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Effective cellulose nanocrystal imaging using transmission electron microscopy

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ABSTRACT

Characterization of cellulose nanocrystals (CNCs) is often complex and tedious. With their increased use for biological materials, polymer reinforcing agents, and other applications, better characterization methods of CNCs are needed to ensure product quality. However, because of their small size, hydrogen bonding, and low electron density, individual CNCs are difficult to image with high resolution and magnification using electron microscopy. Methods to help counter these challenges include staining for increased contrast and techniques to increase dispersion. This work tested several stains, dispersing agents, and sample supports to find a consistent method of individualizing CNCs and providing good contrast for imaging in transmission electron microscopy (TEM). The most consistent method found uses a low concentration of CNCs, bovine serum albumin as a dispersing agent, and Nanovan® as the contrasting stain on a silicon monoxide-coated Formvar TEM grid.

1. Introduction

Cellulose nanocrystals (CNCs) are derived from the crystalline regions found in cellulose (Habibi, Lucia, & Rojas, 2010; Kaushik, Fraschini, Chauve, Putaux, & Moores, 2015). Their size can vary greatly depending on source material and extraction method; hence, accurate characterization of individual crystals is important (Habibi et al., 2010). Transmission electron microscopy (TEM) is a well-used method for imaging CNCs but comes with major challenges including the sample preparation factors dispersibility and contrast (Kaushik et al., 2015).

Dispersibility issues arise due to the attractive forces between the CNCs. The crystalline regions of cellulose are held together through inter- and intra-molecular hydrogen bonds and van der Waals forces which are difficult to break (Kaushik et al., 2015; Moon, Martini, Nairn, Simonsen, & Youngblood, 2011). Hydrogen bond stacking and van der Waals forces can hold CNCs together in bundles, especially during the drying on a surface such as a TEM grid, which is difficult to avoid if no other method than sonication is being used for dispersion (Espinosa, Kuhnt, Foster, & Weder, 2013; Hosseinidoust, Alam, Sim, Tufenkji, & van de Ven, 2015; Kaushik et al., 2015; Postek et al., 2011; Qua, Hornsby, Sharma, & Lyons, 2011). Other methods to prevent agglomeration during drying involve altering the pH to allow electrostatic forces to disperse the CNCs or by adding an adsorbing dispersion agent such as bovine serum albumin (BSA) to sterically hinder agglomeration during drying (Kaushik, Chen, Van de Ven, & Moores, 2014; Michen et al., 2015).

The contrast in TEM is affected by electron density of the material and its size. Cellulose is made of mostly carbon, so its electron density is low which limits contrast (Kaushik et al., 2014; Kaushik et al., 2015). CNCs also average between 5 and 20 nm in diameter, which is not much depth for transmitting electrons to interact with the sample. In addition, TEM sample substrates are typically carbon-based so both the sample and the substrate will transmit to the detector similar electron outputs, meaning the contrast between sample and substrate is low (Habibi et al., 2010). A stain containing a heavy metal salt, such as uranyl acetate, is often used to increase contrast. Uranyl acetate is one of the most widely used heavy metal negative stains, but it is relatively expensive, toxic, and radioactive, which introduces restrictions on its use (Ikeda, Inoue, Kanematsu, Horiuchi, & Park, 2011; Kaushik et al., 2014; Kaushik et al., 2015). Other stains are available but have not been widely explored. Though staining benefits imaging contrast other imaging side effects can occur, including aggregation of the sample during drying and measurement inaccuracy during image analysis (Kaushik et al., 2014).

Table 1 outlines various methods used by other researchers for TEM imaging of CNCs, which influenced the work discussed in this paper. In general, lower magnifications produce higher contrast, but are not at an

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Table 1
TEM imaging techniques previously used for CNCs (key below).

Source	Dispersion Method (Dispersant), concentration	Stain	Grid Type	Grid Drying Method	Voltage (kV)	Mag Acceptable for Measurements	Contrast	Dispersion	Stain Comments	Reference
W	US, pH3 (W), 0.1–0.3 w/w%	UA or none	SiO, C, F	–	L or H	G	G	G	NA	Kaushik et al. (2014)
Cotton	(W), 1–2%	UA	C	–	L	G	G	G	S, T	Montanari, Roumani, Heux, & Vignon (2005)
Flax	(W)	1% UA**	CC	Rm Temp	M	G	M	M	S	Qua et al. (2011)
Wood	(CM), 5 mg/mL	UA***	Cu	Overnight	M	G	M	P	S, T, C	Zhou et al. (2015)
Wood	(W), 0.1 w/w%	2% UA*	GC	–	M	G	P	G	T	Hosseinidoust et al. (2015)
Cotton	US (W)	2% AM	Cu	–	L	G	P	G	T	Choi (2006)
Wood	US or S (W), 0.01 wt%	2% UA	F	–	M	M	G	M	S, C	de Paula, Mano, Duek, & Pereira (2015)
Tunicate, Cotton	S (W), 0.1 mg/mL	–	CC	70C 2 h	L	M	G	M	S, C	Jorfi, Roberts, Foster, & Weder (2013)
Cassava Bagasse+	HV	1% AM	Cu	Rm Temp	M	M	G	M	T	Wicaksono, Syamsu, Yuliasih, & Nasir (2013)
Tunicate	(W), 0.1 mg/mL	–	CC	70C 2 h	L	P	G	M	NA	Potter et al. (2014)
Tunicate	BS (DMF), 0.1 mg/mL	None	CC	70C 4 h	L	P	G	M	S	Biyani (2014)
Wood	(W), 0.1 mg/mL	–	CC	70C 1 h	L	P	G	M	S, C	Espinosa et al. (2013)
–	US (CF), 1 w%	2% UA#	CC	–	H	G	G	M	S, C	Kvien, Tanem, & Oksman* (2005)

Key for Table 1

Dispersion		Stain		Grid Type		Voltage	
> Mechanical Dispersion		UA	uranyl acetate	Cu	Copper	H	High (150–200 kV)
S	sonicate (general)	AM	ammonium molybdate	C	Carbon	M	Medium (100–120 kV)
BS	bath sonicator			CC	Carbon Coated	L	Low (60–80 kV)
US	ultrasonicator			F	Formvar		
HV	high velocity mixer			GC	Glow discharged Carbon		
pH3	water adjusted to pH 3 using HCl			SC	SiO coated		
> (Solution Medium)							
(W)	water						
(DMF)	DMF						
(CM)	cell culture media (Mueller-Hinton)						
(CF)	chloroform						

Mag Acceptable		Contrast and Dispersion		Stain related	
G	Mag optimal range (< 300 nm scale)	G	Good	C	crystals
M	Mag acceptable with additional contrast strategies like staining (> 300 nm, < 1 μm scale)	M	Moderate	S	splotchiness/excess
P	Mag too high (> 1 μm scale)	P	Poor	T	stain not targeted onto CNCs

– No information.
+ Nanofibrils, not cellulose nanocrystals.
* Drop of stain on for 5 min, washed 3 x by adding a drop of water for 10 s each (rinse method).
** Drop of stain on for 30 s, then immersed in water (dunk method).
*** Dipped in glutaraldehyde before dipped in UA (study with E.coli).
# CNC solution allowed to drip through the grid, then the grid floated in UA solution for 3 min.