ORNL/TM-2022/2770
CRADA/NFE-19-07867

CRADA Final Report: CRADA Number NFE-19-07867 with Neptune Fluid Flow Systems

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Date: July 2022

OAK RIDGE NATIONAL LABORATORY
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Prepared by
OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee 37831-6283
managed by
UT-BATTELLE, LLC
for the
US DEPARTMENT OF ENERGY
under contract DE-AC05-00OR22725

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Summary

Neptune Fluid Flow Systems is a rising engineering firm with a technology focus in improving cryogenic sample preparation for cryogenic-transmission electron microscopy (cryo-TEM) studies. To advance the R&D of its innovative cryogenic preparation method, Neptune has teamed up with the Center for Nanophase Materials Science (CNMS) at Oak Ridge National Laboratory (ORNL) to achieve the following aims: (1) validate the technical feasibility of CryoSheet sample prep method, (2) design, fabricate and test the cryo-stage support, and (3) experiment and achieve success with the novel sample preparation method on different soft matters.

Description of the Project

Cryo-transmission electron microscopy (Cryo-TEM), a structural characterization technique that studies samples at cryogenic temperatures (generally liquid-nitrogen temperatures), has the potential to dramatically change the current landscape of advanced manufacturing in the U.S. by giving scientists in both industry and academia a much better understanding of the organized structures of novel nanomaterials and polymer composites in solutions, as well as how to control their different morphologies and macroscopic functions at the microscopic level, thus offering promising directions for future research, innovations, and consumer products. To unleash the full potential of cryo-TEM through the complex yet diverse materials space—which spans everything from synthetic precursors, to transient assemblies, battery materials, ionic liquids, nanoparticle bioconjugates and biomimetic such as peptoids currently used in drug delivery, everyday commodities such as paints, synthetic polymers, ice cream, and toothpaste—it is critical to find a more reliable and reproducible method to deposit and vitrify the wide array of soft material samples for study under an electron microscope.

Our team at Neptune Fluid Flow Systems is developing a method to simultaneously deposit and vitrify these beam- and environmentally-sensitive hydrated specimens on cryo-TEM grids in a more reliable, reproducible, and repeatable manner compared to the market standard, which simply deposits and vitrifies the grids in a stepwise fashion and consequently has a high failure rate. This technology, based on the use of sheet nozzles, also has the ability to address the sample thickness issue raised by the materials science and structural biology communities.

As will be discussed in the following section, the work done within this collaboration between the Center for Nanophase Materials Science (CNMS) at Oak Ridge National Laboratory (ORNL) and Neptune Fluid Flow Systems has overcome many of the technological hurdles and demonstrated the market readiness of this technology through total process optimization and prototyping. Most importantly, it has addressed many of the technical unknowns including compatibility and generality of the proposed innovation.

Results

Task 1:
Technical feasibility study on the CryoSheet sample preparation method

Our work has proven the technical feasibility of sheet nozzles in producing, depositing, and vitrifying thin layers of liquid with buffer solutions, detergent solutions, and organic solvents that are commonly encountered in structural biology and materials science research. For example, tests with phosphate-buffered saline (PBS) solution, pH 7.4, showed the sheet jet runs the same as with pure water (**Figure 1**). Previous issues with salt build-up were not observed, even when the jet was left running for days at a sample consumption rate of 150 µL/min (**Figure 3**), and cycled on and off dozens of times. The same holds for KCl and KH_2PO_4, pH 7.3, DHPC, and Tween 20 of concentrations 1% or less. Ethanol and ethylene glycol (which are more viscous than water) ran stably in the jet but at higher gas and liquid pressures (**Figure 2**). During these test runs, we also investigated the effects of force impact, flow rate, and setup geometry of the liquid jet on the vitreous ice layer—both its thickness and its consistency of thickness, and were able to show that we can achieve a high level of user control in all instances. Furthermore, we developed a method for measuring the thickness of the sheets through thin-film interference and are compiling a table of all the parameters necessary to ensure consistent and precise deposition thicknesses. We also determined that the dead volume in our system is largely a product of capillary length from the sample reservoir to the sheet nozzle. Keeping this tube as short as possible while minimizing precooling of sample that have a low viscosity index before deposition onto a grid is critical.

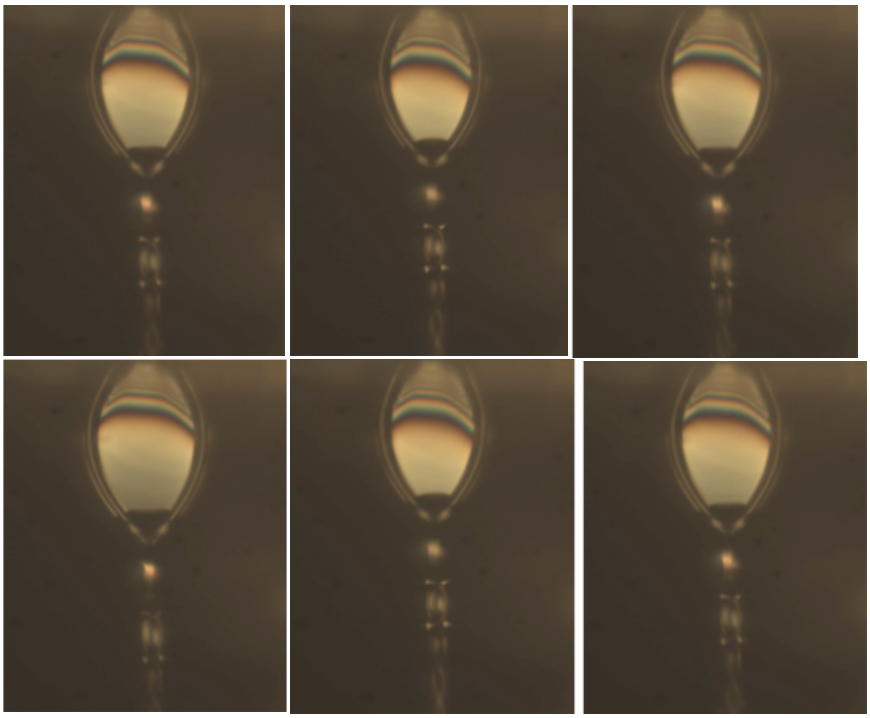

Figure 1: (top) left to right: H_2O, Tween 20, and DHPC. (bottom) left to right: PBS, pH 7.4, KCl and KH_2PO_4, pH 7.3, and ethanol. All done at the same pressure and flow rate. The sheets and their thickness look almost indistinguishable.

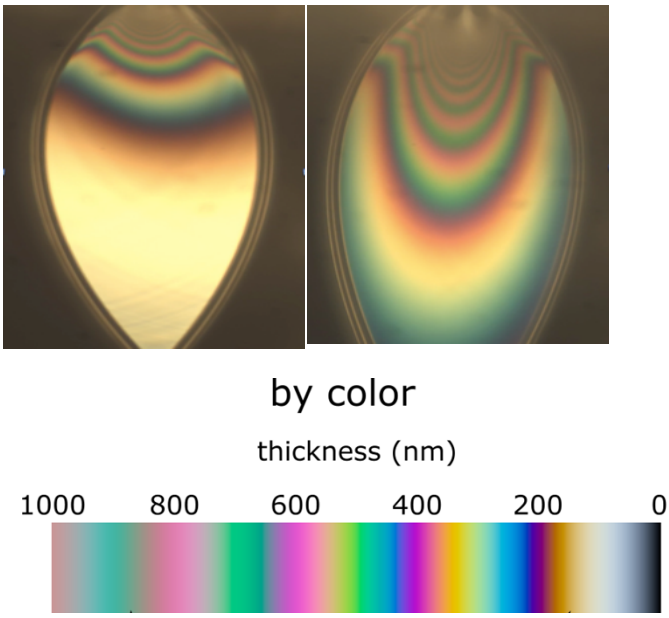

Figure 2: demonstration of thickness control in ethylene glycol: (left) thin sheet, (right) thick sheet. Further, we are able to determine the sheet thickness based on spectral colors.

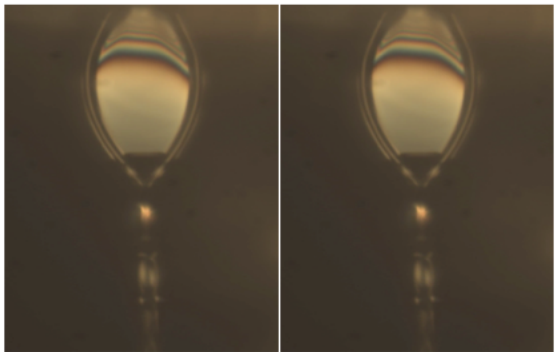

Figure 3: PBS, pH 7.4 solution (left) after 1 min of run time, and (right) after 72 hrs of run time and 100 on-and-off cycles. Zero change in jet is observed.

Figure 4: our two-piece cryo-stage. The top portion with eight grid slots is made of aluminum while the body is stainless steel.

Task 2:
Fabricate and test the cryo-stage support

We have designed and fabricated an aluminum cryo-block v1.0 to maintain corrosion resistance and a very high thermal conductivity, all at a relatively low cost (**Figure 4**). The rotational symmetry of our cryostage allows the placement of new grids into position by externally rotating the stage after each deposition. This arrangement allows for preparation of up to 8 grids in vacuum chamber. Furthermore, a plug thermocouple can be placed directly into the center of the cryostage for monitoring the temperature of the grids before, during, and after sample deposition. Lastly, the top of our cryostage is removable relative to the base to facilitate easy transfer of grids to grid boxes. This setup was used for investigation in the next section.

Task 3:
Prototype of the proposed concept

We validated Neptune's cryo-TEM sample preparation method on different soft matters and conducted a benchmark study against the current plunge-freeze based preparation approach. In particular, we did not observe any sample clogging or drying-up on the nozzle tip when we ran different soft sample types (ionic liquids, protein-embedded nanodiscs, and liposomes) through the sheet nozzles at a constant flow rate for an extended period of time. In fact, we were able to produce a thin sheet of liquid at ambient conditions in every test.

Once we have determined the optimal range of deposition angles on our full setup, we then tested it with some more challenging samples. In the first case, we successfully deposited, vitrified, and imaged a membrane protein-like ionic liquid that contains micellular structures (**Figure 5.L**). This highly viscous sample was previously unable to be imaged due to multiple failed attempts to thin it on TEM grids by our collaborator. At present, optimization of the deposition conditions for high viscosity samples such as liposomes and solubilized membrane proteins is still on-going. Precooling is a critical step when working with low viscosity index solutions (i.e., those whose viscosities are very sensitive to temperature changes). Our tests with protein-embedded nanodiscs (**Figure 5.R**) showed no sign of aggregation or dissociation after going through the sheet nozzle. As shown in **Figure 6**, we were also able to image and confirm our single-walled liposome sample was on average spherical in shape with only a slight 1.2-1 length-width anisotropy.

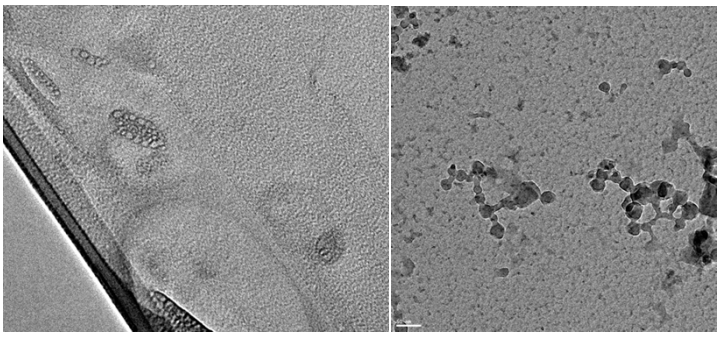

Figure 5: Cryo-TEM images of (left) ionic liquid containing micellular structures, and (right) protein- embedded nanodiscs. (Neptune's unpublished data)

Grids with different thicknesses were prepared both reliably and controllably using our setup. As shown in **Figure 7**, deposited layers as thin as 15nm and up can be made. It is important to note that we can control ice layer thickness as needed to account for changes in sample viscosity due to temperature drops during deposition.

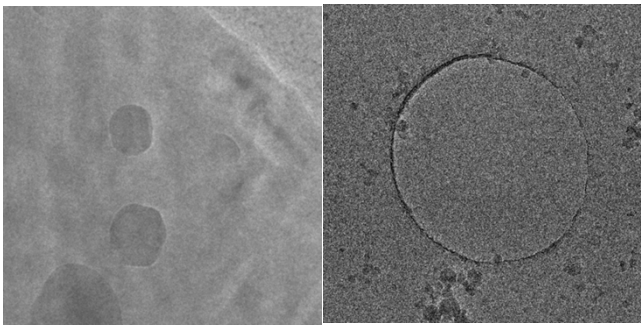

Figure 6: Cryo-TEM images showing that at an ice thickness of 100nm, the 75-nm liposomes (left) were preferentially located over the grid holes, whereas the protein embedded nanodiscs of ~10nm in size embedded in 100 nm thick ice (right) were nowhere to be found over the holes. (Neptune's unpublished data)

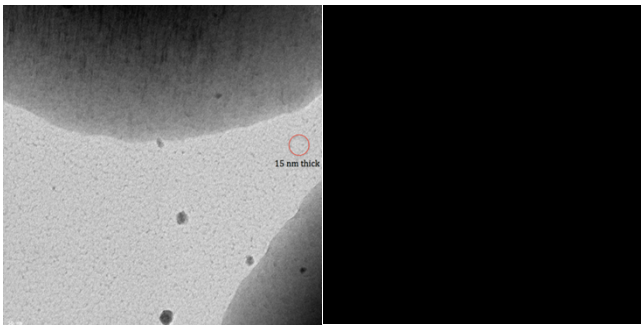

Figure 7: Cryo-TEM images showing (left) a 15nm-thick deposited liquid sheet, and (right) another deposited sheet with an ice thickness that was too high to measure through. (Neptune's unpublished data)

Finally, we examined a sample of worm-like micelles with bound proteins prepared using either the common plunge-freezing method or Neptune's preparation method under an electron microscope. As shown in **Figure 8**, there was a clear difference in the two sets of images, indicating that Neptune's approach was far superior in being able to preserve the sample integrity during the preparation step whereas the plunge-freezing method failed to do so.

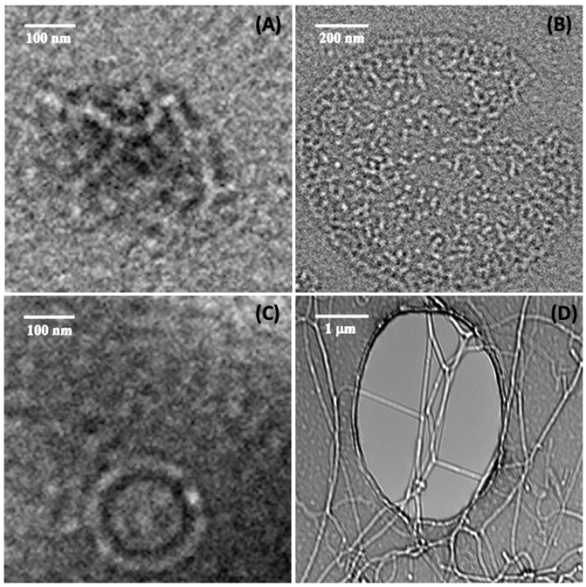

Figure 8: Cryo-TEM images of worm-like micelles with bound proteins. They are prepared via a plunge freezing method (A and B), or Neptune's novel approach (C and D). In the first case, (A) the sample has distinctly formed small clumps that then lump together to form bigger ones, showing clear signs of denaturation of the worm-like micelles. (B) is an image of another clustered clump with more fine details; whereas by switching to Neptune's method, the sample shows up as (C) vesicles and a large network of (D) tubular structures that extend across the entire grid as expected. (Neptune's unpublished data)

Future Directions

In the next few years, Neptune will continue to push forward with its R&D work in the cryo-TEM sample preparation and microfluidics space with funding support from federal grants and product sales. Meanwhile, the company will also carry on with innovating and identifying unmet needs within its technology focus area, with the goal of becoming the major supplier of microfluidic connectors and the world's leader in end-to-end sample management for cryopreservation work. Finally, we look forward to continue collaborating with our technical mentor, Dr. Ilia Ivanov, on various projects.

Related Publications & Awards

McQueen, T. & Liang, W. (2020). "System and Method for Preparing Cryo-EM Grids". U.S. Patent No. 10,770,265B1. Filed 19 March, 2020. Issued 8 September, 2020.
McQueen, T. & Liang, W. (2020). "System and Method for Preparing Cryo-EM Grids". U.S. Patent No. 10,866,172B2. Filed 24 July, 2020. Issued 15 December 2020.
McQueen, T. & Liang, W. (2020). "System and Method for Preparing Cryo-EM Grids". International Patent Appl. No. PCT/US20/23496. Filed 19 March, 2020. *Patent Pending*.
McQueen, T., "Capturing Molecular Actions via Vitrification", ORNL Finalist, Ignite-Off! Competition (2020), Oak Ridge, TN.