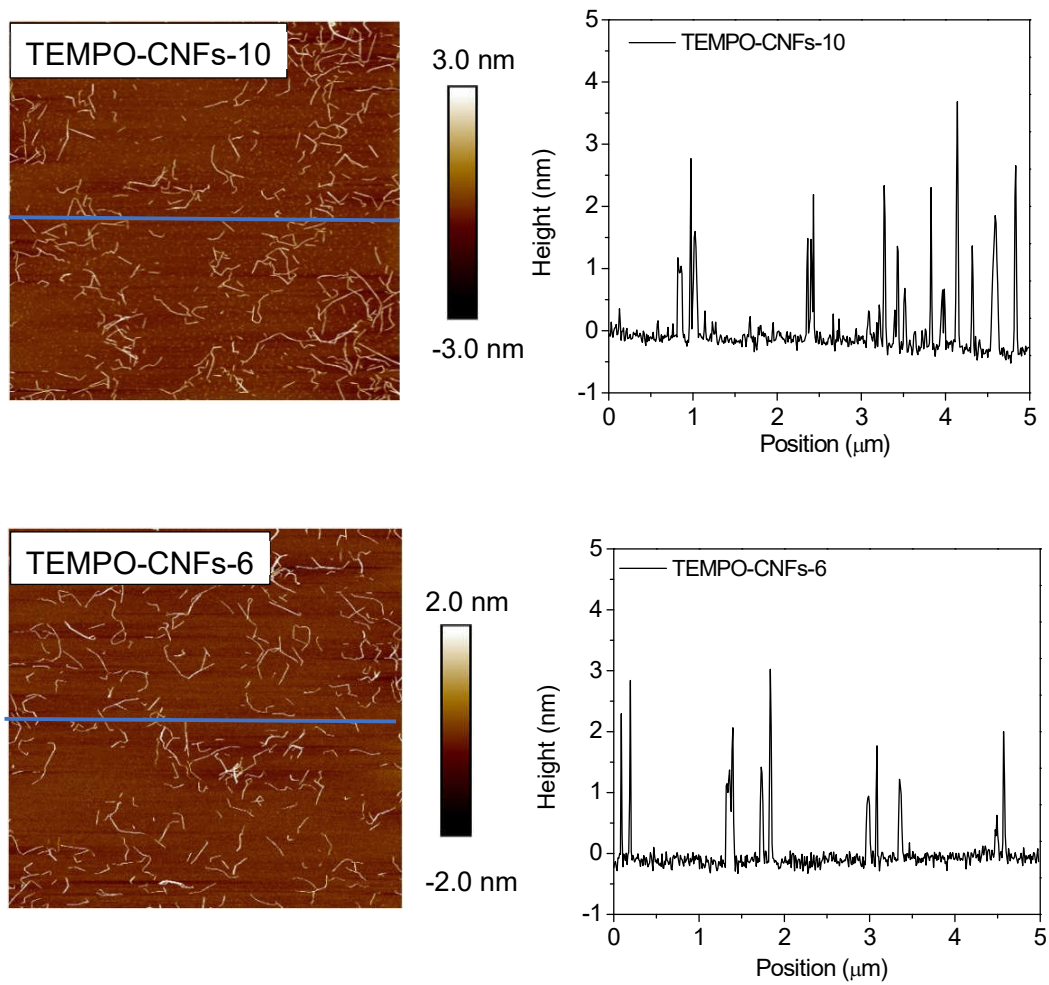


Figure 3-7. Height analysis of TEMPO-CNFs-10 and TEMPO-CNFs-6.

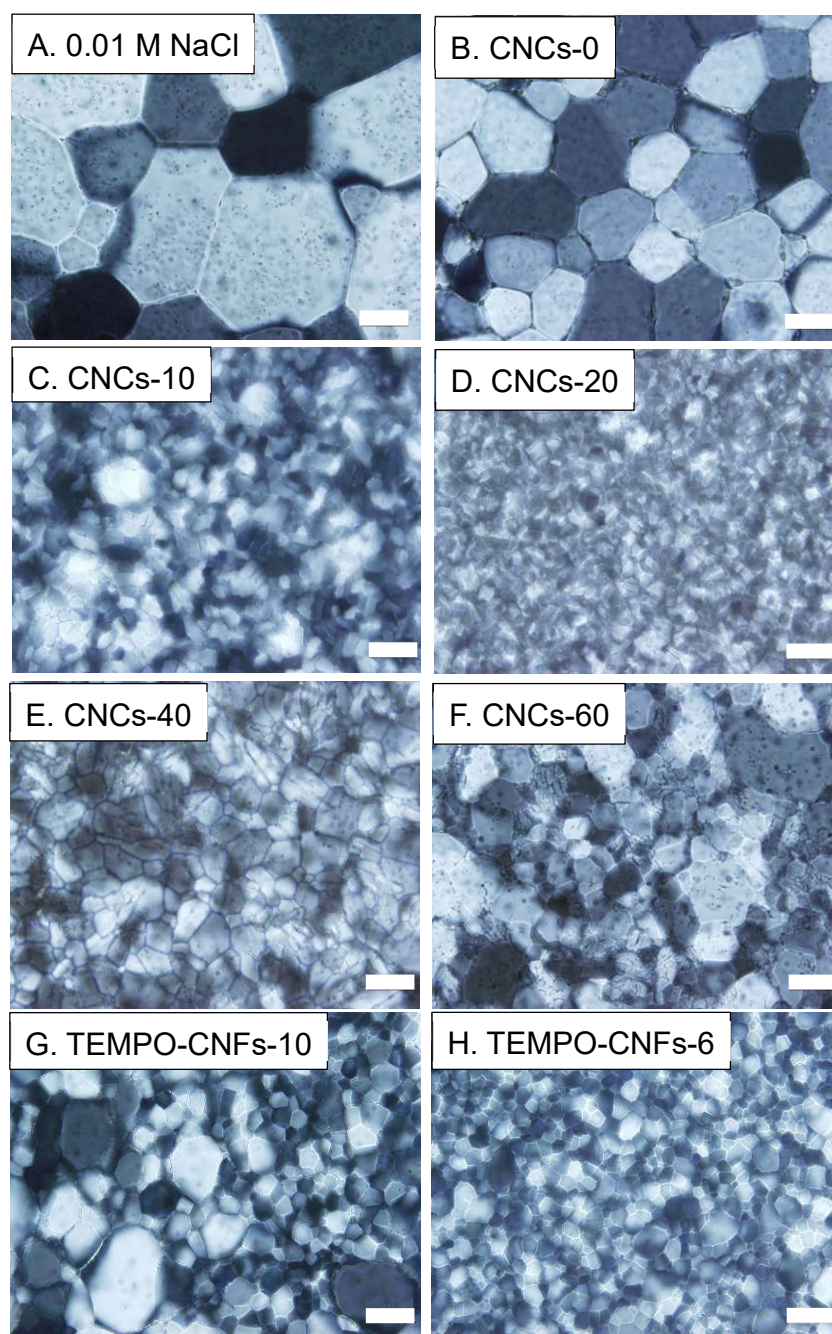


Figure 3-8. Polarized light microscopy images of ice wafers after 30 min annealing at -8°C . CNCs (3.0 mg/mL) and TEMPO-CNFs (1.5 mg/mL) were dispersed in 0.01 M NaCl. Scale bar = 50 μm .

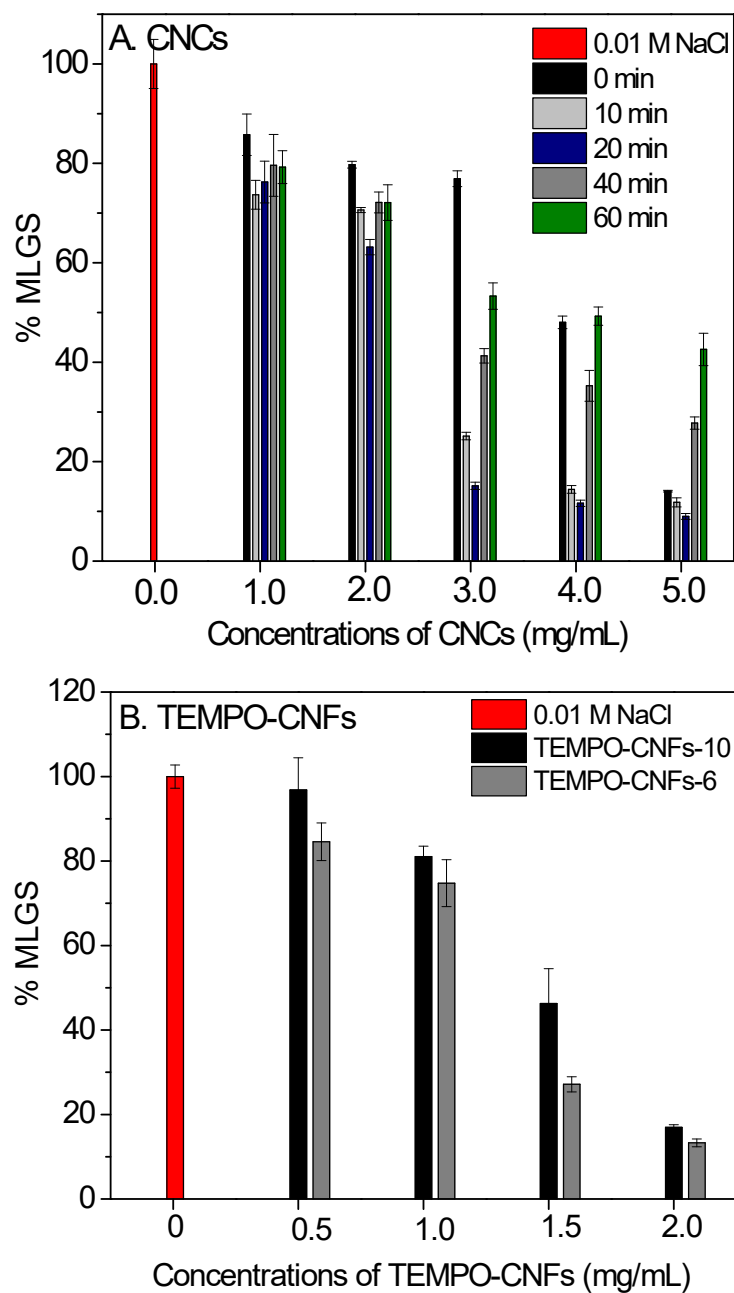


Figure 3-9. IRI activity of (A) CNCs hydrolyzed by HCl for 0-60 min and (B) TEMPO-CNFs with different carboxylate group densities. The 0.01 M NaCl control is the medium used to disperse nanocelluloses. Values are represented as mean $\pm$ standard deviation from three ice wafers.

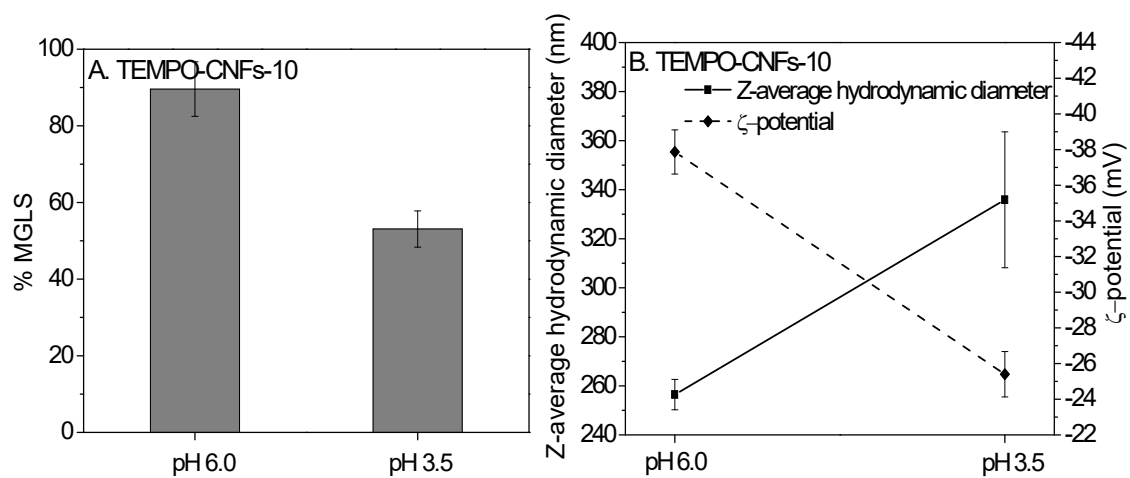


Figure 3-10. (A) IRI activity of TEMPO-CNFs-10 (5.0 mg/mL) at pH 6.0 and pH 3.5 in 0.223 M sucrose; (B) Z-average hydrodynamic diameter and ζ -potential of TEMPO-CNFs-10 (0.5 mg/mL) at pH 6.0 and pH 3.5. Values are represented as mean $\pm$ standard deviation from three replicates.

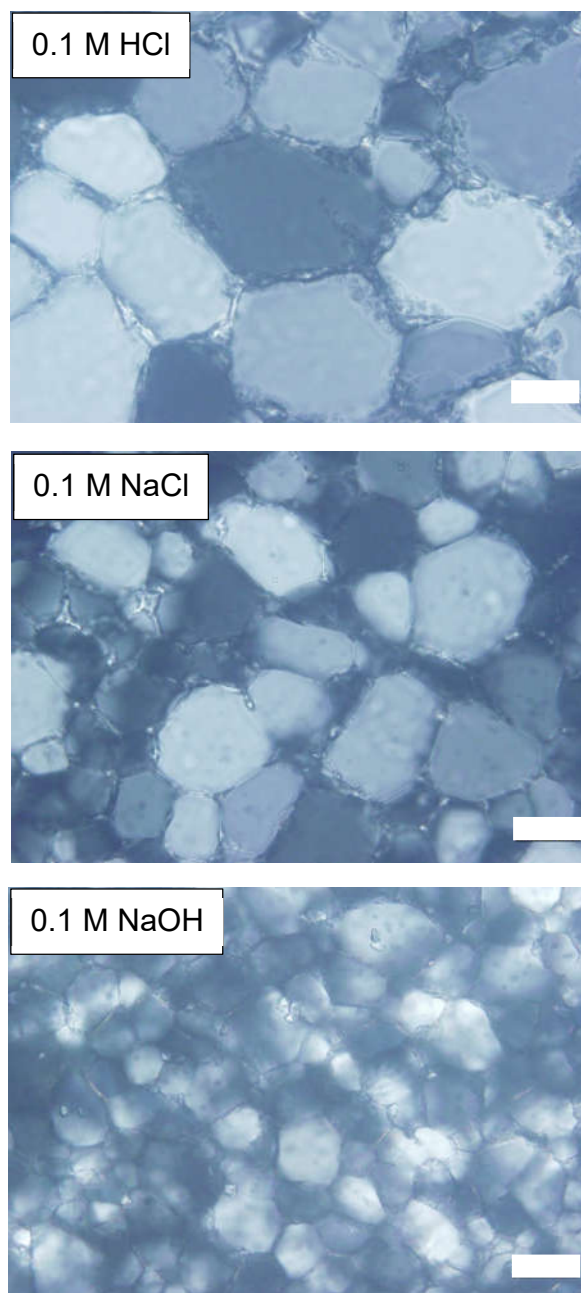


Figure 3-11. Polarized light microscopy images of ice wafers after 30 min annealing at -8°C. 10.0 mg/mL CNCs-0 were dispersed in 0.1 M HCl, 0.1 M NaCl and 0.1 M NaOH. Scale bar = 50 μm.

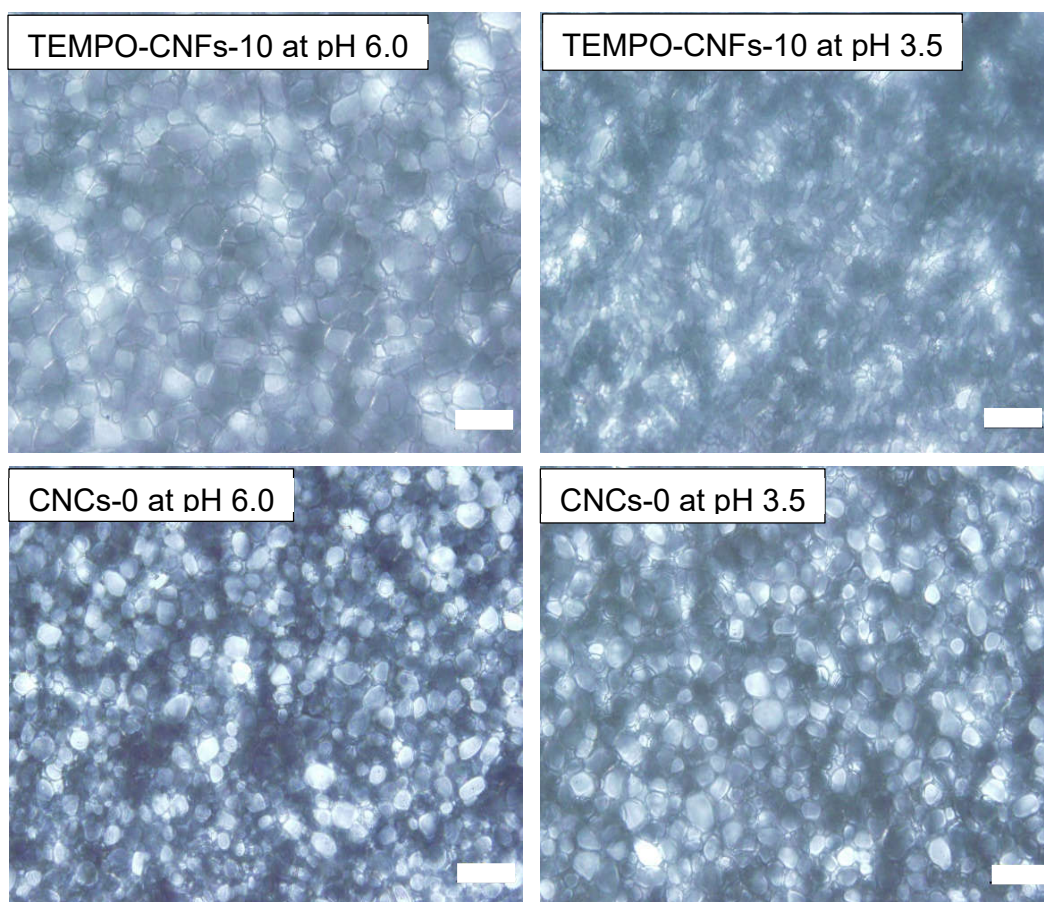


Figure 3-12. Polarized light microscopy images of ice wafers after 30 min annealing at -8°C. 5.0 mg/mL TEMPO-CNFs-10 and CNCs-0 were dispersed in 0.223 M sucrose at pH 6.0 or 3.5. Scale bar = 50 μm .

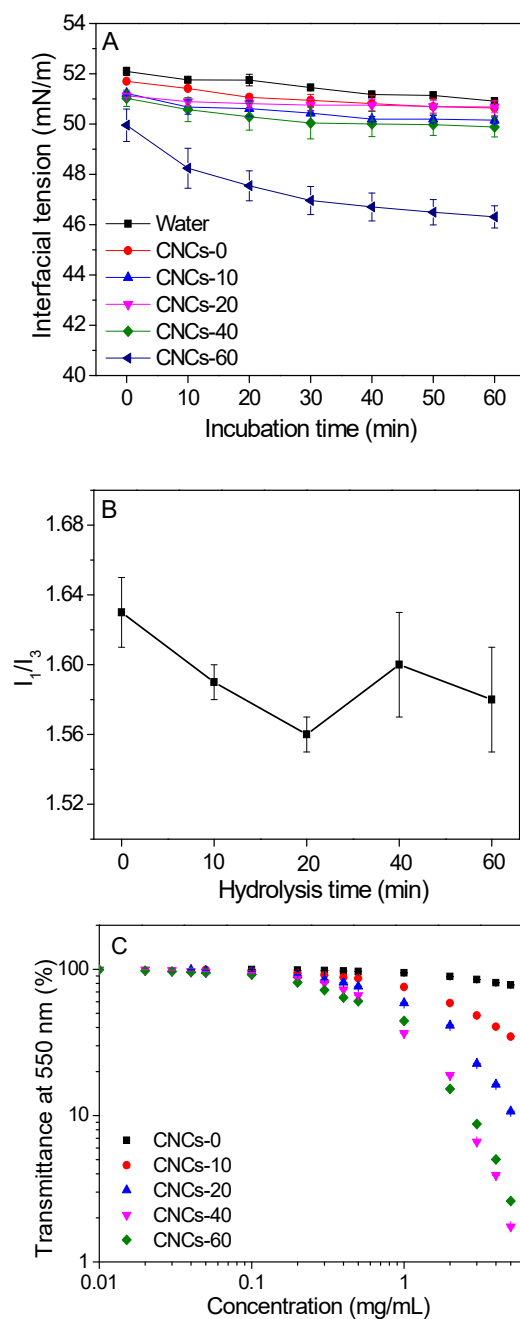


Figure 3-13. Hydrophobicity of CNCs. (A) Dodecane/water interfacial tensions; (B) I_1/I_3 peak intensity ratios in pyrene assay, and (C) aggregation behavior analysis of CNCs with different SCDs. Values are represented as mean $\pm$ standard deviation from three measurements.

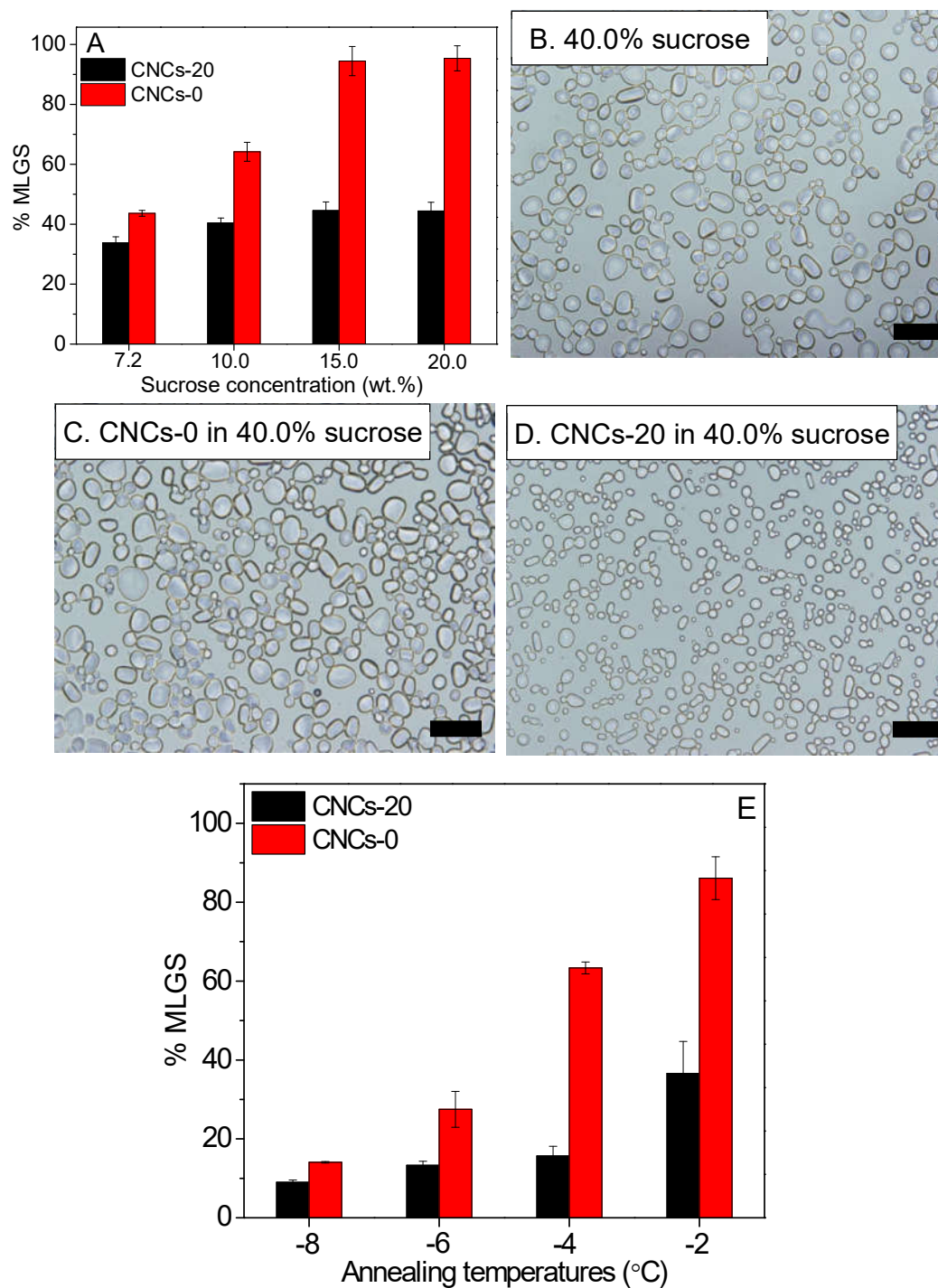


Figure 3-14. The IRI activity of CNCs-0 and CNCs-20 at different unfrozen water fractions after 60 min annealing at -8°C: (A) the %MLGS at sucrose concentrations from 7.2 to 20.0 wt.%; (B) the optical microscopy images of 40.0 wt.% sucrose control, (C) CNCs-0, and

(D) CNCs-20 dispersed in 40.0 wt.% sucrose. The concentration of CNCs was 10.0 mg/mL in all treatments. Scale bar = 50 μ m. (E) The %MLGS of CNCs-0 and CNCs-20 at different annealing temperatures. Both CNCs-0 and CNCs-20 were dispersed in 0.01 M NaCl at 5.0 mg/mL. The %MLGS are represented as mean $\pm$ standard deviation from three ice wafers.

Figure 3-14 continued

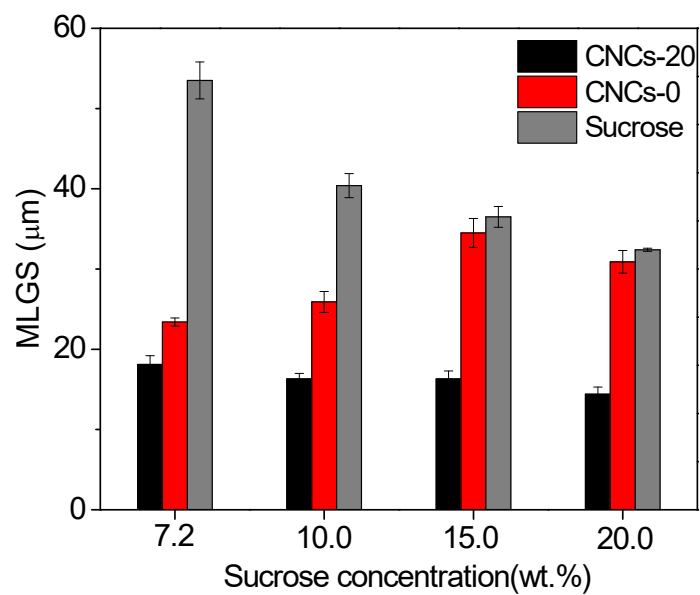


Figure 3-15. Mean largest grain size of 10.0 mg/mL CNCs-20 and CNCs-0 dispersed in different sucrose solutions after 60 min annealing at -8°C . Values are represented as mean $\pm$ standard deviation from three ice wafers.

Chapter 4. Effect of fibril length on the ice recrystallization inhibition activity of nanocelluloses

A version of this chapter was originally published by Teng Li, Min Li, Qixin Zhong, and Tao Wu.

Li, T., Li, M., Zhong, Q., & Wu, T. (2020). Effect of Fibril Length on the Ice Recrystallization Inhibition Activity of Nanocelluloses. *Carbohydrate Polymers*, 116275.

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Min Li: Writing - Review & Editing

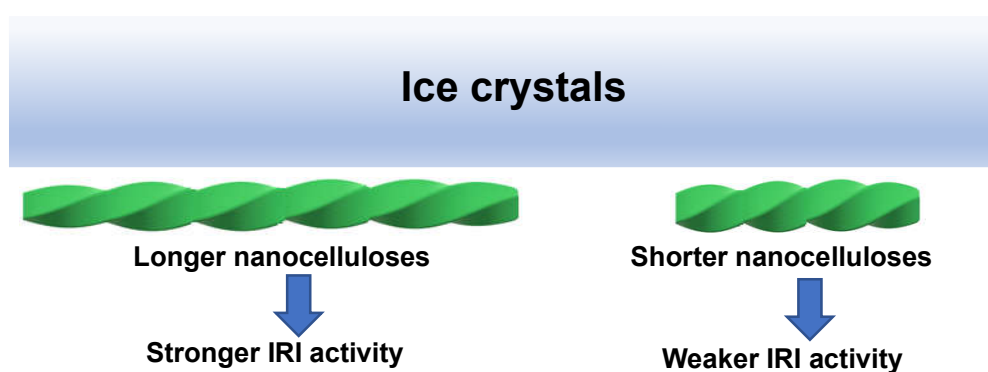
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Tao Wu: Conceptualization, Methodology, Resources, Writing - Review & Editing,

Supervision, Project administration, Funding acquisition

4.1 Abstract

Ice recrystallization inhibition (IRI) activity has been recently discovered on nanocelluloses, which enables potential applications in several fields. This study focused on the effect of fibril length on the IRI activity of nanocelluloses. The 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils (TEMPO-CNFs) with similar surface charge densities (SCDs) and fibril widths, but with different fibril lengths were prepared by sonication treatment. The IRI activity of nanocelluloses was enhanced in TEMPO-CNFs receiving sonication less than 30 min because of the reduction of fibril aggregation. Further increase of sonication time to 60 and 120 min led to a decreased IRI activity due to the reduction of fibril lengths. Our results indicate longer nanocelluloses are more IRI active. The IRI activity was not correlated with the viscosity or gelling properties of nanocellulose dispersions. Research findings are useful in producing nanocelluloses with enhanced IRI activity and in understanding the underlying IRI mechanism.



Keywords: Nanocelluloses; Fibril length; Ice recrystallization inhibition

4.2 Introduction

Cryopreservation is used in the long-term storage of foods, cells, tissues, and organs (Coombs, Holman, Friend, & Hopkins, 2017; Elliott, Wang, & Fuller, 2017). During storage, ice recrystallization leads to the formation of large ice crystals, which are associated with reduced post-thaw cell recovery rates and quality defects of frozen foods (Adapa, Schmidt, Jeon, Herald, & Flores, 2000; Deller, Vatish, Mitchell, & Gibson, 2014). Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs) can bind to a certain or multiple ice planes and exhibit potent ice recrystallization inhibition (IRI) activity (Rahman et al., 2019; Sidebottom et al., 2000). However, large-scale applications of AF(G)Ps are limited by their high production cost. Therefore, many research efforts have been directed to explore inexpensive ice recrystallization inhibitors as AF(G)Ps substitutes (Biggs et al., 2017; He, Liu, & Wang, 2018).

Recently a facial amphiphilic structure with segregated display of hydrophobic and hydrophilic groups has been proposed to be crucial to the antifreeze activity of AF(G)Ps, where hydrophobic groups bind to ice, and the hydrophilic part provides aqueous solubility (Haymet, Ward, & Harding, 1999; Mochizuki & Molinero, 2018; Tachibana et al., 2004; Zanetti-Polzi, Biswas, Del Galdo, Barone, & Daidone, 2019). Several amphiphilic materials such as nisin, zirconium acetate, zirconium acetate hydroxide, and nanofibers self-assembled from safranin O have shown IRI activity (Drori et al., 2016; Mitchell & Gibson, 2015; Mizrahy, Bar-Dolev, Guy, & Braslavsky, 2013). Other studies reported

potent IRI activity in synthesized amphiphilic materials including metallohelices and glycopolymers (Graham, Fayter, Houston, Evans, & Gibson, 2018; Mitchell et al., 2017).

Nanocelluloses are biomass-based materials with various potential applications (Gomez et al., 2016; Markstedt et al., 2015). The facially amphiphilic structure of nanocelluloses has been inferred from their ability to stabilize emulsions (Jimenez Saelices & Capron, 2018; Kalashnikova, Bizot, Cathala, & Capron, 2011; Kalashnikova, Bizot, Cathala, & Capron, 2012). It has been proposed that the (200) crystalline planes of nanocelluloses mostly displaying CH groups are hydrophobic while other planes rich in hydroxyl groups are hydrophilic (Bruehl, Tavares, Carreau, & Heuzey, 2019; Kalashnikova et al., 2012). Based on these findings, we hypothesized that nanocelluloses are IRI active, which has been proved in our previous study showing the effectiveness of 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils (TEMPO-CNFs) and cellulose nanocrystals (CNCs) in inhibiting ice recrystallization (Li, Zhao, Zhong, & Wu, 2019).

Elucidating the structure-IRI activity relationships is important to the rational design of ice recrystallization inhibitors and understanding the underlying IRI mechanisms. Significant research efforts have been directed to understand the structure-IRI activity relationships of AF(G)Ps, PVA, and low molecular weight compounds (Balcerzak, Ferreira, Trant, & Ben, 2012; Capicciotti et al., 2012; Congdon, Notman, & Gibson, 2013; Deller, Carter, Zampetakis, Scarpa, & Perriman, 2018). Surface charge density (SCD) and fibril length are two of the most important structural parameters of nanocelluloses. Our previous

study has demonstrated that the IRI activity of nanocelluloses is enhanced by reducing SCD until fibril aggregation occurs (Li et al., 2020). Similar to the SCD, fibril length is a significant parameter determining the emulsion stabilization ability of nanocelluloses (Kalashnikova, Bizot, Bertoncini, Cathala, & Capron, 2013). Particularly, longer nanocelluloses result in a less dense surface coverage on emulsion droplets and thus can stabilize emulsion with a smaller nanocellulose : oil ratio (Kalashnikova et al., 2013). Considering the potential similar role of amphiphilicity in emulsion stabilization and IRI, we hypothesize fibril length also plays an important role in the IRI activity of nanocelluloses.

In this study, nanocelluloses with similar SCDs and fibril widths, but with varied fibril lengths were prepared by sonication, characterized for physicochemical properties by atomic force microscope and other tools, and compared for IRI activity by the splat assay. In addition to mechanistic understanding the IRI activity of nanocelluloses, findings of this study are important to produce nanocelluloses with enhanced IRI activity through controlling material morphology.

4.3 Materials and methods

4.3.1 Materials

Commercial cellulose nanofibrils (CNFs), 3.0 wt.% in the slurry form, were purchased from the Process Development Center at the University of Maine (Orono, ME) and were used as received. Physicochemical characteristics of CNFs were reported in our previous

publication (Li et al., 2019). Polyethylene glycol (PEG) with a number-average molecular weight of 8,000 Da and other chemicals were purchased from Thermal Fisher Scientific (Waltham, MA). De-ionized water was used throughout the experiments.

4.3.2 Preparation of TEMPO-CNFs with similar SCDs and varied fibril lengths

TEMPO-CNFs were prepared with TEMPO-catalyzed oxidation of commercial CNFs according to a literature method (Shinoda, Saito, Okita, & Isogai, 2012). Briefly, 167 g CNFs slurry was mixed with 0.50 g NaBr and 0.08 g TEMPO in 338 mL water. Then NaClO was added at a level of 10.0 mmol/g dry CNFs. The mixture was stirred at 22°C with pH maintained at 10.0 ± 0.2 by adding 0.5 M NaOH until no pH decrease was observed. After oxidation, the TEMPO-CNFs (white fibril aggregates) were collected by centrifugation at 10,000 g and 4°C for 10 min with a Sorvall RC5B-Plus centrifuge using a SS 34 centrifuge rotor (Thermo Fisher Scientific, Waltham, MA). TEMPO-CNFs were re-dispersed in 250 mL water and dialyzed against excessive water to neutral pH at 4°C in a dialysis tubing with a molecular weight cut-off of 6,000-8,000 Da (Thermo Fisher Scientific, Waltham, MA). The fibril length of TEMPO-CNFs was reduced by a high-intensity ultrasound (VCX- 750, Sonics and Materials, Newton, CT) treatment (Zhou, Saito, Bergstrom, & Isogai, 2018). TEMPO-CNFs were dispersed in water at 5.0 mg/mL in a 100 mL beaker and sonicated at 60 % pressure amplitude for 1, 5, 10, 30, 60, and 120 min in a pulse mode (59 s on and 59 s off). During sonication, the beaker was placed in an ice-water

bath to avoid excessive heating. The obtained TEMPO-CNFs were labeled as TEMPO-CNFs-X, with X being the sonication time in min.

4.3.3 Transmittance of TEMPO-CNFs dispersions

TEMPO-CNFs were dispersed in 0.01 M NaCl at a final concentration of 1.0 mg/mL and pH adjusted to 6.0 using 0.1 N HCl. The % transmittance of dispersions was measured at 550 nm with an Evolution 201 UV-VIS spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

4.3.4 Z-average hydrodynamic diameter and polydispersity index (PDI)

TEMPO-CNFs were dispersed in 0.01 M NaCl at a final concentration of 0.5 mg/mL and pH adjusted to 6.0 using 0.1 N HCl. The Z-average hydrodynamic diameter and PDI were measured using a dynamic light scattering (DLS) system (Nano ZS, Malvern Instruments, Worcestershire, UK) as in our earlier study (Li et al., 2019).

4.3.5 Observation of fibril aggregates in TEMPO-CNFs-0.

TEMPO-CNFs-0 were dispersed in 0.01 M NaCl at 5.0 mg/mL. A 10 μ L dispersion was deposited on a microscope glass slide and covered by a cover slide. The polarized image was taken by a polarized light microscope (BX51, Olympus, Tokyo, Japan) with a built-in digital camera (DP 70, Olympus, Tokyo, Japan) under 100 $\times$ magnification.

4.3.6 Morphological analysis of TEMPO-CNFs

The morphology of TEMPO-CNFs was analyzed using atomic force microscopy (AFM; model Multimode VIII, Bruker Corp., Santa Barbara, CA). Briefly, TEMPO-CNFs

were dispersed in 0.01 M NaCl at a final concentration of 0.05 mg/mL and pH adjusted to 6.0 using 0.1 N HCl. Then 30.0 μ L of the dispersion was spin-coated on a fresh peeled mica surface, followed by air drying for 2 h before scanning. AFM images were acquired using a ScanAsyst-AIR probe (maximum tip radius 12 nm) in the ScanAsyst-Air mode according to a reference method (Li et al., 2019). Images were flattened using the Nanoscope Analysis software (Bruker Corp, Santa Barbara, CA). The number frequency distribution of fibril length was analyzed using the ImageJ software (National Institutes of Health, Bethesda, MD). At least 150 individual fibrils were used in the analysis and the length-weighted average length (L) was calculated with the following Eq (1):

$$L = \frac{\sum_{i=1}^n N_i L_i^2}{\sum_{i=1}^n N_i L_i} \quad (1)$$

where N_i is the number of fibrils with length L_i . Because the radius of AFM tip used was larger than the fibril width, the fibril width determined by AFM represented the tip geometry rather than the fibril width (Arcari et al., 2019). Therefore, the fibril widths were measured from the fibril height on AFM images (Zhou et al., 2018). The height of individual fibrils was analyzed by the Nanoscope Analysis software and at least 50 individual fibrils were measured to calculate the height-weighted average height (H) using Eq (2):

$$H = \frac{\sum_{i=1}^n N_i H_i^2}{\sum_{i=1}^n N_i H_i} \quad (2)$$

Where N_i is the number of fibrils with height H_i . Length/height-weighted average values were calculated to reflect the impact of fibrils with long lengths and/or large heights.

4.3.7 Degree of polymerization (DP) of TEMPO-CNFs

The relative viscosity (η_{rel}) of nanocellulose-cupriethylenediamine hydroxide (CED) solutions was measured using an AVS 370 Ubbelohde capillary viscometer (SI Analytics, Weilheim, Germany) with a capillary diameter of 0.84 mm. The C6-aldehyde groups of TEMPO-CNFs were reduced to hydroxyl groups by NaBH_4 before viscosity measurements according to a referenced method (Shinoda et al., 2012). Lyophilized nanocelluloses were dissolved in 0.5 M CED at 2.0 mg/mL. The temperature of viscometer and samples was precisely controlled at $25 \pm 0.1^\circ\text{C}$ using a Schott CT 52 water bath (SI Analytics, Weilheim, Germany). The efflux time of nanocelluloses-CED solutions and 0.5 M CED was recorded. The η_{rel} was calculated from Eq (3):

$$\eta_{\text{rel}} = t_s / t_0 \quad (3)$$

where t_s and t_0 are the efflux time of nanocellulose-CED solutions and 0.5 M CED, respectively. According to the obtained η_{rel} , the product of intrinsic viscosity and nanocellulose concentration ($[\eta] \times C$) was extracted from a predetermined table in the ISO 5351:2010 standard, and the DP of nanocelluloses was calculated from Eq (4) (Shinoda et al., 2012):

$$DP = 1.75 \times [\eta] \quad (4)$$

4.3.8 Surface charge density of nanocelluloses

The SCD of nanocelluloses was measured by a conductometric titration (Saito & Isogai, 2004). The pH of TEMPO-CNFs dispersion (1.0 mg/mL, 50 mL) was adjusted to

2.5 using 1.0 N HCl and stirred for 10 min to protonate carboxylate groups on nanocelluloses. Then the dispersion conductivity during titration with 0.01 M NaOH was monitored with a YSI 3200 conductometric meter using a 3256 cell (YSI Incorporated, Yellow Springs, OH). The SCD of TEMPO-CNFs was calculated as the carboxylate group density using Eq (5):

$$\text{Carboxylate group density } \left(\frac{\text{mmol}}{\text{g}} \right) = \frac{0.01 \times (V_2 - V_1)}{m_{\text{TEMPO-CNFs}}} \quad (5)$$

As shown in **Figure 4-1**, V_1 and V_2 are the volume of NaOH at the first and second equivalent points in titration curve, respectively, and $m_{\text{TEMPO-CNFs}}$ is the mass of TEMPO-CNFs (0.05 g).

4.3.9 IRI activity of nanocelluloses

The IRI activity of TEMPO-CNFs was determined with the splat assay (Knight, Hallett, & Devries, 1988). TEMPO-CNFs were dispersed in 0.01 M NaCl with pH adjusted to 6.0 using 0.1 N HCl. PEG was dissolved in 0.01 M NaCl at 2.0 mg/mL with pH adjusted to 6.0 using 0.1 N HCl and used as a negative control. A sample droplet (ca. 10 μL) was dropped from a 1.4 m height above a glass slide chilled with dry ice using a 3 mL syringe (Becton & Dickinson., Franklin Lakes, NJ). An ice wafer was formed instantly when the droplet hit the chilled glass slide. Then the glass slide was quickly transferred into an HCS 302 cryostage (Instec Instruments, Boulder, CO) and incubated at -8.0°C under the purge of N_2 for 30 min. Three images from each ice wafer were obtained using a polarized light microscope (BX51, Olympus, Tokyo, Japan) with a built-in digital camera (DP 70,

Olympus, Tokyo, Japan) under a 400 $\times$ magnification. Ten largest ice crystals in each image were measured for the single largest length in any axis. The average of the single largest length was calculated from two randomly selected images of each ice wafer. The mean largest grain size (MLGS) was calculated from three independent ice wafers. The %MLGS was obtained by dividing the MLGS of a sample to that of 0.01 M NaCl.

4.3.10 Apparent viscosity, storage, and loss moduli of TEMPO-CNFs dispersions before and after freezing-thawing treatment

TEMPO-CNFs after sonication for different durations were dispersed at a final concentration of 2.0 mg/mL in 40 mL of 0.01 M NaCl with pH adjusted to 6.0 using 0.1 N HCl in 50 mL centrifuge tubes. The centrifuge tubes were immersed into an ethanol-dry ice bath for 10 min and kept in a -8 $^{\circ}$ C water-glycol bath for 30 min, followed by thawing in a 4 $^{\circ}$ C refrigerator for 48 h. The shear viscosity, storage modulus (G'), and loss modulus (G'') of TEMPO-CNFs dispersions before and after the freezing-thawing treatment were measured using a rheometer (AR 2000, TA Instruments, New Castle, DE) equipped with a 40 mm diameter crosshatched plate. The gap between two plates was fixed at 1.0 mm. The temperature was maintained at 25.0 $\pm$ 0.1 $^{\circ}$ C. Viscosity profiles were collected by steady state flow measurements over a shear rate range of 0.01 s $^{-1}$ -1000 s $^{-1}$ with 5 data points collected per decade of shear rate. The frequency sweep tests were performed at a range of 0.01-10 Hz and an oscillatory strain magnitude of 0.1%.

4.4 Results and discussion

4.4.1 Characteristics of TEMPO-CNFs after sonication.

The TEMPO-CNFs-0 dispersion had a turbid appearance (**inset in Figure 4-2A**), the lowest transmittance (**Figure 4-2A**), and the largest hydrodynamic diameter (**Table 4-1**). The polarized light microscopy image of TEMPO-CNFs-0 demonstrated the existence of fibril aggregates (**Figure 4-3**), which agreed with a previous report that TEMPO-CNFs were still severely aggregated after TEMPO-catalyzed oxidation without homogenization treatment (Saito, Nishiyama, Putaux, Vignon, & Isogai, 2006). With the increase of sonication time from 0 to 120 min, the TEMPO-CNFs dispersion gradually became less turbid, the transmittance increased from $9.1 \pm 0.0\%$ to $87.3 \pm 0.0\%$ (**Figure 4-2A**), and the hydrodynamic diameter decreased from 4812 ± 270 nm to 80 ± 0 nm. Meanwhile, the size distribution changed from a multimodal or a bimodal with larger PDIs to a unimodal distribution with smaller PDIs (**Figure 4-2B** and **Table 4-1**). The AFM analysis showed that sonication reduced the fibril aggregation and fibril length (**Figure 4-4**). Because of aggregation, fibril height and length in TEMPO-CNFs-0 were not analyzed. Broad fibril length distributions were observed in TEMPO-CNFs-1/5/10, which contained fibrils longer than 800 nm (**Figure 4-5**). In TEMPO-CNFs-30/60/120, fibrils longer than 600 nm disappeared, and the relative frequency of short fibrils increased. The average fibril length decreased from 409 nm in TEMPO-CNFs-1 to 202 nm in TEMPO-CNFs-120 (**Table 4-1**). The corresponding DP decreased from 242 ± 0 to 161 ± 4 (**Table 4-1**). The width of

TEMPO-CNFs was not significantly affected by the sonication treatment. As shown in **Figure 4-6**, the height of individual fibrils was in the range of 2.0 nm-3.5 nm, regardless of sonication time. The average height of TEMPO-CNFs after different sonication durations was also similar (**Table 4-1**). The carboxylate group density was 1.65 ± 0.05 mmol/g in TEMPO-CNFs-0 and remained almost unchanged during sonication treatment. These results showed TEMPO-CNFs with similar SCDs and widths, but with different fibril lengths were successfully obtained.

4.4.2 Effects of fibril length on the IRI activity of nanocelluloses.

The representative polarized light microscopy images of 0.01 M NaCl, PEG, and TEMPO-CNFs with varied sonication times are shown in **Figure 4-7**. A smaller size of ice crystals indicates stronger IRI activity. Large ice crystals were observed in 0.01 M NaCl and 2.0 mg/mL PEG, which were blank and negative control, respectively. Large ice crystals were also observed in TEMPO-CNFs-0. It indicated that TEMPO-CNFs-0 barely inhibited ice recrystallization. This was caused by the severe fibril aggregation (**Figure 4-3**) that limited the number of fibrils available for IRI. For treatments with TEMPO-CNFs sonicated for different durations, the size of ice crystals decreased continuously in the initial 30 min and then increased at 60 and 120 min. The increase of IRI activity (decrease of ice crystal size) in the initial 30 min could be explained by the reduction of fibril aggregation, which released more fibrils to inhibit ice recrystallization. The decrease of IRI activity at 60 and 120 min could be attributed to the decrease of fibril length caused by

sonication (**Table 4-1**). The findings are analogous to the stronger IRI activity of PVA with a longer chain length (Congdon et al., 2013; Inada & Lu, 2003).

Figure 4-8 shows the relationship between %MLGS and concentration of nanocelluloses. A smaller %MLGS value represents stronger IRI activity. At concentrations of 1.0 and 2.0 mg/mL, %MLGSs of TEMPO-CNFs decreased with the increase of sonication time and reached the minimum value at 30 min. Increases of %MLGSs were observed at 60 and 120 min. At 3.0 and 4.0 mg/mL, %MLGSs of all TEMPO-CNFs were similar, probably indicating a saturated surface coverage of ice crystals by nanocelluloses. The TEMPO-CNFs-30 demonstrated the strongest IRI activity with an %MLGS < 30% at 2.0 mg/mL, whereas at least a concentration of 3.0 mg/mL was needed for other sonicated TEMPO-CNFs. These results confirmed that initial increase of IRI activity was caused by ultrasound-induced disruption of fibril aggregates and the later decrease was caused by the ultrasound-induced fragmentation of nanocelluloses.

4.4.3 Relationship between IRI activity and gelling properties of TEMPO-CNFs.

Nanocelluloses can form gels after freezing and thawing, which is ascribed to the freezing-induced fibril aggregation (Jiang & Hsieh, 2014b; Lewis, Hatzikiriakos, Hamad, & MacLachlan, 2019). The porous gel structures can be observed from lyophilized aerogels, and the porosity is dependent of nanocellulose concentration (Jiang & Hsieh, 2014a). Generally, a denser and more ordered aerogel structure is developed at a higher nanocellulose concentration (Jiang & Hsieh, 2014a, 2014b). The IRI activity of

nanocelluloses likely results from this gel structure, which confines the dimension of initially formed ice crystals and acts as a physical barrier to inhibit ice growth. Therefore, viscosity and viscoelasticity of nanocellulose dispersions were determined to understand the relationship between this gel structure and IRI activity. The apparent viscosities of nanocellulose dispersions before and after the freezing-thawing treatment are shown in **Figure 4-9A, B**. One order increase in apparent viscosity was found for all sonicated dispersions after the freezing-thawing treatment, which was caused by freezing-induced fibril aggregation (Lewis et al., 2019). Larger values of G' than G'' indicated TEMPO-CNFs-1/5/10 became gels after the freezing-thawing treatment (**Figure 4-9C**). TEMPO-CNFs-0/1/5 were more viscous than other samples before and after the freezing-thawing treatment. The order of apparent viscosity and G' of samples did not match with the order of their IRI activity. For instance, TEMPO-CNFs-30 demonstrated the strongest IRI activity but had a lower viscosity and G' than TEMPO-CNFs treated for a shorter sonication time. The results indicated that the IRI activity of nanocelluloses was not correlated with their macroscopic viscosity or viscoelasticity. Gelation is also not essential for the IRI activity of low molecular weight carbohydrate-based materials (Capicciotti et al., 2012). Therefore, the IRI activity of nanocelluloses did not result from the freezing-induced gelation, and the fibril length dependence of IRI activity of nanocelluloses may result from the interactions between nanocelluloses and ice crystals because longer fibrils have more binding units.

4.5 Conclusions

Sonication led to a disruption in the aggregation of TEMPO-CNFs and a reduction of fibril length but not SCD and width. An increase of sonication time in the initial 30 min improved the IRI activity of TEMPO-CNFs because of disruption of fibril aggregation, whereas fragmentation of nanocelluloses after 60 min and 120 min sonication lowered the IRI activity of TEMPO-CNFs. The IRI activity of TEMPO-CNFs as affected by fibril length was not correlated with the viscosity and viscoelasticity of dispersions, indicating TEMPO-CNFs interacted with ice crystals to inhibit the ice recrystallization. The research findings are useful in the production of nanocelluloses with enhanced IRI activity and provide a new direction to understand the exact IRI mechanisms of nanocelluloses.

4.6 Abbreviations used in this chapter

CNF, cellulose nanofibrils; CNCs, cellulose nanocrystals; TEMPO-CNFs, 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils; SCD, surface charge density; IRI, ice recrystallization inhibition; PVA, polyvinyl alcohol; (%) MLGS, (percentage) mean largest grain size; AFPs, antifreeze proteins; AFGPs, antifreeze glycoproteins; AFM, atomic force microscopy; DLS, dynamic light scattering

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Appendix

Table 4-1. Physicochemical properties of TEMPO-CNFs after sonication for 0-120 min.

Values are represented as mean $\pm$ standard deviation from three replicates.

Sonication time (min)	Carboxylate group density (mmol/g)	Hydrodynamic diameter (nm)	PDI	DP	Fibril length (nm)	Fibril height (nm)
0	1.65 $\pm$ 0.05	4812 $\pm$ 270	0.54 $\pm$ 0.18	242 $\pm$ 0	NA	NA
1	1.60 $\pm$ 0.00	1760 $\pm$ 150	0.98 $\pm$ 0.03	227 $\pm$ 0	409	3.1
5	1.60 $\pm$ 0.00	336 $\pm$ 25	0.65 $\pm$ 0.07	204 $\pm$ 0	405	3.0
10	1.60 $\pm$ 0.00	214 $\pm$ 11	0.51 $\pm$ 0.07	197 $\pm$ 0	349	3.2
30	1.60 $\pm$ 0.00	126 $\pm$ 0	0.30 $\pm$ 0.01	182 $\pm$ 0	286	3.1
60	1.60 $\pm$ 0.00	99 $\pm$ 0	0.29 $\pm$ 0.01	166 $\pm$ 0	243	3.0
120	1.60 $\pm$ 0.00	80 $\pm$ 0	0.23 $\pm$ 0.01	161 $\pm$ 4	202	3.1

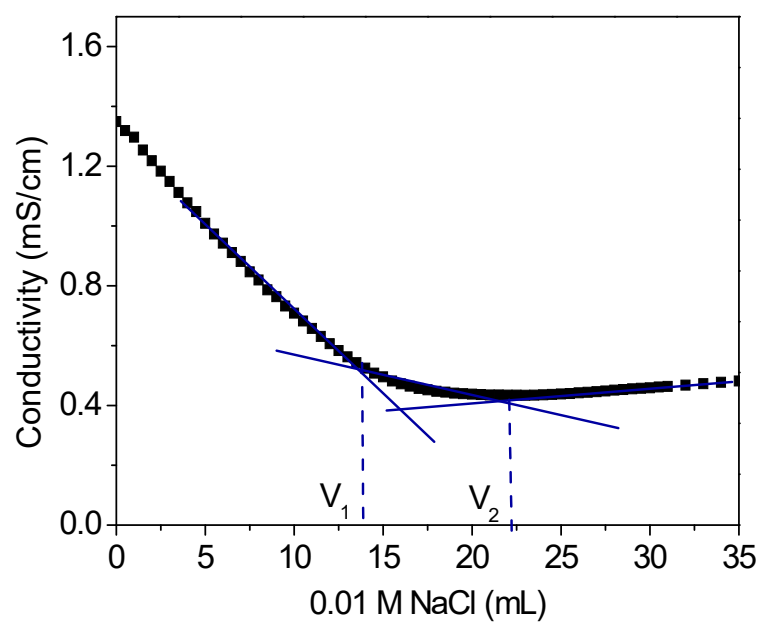


Figure 4-1. A typical conductometric titration curve of TEMPO-CNFs.

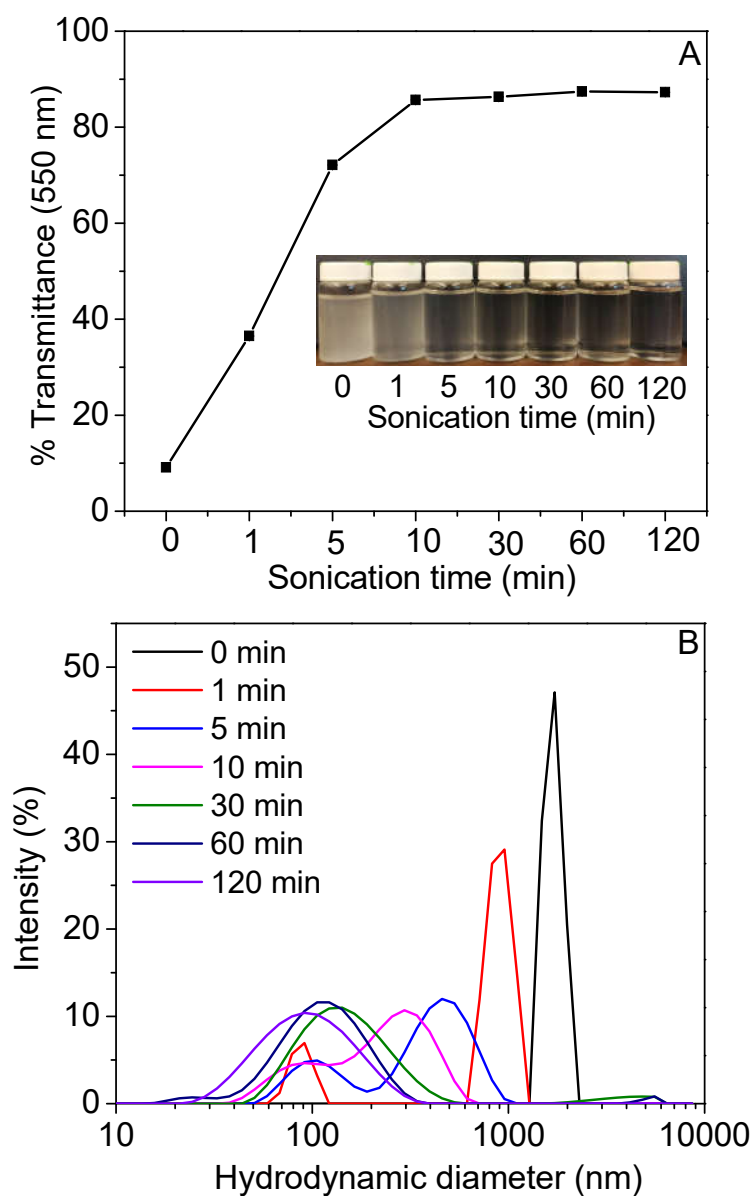


Figure 4-2. (A) Effects of sonication time on the % transmittance and appearance (inset in A), and on (B) Z-average hydrodynamic diameter distribution of TEMPO-CNFs dispersions. TEMPO-CNFs were dispersed in 0.01 M NaCl at a concentration of 1.0 mg/mL and 0.5 mg/mL in A and B, respectively.

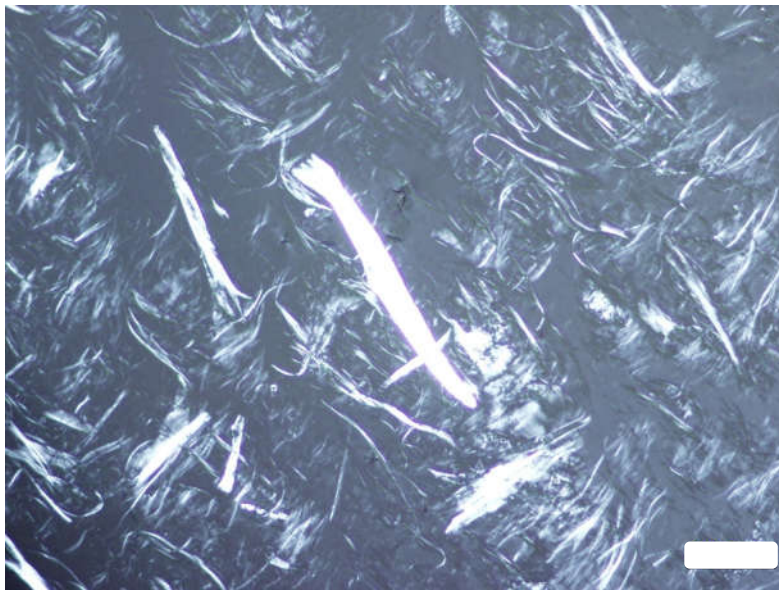


Figure 4-3. The polarized light microscopy image of TEMPO-CNFs-0. TEMPO-CNFs-0 were dispersed in 0.01 M NaCl at 5.0 mg/mL. The scale bar = 200 μm .

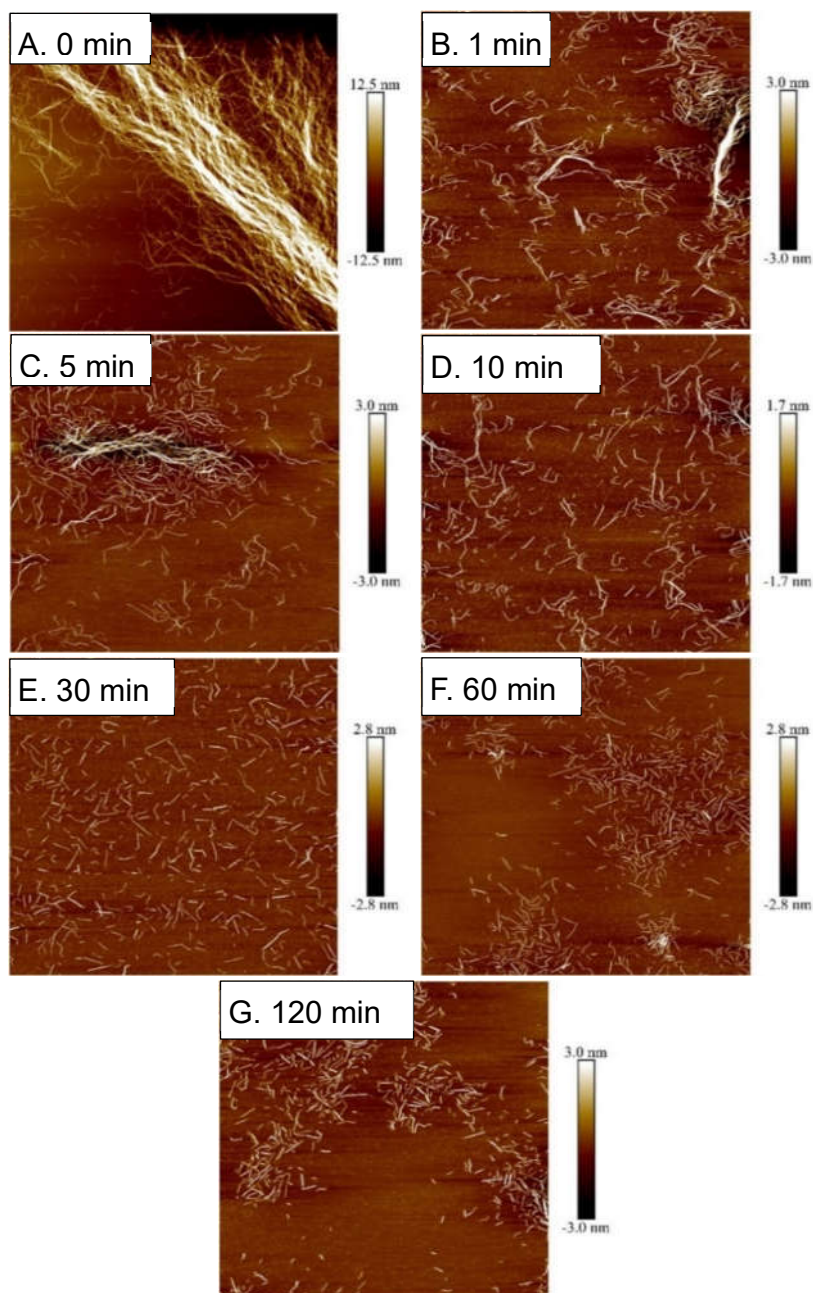


Figure 4-4. AFM morphology of TEMPO-CNFs after sonication for (A) 0 min, (B) 1 min, (C) 5 min, (D) 10 min, (E) 30 min, (F) 60 min, and (G) 120 min. The images are scanned at $5\ \mu\text{m} \times 5\ \mu\text{m}$. TEMPO-CNFs were dispersed in 0.01 M NaCl at a concentration of 0.05 mg/mL.

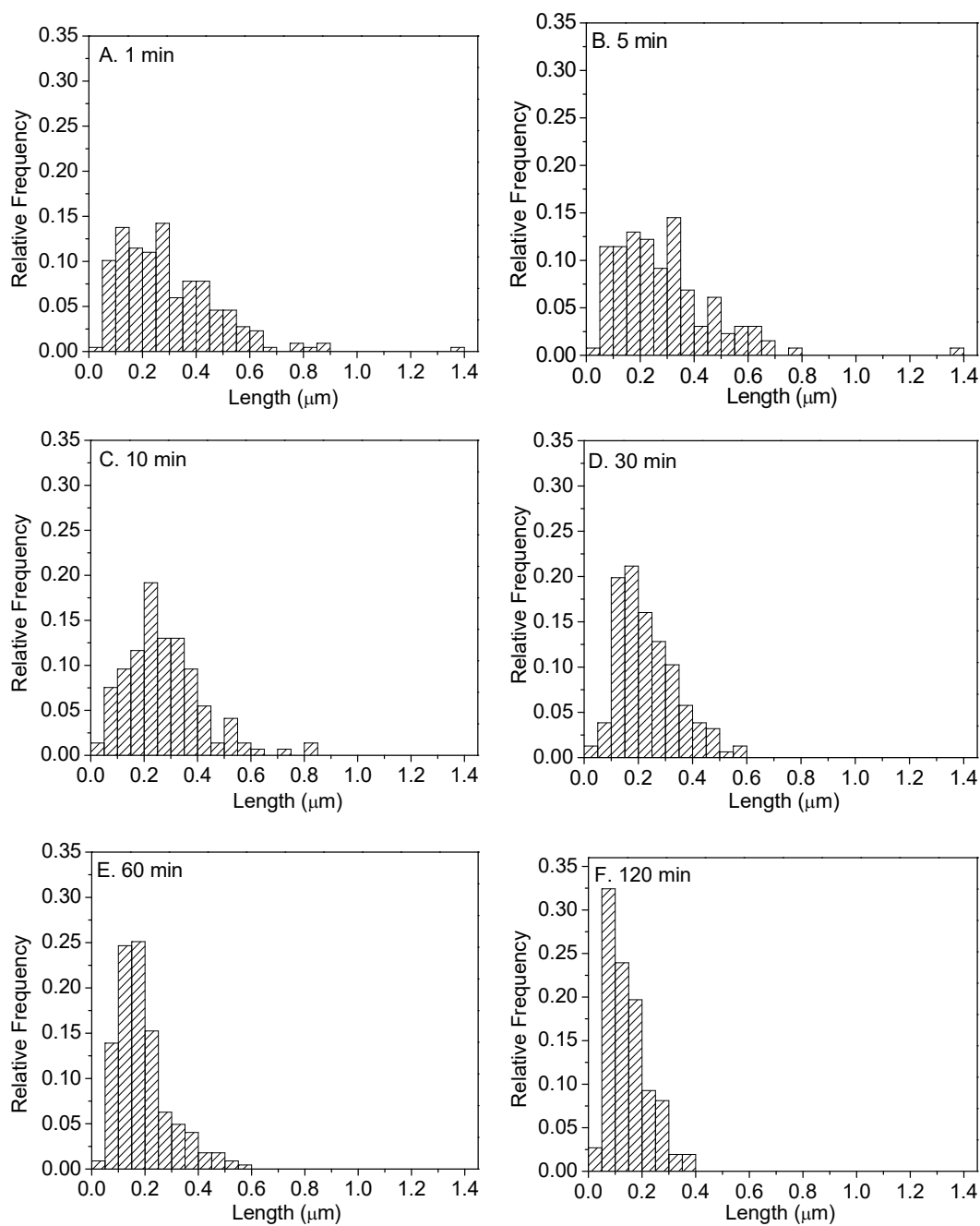


Figure 4-5. The fibril length frequency distribution of TEMPO-CNFs after sonication for (A) 1 min, (B) 5 min, (C) 10 min, (D) 30 min, (E) 60 min, and (F) 120 min.

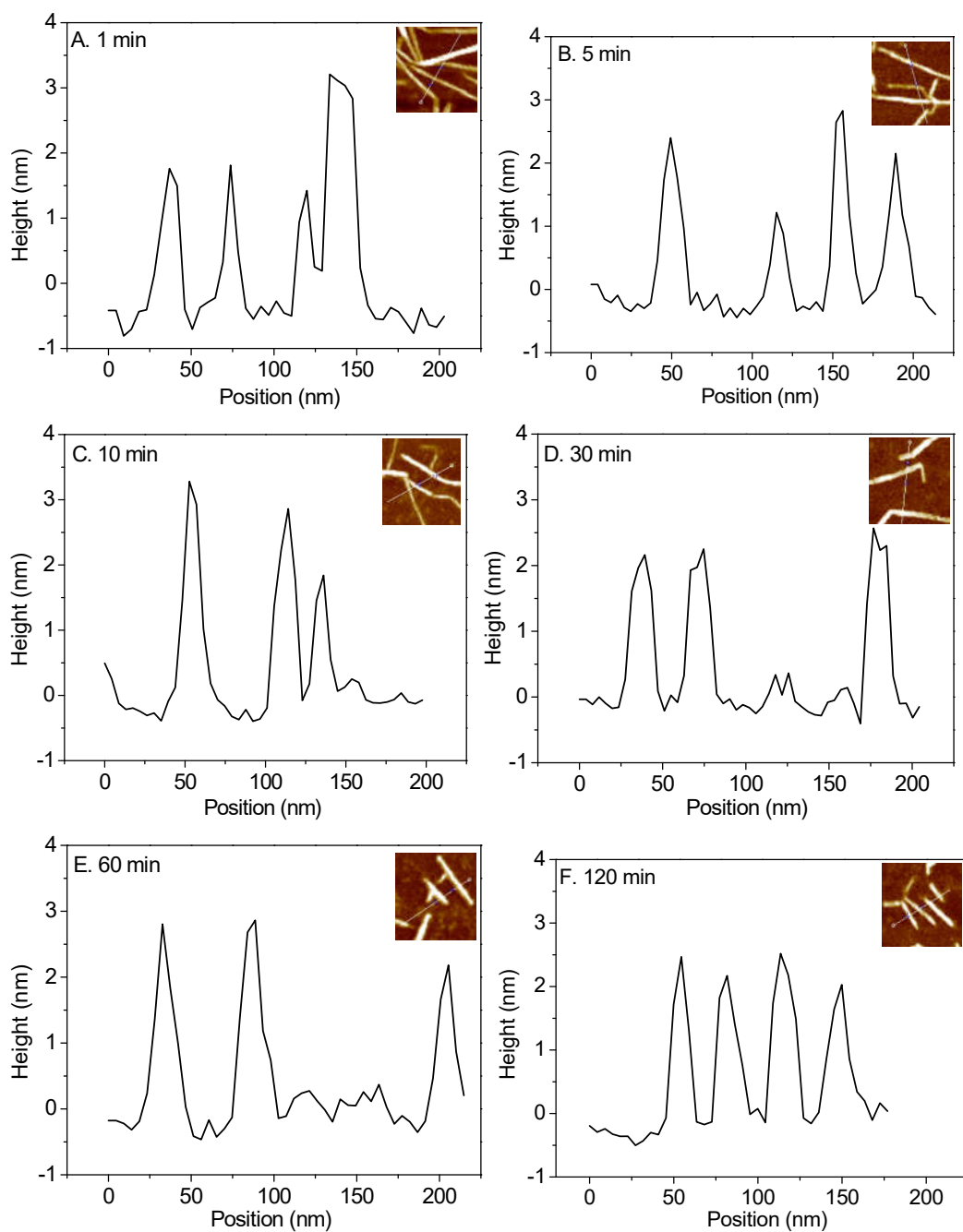


Figure 4-6. The fibril height of TEMPO-CNFs after sonication for (A) 1 min, (B) 5 min, (C) 10 min, (D) 30 min, (E) 60 min, and (F) 120 min.

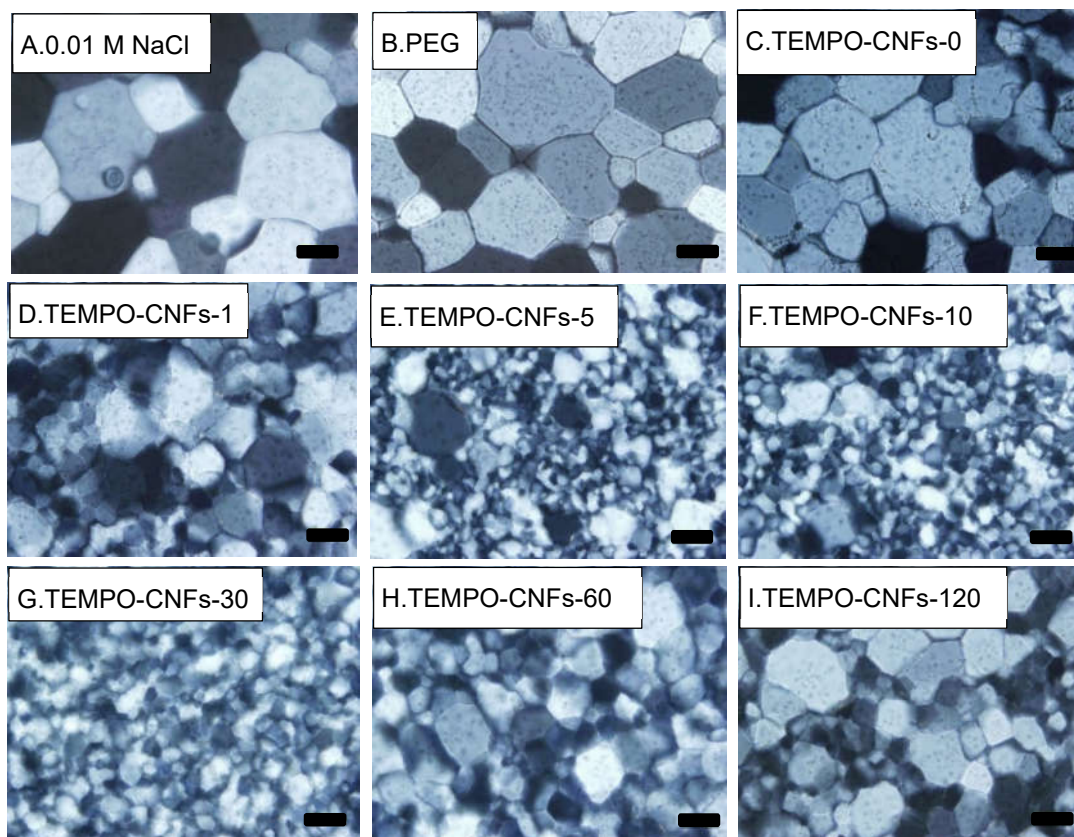


Figure 4-7. Polarized light microscopy images of ice crystal wafers after 30 min annealing at -8°C: (A) 0.01 M NaCl, (B) PEG (negative control), TEMPO-CNFs sonicated for (C) 0 min, (D) 1 min, (E) 5 min, (F) 10 min, (G) 30 min, (H) 60 min, and (I) 120 min. PEG and TEMPO-CNFs (2.0 mg/mL) were dispersed in 0.01 M NaCl. Scale bar = 50 μm.

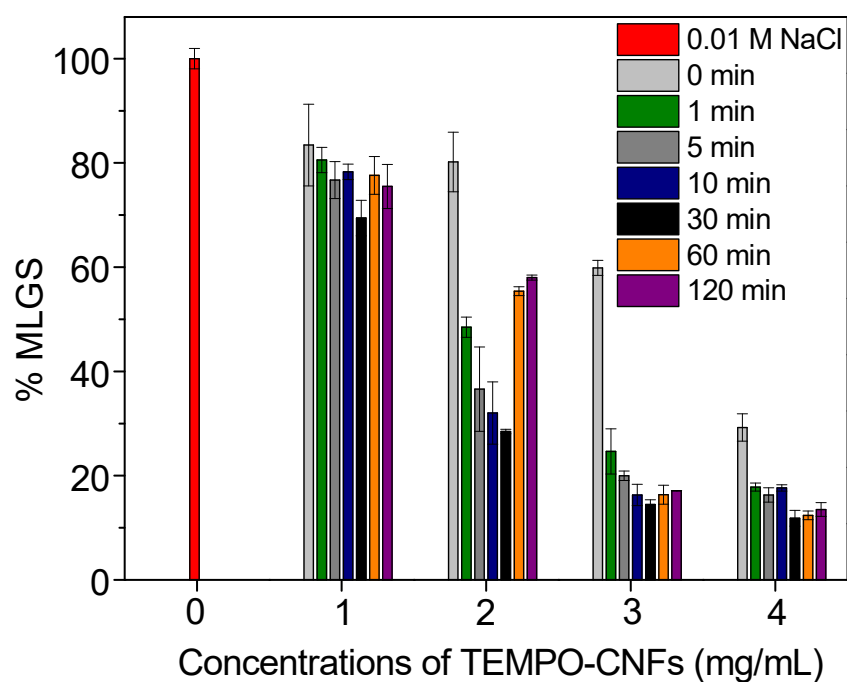


Figure 4-8. The %MLGS of treatments with TEMPO-CNFs sonicated for 0-120 min, with comparison to dispersion medium (0.01 M NaCl). Values represent mean $\pm$ standard deviation from three ice wafers.

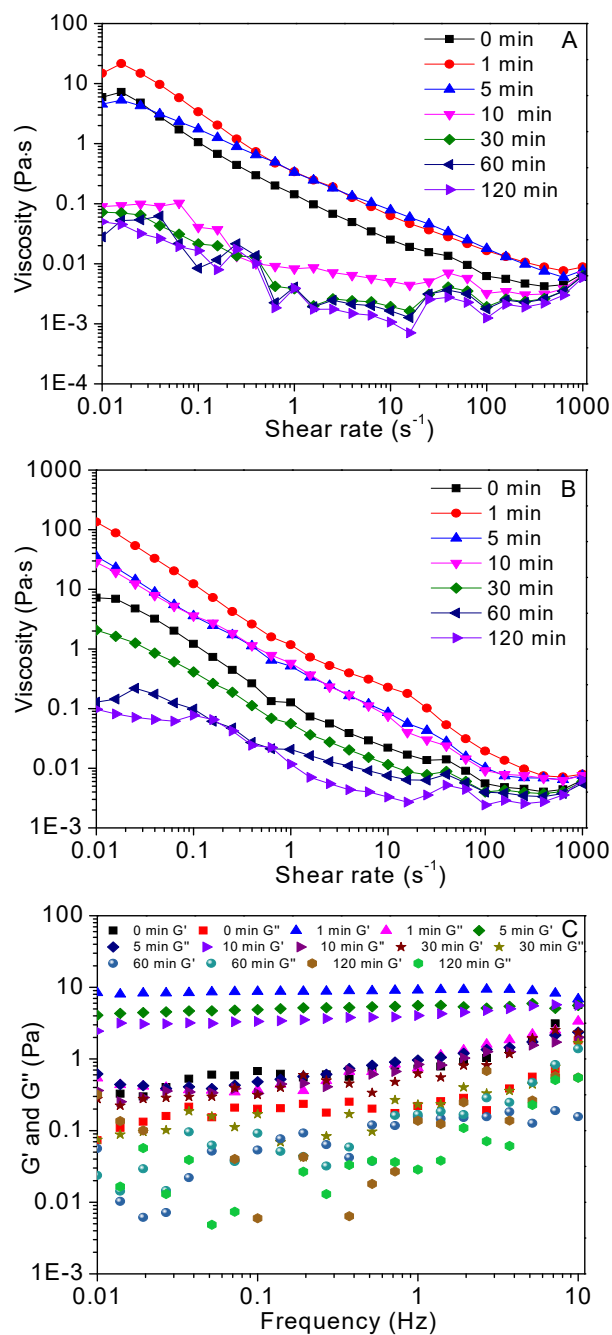


Figure 4-9. Apparent viscosity of TEMPO-CNFs dispersions (A) before and (B) after the freezing-thawing treatment; (C) storage (G') and loss moduli (G'') of TEMPO-CNFs dispersions after the freezing-thawing treatment. TEMPO-CNFs were dispersed at 2.0 mg/mL in 0.01 M NaCl.

Chapter 5. Electrosterically stabilized cellulose nanocrystals demonstrated ice recrystallization inhibition and cryoprotection activities

A version of this chapter has been submitted to *Carbohydrate Polymers* by Teng Li, Min Li, Vermont P. Dia, Scott Lenaghan Qixin Zhong, Tao Wu

Author contribution statement of the submitted version:

Teng Li: Methodology, Investigation, Formal analysis, Validation, Visualization, Writing - Original Draft,

Min Li: Writing - Review & Editing

Vermont P. Dia: Resources, Methodology, Writing - Review & Editing

Scott Lenaghan: Writing - Review & Editing

Qixin Zhong: Resources, Funding acquisition, Writing - Review & Editing, Supervision

Tao Wu: Conceptualization, Methodology, Formal analysis, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition

5.1 Abstract

Ice recrystallization inhibitors have emerged as novel cryoprotectants to improve cell viability for cryopreservation. Nanocelluloses were identified as new materials for ice recrystallization inhibition (IRI); however, conventional nanocelluloses aggregate and lose IRI activity at high ionic strengths, which limit their application as cryoprotectants. In this study, we synthesized a novel group of nanocelluloses - electrosterically stabilized cellulose nanocrystals (ECNCs), which remained dispersed and IRI-active at high ionic strengths. ECNCs improved the post-thaw viability of HCT-116 colorectal cancer cells in slow/fast freezing-slow thawing protocols in the presence of 1-20% dimethyl sulfoxide (DMSO), as well as in slow/fast freezing-fast thawing protocols at reduced DMSO concentrations. The effectiveness in cryoprotection did not match the IRI activity in ECNCs, polyethylene glycol (PEG), and polyvinyl alcohol (PVA); and in ECNCs with different surface charge densities. Overall, ECNCs demonstrated IRI and cryoprotection activities, but the mechanism of cryoprotection remains unknown.

KEYWORDS: Electrosterically stabilized cellulose nanocrystals; Ice recrystallization inhibition; Cryoprotection

5.2 Introduction

Cryopreservation of cells is typically conducted at ultra-low temperatures (typically -80°C or in liquid N₂) with cryoprotectants added to improve cell viability (Briard, Jahan, et al., 2016; Lovelock, 1954). During freezing, salts are excluded from ice crystals, leading to an increase of salt concentration and therefore osmotic pressure (OP) in unfrozen media (Lovelock, 1953). Consequently, an OP imbalance is created across cell membranes, which results in cell dehydration and volume shrinkage. Dehydration of cells can create a large mechanical stress that results in cell membrane leakage and cell death (Lovelock, 1954; Mazur, 1970; Wolfe & Bryant, 1999). The second major cause of cell death during cryopreservation is intracellular/extracellular ice recrystallization. Ice crystals are formed inside cells during freezing and can grow into large crystals via an intracellular ice recrystallization process during storage and thawing (Mazur, Leibo, & Chu, 1972). Intracellular ice recrystallization induces structural damages in cytoplasm and membranes, especially at slow thawing rates that facilitate ice recrystallization (Bank, 1973; Mazur, 1965; Mazur et al., 1972). Concurrently, extracellular ice recrystallization also causes mechanical damages to cells in cryopreservation (Fujikawa & Miura, 1986; Takamatsu & Zawlodzka, 2006).

Use of cryoprotectants is essential to improve the cell viability in cryopreservation (Fuller, 2004). Cryoprotectants can be divided into penetrating and non-penetrating materials depending on their ability to penetrate cell membranes. Penetrating

cryoprotectants include dimethyl sulfoxide (DMSO), glycerol, and propylene glycol (Elliott, Wang, & Fuller, 2017). Among them, DMSO is highly effective and widely used across species (Elliott et al., 2017). It has been proposed that DMSO protects cells by buffering the OP imbalance (Lovelock, 1954; Lovelock & Bishop, 1959). Though DMSO is quite effective, DMSO is cytotoxic and must be removed after thawing in certain applications (Morshed, Jain, & McMartin, 1998; Timm, Saaby, Moesby, & Hansen, 2013). Non-penetrating cryoprotectants include small molecular weight carbohydrates such as sucrose and trehalose; polymers such as PEG, poly(vinyl pyrrolidone) (PVP), and hydroxyethyl starch; proteins such as serum proteins, milk proteins, and egg yolk (Bergeron & Manjunath, 2006; Elliott et al., 2017). Several mechanisms of these cryoprotectants have been proposed, including vitrification (formation of an amorphous glassy state) (Takahashi, Hirsh, Erbe, & Williams, 1988) and stabilization of cell membranes by cryoprotectant-membrane interactions (Crowe et al., 1988). Still, the exact mechanism of cryoprotection by non-penetrating cryoprotectants remains unknown.

Because of the role of intra and extracellular ice recrystallization on decreasing cell viability, materials with ice recrystallization inhibition (IRI) activity have been explored as cryoprotectants. Carbohydrate-based small molecules with potent IRI activity have been synthesized and proven to improve the recovery of red blood cells at reduced concentrations of glycerol (Briard, Poisson, et al., 2016; Capicciotti et al., 2015). IRI active polymers such as polyvinyl alcohol (PVA) and polyproline have been shown to enhance

the post-thaw recovery of red blood cells (Deller, Vatish, Mitchell, & Gibson, 2014), A549 cell suspensions (Deller, Pessin, Vatish, Mitchell, & Gibson, 2016), and cell monolayers (Graham et al., 2017). Carboxylated ϵ -poly-L-lysine is also IRI active and improved the viability of L929 cells and rat stem cells in the absence of DMSO (Matsumura & Hyon, 2009). The IRI activity was also found in graphene oxide and oxidized quasi-carbon nitride quantum dots, and their cryoprotective effects on horse sperms and red blood cells have been reported (Bai et al., 2017; Geng et al., 2017). Recently, ice recrystallization inhibitors that penetrate the cell membrane have been explored as well. Arylglycoside-based small molecules can penetrate endothelial cells of human umbilical veins and inhibit intracellular ice recrystallization, and their cryoprotective effect was observed in red blood cells (Poisson, Acker, Briard, Meyer, & Ben, 2019). More recently, type III antifreeze proteins were delivered into A549 cells by cell-penetrating peptides, which improved cell viability compared to cells cryoprotected by DMSO alone (Tomas, Bailey, Hasan, & Gibson, 2019). However, there are some materials that are highly IRI active, but not effective in the cryoprotection of certain cells. For example, anti-freeze glycoproteins (AFGPs) and PVA are highly IRI active, but they were not effective in the cryoprotection of rat cardiac explants and platelets (Six, Lyssens, Devloo, Compernelle, & Feys, 2019; Wang, Zhu, Yang, Layne, & Devries, 1994). In addition to the variation among cell lines (Elliott et al., 2017), the ineffectiveness of AFGPs as a cryoprotectant may be explained by the dynamic ice shaping (DIS) effect, as a result of AFGP absorption promoting recrystallization as

needle-shaped ice crystals (Graham, Liou, Walker, & Davies, 1997) that can pierce and kill cells (Carpenter & Hansen, 1992). However, DIS may not be a universal mechanism. For example, PVA does not have a strong DIS effect but is also not an effective cryoprotectant (Budke & Koop, 2006). The discrepancy has also been observed for synthetic polyampholytes that have much weaker IRI activity than that of PVA, but can improve the recovery of red blood cells more effectively, which indicates polyampholytes may have other cryoprotection mechanisms (Bailey et al., 2019). Therefore, the exact cryoprotection mechanism is to be studied for each IRI material. Additionally, several studies found that IRI active materials alone cannot provide high post-thaw cell viability and DMSO must be used as a cryoprotectant (Deller et al., 2016; Tomas et al., 2019).

Recently, nanocelluloses, including cellulose nanocrystals (CNCs) and 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils (TEMPO-CNFs) were found to possess IRI activity (Li, Zhao, Zhong, & Wu, 2019). However, CNCs and TEMPO-CNFs aggregated and lost IRI activity at high ionic strengths (Li et al., 2019). Thus, the use of nanocelluloses in physiological conditions is limited. This issue can be addressed by using a novel class of nanocelluloses - ECNC, which can remain dispersed at a high ionic strength (Yang, Alam, & Van de Ven, 2013). ECNCs are characterized by a rodlike, highly crystalline backbone, which is similar to CNCs, but differs with protruding dicarboxylated cellulose chains at both ends, which provide steric interactions to prevent aggregation at a high ionic strength (Safari, Sheikhi, & Van de Ven, 2014; Yang et al., 2013). Due to the

backbone similarity between ECNCs and conventional CNCs, we hypothesize that ECNCs may have IRI activity. Using of ECNCs as IRI active material may be advantageous because of their biocompatibility and sustainability.

In this study, we synthesized ECNCs from natural celluloses with a two-step oxidation process and evaluated their colloidal stability and IRI activity in cell culture media. Since many IRI active materials have been tested as cryoprotectants, the cryopreservation effectiveness of ECNCs was evaluated under different freezing/thawing conditions and at varied DMSO concentrations. The relationship between the IRI activity and cryopreservation effectiveness of ECNCs was discussed with an aim to shed light on the cryoprotection mechanism of IRI materials.

5.3 Materials and methods

5.3.1 Materials

Dry wood pulp sheets (mixture of softwood and hardwood) were sourced from Kimberly-Clark Corporation (Irving, TX). Commercial CNCs and TEMPO-CNFs were purchased from the Process Development Center at University of Maine (Orono, ME), with characteristics reported in our previous study (Li et al., 2019). PEG with a number average molecular weight of 6,000 Da and PVA (99+% hydrolyzed) with a weight average molecular weight of 89,000 Da were purchased from Sigma-Aldrich Corp. (St. Louis, MO). HCT-116 human colon cancer cell line was purchased from the American Type Culture

Collection (Manassas, VA). All other reagents were purchased from Fisher Scientific (Pittsburgh, PA), and deionized (D.I.) water was used in all experiments unless specified.

5.3.2 Synthesis of ECNCs

ECNCs were prepared as described previously (Sheikhi, Yang, Alam, & Van de Ven, 2016). Briefly, 4 g dry pulp was soaked in 300 mL water for 24 h and thoroughly dispersed by a blender (model 31BL92, Dynamic corporation of America, New Hartford, CT). Excessive water was removed by filtering with filter cloth (Millipore, Burlington, MA). The resulting pulp was mixed with 5.33 g of NaIO_4 , 15.6 g of NaCl , and water to a final volume of 266 mL in a 500 mL beaker. The beaker was wrapped with aluminum foil to block light. The oxidation reaction was performed at 22°C with constant stirring for 96 h. Thereafter, the NaIO_4 oxidized pulp was collected and washed 3 times by centrifugation at 10,000 g for 10 min using a LYNX 6000 centrifuge (Thermo Scientific, Waltham, MA) with a F14 rotor. The dry mass content in wet NaIO_4 oxidized pulp was measured after drying wet pulp at 105°C for 5 h. The aldehyde group density of NaIO_4 oxidized pulp was 8.8 mmol/g, measured using the hydroxylamine hydrochloride method (Kim, Kuga, Wada, Okano, & Kondo, 2000). The NaIO_4 oxidized pulp (2 g on a dry weight basis) was dispersed in 100 mL water (including the moisture in the NaIO_4 oxidized pulp) with 5.86 g NaCl and 9.4 g 30% H_2O_2 . NaClO_2 (80%) was added at levels of 3.525, 1.763 and 0.881 g to prepare ECNCs with varied surface charge densities. The mixture was stirred at room temperature and reacted for 22 h. The pH of the mixture was maintained at 6.0 by adding

0.5 M NaOH continually during the first 6 h, left unmonitored (little pH change after 6 h), and then adjusted to 6.0 again at the end of the reaction. The resulting dispersion was centrifuged at 27,000 g for 10 min to collect the supernatant. Ethanol was added into the supernatant at 16, 26, and 40 wt.% for the treatments added with 3.525, 1.763 and 0.881 g of NaClO₂ (80%), and white precipitate (ECNCs) was collected by centrifugation at 10,000 g for 10 min. ECNCs were re-dispersed in 20.0 mL water and dialyzed against 2,500 mL water in a dialysis tubing with a molecular weight cut-off of 6,000-8,000 Da (Thermo Fisher Scientific, Waltham, MA). Water in the reservoir was changed every 2 h until reaching conductivity below 3.0 µS/cm. The resulting ECNCs dispersion was concentrated using a rotary evaporator at 55°C and stored at 4°C. The ECNC concentration in the concentrated ECNCs dispersion was measured after drying at 105°C for 5 h.

5.3.3 Determination of surface charge density of ECNCs

The surface charge (carboxylate group) density of ECNCs was determined using a conductometric titration method (Li et al., 2019). The pH of ECNCs dispersion (50 mL, 1.0 mg/mL) was adjusted to 2.5 with 1.0 N HCl to protonate carboxylate groups. The protonated ECNCs were titrated with 0.05 M NaOH and the conductivity was monitored with a YSI 3200 conductivity meter with a 3256 cell (YSI Inc, Yellow Springs, OH). The carboxylate group density of the sample was determined from the conductometric titration curve (**Figure 5-1**) and calculated with equation (1):

$$\text{Carboxylate group density (mmol/g)} = \frac{0.05 \times (V_2 - V_1)}{m} \quad (1)$$

Where V_1 and V_2 are the NaOH volumes (mL) at the first and second equivalent point, respectively, and m is the mass of ECNCs (0.05 g on a dry weight basis). ECNCs are designated as ECNCs-X, with X being the carboxylate group density (mmol/g).

5.3.4 Atomic force microscopy (AFM) analysis of nanocelluloses

Nanocelluloses were dispersed in water or PBS at 1.0 mg/mL. A freshly peeled mica surface was firstly spin-coated with 20 μ L of 1 mM spermidine, and then with 30 μ L of a nanocellulose dispersion at 2,000 rpm for 60 s using a P6700 spin coater (Specialty Coating Systems Inc., Indianapolis, IN). All the samples were air-dried for 2 h before scanning using a ScanAsyst-AIR probe in the ScanAsyst-Air mode (model Multimode VIII, Bruker Corp., Santa Barbara, CA). All AFM images were flattened using the Nanoscope Analysis software (Bruker Corp, Santa Barbara, CA). The length of individual ECNCs fibrils was measured using Image J software (National Institutes of Health, Bethesda, MD) and the length-weighted average fibril length (L_L) was calculated from 150 fibrils from 3 AFM images (50 fibrils from each image) using equation (2). The fibril width was measured from the fibril height on AFM images. The width-weighted average fibril width (W_w) was calculated from 60 fibrils from 3 AFM images (20 fibrils from each image) using equation (3).

$$L_L = \frac{\sum_{i=1}^n N_i L_i^2}{\sum_{i=1}^n N_i L_i} \quad (2)$$

Where, N_i is the number of fibrils with length L_i .

$$W_w = \frac{\sum_{i=1}^n N_i W_i^2}{\sum_{i=1}^n N_i W_i} \quad (3)$$

Where, N_i is the number of fibrils with width W_i .

5.3.5 Determination of Z-average hydrodynamic diameter (D_h) and ζ -potential of nanocelluloses

The D_h and ζ -potential were determined using a Malvern dynamic light scattering system (Nano ZS, Malvern Instruments, Malvern, UK) (Li et al., 2019). Nanocelluloses were dispersed at a concentration of 0.5 mg/mL in water containing 0-0.08 M NaCl, or PBS (0.137 M NaCl, 0.0027 M KCl, 0.01M Na₂HPO₄ and 0.0018 M KH₂PO₄). To evaluate the properties of nanocelluloses in the culture media for cryopreservation, nanocelluloses were also dispersed at a concentration of 0.5 mg/mL in PBS containing 1.0-20% DMSO. For ζ -potential, only nanocelluloses dispersed in water at a concentration of 0.5 mg/mL were measured.

5.3.6 Determination of IRI activity in PBS by the standard splat assay

The IRI activity of ECNCs in PBS was determined with the standard splat assay (Knight, Hallett, & Devries, 1988). A microscope glass slide was chilled on the surface of a metal block, which was surrounded by dry ice in a foam box for at least 30 min. A sample droplet was dropped from 1.4 m above the pre-chilled glass slide. Upon hitting the glass slide, a thin ice wafer was formed. Then the glass slide was immediately transferred into an HCS 302 cryostage (Instec Instruments, Boulder, CO) and incubated at -8.0 °C under the purge of N₂ for 30 min. Three images from each ice wafer were obtained on a polarized light microscope (BX51, Olympus, Tokyo, Japan) with a built-in digital camera (DP 70,

Olympus, Tokyo, Japan). One image was randomly selected from three images of each ice wafer, and the grain size of the 10 largest ice crystals in the image was measured using Image J (National Institutes of Health, Bethesda, MD). The mean largest grain area (MLGA) of each sample was calculated from 3 independent ice wafers. The percentage MLGA (MLGA%) was calculated by dividing the MLGA of a sample to that of PBS.

5.3.7 Cryopreservation of HCT-116 cell line

Cells were propagated in a 250 mL cell culture flask (Greiner Bio-One, Kremsmünster, Austria) with 15.0 mL complete minimum essential medium (MEM) containing 10% fetal bovine serum and 1% Penicillin-Streptomycin in a 37°C incubator (Heracell 150i, Thermo Scientific, Waltham, MA) with 5% CO₂ for 72 h. The medium was aspirated out by a 2 mL aspirating pipette (model 229262, Celltreat, Pepperell, MA) connected to a vacuum aspirator (model F19917-0250, Bel-Art-SP Scienceware, Wayne, NJ). One mL trypsin (0.25%) was added to rinse the flask quickly and dead cells were removed by an aspirating pipette. Then 2.0 mL trypsin was added, and the flask was incubated in the incubator for 3 min to detach living cells, followed by adding 4.0 mL pre-warmed complete MEM to deactivate trypsin. Cell pellet was collected by centrifuging the cell suspension at 130 g for 5 min at 4°C. Thereafter, 12.0 mL pre-warmed complete MEM was added to resuspend cells. Cell suspension (1.0 mL) was transferred into 15 mL centrifuge tube and centrifuged to remove the medium. Cell pellets were resuspended in PBS containing cryoprotectants to a total volume of 1.0 mL and transferred into 1.8 mL cryovials. Cell viability was

evaluated in four freezing-thawing protocols including slow freezing-slow thawing, fast freezing-slow thawing, slow freezing-fast thawing, and fast freezing-fast thawing. The fast freezing was conducted by directly submerging cryovials in liquid N₂. The slow freezing protocol was conducted by freezing cryovials with a Corning CoolCell cryovial container (Corning Inc, Corning, NY) in a -80°C freezer for 120 min, which according to the manufacturer, had a cooling rate of -1°C/min. After fast and slow freezing, the cells were stored in liquid N₂ for 60 min. The fast thawing protocol was performed by submerging cryovials in a 37°C water bath (18 L shaking water bath, VWR, Radnor, PA) for 3 min, with an average thawing rate of 77.7°C/min as determined using a thermometer. The slow thawing protocol with an estimated rate of 7.2°C/min was conducted by thawing cryovials in a 25°C Benchmark H2200-HC incubator (Edison, NJ) for 30 min. The cell viability was measured after thawing using the trypan blue staining method. Cell suspension (10 µL) was mixed with 0.4% trypan blue (10 µL) and stored at 22°C for 1 min. Then, 10 µL mixture was loaded on a counting chamber slide and the cell viability was determined with a Countess II automated cell counter (Life Technologies, Bothell, WA). Percentage of cell viability (viability %) was calculated by dividing the number of living cells over total number (live + dead) of cells (Song & Hui, 2019). Two cryovials were used in each treatment (Deller, 2013), and three measurements of viability were performed for each vial.

5.3.8 Thirty-day cryopreservation of HCT-116 cell line

PBS containing 3% DMSO and 10.0 mg/mL ECNCs-8.0 was used as a culture medium in the slow freezing-fast thawing protocol. PBS containing 10% DMSO and 10.0 mg/mL ECNCs-8.0 was used as a culture medium in the fast freezing-slow thawing protocol. After freezing, samples were stored in liquid N₂ in a JR cryogenic storage system (Thermo Fisher Scientific, Waltham, MA). On day 1, 3, 7, 14, and 30, cells were thawed to determine the viability using the methods described earlier.

5.3.9 Determination of IRI activity in culture media in the slow thawing process

The IRI activity of ECNCs, PEG, and PVA in PBS with 10% DMSO was measured using a protocol simulating a slow thawing process. After preparation of ice wafers following the above splat assay, glass slides were transferred immediately onto the cryostage with temperature set at -30.0°C. Thereafter, temperature was increased from -30 to -5°C at 7.2°C/min (average rate of slow thawing). This temperature range was selected because preliminary tests showed that ice crystals started to grow around -30°C and started to melt around -5°C. Images of ice wafers were obtained every 5°C from -30°C to -5°C. Three ice wafers were analyzed for each sample. The MLGA of each sample was calculated following the method described earlier.

5.3.10 Statistics analysis

Independent samples t-test was used to analyze the difference of cell viability between treatments and the control in different freezing/thawing conditions and at varied DMSO

concentrations. One-way analysis of variance was used to analyze the difference of cell viability among different storage time treatments; among PVA, ECNCs-8.0, and PEG treatments following the fast freezing-slow thawing protocol; and among ECNCs with different surface charge densities ($p < 0.05$). All analyses were performed using the SPSS® Statistics software (IBM, Armonk, NY).

5.4 Results and discussion

5.4.1 Physicochemical properties of ECNCs

Three ECNCs with the carboxylate group density of 8.0, 5.5, and 3.3 mmol/g were synthesized when 3.525, 1.763, and 0.881 g of NaClO₂ (80%) was used, respectively (**Table 5-1**). Hereafter, the ECNCs are designated as ECNCs-X, with X being the carboxylate group density (mmol/g). The surface charge density range of 3.3-8.0 mmol/g in ECNCs was much higher than that of 0.4 mmol/g in CNCs and 1.5 mmol/g in TEMPO-CNFs determined in our previous study (Li et al., 2019). Compared with the ζ -potential of -31.5 ± 1.4 mV in CNCs and -33.3 ± 0.3 mV in TEMPO-CNFs (Li et al., 2019), ECNCs had ζ -potentials ranged from -28.2 ± 1.1 to -45.0 ± 2.3 mV. The ζ -potential results agreed with that of surface charge density, with the magnitude following the decreasing order of ECNCs-8.0 > ECNCs-5.5 > ECNCs-3.3. The three ECNCs had a similar L_L and W_W (**Table 5-1**).

The morphologies of ECNCs-8.0, CNCs and TEMPO-CNFs in water and PBS are compared in **Figure 5-2**. In water, ECNCs-8.0 were rodlike fibrils without much lateral

aggregation. CNCs were also rodlike, but with severe lateral aggregation. TEMPO-CNFs were nanofibrils with a length of up to several hundred nanometers, and they were not aggregated. When dispersed in PBS, ECNCs-8.0 fibrils were evenly distributed on the mica surface without much aggregation, whereas aggregation was evident for CNCs and TEMPO-CNFs. The D_h of CNCs and TEMPO-CNFs increased with the increase of ionic strength, while that of ECNCs-8.0 initially decreased at 0.02 M NaCl and remained constant at higher ionic strengths (**Figure 5-3A**). The decrease of D_h of ECNCs at 0.02 M NaCl was caused by the shrinkage of protruding cellulose chains due to the salt-induced charge screening effect (Yang et al., 2013). The D_h of nanocelluloses was also evaluated in PBS containing up to 20% DMSO (**Figure 5-3B**). DMSO had little effect on the D_h of three nanocelluloses in PBS, although a slight size increase was observed for ECNCs-8.0 at 20% DMSO. ECNCs-8.0 had much smaller D_h than other nanocelluloses in NaCl solution and PBS containing DMSO. Both AFM and D_h results demonstrated a good colloidal stability of ECNCs-8.0 at the studied ionic strength range.

5.4.2 IRI activity of ECNCs

The IRI activity of ECNCs with varied surface charge densities in PBS is shown in **Figure 5-4**, with a smaller MLGA (%) representing a stronger IRI activity. The IRI activity increased with the increase of ECNCs concentration. All three ECNCs demonstrated a stronger IRI activity than PEG (the negative control) at the same concentration.

Numerically, ECNCs-3.3 were more IRI-active than the other two ECNCs, which can be ascribed to its lower surface charge density (Li et al., 2020).

5.4.3 Cryoprotection effectiveness of ECNCs

The cryoprotective effect of ECNCs was tested in four protocols, including slow freezing-slow thawing, fast freezing-slow thawing, slow freezing-fast thawing, and fast freezing-fast thawing (**Figure 5-5**). Only ECNCs-8.0 were tested here because it had the highest yield after synthesis. In the absence of DMSO, the addition of 10.0 mg/mL ECNCs-8.0 failed to achieve cell viability of more than 20% in any of the four protocols. A similar result has been reported for IRI-active PVA, which had no cryoprotective effect on A549 cells in the absence of DMSO (Deller et al., 2016). In the presence of DMSO, 10.0 mg/mL ECNCs-8.0 significantly improved cell viability from 1.2-81.0% in controls to 16.7-93.2% in treatments at most DMSO concentrations in the slow freezing-slow thawing protocol (**Figure 5-5A**). Improvement from 0.0-56.7% to 0.8-82.5% was observed at all DMSO concentrations in the fast freezing-slow thawing protocol (**Figure 5-5B**). ECNCs-8.0 also significantly improved the cell viability from 11.8-57.3% to 35.8-76.0% in the slow freezing-fast thawing protocol at DMSO concentrations of 1-3% (**Figure 5-5C**). For the fast freezing-fast thawing protocol, significant increases of cell viability from 0.0-51.2% to 3.0-69.5% were observed at DMSO concentrations of 1-5% (**Figure 5-5D**). Lowering DMSO concentration can minimize the osmotic shock associated with the removal of high concentrations of DMSO and reduce the potential toxicity in biomedical applications

(Meryman & Hornblower, 1972; Whittingham, Mazur, & Leibo, 1972). The cryoprotective effect of ECNCs-8.0 was also observed after cryo-storage for up to 30 days, with no appreciable difference of cell viability ($p > 0.05$) observed following the slow freezing-fast thawing and fast freezing-slow thawing protocols (**Figure 5-6**).

5.4.4 Correlation between IRI activity and cryoprotection effectiveness of ECNCs

In the standard splat assay, the IRI activity is evaluated at a constant temperature, which does not reflect the temperature increase profile in a thawing process where ice recrystallization is most prominent. Thus, the IRI activities of ECNCs-8.0, PVA, and PEG were evaluated in PBS containing 10% DMSO in a condition that simulated the fast freezing-slow thawing protocol. The MLGA of each sample was measured every 5°C in the temperature range from -30 to -5°C. As shown in **Figure 5-7**, without an ice recrystallization inhibitor, the blank control (PBS with 10% DMSO) demonstrated the largest MLGA range of 93.2-488.1 μm^2 . ECNCs-8.0 demonstrated a smaller MLGA range than that of PEG at same concentration for most temperatures. A potent IRI activity was observed on PVA, which had a smaller MLGA range at 3.0 mg/mL than that of ECNCs-8.0 and PEG at 40.0 mg/mL. Therefore, the IRI activity of inhibitors observed in the standard splat assay is also maintained in the thawing process.

The cryoprotection effectiveness (**Figure 5-8**) however did not match with the IRI activity of PVA, ECNCs, and PEG (**Figure 5-7**). PVA was most IRI active, but not particularly effective in improving the cell viability after freezing-thawing, and even

reduced the cell viability at 1.0-10.0 mg/mL. A previous study showed 5.0 and 10.0 mg/mL PVA reduced the viability of A549 cells (Deller et al., 2016). Although higher concentrations of ECNCs were more IRI active, the cell viability was not improved significantly with the increase of ECNCs concentration from 10.0 to 40.0 mg/mL. PEG was not IRI active, but improvement of viability was observed. Both ECNCs-8.0 and PEG were less IRI active than PVA, but more effective in cryoprotection. These results showed that the cryoprotection effectiveness of PVA, ECNCs-8.0, and PEG were not attributable to the degree of their IRI activity. The similar phenomenon was also observed in ECNCs with different surface charge densities. These three ECNCs had similar morphology, L_L and W_W (**Figure 5-9, Table 5-1**), as well as good colloidal stability in PBS (**Figure 5-9**). ECNCs-8.0 were less IRI active than ECNCs-3.3 and ECNCs-5.5, as evidenced by the larger MLGA values at most temperatures (**Figure 5-10A**), but no significant difference of cell viability was observed among the three ECNCs (**Figure 5-10B**). There is one possible explanation for these results: IRI is not the only cryoprotection mechanism of ECNCs. Given the amphiphilic nature of nanocelluloses (Capron, Rojas, & Bordes, 2017), ECNCs may stabilize the cell membranes by hydrophobic interactions between hydrophobic backbones of ECNCs and cell membranes, which has been proposed as a cryoprotection mechanism for amphiphilic polymers (Rajan, Hayashi, Nagashima, & Matsumura, 2016). These interactions may protect cell membranes from mechanical damages resulting from ice recrystallization and dehydration. Additionally, since the cryoprotection effectiveness

is highly dependent on the specific cell line (Elliott et al., 2017), ECNCs should be evaluated in more cells to determine cryoprotection effectiveness. The cellular function after cryopreservation should also be evaluated (Ezzati, Shanehbandi, Hamdi, Rahbar, & Pashaiasl, 2020).

5.5 Conclusions.

ECNCs with varied surface charge densities were prepared by a two-step oxidation process. Compared with conventional nanocelluloses, AFM and D_h results demonstrated a good colloidal stability of ECNCs at increased ionic strengths and in PBS containing 1-20% DMSO. The IRI activity of ECNCs was stronger than that of PEG but weaker than that of PVA. ECNCs with a lower surface charge density demonstrated a stronger IRI activity. In the absence of DMSO, the addition of 10.0 mg/mL ECNCs-8.0 failed to achieve cell viability of more than 20% in any of the four freezing-thawing protocols. In the presence of 1-20% DMSO, 10 mg/mL ECNCs significantly enhanced the cell viability in slow/fast freezing-slow thawing protocols. Significant improvement of cell viability was also observed in the slow/fast freezing-fast thawing protocols at reduced DMSO concentrations. The cryoprotection effectiveness of ECNCs did not match with their IRI activity. ECNCs-8.0 and PEG were more effective in cryoprotection but less IRI active than PVA; ECNCs with varied surface charge densities demonstrated similar cryoprotection effects but ECNCs-3.3 were more IRI active. Overall, ECNCs demonstrated IRI activity and cryoprotection effects, and the clear cryoprotection mechanisms are to be studied.

5.6 Acknowledgements

We thank Professor Siqun Wang from the Center for Renewable Carbon at University of Tennessee, Knoxville for providing the dry pulp sheets. We thank Yang Lin and Andrea Nieto-Veloza for their kind help in cell work. This work is supported by the USDA National Institute of Food and Agriculture Hatch Project 1016040, TEN 00487, and 223984.

5.7 Abbreviations

ECNCs, electrosterically stabilized cellulose nanocrystals; CNCs, cellulose nanocrystals; TEMPO-CNFs, 2, 2, 6, 6-tetramethylpiperidine-1-oxyl oxidized cellulose nanofibrils; DMSO, dimethyl sulfoxide; IRI, ice recrystallization inhibition; PEG, poly(ethylene glycol); PVP, poly(vinyl pyrrolidone); PVA, poly(vinyl alcohol); MEM, minimum essential medium; D_h , Z-average hydrodynamic diameter; MLGA (%), (percentage) mean largest grain area; AFM, atomic force microscopy; DIS, dynamic ice shaping.

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Appendix

Table 5-1. Physicochemical properties of ECNCs with varied surface charge densities.

Values of carboxylate group density and ζ -potential are represented as mean $\pm$ standard deviation of three replicates. Length-weighted average length and width-weighted average width were determined from 150 and 60 fibrils, respectively. Mean values not sharing a same letter are significantly different ($p < 0.05$).

Sample code	Carboxylate group density (mmol/g)	ζ -potential at pH 6.0 (mV)	Length-weighted average length (nm)	Width-weighted average width (nm)
ECNCs-8.0	8.0 ± 0.0	-45.0 ± 2.3^a	123.7 ± 3.2^a	1.69 ± 0.09^a
ECNCs-5.5	5.5 ± 0.0	-33.5 ± 0.2^b	123.0 ± 1.3^a	1.71 ± 0.04^a
ECNCs-3.3	3.3 ± 0.0	-28.2 ± 1.1^c	124.2 ± 0.8^a	1.67 ± 0.04^a

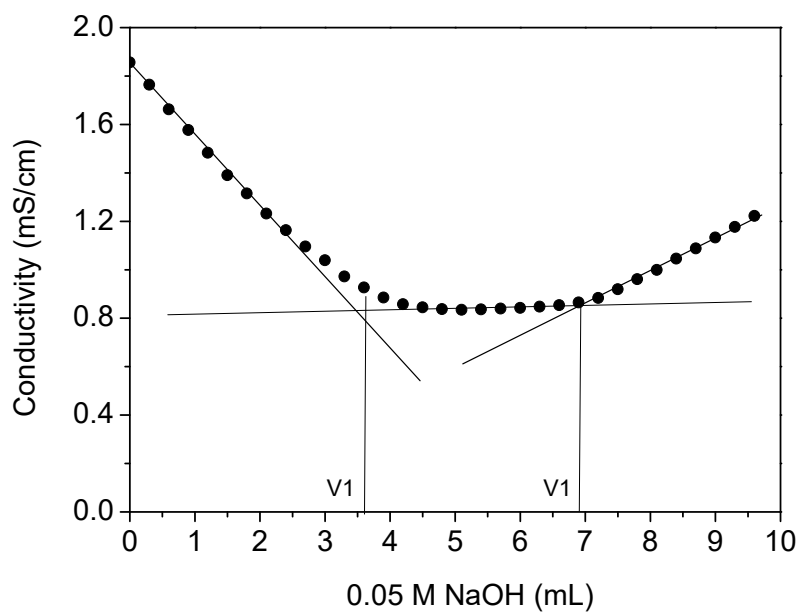


Figure 5-1. Typical conductometric titration curves of protonated ECNCs titrated with 0.05 M NaOH. The pH of ECNCs dispersion (50 mL, 1.0 mg/mL) was adjusted to 2.5 with 1.0 N HCl to protonate carboxylate groups. The carboxylate group density was calculated based on the amount of NaOH consumed in the titration.

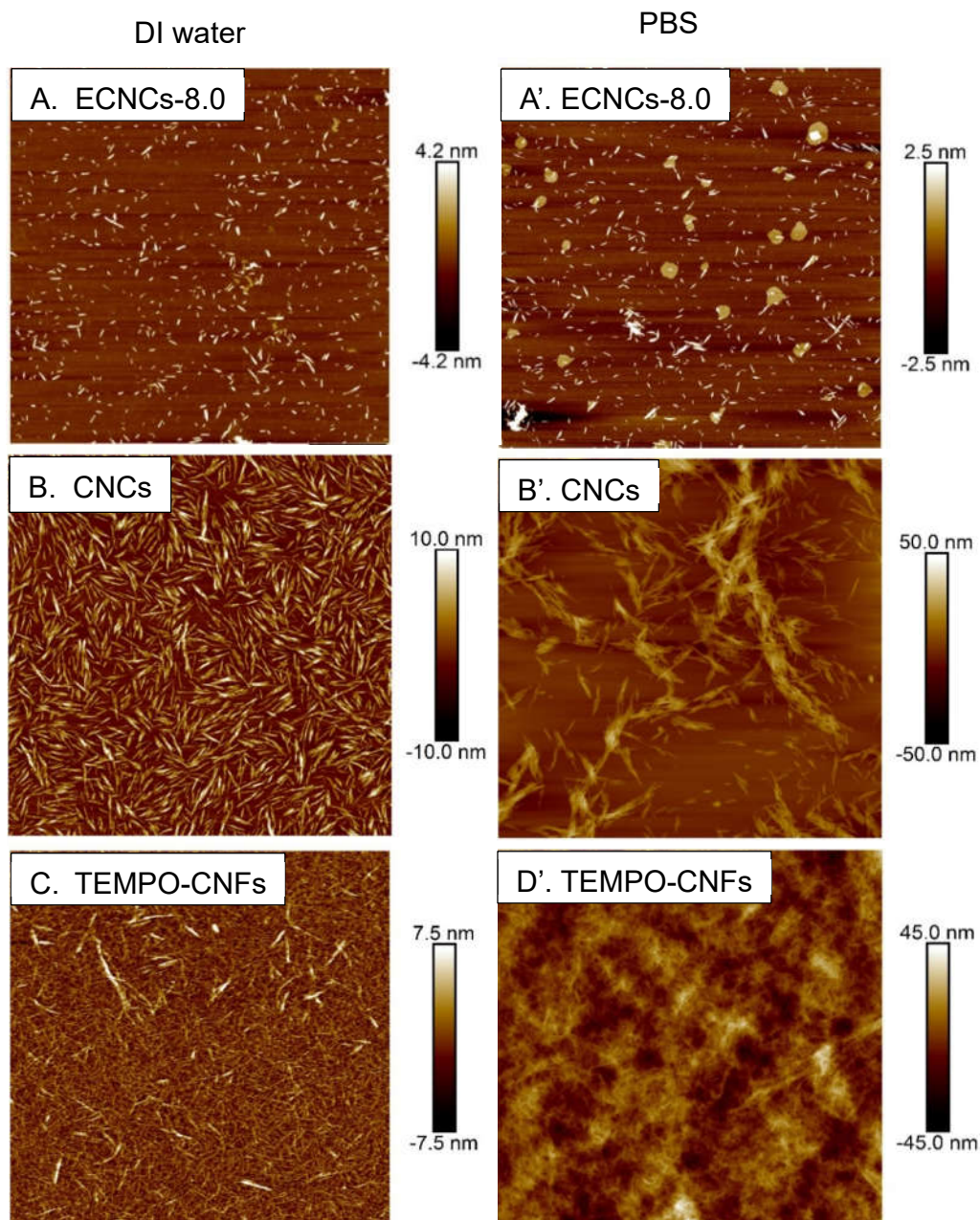


Figure 5-2. Atomic force microscopy images of ECNCs-8.0, CNCs, and TEMPO-CNFs dispersed in water (A, B, and C) or PBS (A', B', and C'). The dimension of images is $5\text{ }\mu\text{m} \times 5\text{ }\mu\text{m}$.

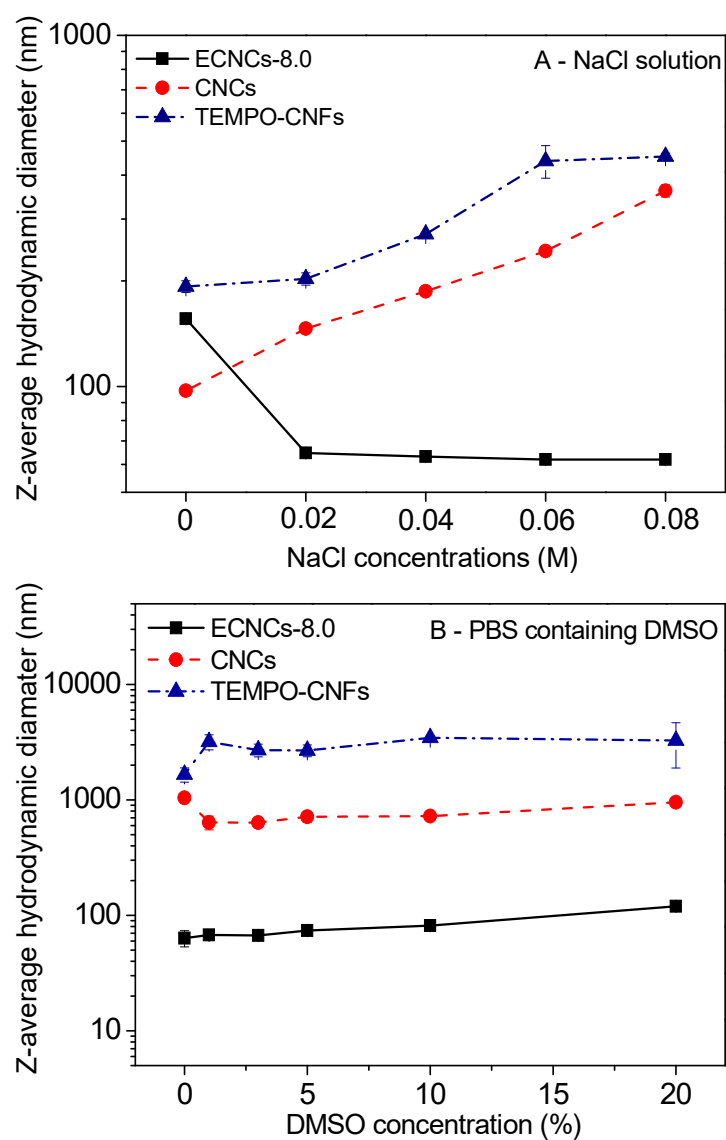


Figure 5-3. Z-average hydrodynamic diameters of ECNCs-8.0, CNCs and TEMPO-CNFs in (A) 0-0.08 M NaCl at pH 6.0 and in (B) PBS containing 1, 3, 5, 10, and 20% DMSO at pH 7.4. The concentration of nanocelluloses was 0.5 mg/mL for all samples. Values are represented as mean $\pm$ standard deviation of three replicates.

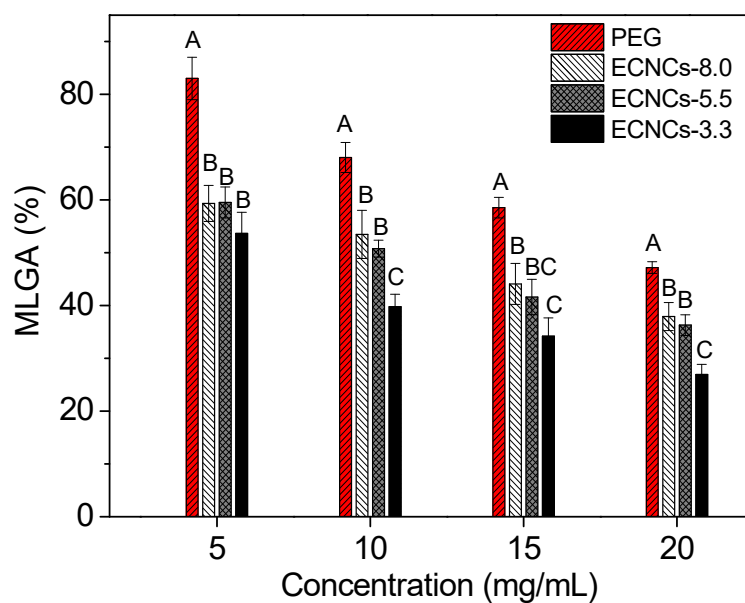


Figure 5-4. IRI activities (MLGA%) of ECNCs with varied surface charge densities in PBS analyzed by a standard splot assay. Values are represented as mean $\pm$ standard deviation of three ice wafers. Statistics analysis was conducted to compare the MLGA(%) difference among PEG and the three ECNCs at the same concentration. Mean values not sharing a same letter are significant different ($p < 0.05$).

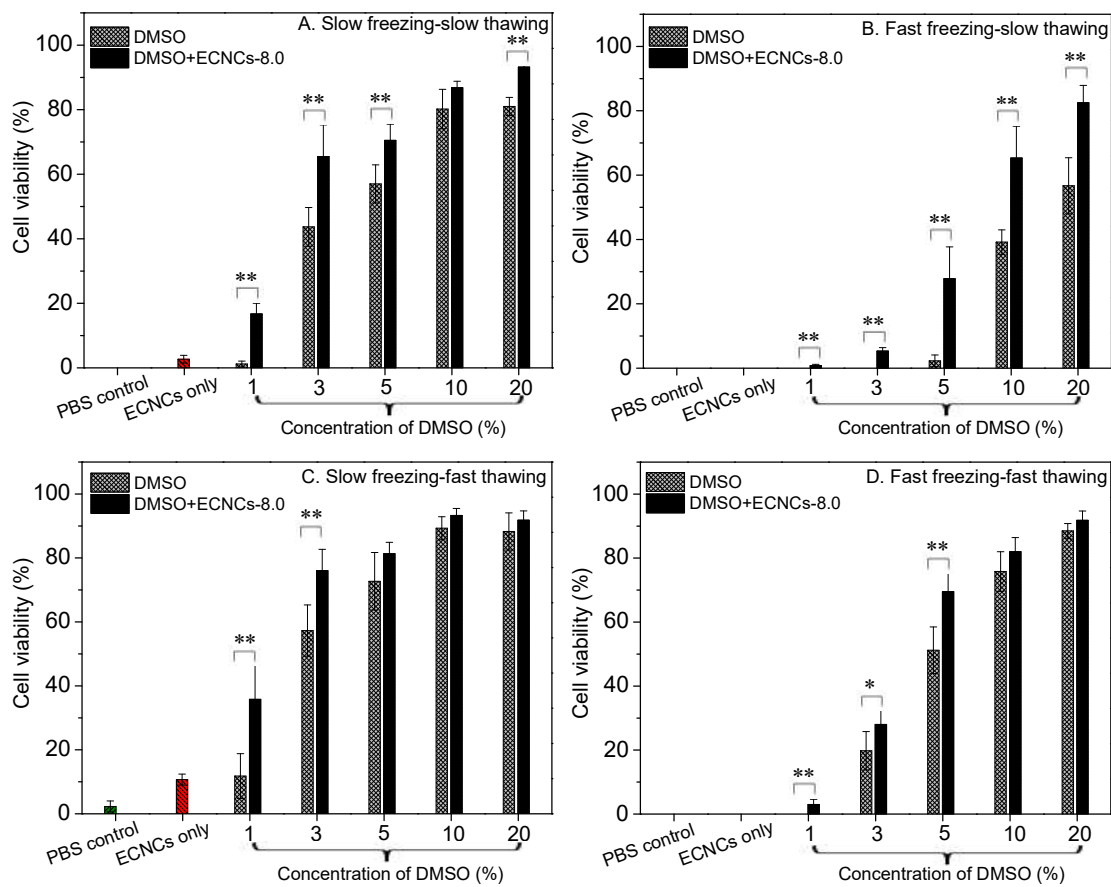


Figure 5-5. Viability of HCT-116 cells in PBS control, PBS with 10.0 mg/mL ECNCs-8.0, and PBS containing 1-20% DMSO with or without 10.0 mg/mL ECNC-8.0, following different freezing-thawing protocols, with significant differences at $p < 0.05$ and $p < 0.01$ labeled for * and **, respectively. Slow freezing: temperature was decreased at $-1^{\circ}\text{C}/\text{min}$ to -80°C , then kept in liquid N_2 for 60 min. Fast freezing: cryovials were submerged into liquid N_2 and kept for 60 min. Slow thawing: cryovials were kept in a 25°C air incubator for 30 min. Fast thawing: cryovials were kept in a 37°C water bath for 3 min.

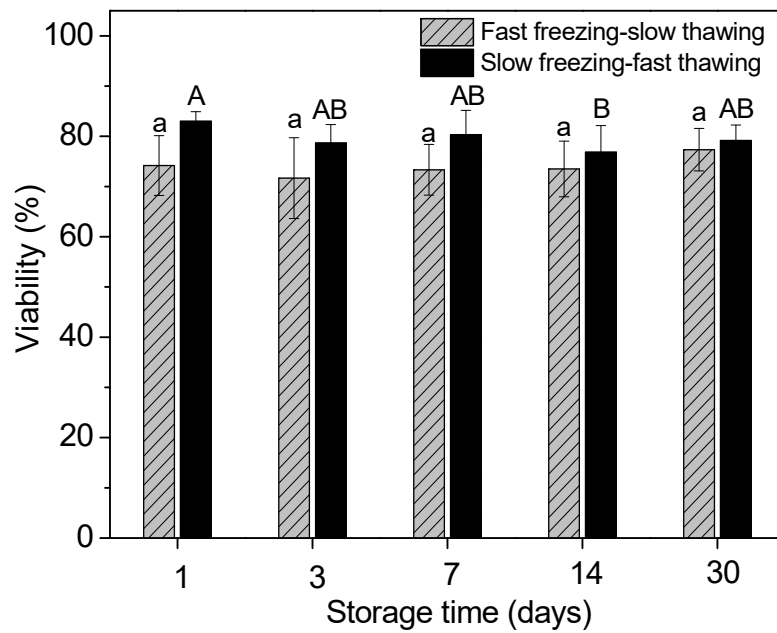


Figure 5-6. Viability of HCT-116 cells after storage for 30 days in a medium containing DMSO and 10.0 mg/mL ECNC-8.0. The concentration of DMSO in the medium was 3% and 10%, respectively, in the slow freezing-fast thawing and fast freezing-slow thawing protocol. Mean values that do not share a same letter (lowercase for fast freezing-slow thawing treatments; uppercase for slow freezing-fast thawing treatments) are significantly different ($p < 0.05$).

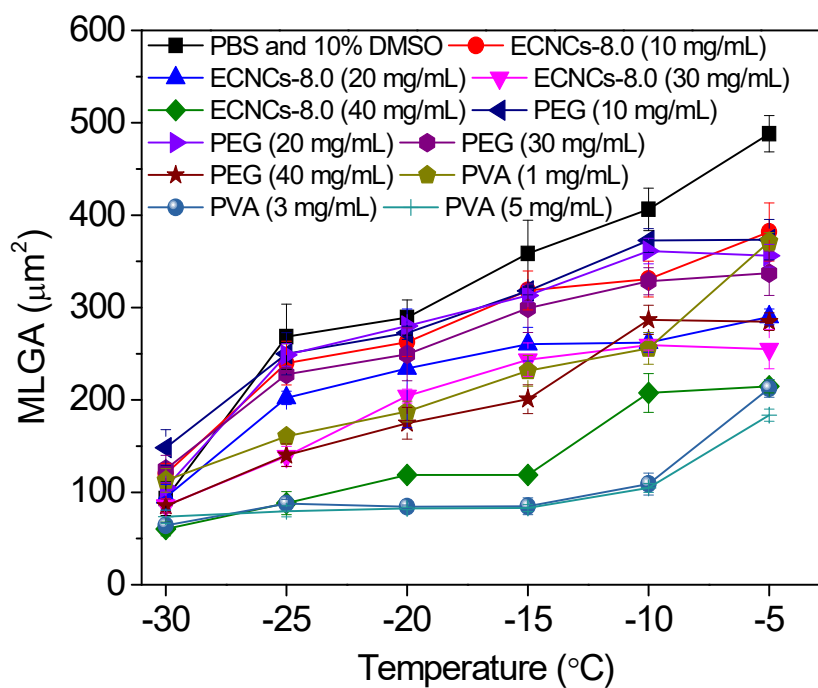


Figure 5-7. Mean largest grain area (MLGA) of ice crystals formed from PBS containing 10% DMSO and various amounts of ECNCs, PEG, or PVA. The temperature was increased from -30°C to -5°C at a rate of 7.2 °C/min. Values are represented as mean $\pm$ standard deviation of three ice wafers.

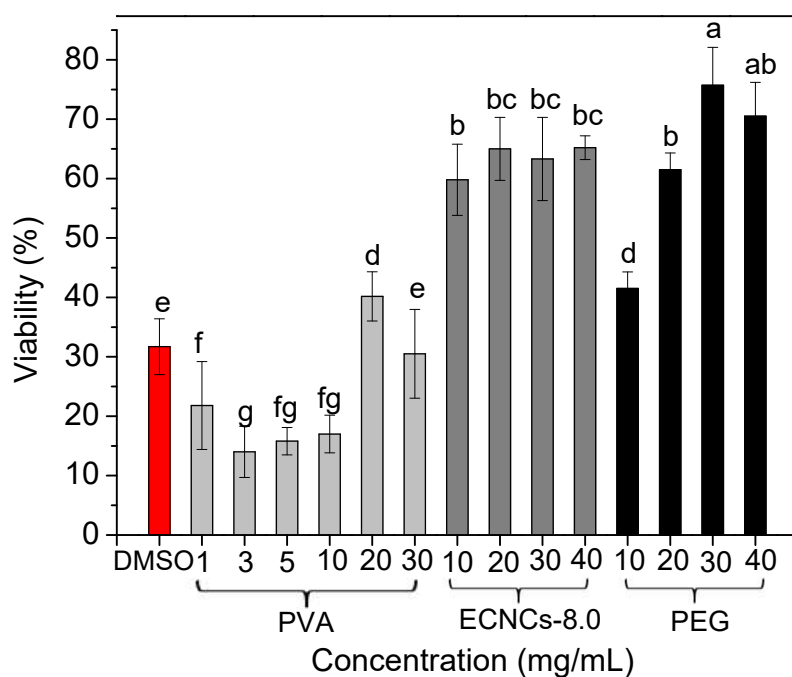


Figure 5-8. Viability of HCT-116 cells following fast freezing-slow thawing treatment of cultures in PBS containing 10% DMSO and various amounts of PVA, ECNCs-8.0, or PEG.

Mean values that do not share a same letter are significantly different ($p < 0.05$).

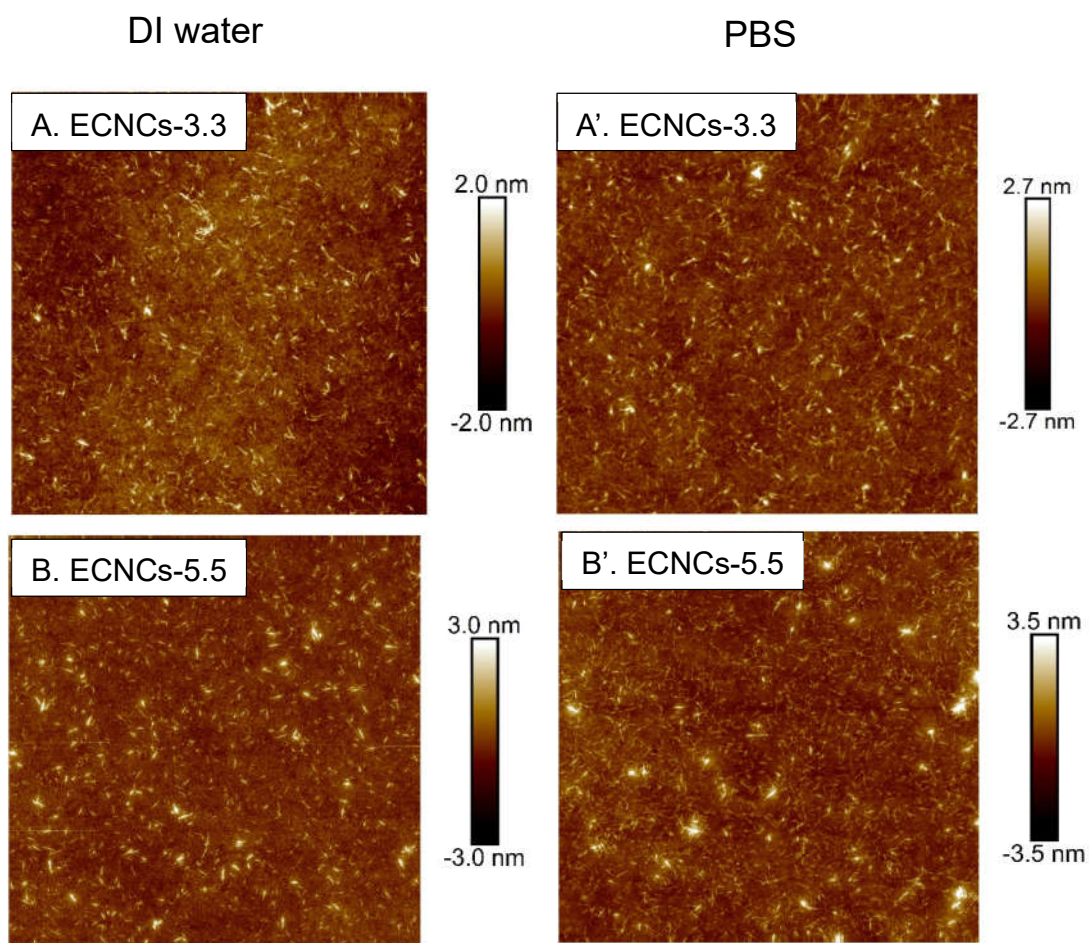


Figure 5-9. Atomic force microscopy images of ECNCs-3.3 and ECNCs-5.5 dispersed in water or PBS. The concentration of ECNCs was 1.0 mg/mL. ECNCs dispersions were spin-coated on mica surfaces which were pre-coated with 1 mM spermidine. Samples were air-dried 2 h before scanning. The dimension of images is 5 μm $\times$ 5 μm .

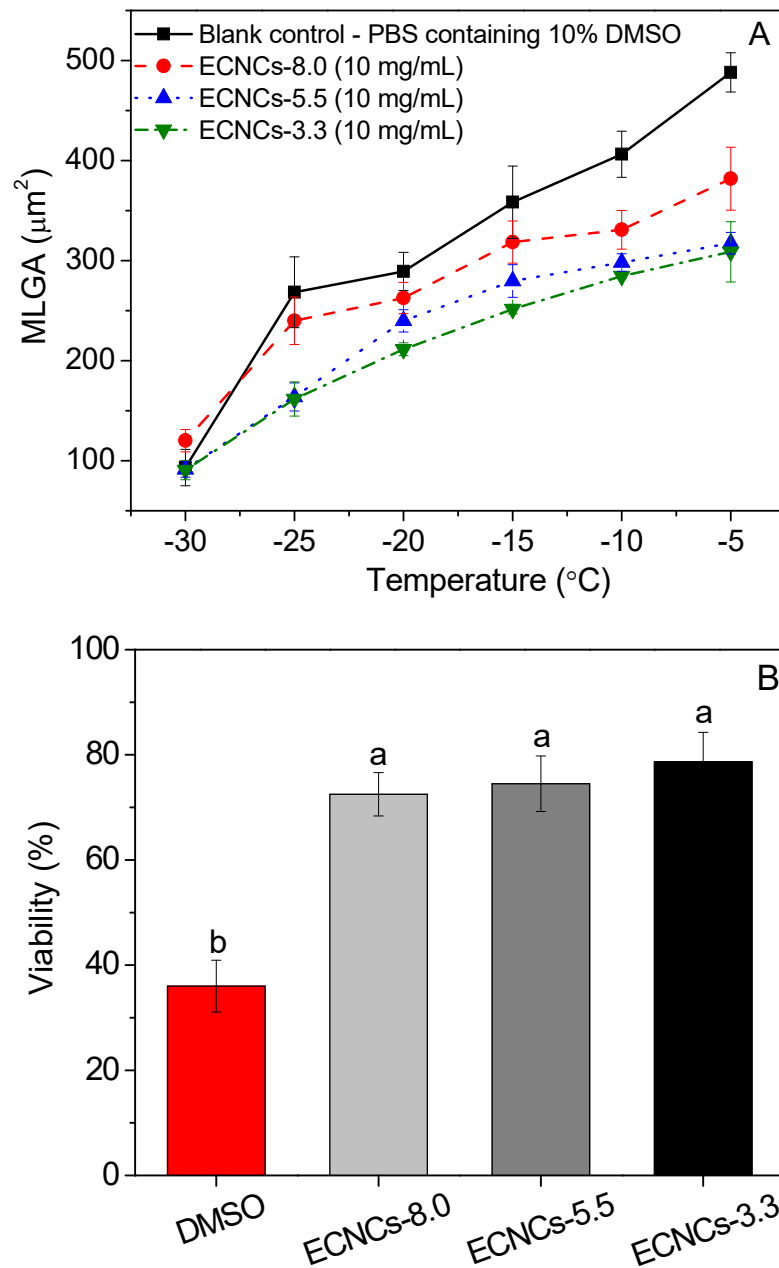


Figure 5-10. (A) The mean largest grain area (MLGA) of ice crystals formed from PBS containing 10% DMSO and 0 or 10 mg/mL ECNCs and (B) the viability of HCT-116 cells in media with comparable compositions, following a fast freezing-slow thawing protocol. Mean values that do not share a same letter are significantly different ($p < 0.05$).

Chapter 6. Concluding remarks and future work

6.1 Conclusions

Nanocelluloses were found to inhibit IR effectively in 0.01 M NaCl at the level of mg/mL without observable TH or DIS activity. The IRI activity of nanocelluloses was impaired by the increases of salt concentration and annealing temperature, which were caused by fibril aggregation and increased ice recrystallization rate, respectively

CNCs with varied SCDs were prepared by varying the duration of HCl hydrolysis, which cleaved the surface sulfate groups without causing a significant change of DP. TEMPO-CNFs with varied SCDs were prepared by varying the amount of NaClO used in the TEMPO-catalyzed oxidation. Physicochemical and morphological analyses confirmed that the prepared nanocelluloses had similar DPs or fibril lengths but varied SCDs. Without causing fibril aggregation, reducing the SCD of CNCs led to an increase of IRI activity with a better resistance to higher unfrozen water fraction and higher annealing temperatures. TEMPO-CNFs with a lower carboxylate group density demonstrated a stronger IRI activity. Lowering pH to protonate the carboxylate groups of TEMPO-CNFs also enhanced the IRI activity. The surface charge effect indicates that the IRI activity of nanocelluloses is associated with their amphiphilicity and higher hydrophobicity leads to an increase of IRI activity until fibril aggregation occurs.

TEMPO-CNFs with similar SCDs and fibril widths, but with different fibril length were prepared by sonication treatments. The IRI activity was enhanced in TEMPO-CNFs receiving sonication less than 30 min because of the reduction of fibril aggregation. Further

increase of sonication time to 60 and 120 min led to the decreased IRI activity due to the reduction of fibril lengths. Our results indicate longer nanocelluloses are more IRI active. The IRI activity was not correlated with the viscosity or gelling properties of nanocellulose dispersions.

Electrosterically stabilized cellulose nanocrystals (ECNCs) were synthesized and studied as a cryoprotectant for HCT-116 cell line under different cryopreservation protocols. ECNCs demonstrated good colloidal stability and observable IRI activity in PBS with DMSO up to 20%. ECNCs improved cell viability at reduced concentrations of DMSO, indicating ECNCs might be used in cryopreservation. The cryoprotection effectiveness did not match the IRI activity in ECNCs, polyethylene glycol, and polyvinyl alcohol; and in ECNCs with different SCDs.

This dissertation has demonstrated that nanocelluloses are IR inhibitors with potentials for cryopreservation application.

6.2 Future work

Although the SCD/fibril length-IRI activity relationships have been elucidated, the molecular mechanism of IRI is still unknown for nanocelluloses. MDS may be a powerful tool to study the interactions between nanocelluloses and ice, which are necessary to understand the exact IRI mechanism. Although the cryoprotection effect of the ECNCs on HCT-116 has been demonstrated, the cryopreservation mechanism of ECNCs remains unknown and should be investigated. Besides, the cryoprotection effectiveness of ECNCs

should be evaluated in more cell lines. Additionally, the cellular function after cryopreservation by ECNCs should be evaluated. Foods are complex systems containing proteins, fats, polysaccharides, and low-molecular-weight solutes. To apply nanocelluloses in food, it is necessary to understand the how nanocelluloses-food ingredients interactions affect IRI.

VITA

Teng Li was born on July 1989, in Haining, Zhejiang province, China. After graduated from Haining Senior High School in 2008, he entered Shanghai Ocean University and obtained a bachelor's degree of Food Science and Technology in 2012. Thereafter, Teng obtained his master's degree in Nanjing Agricultural University, where his thesis focused on the water dynamics in tofu and fermented rice by time-domain NMR. After graduation in 2015, he worked as an application engineer in Shanghai Niumag corporation. In August of 2017, he became a doctoral student in Food Science under the advising of Dr. Tao Wu and Dr. Qixin Zhong. Teng has been working on the project “ice recrystallization inhibition by nanocelluloses.” He has a work offer from Zhejiang Gongshang University.