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Applications and Design of Reinforced Silicon Nitride Windows for *In Situ* Liquid Transmission Electron Microscopy

Madeline J. Dukes,[1] Albert D. Dukes, III,[2] Kate L. Klein,[3] Rebecca Thomas,[1] Deborah F. Kelly,[4] and John Damiano[1]

[1] Protochips, Inc. Raleigh, NC 27606, USA
[2] Lander University, Department of Physical Sciences, Greenwood, SC 29649, USA
[3] Department of Mechanical Engineering, University of the District of Columbia, Washington, DC 20008, USA
[4] Virginia Tech Carilion Research Institute, Virginia Tech, Roanoke, VA 24016, USA

In situ transmission electron microscopy (TEM) imaging through liquids has gained significant interest for a broad range of biological and material science applications [1, 2]. Liquid samples can be confined in a closed cell created by stacking two silicon nitride (SiN) microchips together, creating an isolated liquid chamber within the vacuum system of the microscope. However, creating and maintaining thin, uniform liquid specimens is challenging due to the pressure differential between the liquid-filled cell and the column vacuum. This issue causes significant bulging to occur across the thin membranes [3, 4]. We have shown that reinforcing the SiN membrane reduces membrane bulging while maintaining a suitably large imaging area [1]. Here we describe two types of patent-pending microchip designs with reinforced membranes that further improve the uniformity and area available for sample observation.

Imaging windows etched into the SiN microchips consist of a 200-nm thick SiN film that is etched down to 50-nm in discrete regions creating wells that can accommodate 150-nm of liquid thickness. The first type of microchip design maintains the 200-nm thick nitride frame of the imaging window with arrays of "microwells" for sample confinement. The array of etched microwells can vary in dimension and number as well as the dimensions of the overall imaging window. We have demonstrated arrays of up to 16×16 microwells with dimensions of 10×10 μm can be readily fabricated across a 400×400 μm window (Fig. 1A). Using these designs, significant bulging was not observed even with large window dimensions. This effect may be attributed collectively to the 200-nm thick frame and adhesive forces that exist between the stacked microchips as they come into contact in the absence of any spacers. As samples are confined within the microwells, identifying regions of interest is simplified and data can be acquired from independent microwells.

Employing the microwell-containing microchips for *in situ* TEM imaging, we were able to image a variety of biomedically relevant macromolecules, such as rotavirus particles synthesizing viral mRNAs (Fig. 1B) and liposome vehicles for drug delivery (Fig. 1C). In addition to using the microwells to study biological macromolecules, we were able to visualize real-time processes occurring within individual microwells such as electron beam induced *in situ* growth of lead nanoparticles from a lead nitrate (Pb(NO3)2) solution (Fig. 2). The rate of growth is dependent on both the electron beam intensity and the concentration of the lead nitrate. Nucleation of the lead particles occurs rapidly upon exposure to the electron beam, which acts as the reducing agent. In samples with a higher concentration of lead nitrate, nucleation is followed by a period of particle growth. Ostwald ripening was observed to begin after approximately 7-8 minutes of exposure to the electron beam. Following the Ostwald ripening, the lead nanoparticles begin a period of sustained growth resulting in larger structures with varying morphologies.

Using the second type of microchip design, the majority of the window is etched leaving behind square posts of 200-nm thick nitride (Fig. 3). Arrays of posts in static cells and posts within flow channels were produced. The posts prevent the membranes from collapsing as well as providing an adhesion point, yielding uniform cell thickness. This design is particularly useful for imaging non-liquid viscous materials such as gels, creams, and emulsions in a native, hydrated state because the posts limits the amount of material that is deposited onto the thin (50 nm) region of the window when the top microchip is positioned onto a thinly spread layer of sample. The material is confined between the windows and imaged in air without introducing artifacts due to dehydration or freeze-fracture preparation.

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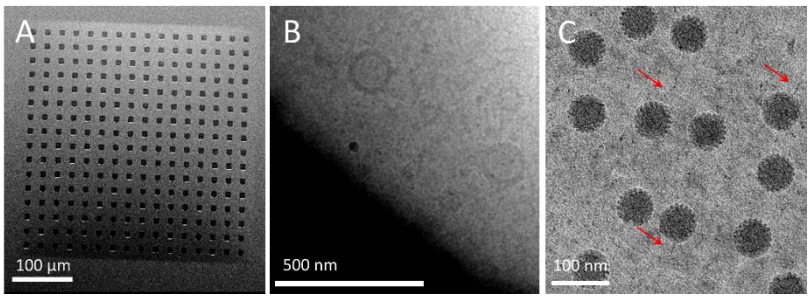

Figure 1: Microwell array etched SiN windows for imaging biological macromolecules. (A) SEM image of a microchip containing a 16×16 array of 10×10 µm microwells etched onto a 400×400 µm SiN window. Each well contains 150 nm of liquid. (B) TEM image of peglylated liposomes in solution. The dark region at the left of the image is the edge of a microwell. (C) Transcribing rotavirus particles imaged in solution with TEM and contained in a microwell array microchip. The arrows indicate RNA strands exiting the rotavirus particles.

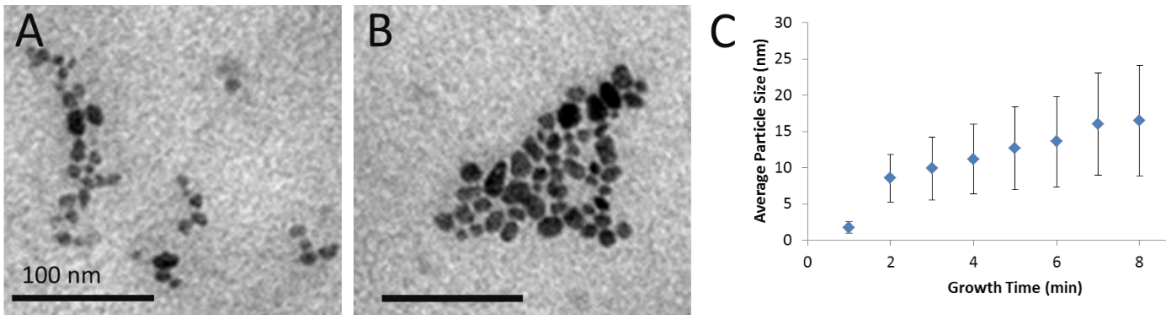

Figure 2: Time series of a beam induced growth of lead nanoparticles in solution imaged with TEM. A solution of 40 mM solution $Pb(NO_3)_2$ dissolved in water was contained in the microwells of the E-chip. (A) Lead nanoparticles after 2 minutes of beam exposure. (B) Lead nanoparticles after 8 minutes of beam exposure. (C) Nanoparticle growth over time. The average size for each time point was calculated from the average diameter of the individual particles at each time point. The total number of particles at each time point was 70-250.

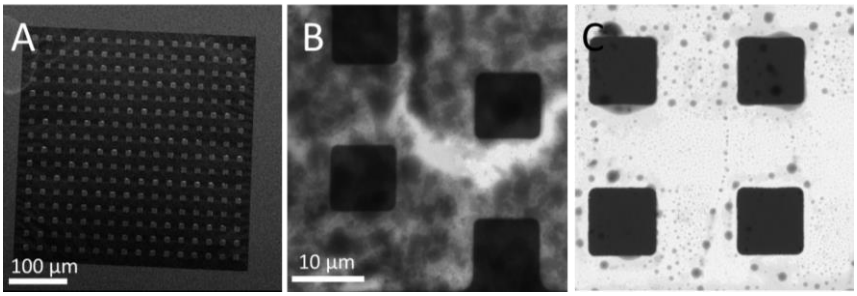

Figure 3: SiN windows etched with reinforced posts. (A) SEM image of a 16x16 array of 10×10 µm posts in a 400x400 µm etched SiN window. (B) TEM image of commercially available sunscreen imaged in a hydrated state using a post-reinforced SiN microchip. (C) TEM image of commercially available cosmetic cream imaged in a hydrated state using a post reinforced SiN microchip.

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