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Cryo-Scanning Electron Microscopy Analysis for the Structural Evolution of Cellulose Nanocrystals based Hydrogels

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Cellulose nanocrystals (CNCs) are highly crystalline spindle-shaped particles that have received much attention due to their remarkable properties such as ease of surface functionalization, mechanical properties, bio-sustainability, bio-renewability, relatively lower production cost, and low cytotoxicity [1-2]. Therefore, many studies have been conducted to use these attractive nanomaterials in the matrix of polymer gels (i.e. hydrogel) for improving the gel properties [3-4]. However, the influence of CNCs on hydrogel structure is not yet fully understood since there are two major challenges when examining the structure of CNC-based hydrogels using electron microscopy (EM) technique. First, high water content materials such as hydrogels should be characterized at high vacuum operating conditions but this approach causes the liquid to evaporate, altering the original hydrogel structures during imaging. Due to this phenomenon, freeze dried methods combined with thin-metal coating have been used widely for hydrogel structure analysis using scanning electron microscopy (SEM). The second challenge is that both of the CNCs and the hydrogel have similar electron densities, and therefore poor contrast between the CNCs, polymer network and water prevents the resolution of the individual components. As result, standard EM technique cannot be applied to the hydrogel composite materials [5-6]. Therefore, in order to observe the changes of hydrogel structure with the distribution of CNCs directly without any pretreatment of samples, it is critical to develop the cryo-SEM characterization technique with new sample preparation methods.

In this study, two different CNC based hydrogel nanocomposites were used to understand the effect of CNCs on hydrogel structure and its distribution. The first hydrogel was poly(4-vinylbenzenesulfonic acid sodium salt) (PVBS) to which the CNCs (2% w/v) were added to enhance the adsorption characteristics and rheological properties. The second hydrogel was agarose (12.5% w/v) gel prepared in phosphate buffered saline (PBS), which has can be used in mammalian cell culture. CNC was added to the agarose gel to increase rigidity and create 3D culture scaffolds.

To preserve the structure of the CNC-based hydrogels closer to their native state, a freeze fracture method were used in this study (Figure 1a). First, a piece of gel sample was placed onto one of small copper-based rivets and the second rivet is carefully placed on top of the sample. Then, the ensemble of two rivets was frozen by plunging it vertically into liquid nitrogen (LN₂). The frozen hydrogel sample was then fractured by two tweezers to expose the internal structure of hydrogel (Figure 1b). To remove ice crystals from the revealed fracture surface, it was sublimated for 20 min at -100°C and subsequently sputter-coated with platinum in cryo-coater (Leica ACE600). After this, the sample was eventually transferred by VCT100 shutter (Figure 1c) in the cryo-stage SEM (Zeiss Nvision40) with temperature controller and re-sublimated for 20min at -100°C until any remaining ice crystals were removed during transfer process (dual sublimation process). Then, the sample surface was observed under an

accelerating voltage of 3 keV at $-140\text{ }^{\circ}\text{C}$ using the in-lens EsB (energy and angle selective backscattered electron) detector (Figure 1d).

As shown in Figure 2a, the CNCs used for this study appeared as long needle-like structures approx. 200 nm in length and 6 nm in width by atomic force microscopy (AFM). The PVBS hydrogel without CNC exhibited a honey-comb matrix, with pores $\sim 30\text{ }\mu\text{m}$ in diameter, which were defined by thin and flat faces (Figure 2b). Image at higher magnifications revealed that the broken flat wall is composed of close-packed nanofibers (Figure 2b, inset). For the agarose gel, a smaller honey-comb matrix, with pores $\sim 3\text{ }\mu\text{m}$ size, defined by polyhedral chambers with flat faces was observed (Figure 2c). At higher magnifications, the chamber walls are composed of entanglements of nanofibers (Figure 2c, inset). However, the addition of CNCs to each polymer did affect the final structure of both hydrogels: for PVBS, its chamber size became smaller, with the individual CNCs visible in the chamber walls as seen in the inset of Figure 2d. In the case of agarose gel, the ball-like structures were formed within complex fibril-like composites (Figure 2e) and as the inset shows, CNC-like structures were present on their surface. These results suggest that CNCs are evenly dispersed throughout the hydrogel matrix and can affect the final structure of hydrogels by altering the size and morphology of the hydrogels significantly.

In summary, freeze fracture methods and dual sublimation techniques were developed for successful characterization of two different hydrogels with/without CNCs by cryo-SEM. Notably, CNC structures were successfully visualized in the hydrogel matrix. The changes that were observed in the structure of the hydrogels after adding CNCs may affect the properties of the hydrogel and therefore their final applications: smaller chamber size in PVBS and ball-like shapes in agarose gel. In near future, cryo-SEM/FIB techniques will be developed for characterization of various types of hydrogels along with novel biomedical applications.

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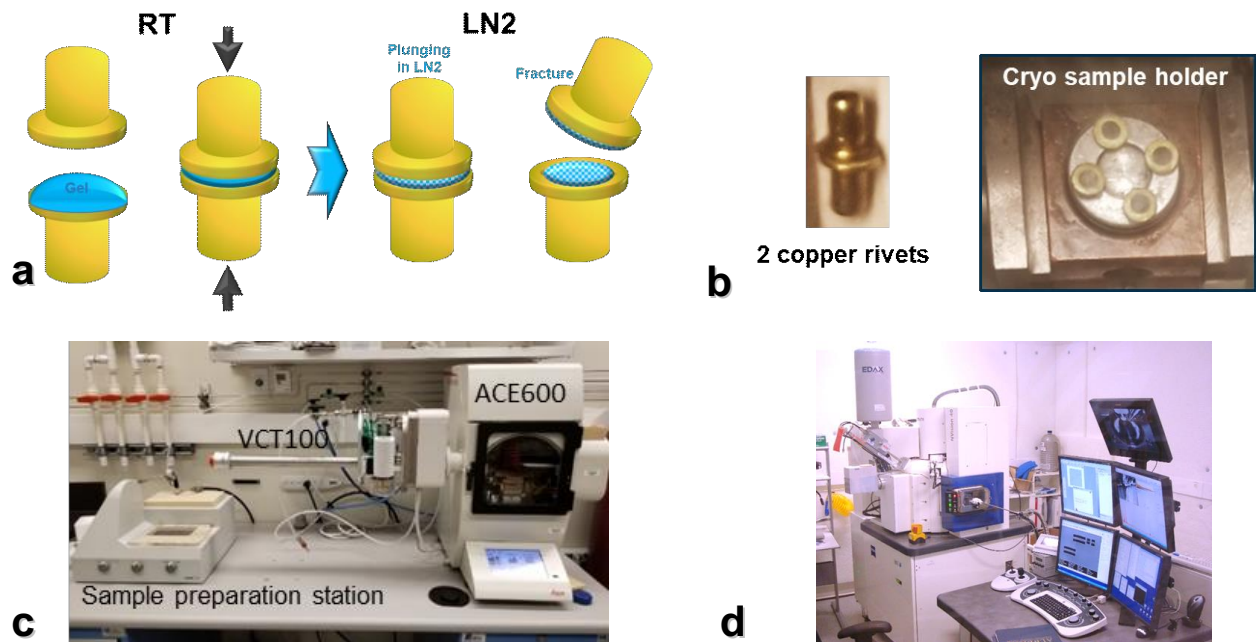


Figure 1. Cryo-SEM: a) schematic of freeze fracture method, b) photos of copper rivets and cryo sample holder c) sample preparation station, cryo transfer system (VCT100) and cryo coater (ACE600), and d) ZEISS NVision40 cryo-SEM/FIB system.

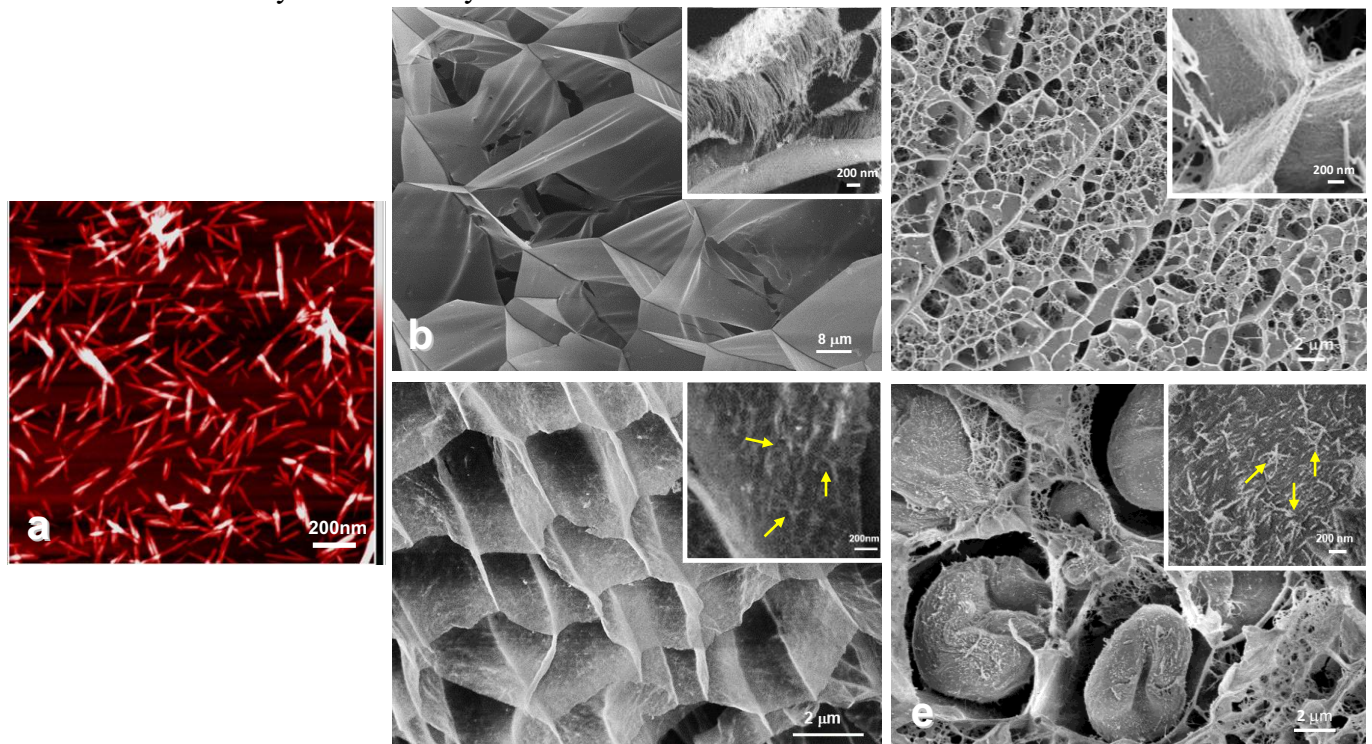


Figure 2. AFM and Cryo-SEM images of CNC based hydrogels: a) AFM image of CNCs and cryo-SEM images of b) PVBS hydrogel, c) agarose gel, d) CNC based PVBS hydrogel, and e) CNC based agarose gel. Insets are high-resolution images and yellow arrows indicate CNCs.