Characterization of Extracellular Vesicles Produced from Glycocalyx-Engineered Cells *in vitro*

CNF Project Number: 2272-14

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Primary Source(s) of Research Funding: National Science Foundation Graduate Research Fellowship

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Primary CNF Tools Used: Malvern NS300 NanoSight

Abstract:

Extracellular vesicles are key mediators of intercellular communication and are a rising area of biomedical research in disease diagnostics and therapeutics. However, surprisingly little is known about the glycobiology of extracellular vesicles, despite the fact that the cell glycocalyx dictates many of a cell's interactions. Specifically, very little is known about the role of the glycocalyx in EV production and function or whether EVs can be engineered through rational manipulation of the glycocalyx. Here we describe recent efforts to investigate the effects of glycocalyx engineering on the production of extracellular vesicles *in vitro*.

Summary of Research:

Extracellular vesicles (EVs) play a key role in intercellular communication. They have been shown to carry a wide range of cargoes, including DNA, coding and non-coding RNAs, and proteins. Because of the diverse nature of their cargoes, and their innate biocompatibility, EVs have quickly become a prominent focus in numerous areas of biomedical engineering research, including disease pathogenesis, diagnostics, drug delivery, and targeted therapies.

However, there are still many aspects of EV biology which are still poorly understood. One largely unexplored area is the significance of the glycocalyx — a polymer meshwork of proteins, nucleic acids, and glycans, which dictates numerous cellular interactions — on EV biogenesis and function. Specifically, the capacity for the production of rationally designed EVs through engineering of the glycocalyx remains poorly understood. It has been previously shown that engineering the glycocalyx can result in membrane morphologies which are favorable for the formation of certain types of EVs [1]. This report summarizes research from the last year which demonstrates that glycocalyx engineering, specifically be the overexpression of the mucin glycoprotein MUC1, results in an increase in production of EVs with size characteristics consistent with classical exosomes and microvesicles.

To engineer the glycocalyx, MCF10A cells were genetically engineered to overexpress a MUC1.mOxGFP construct on the cell membrane, hereafter referred to as MCF10A-1E7 cells. Expression of MUC1-mOxGFP in MCF10A-1E7s was tied to a tetracycline-inducible promoter, and cells were treated with doxycycline (Dox) for 24 hours at a concentration of either 0.1 ug/mL or 1 ug/mL to induce MUC1-mOxGFP overexpression. MCF10A cells engineered with only the promoter but no MUC1-mOxGFP construct, hereafter referred to as MCF10A-rtTA cells, were used as a negative control. After Dox treatment, the cells were switched to serum-free media and cultured at 37°C, 5% CO_2 for 15 hr to 18 hr. EV-containing media was harvested and EV concentration was measured by nanoparticle tracking analysis (NTA) using the Malvern NS300 NanoSight.

Figure 1 shows that media harvested from MCF10A-1E7 cells contained a higher concentration of EVs of sizes consistent with exosomes (50-150 nm) and microvesicles (100-1000 nm) compared to MCF10A-rtTA cells. Additionally, these data suggest that EV production in MCF10A-1E7 cells increased in a Dox concentration-dependent manner. These findings are further supported by Figure 2, which shows the EV concentrations of each of nine fractions obtained by density gradient ultracentrifugation

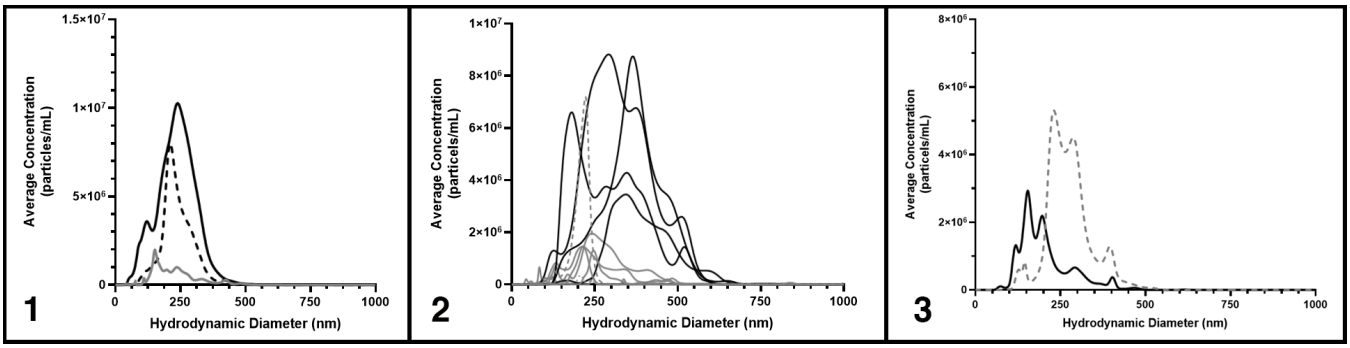

Figure 1. left: EV preparations from glycoengineered MCF10A cells. EV particle concentrations measured by NTA from MCF10A-rtTA cells (solid gray line), MCF10A-1E7 cells induced with 0.1 ug/mL Dox (dashed black line), and MCF10A-1E7 cells induced with 1 ug/mL Dox (solid black line). Curves represent the average reported concentration from five video recordings.

Figure 2, middle: Density gradient centrifugation of EVs from MCF10A-1E7 cells. EV particle concentrations measured by NTA from deionized water (neg. control, gray dotted line); 240 nm NIST nanospheres (pos. control, gray dashed line); density gradient fractions F1, F2, F7, F8, and F9 (solid gray lines); and density gradient fractions F3, F4, F5, and F6 (solid black lines). Curves represent the average reported concentration from five video recordings

Figure 3, right: Triton X-100 Treatment of EVs from MCF10A-1E7 cells. EV particle concentrations measured by NTA from mock-treated sample (neg. control, gray dotted line) or sample treated with Triton X-100 (solid black line). Curves represent the average reported concentration from five video recordings.

of EVs from MCF10A-1E7 cells using a protocol adapted from Jeppesen, et al. [2]. The vast majority of EVs were contained in fractions F3, F4, F5, and F6, which is consistent with previously published data of exosome samples [2]. Deionized water and NIST 240 nm nanospheres were used as negative and positive controls for EVs, respectively.

In order to validate that the particles analyzed by NTA were biological in origin, EVs from MCF10A-1E7 cells were treated with 0.1% Triton X-100 at room temperature for 10 min to solubilize lipid membranes. Mock-treated MCF10A-1E7 EVs were used as a negative control. Figure 3 shows the dramatic reduction in both the EV concentration and the average EV size profile following Triton X-100 treatment, demonstrating that the samples do in fact contain EVs bound by biological lipid membranes.

Conclusions and Future Steps:

Altogether, these data provide the first evidence that EV production can be controlled by engineering the glycocalyx of cells. Further experiments are needed to conclusively prove that the particles detected by NTA contain the classical exosome or microvesicle markers, such as CD63, ALIX, TSG-101, and Annexin A1.

Additionally, future experiments will strive to study the glycobiology of these engineered EVs. More NTA experiments will be conducted at the CNF as part of an effort to reliably segregate exosomes and microvesicles in order to more precisely study the exosome and microvesicle glycocalyx properties.

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NanoScale Hole Patterns Etched into Glass for Spectral Sensing

CNF Project Number: 2898-20

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Primary CNF Tools Used: ASML 300C DUV, GCA 5x Autostep i-line stepper, Oxford 81 / Oxford 100 etchers

Abstract:

Over the past several years the ASML 300C DUV and GCA 5x Autostep i-line stepper have been used to produce pillar and hole features with diameters ranging from 232 nm to 816 nm on fused silica and silicon wafers as part of an academic account. These wafers have been further processed to include front and backside patterned metals layers in an academic cleanroom in New York City and then sent to our contract manufacturer to process them into spectral sensors. This report details how this process has been exported over to a complete manufacturing process for scale. This manufacturing process is split into parts. The first part consists of contract work at CNF where nanoscale hole features are patterned with the ASML 300C DUV and etched with the Oxford 81 and Oxford 100 etchers on an industrial account created this past year. The wafers are then further processed by a series of contract manufacturers to ultimately produce spectral sensors at scale. The initial results of the process have produced near identical results to the older process with a small decrease in throughput.

Summary of Research:

In previous years, a process for patterning nanophotonic pillar and hole structures was developed at CNF which used the ASML 300C DUV stepper as well as the GCA 5xAutostep i-line stepper. These features were etched into the substrate material using the patterned resist as an etch mask. The ASML 300C DUV stepper process has been used to pattern 4" borosilicate float glass wafers ("borofloat"), 4" fused silica wafers, and 4" silicon wafers. Pillar features like those shown in Figure 1 were fabricated with diameters ranging from 232 nm to 816 nm. Hole features were fabricated with design diameters ranging from 306 nm to 446 nm. Optimal depth of focus (DOF), exposure dose, and etch time were determined for nanophotonic patterns in fused silica by varying these parameters incrementally and examining the resultant features. Photonic crystal geometry was examined in the SEM and photonic crystal performance was assessed optically via extraction of waveguided light.

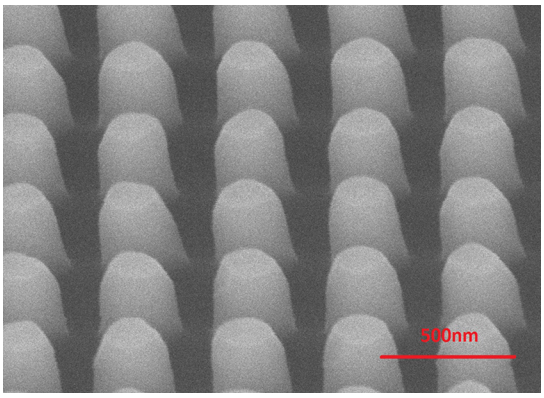

Figure 1: SEM image of photonic crystal pattern, nominally with 270 nm pillar features, fabricated fused silica with process developed with ASML 300C DUV stepper.

For recent applications, nanophotonic patterning is mainly focused on holes versus pillars because pillars are more likely to become damaged in a way that renders them useless for our spectral application during further processing and wafer handling.

In recent years, processing steps have been added to the wafer after nanophotonic crystal pattering to include both front and back side aluminum reflector layers which were developed in an academic cleanroom in New York City. These added layers are combined with single edge angled polishing after die singulation as shown in Figure 2, which is done in Asia. Additional front and backside

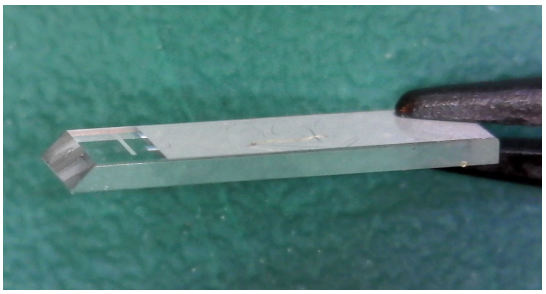

Figure 2: Diced and polished fused silica die with pattered Al reflectors on both sides in addition to the nanophotonic pattern and 45° edge polish.

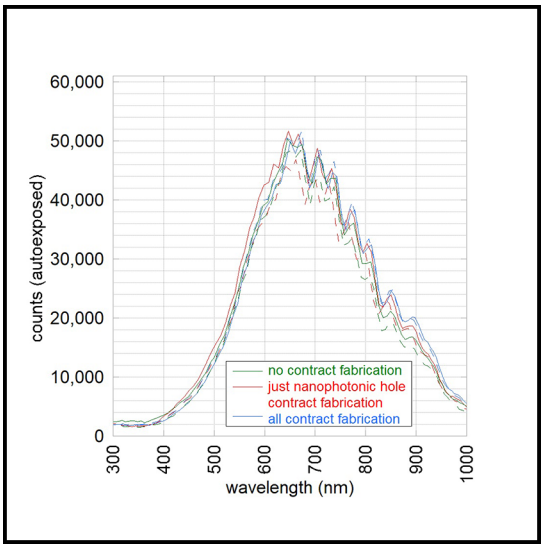

Figure 3: Overlay plot comparing dies with different amounts of contract manufacturing. Data has been normalized to they have the same peak values.

Figure 4: Example of spectrometer batch made with contract manufacturing process that includes fabrication steps at CNF.

patterned black absorber layers were also added to the process in Asia for better light handling. The dies are then built into spectral sensor housing with commercial off-the-shelf linear detectors to make spectrometers.

This last year, the complete process was exported to contract manufacturing, which utilizes the CNF ASML 300C DUV for submicron patterning of the photonic holes that are then etched using the Oxford 81 and Oxford 100 etchers. The nanophotonic patterning that is etched into fused silica wafers at CNF is done by a local CNF user who does contract work. The wafers are then transferred over to a local foundry for front and back side metal patterning. The wafers are then sent to our contract manufacturer in Asia where the additional patterned absorber layers are applied to the front and back side, the wafers are diced, and the die edges are polished at an angle. These dies are then built into spectrometers. Each 4" wafer produces just over 200 dies, allowing in turn which can be built into 200 spectrometers. This process allows for ease of spectrometer scale production into the thousands.

Comparisons were made between wafer dies that had nanophotonic patterns and metal that been made completely without contract manufacturing, dies that had only the nanophotonic patterns made by contract manufacturing, and dies that had both the nanophotonic patterns and metal done by contract manufacturing. Figure 3 shows an overlay of the spectrums produced with all these three different categories of without, partial, and complete contract manufacturing responding to a halogen lamp. The data was taken at differing integration times so that the peak value for each spectrum is normalized to the same value. It was found that the dies made without contract manufacturing had integration times of 0.12-0.14 ms, while the dies with just the nanophotonic pattern made by contract fabrication had integration times of 0.20 ms, and the dies made completely with contract manufacturing had integration times of 0.20-0.22 ms. So there is some small decrease in the brightness of the dies made with contract manufacturing that could be related to small variation in the process. This small variation in brightness is small enough to not be a concerning issue.

The use of the DUV capabilities at CNF has allowed high resolution, robust production of nanophotonic patterns for commercial quantities. Ideally all of Chromation's wafer fabrication would be done at a single foundry, but the DUV lithography capability that CNF has is hard to find elsewhere. Furthermore, the additional layers added to the wafers at our contract manufacturer in Asia was a process specialty designed for our applications that could not be achieved in a conventional cleanroom. A commercial process that includes contract work at CNF is an effective way of producing Chromation's nanophontic spectrometer at scale. Figure 4 shows a batch of spectrometers made with this manufacturing process.

300 mm E-Beam Lithography

CNF Project Number: 2931-21

Principal Investigator & Remote User: Craig McGray

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Primary Source of Research Funding: Private/Corporate
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Primary CNF Tools Used: JEOL 9500

Abstract:

We require small capacitors on 300 mm oxidized silicon wafers for SEM applications. To fabricate these capacitors, we used the JEOL 9500 system to pattern small (~50nm) features for subsequent metallization and liftoff.

Summary of Research:

Two wafers were coated with a bilayer electron-beam resist and patterned with a 9-die dose array on the JEOL 9500. The wafer was then developed and removed from the CNF for plasma descum, metallization, and liftoff elsewhere.

None of the 50 nm critical features were resolved at any of the attempted nine electron beam doses. An example site of a 50 nm feature is shown in Figure 1. Further, many of the larger features exhibited loss of adhesion, as shown in Figure 2.

Conclusions and Future Steps:

We hypothesize that the bottom layer of electron-beam resist may have been incompletely developed, such that the Cr-Au metallization layer did not adhere well to the underlying oxide. We propose repeating the lithography with a 100 mm oxidized silicon wafer, such that electron microscopy can be performed at the CNF immediately after development to determine if any resist remains in the exposed areas of the wafer.

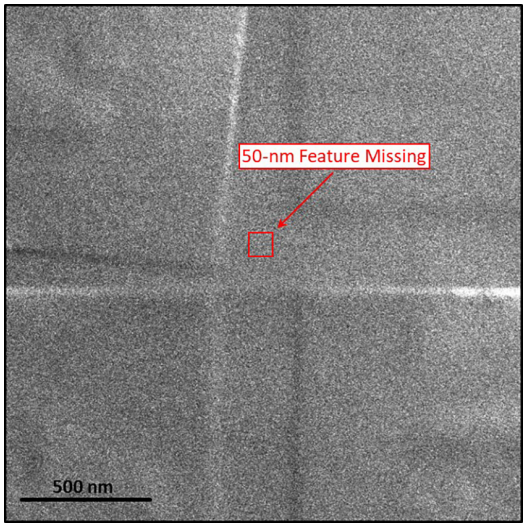

Figure 1: Electron micrograph of the location of a 50 nm critical feature that was not resolved by the electron-beam lithography process.

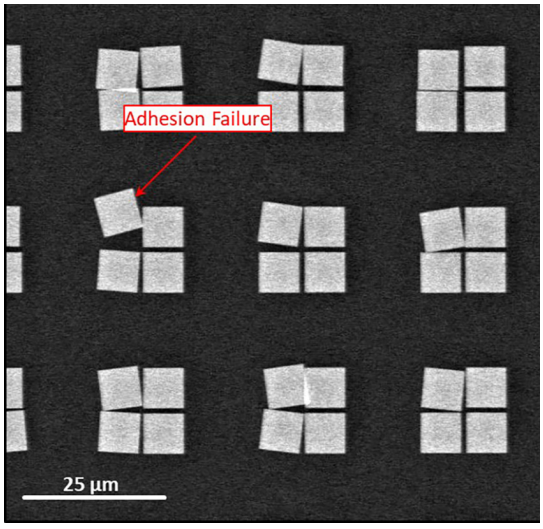

Figure 2: Electron micrograph showing 5 µm features that delaminated from the substrate after liftoff.

Characterization of Additively Manufactured High Aspect Ratio Microchannels via Two-Photon Polymerization

CNF Fellowship

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Primary CNF Tools Used: NanoScribe Photonic Professional GT2

Abstract:

Two-photon polymerization and its application to additive manufacturing represents an unprecedented ability to develop nanometer scale 3D and 2D designs. Such microchannels have a variety of research applications, including creating high aspect ratio microchannels for microfluidic devices [1] and making architected gas diffusion layers [2] among a host of other applications. The NanoScribe Photonic Professional GT2 was utilized to develop high aspect ratio microchannels and better characterize the printing and developing process.

Summary of Research:

Two-photon polymerization offers the ability to create structures with delicate features, in the range of hundreds of nanometers. In the case of the NanoScribe Photonic Professional GT2, the manufacturer reported minimum feature size is on the order of 160 nm [3]. Given these capabilities, this 3D printer has a number of applications, among which are the development of microfluidic channels. The printing process itself relies on selectively polymerizing portions of a resin bubble, bonding the print to a substrate, typically glass or silicon. As part of this process, unpolymerized resin remains in the printed object and must be removed during a process called development, wherein a solvent is used to remove this excess resin.

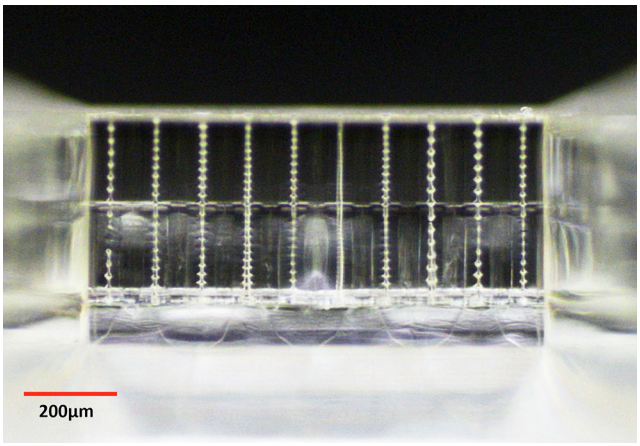

Figure 1: Fully developed microchannels, 400 µm in height, with varying channel diameter.

High aspect ratio microchannels, of up to 100:1 (length: diameter) were printed and sampled for appropriate development. As this is a transport limited process, various methods such as sonication, stirring, and long duration development were attempted. Additionally, the age of the resin and storage method were examined with IP-S resin, a type suited to medium size features (at 200 nm layer height).

In the first case, samples were developed using the suggested 20 minutes of development with propylene glycol methyl ether acetate (PGMEA) to dissolve uncured resin and a subsequent bath of isopropyl alcohol for five minutes to remove the PGMEA. After this, samples were optically characterized using the Nikon Wild light microscope to determine channel development. At this point no samples displayed complete development, requiring modifications to the development procedure. Further, it was determined that the light from the microscope was causing some degree of polymerization of the undeveloped resin. In effect, this prevents further development by clogging channels, meaning it was not possible to optically characterize the samples with unfiltered light.

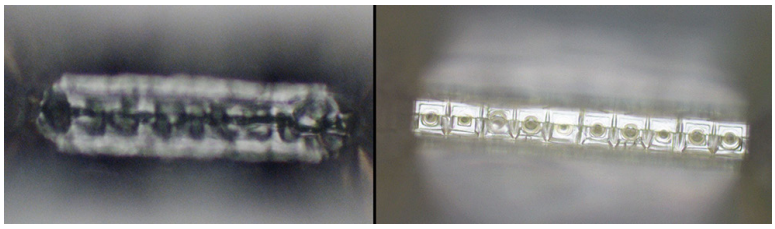

Figure 2: Underside of the microchannels. At left, resin that has been partially cured by a light microscope, inhibiting channel development. At right, fully developed channels from the sample in Figure 1 after 48 hours in PGMEA.

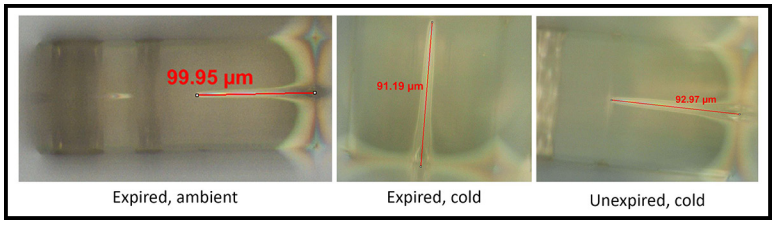

Figure 3: IP-S development performance for the three resin types examined; all showed similar results.

Therefore, it is recommended that for applications where there is not a suitable light source, samples should be tested for an appropriate development cycle with articles not meant for production.

Figure 2 is illustrative of the impact of a light microscope on inhibiting channel development. Partial curing of the resin created a block of material which could not be cleared via extended development (>72 hours) and sonication.

Additionally, the build method, such as creating a totally solid versus shell and scaffold design must be considered for extended development. Long duration development (>24 hours) will produce vacancies in the shell and scaffold designs as the PGMEA develop creates small holes in the shell and dissolves resin within the scaffold. For critical sections, a solid development method must be used, as seen in Figure 1. Here the surrounding structure is shell and scaffold, reducing printing time, while the area surrounding the microchannels is solid.

Another set of tests examined the impact of age and storage conditions of IP-S resin. Resins used in the GT2 printer have short shelf lives and are recommended to be stored cold. Prior to use, the resin should be brought to room temperature. Print quality and development time were explored with expired, cold resin (stored just above 0°C), expired, warm resin (stored at ambient), and unexpired, cold resin (stored in similar conditions to the expired, cold resin). A variety of geometries were printed, demonstrating micron-scale walls, overhangs, and microchannels via the 25X objective lens. All samples were developed for the NanoScribe-recommended 20 minutes in PGMEA and five minutes in isopropanol. Per the previous study, this time was known to be inadequate for complete channel development, however this could be accentuated with the different resins, as there is the possibility that some self-polymerization could occur within the expired materials.

Ultimately the results of testing showed that all resins with the suggested development procedure produced similar results. In fact, in this small sample, the expired, ambient temperature resin exhibited the greatest channel development. This seems to indicate that there is not a significant drawback for using this older resin for short development cycles. Longer development times were not trialed, and these results should not be extrapolated to out beyond short development cycles (<1 hour).

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