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Multi-Layer Micro-Nanofluidic Device for Isolation and Capture of Extracellular Vesicles Derived from Liposarcoma Cell Conditioned Media

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Abstract

We report on isolation, capture, and subsequent elution for analysis of extracellular vesicles derived from human liposarcoma cell conditioned media, using a multi-layer micro-nanofluidic device operated with tangential flow separation. Our device integrates size-based separation followed by immunoaffinity-based capture of extracellular vesicles in the same device. For liposarcomas, this is the first report on isolating, capturing, and then eluting the extracellular vesicles using a micro-nanofluidic device. The results show a significantly higher yield of the eluted extracellular vesicles (~84%) compared to the current methods of ultracentrifugation (~6%) and ExoQuick-based separations (~16%).

Keywords

Extracellular vesicles; exosomes; microfluidics; nanofluidics; liposarcoma; cancer

I. INTRODUCTION

LIPOSARCOMA (LPS) is the most common soft tissue sarcoma and accounts for approximately 16% of all the sarcomas [1]. Current methods for diagnosis rely on invasive tissue biopsies as standalone procedures or in combination with imaging approaches to identify tumor mass and location [2]. One of the emerging molecular targets for clinicians is the evaluation of the *MDM2* DNA sequence copy numbers to distinguish between dedifferentiated liposarcoma (DDLPS) and benign lipoma [3], [4].

The anatomical location of LPS and challenges with single tissue biopsies [5], [6], tumor heterogeneities [7], and the potential for distant metastases (10–50%) [8] leads to poor prognosis and thereby poor overall survival rates [9].

These challenges prompted researchers to explore the use of circulating biomolecules like miRNA and DNA that could serve as potential biomarkers for early stage detection and are relatively easy to access through body fluids [10], [11]. Recently, it was shown that extracellular vesicle (EV) derived miR-25-3p, miR-92-a-3p, and *MDM2* DNA correlate with the development and progression of DDLPS [12], [13]. EVs are cell-derived sub-micron (30–150 nm) structures which are released into a variety of body fluids (blood serum, urine, saliva, breast milk) [14]–[16], carrying a diverse array of proteins, miRNA, and DNA. Since the material packaged within the EVs originates from a cell, the analysis of EV-contained cargo enables identification of originating cells and also presents a snapshot of the host cell state at the time of EV generation [17], [18]. However, a key challenge in utilizing EVs for routine diagnostics lies in the isolation, capture, and extraction of this cargo.

One existing method for EV isolation is ultracentrifugation, which requires several centrifugation steps at progressively higher spin speeds [19]. This technique is time-consuming (3–4 hours), requires large sample volumes (~15 mL), and significant technical expertise for successful outcomes. Moreover, the lower recovery rates (~5%) [20], [21] and inability to distinguish between vesicle sub-types [22] leads to inaccuracies in the EV composition [23]. Furthermore, this method is not appropriate for cases in which sample volume may be limited or hard to obtain e.g., for pediatric patients.

In addition to ultracentrifugation, precipitation technologies using proprietary kits such as ExoQuick [24] can also be used to isolate EVs. Although the recovery rates (~16%) are higher than ultracentrifugation, this method requires longer isolation times (~14 hours) and are comparatively expensive (\$50/test). Furthermore, the precipitation reagent for ExoQuick may lead to co-isolation of other EV sub-types [25]. In addition to the clinical and healthcare costs that go with high infrastructure requirements and reagent costs, the presence of artifacts which are not completely removed during the isolation process further adds on to the challenges of realizing tumor-derived EVs as biomarkers in clinical settings.

In recent times, microfluidic systems have attracted attention due to their ability to precisely manipulate microliter-scale samples for separation of biological particles over a time scale of several minutes. For instance, Davies *et al.* developed a microfluidic filtration device comprising a photo-patterned porous polymer monolith membrane to isolate EVs from blood [26]. With a sample volume of 240 μ L, the microfluidic device showed limitations like

formation of gas bubbles near the electrodes, fabrication complexities associated with the membrane, and lower purity RNA compared to ultracentrifugation. Cho *et al.* [27] used electromigration to isolate EVs. During the migration, a track etched polycarbonate membrane with 30 nm pores captured biomolecules with diameter larger than the pore size while allowing biomolecules smaller than the pore size to pass through. This method allowed a relatively fast EV isolation (~30 min) with a 65% EV recovery. Though this system was able to operate 10X faster and recover 14X more EVs compared to ultracentrifugation, the purity of isolated EVs was lower compared to ultracentrifugation [27]. Similarly, Dehghani *et al.* [28] developed a microfluidic device that captured EVs on the membrane and allowed proteins to pass through under an applied pressure difference. They showed that ultrathin membranes ($<1\ \mu\text{m}$) were better suited than track-etched membranes (6–10 μm thick), since under high transmembrane pressure (68.94 kPa) the pores of the track-etched membranes were clogged by EV sized particles. Moreover, the capture of EVs on the membrane as reported by Dehghani *et al.* could also lead to co-isolation of different EV sub-types, thereby posing difficulties while quantifying the EV cargo.

In this paper, we report on a multi-layer micro-nanofluidic device that integrates the multiple unit operations of isolation, capture, and elution of EVs to less than 1 hour of total operation time while using ~300 μL of the sample to recover >80% of the EVs. Moreover, in our device, EVs remaining on the nanocapillary array membrane (NCAM) were not used for analysis but only the ones isolated and captured through the NCAM permeate.

II. MATERIALS AND METHODS

A. Device Fabrication

The micro-nanofluidic device was fabricated using polydimethylsiloxane (PDMS) with soft lithography [29]. The channel design in AutoCAD (Autodesk) was printed on a 5"×7" PET transparency film as a mask (CadArt Services Inc.). The microchannel features of both the injection and separation channel (3 cm (L) × 500 μm (W) × 150 μm (H)) were patterned on a n-type 4" silicon wafer with standard UV lithography to generate a negative mold for casting PDMS. The n-type 4" silicon wafer (University Wafers, USA) was cleaned with piranha solution (3-parts H_2SO_4 and 1-part H_2O_2) for 30 minutes, followed by a de-ionized water rinse and dried under a steady stream of nitrogen (N_2). Next, the wafer was coated with SU-8 2050. The SU-8 photoresist (PR) was first spun at 500 rpm for 7 s followed by a spin at 1140 rpm for 30 s to achieve a final thickness of 150 μm . After spin coating, the Si wafer was placed on a hotplate for baking at 65°C for 5 minutes with a ramp to 95°C for an additional 30 minutes. A UV contact aligner (EV Group 620 contact aligner; wavelength of 365 nm at 15 mW/cm²) was used to expose the PR coated Si wafer for 17 s. After exposure, the Si wafer was removed from the aligner and placed on the hotplate for another two-step bake starting at 65°C for 5 minutes followed by at 95°C for 12 minutes. The exposed and baked pattern was developed in SU-8 developer with the Si wafer immersed in a developer bath for 15 minutes with gentle manual agitation. The developed Si wafer was rinsed with isopropyl alcohol (IPA) and dried under steady stream of N_2 to yield the negative microchannel features at 150 μm height. The Si wafer mold was to be reused and therefore after drying, the wafer was hard baked on a hotplate at 175°C for 20 minutes.

Silicone elastomer base and curing agent (Ellsworth Adhesives) were mixed at a ratio of 10:1, poured on the above prepared silicon master, degassed, and cured at 65°C for 5 hours. The cured PDMS was peeled from the silicon master and cut into individual devices (Fig. 1). Next, to access the injection channel and the separation channel, through-holes were punched with 1.5 mm biopsy punches on the PDMS with the injection channel.

The nanofluidic separator was a polycarbonate track-etched NCAM (GE Water & Process Technologies) with nominal capillary diameter of 200 nm, a nominal thickness of 10 μm and a manufacturer reported nanocapillary (or nanopore) density of 3×10^8 pores/ cm^2 .

A 5 mm $\times$ 5 mm NCAM section was oxygen plasma treated at 600 mTorr for 3 minutes using oxygen plasma chamber (Harrick Plasma, Model: Plasma Cleaner PDC-001, RF power: 30 W). The oxygen plasma treated NCAM was then silanized with (3-Aminopropyl) triethoxysilane (APTES; Sigma Aldrich) solution (5% v/v) in double distilled water placed on a hotplate set at 80°C for 20 minutes [30]. The silanized NCAM and the PDMS monolith with the separation channel were oxygen plasma treated at 600 mTorr for 60 s and bonded to each other by placing the NCAM over the middle of the separation channel along the length by visual inspection. Finally, the PDMS containing the monolith of the injection channel and the NCAM-separation channel were treated again with oxygen plasma at 600 mTorr for 60 s. The treated surfaces were then bonded with their channels aligned perpendicular by visual inspection. Fig. 1 inset shows a cross-section of the bonded microchannel with the NCAM sandwiched. Notably, the small gap between the PDMS monolith and the NCAM arises due to the razor-blade cut for the cross-section needed for electron microscopy imaging. Flow tests with fluorescent dyes did not show any leaks between the injection and separation channel around the NCAM in 1 h of testing.

B. Separation Channel Surface Modification

A schematic for the functionalization process flow is shown in Fig. 2A. The micro-nanofluidic device was placed in an oxygen plasma chamber at 600 mTorr for 4 minutes. After plasma treatment, the device was moved to a glove bag continuously purged by dry nitrogen. APTES solution (2% v/v) in 200 proof ethanol (Decon Labs Inc.) was flushed through the separation channel. The APTES flush was repeated every 3 minutes for 15 minutes and the device was left inside the nitrogen glove bag for 2 hours with 10 μL of the APTES solution placed at both ports of the separation channel to minimize effects of evaporative drying on silanization [31], [32]. After 2 hours, 20 μL of 200 proof ethanol was flushed through the separation channel. Next, a 5% glutaraldehyde solution in 1X phosphate buffer saline (PBS; Corning) was flushed through the separation channel every 3 minutes for 15 minutes and left to incubate for 30 minutes. The incubation was followed by a flush with 1X PBS three times. Finally, 50 $\mu\text{g/mL}$ of primary antibody anti-CD63 (Ancell) diluted in 1X PBS was flushed through the separation channel and left to incubate at 4°C for 8 hours. After 8 hours of incubation, the separation channel was flushed with 1X PBS to remove any physisorbed antibody. Finally, the separation channel was filled with 1X PBS followed by 50 μL of 1X PBS placed at both ports of the separation channel to avoid drying and the device was stored at 4°C until further use [33].

C. Extracellular Vesicle Capture and Elution

Human liposarcoma cell line Lipo246 was established as previously reported [34]. Liposarcoma cells were grown in Dulbecco's modified eagle medium (DMEM; ThermoFisher Scientific) supplemented with 10% (v/v) fetal bovine serum (FBS; ThermoFisher Scientific). For LCCM, the Lipo246 cells were incubated in serum-free media for 48 hours and then the conditioned media was collected (LCCM). The LCCM was stored at -80°C until further use. Before using, the LCCM was thawed by placing it in a water bath at 37°C for 30 minutes. After thawing, the LCCM was loaded in a syringe for perfusion to the inlet of the injection channel using a syringe pump (Harvard Apparatus) with the LCCM containing syringe connected to the device *via* a tubing (0.8 mm inner diameter; US Plastic) using barbed nylon connectors (Cole-Parmer). The outlet of the injection channel and inlet and outlet of the separation channel were open to the atmosphere. The transmembrane pressure (TMP) was governed by the inlet flow rate with actual TMP calculated by using measured flow through the separation channel [35]. The syringe pump was operated for different flow rates in the 10–25 $\mu\text{L}/\text{min}$ range in increments of 5 $\mu\text{L}/\text{min}$. All results are reported for 3 distinct devices tested for each test condition. The total perfusion time was fixed for 30 minutes and therefore, the minimum volume of LCCM perfused within the injection microchannel was 300 μL , while the maximum volume perfused was 750 μL over each 30-minute test. For each test, 1 mL of LCCM was drawn into the syringe to take into account the 149 μL dead volume arising from the tubing and connectors.

After 30 minutes, the separation channel was flushed five times with 1X PBS to rinse the channel and remove loose EVs and particles i.e., those not captured by the CD-63 antibody immobilized within the separation channel. Next, the separation channel was flushed with 60 μL of pH 2.2 glycine-HCl buffer to elute the captured EVs [33] and the entire 60 μL elution fluid was collected in an Eppendorf tube. In order to ensure complete collection of EVs through elution, air was pushed through the separation channel using a pipette and any remaining solution was collected and added to the Eppendorf tube. Next, 120 μL of Tris-HCl buffer was added to the Eppendorf tube to raise the pH of the glycine-HCl buffer containing the EVs to $\sim$ pH 7.4. The captured EV solution was stored at -80°C until further use.

D. Confirmation of Extracellular Vesicle Capture and Release

The DiO dye (ThermoFisher Scientific) was flushed through the separation channel and incubated at room temperature for 10 minutes after the expected capture of the EVs following LCCM perfusion through the injection channel and subsequent isolation of the EVs. The tagging of EVs to the anti-CD63 antibody was verified through confocal microscopy examination (A1R, Nikon). Post DiO-tagging, an increase in fluorescence in the separation channel was observed (Fig. 2B (a)). Following elution using glycine-HCl buffer, fluorescence decreased, suggesting successful elution of EVs (Fig. 2B (b)).

Scanning electron microscope (SEM) imaging showed particle accumulation on the NCAMs suggesting that not all EVs and LCCM particles pass through (Fig. 2C). Based on total volume of fluid collected for each tested flow rate over the fixed 30-minute test, it was noted that only $\sim$ 4.2% of incoming fluid volume is the permeate across the NCAM and used for isolation of EVs.

E. Ultracentrifugation and ExoQuick Isolation

EVs were isolated using ultracentrifugation protocol reported by He and colleagues [36]. For ExoQuick isolation of EVs, manufacturer's (ExoQuick-TC from System Biosciences) instructions were followed. For both ultracentrifugation and ExoQuick, 15 mL of LCCM was used for isolating EVs.

F. Recovery Rate Estimation

The recovery rate was estimated as the ratio of (EVs in the collection vial)/(EVs in the original LCCM). The total number of EVs was estimated for each collected volume after a nanoparticle tracking analysis (NTA; Malvern NanoSight NS300). The total particle count was obtained by adding the counts for each size, in increments of 1 nm, from 0.5 nm to 199.5 nm.

G. DNA Isolation and Quantification

Trizol LS reagent was used for DNA extraction from the EVs eluted from the micro-nanofluidic devices. DNA yield were determined using a Cytation3 reader (BioTek).

H. MDM2 DNA Sequencing

MDM2 DNA sequencing was analyzed by PCR amplification and subsequent DNA sequencing of exons 1, 6, 10. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's specifications. DNA sequencing was performed by the Genomic Shared Resource at the Ohio State University Comprehensive Cancer Center.

III. RESULTS

A. Flow Rate Dependence on Extracellular Vesicle Capture

LCCM was perfused in the injection channel at different flow rates, ranging from 10 $\mu\text{L}/\text{min}$ to 25 $\mu\text{L}/\text{min}$, in increments of 5 $\mu\text{L}/\text{min}$, to evaluate the effect of inlet flow rate on capture efficiency. At 10 $\mu\text{L}/\text{min}$, a maximum recovery rate of $84 \pm 2\%$ was observed. For flow rates ranging from 15–25 $\mu\text{L}/\text{min}$ recovery rates of $\sim 20\%$ (no statistical differences within experimental error) were observed (Fig. 3). For data reported in Figures 4 and 5, LCCM was perfused through the injection channel at 10 $\mu\text{L}/\text{min}$.

B. Isolation of Extracellular Vesicles From LCCM

The size distribution of the EVs captured and eluted from the micro-nanofluidic device was compared with the commonly used methods of ultracentrifugation and ExoQuick-based separations (Fig. 4). As seen in Fig. 4, the fluid collected from the micro-nanofluidic device shows two major peaks for particle sizes. The first significant peak was noted at 129.5 nm with the NTA analysis showing 4.31×10^7 particles/mL. A second peak with 0.71×10^7 particles/mL was also observed at 261.5 nm. By contrast, multiple peaks with lower particle per unit volume counts were noted for both the ultracentrifugation and ExoQuick-based EV isolation.

EVs are a rich source of biomarkers containing various bioactive materials including proteins and genetic materials [37]. For DDLPS, *MDM2* DNA is considered a key biomarker [38]. Therefore, Sanger Sequencing for *MDM2* showed the appropriate sequence with the entire exons [12], confirming the presence of *MDM2* DNA in the isolated EVs (Fig. 5).

IV. DISCUSSION

In the fluorescence characterization images of the EVs in the separation channel (Fig. 2B (a)), we observed localized spots of high fluorescence within the microchannel suggesting that the surface modification is non-uniform. Moreover, the fluorescence images show only the EVs captured on the bottom surface of the separation channel as the visualization is through an inverted confocal microscope.

It is worth noting that for most of the immunoaffinity based microfluidic devices for EV isolation, researchers have reported a finite residence time for the EVs in contact with the functionalized substrate to allow adequate capture. Moreover, the change in capture of EVs with shorter or longer residence time, remains unknown. In order to evaluate effect of these residence times, the injection channel flow rate was varied from 10 – 25 $\mu\text{L}/\text{min}$ to yield volumetric flow rates ranging from 0.42 $\mu\text{L}/\text{min}$ to 1.03 $\mu\text{L}/\text{min}$ in the separation channel, respectively. Based on previous studies using antibodies like CD9, CD63, and CD81 to capture the EVs from various cancers, the residence time of these EVs in their devices have been shown to vary from 5 minutes to 40 minutes [33], [39]–[41]. For the separation channel volume of 2.25 μL , the residence time varied from 5.3 minutes at 10 $\mu\text{L}/\text{min}$ injection channel flow rate to 2.2 minutes at 25 $\mu\text{L}/\text{min}$ injection channel flow rate. Since, no standard time or incubation duration has been reported in the studies so far, the data reported here presents the first tabulation of EV recovery as a function of injection channel flow rate. For the data reported here, the residence time of 5.3 minutes (at 10 $\mu\text{L}/\text{min}$) was found to yield the highest capture-release EV efficiency, which is near the lower end of the previously reported residence times.

SEM images of the EVs revealed that the size ranges from 125 nm to 165 nm, and falls within the previously reported size range for EVs [42], [43] (Fig. 2C). As seen in Fig. 4, for ultracentrifugation-based separation, peaks with particle sizes <30 nm were observed suggesting the aggregation of proteins and nucleosome fragments, as previously reported [21]. Moreover, for our device no particle size less than 30 nm were observed suggesting a potential lack of protein aggregation. As part of future work, further quantification of these particles may be required.

Interestingly, the peak at 261.5 nm for our device (Fig. 4) could be attributed to the discrepancy in the NCAM manufacturing process or to the tendency of the EVs to aggregate as seen in Fig. 2C or to a combination of these factors. Based on our previous experience with NCAMs, the uncertainty in the NCAM diameter is on the order of 20% of the nominal capillary diameter as reported by the manufacturer, with possible variations in the uncertainty depending on nominal capillary diameter and specific manufacturing batch [44], [45]. Further investigation of the potential particle aggregation or quantifying the

deficiencies in NCAM operation will be needed to resolve these observations; however, such an investigation is beyond the scope of this paper.

In line with past reports, the data in Fig. 4 is reported as particles per unit volume (particle concentration). However, it is worth noting that the use of EVs is in extracting the cargo from within the EVs. While concentrations are useful, the purpose of a microfluidics-enabled device is to (a) minimize sample volume, (b) permit faster sample processing, and (c) allow capture of most EVs compared to other macro-scale methods. Therefore, as an alternate reporting metric we present the actual numbers of EVs that were isolated, captured, and then eluted from the micro-nanofluidic device reported here (Table I). Table I shows that the micro-nanofluidic device recovered ~10 times more EVs compared to ultracentrifugation and ~4 times more EVs compared to ExoQuick with reduced sample volume (~15X). Moreover, the processing time was reduced by almost 85% to ~30 minutes compared to ultracentrifugation (3–4 hours) and 94% compared to ExoQuick (~12 hours).

One of the molecular hallmarks of DDLPS is elevated levels of *MDM2* DNA [3], [46]. In particular, one of the important targets of molecular aberration in well differentiated and dedifferentiated liposarcoma is the increase in MDM2 protein expression as also the amplification of the *MDM2* [47]. Studies have shown that amplification of *MDM2* leads to ubiquitination and degradation of the tumor suppressor p53, thereby increasing the probability of metastatic disease [48], [49].

As shown in Fig. 5, *MDM2* DNA was verified for structural integrity showing that the isolation, capture, and elution process does not damage the internal EV cargo. The detection of three of the exons (exons 1, 6, and 10) belonging to *MDM2* DNA by sequencing verified that it is *MDM2* DNA that was isolated and was same as the previously reported sequence for *MDM2* DNA derived from the EVs of Lipo246 cell line using ultracentrifugation [12]. Moreover, when the eluted samples from 3 devices was pooled and total DNA quantified, we found ~27 ng/ μ L DNA isolated from the EVs using the micro-nanofluidic device with a total of 300 μ L of LCCM perfused through the injection channel over the 30 minute isolation time as opposed to ~5 ng/ μ L DNA from the EVs isolated using ExoQuick (requiring 30 mL of LCCM and 14 hours isolation time). In summary, the micro-nanofluidic device reported here shows significant advances in reducing operation time compared to ultracentrifugation and precipitation-based EV isolation, shows viability for capturing sufficient EVs from significantly reduced sample volumes, and clearly shows device operation for cell conditioned media for a variety of operational parameters. However, the device does require further optimization for device geometry and isolation and capture efficiency with likely supporting numerical models to quantify and understand all details for device performance. Such an optimization effort is part of on-going work and beyond the scope of the results reported here. With the goal of point-of-care use for these microfluidics devices, use of samples from pathologies other than cancer [50] relevant to EVs present a growing opportunity for microfluidic systems isolating and capturing EVs. Furthermore, device operation will need to be validated on actual biofluid samples from patients for acceptance in clinical settings.

V. CONCLUSIONS

This is the first report demonstrating the isolation, capture, and elution of liposarcoma EVs using a micro-nanofluidic device. We were able to isolate almost ten times the amount of EVs than ultracentrifugation and four times the amount of EVs compared to ExoQuick, demonstrating a significant advance compared to existing state-of-art methods. We also found approximately a five-fold increase in the amount of DNA isolated from the EVs captured using our devices compared to EVs isolated using ExoQuick. The increased amount of DNA enables an opportunity for more rigor and additional analyses that were not possible with the limited amounts of EV-contained DNA obtained previously. Moreover, the processing time was reduced by almost 85% compared to ultracentrifugation and 94% compared to ExoQuick once again providing significant advances in the potential for rapid diagnostics.

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Fig. 1.

Exploded view of microfluidic channels separated by a nanocapillary array membrane (NCAM). The dotted line represents the cross-section that was utilized for scanning electron microscopy (SEM) characterization of the device, as seen inset. The inset shows SEM image showing cross-section of the microfluidic channel and the NCAM (dotted line). Cutting marks and damage to bottom PDMS/NCAM interface from SEM sample preparation are seen.

Fig. 2.

(A) Process depicting the functionalization of separation channel with anti-CD63 antibody.

(B) Fluorescence verification for capture and elution of EVs: (a) Following passage of LCCM, the EVs tag to the anti-CD63 antibody on the surface of the separation channel (green); (b) Following elution with pH 2.2 glycine-HCl buffer, there is a decrease in EV fluorescence (Little to no green). (C) SEM image showing EVs on the NCAM allowing visual inspection. EV diameter estimated to be between 125 nm and 165 nm.

Fig. 3.

Recovery rates in 30 minutes for different injection channel flow rates (10, 15, 20 and 25 $\mu\text{L}/\text{min}$). Error bars are $\pm$ Standard Error of the Mean.

Fig. 4.

Size distribution obtained by NTA for EV isolated using ultracentrifugation (UC), ExoQuick, and the micro-nanofluidic device. The peaks (dotted blue circle) are EVs derived from LCCM. The peak (dotted red circle) is an artifact of the cell growth media. The peaks (dotted black circle) suggest aggregation of EVs also seen as clumps in Figure 2C.

Fig. 5. EVs derived from LCCM contain MDM2. DNA sequencing on the entire exons 1, 6, and 10 of MDM2 confirms integrity of bio-cargo preserved during microfluidic EV isolation.

TABLE I

TOTAL EV YIELD FROM DIFFERENT ISOLATION TECHNIQUES

EV Isolation Process	Total Number of EVs Isolated
Ultracentrifugation (UC)	6.9×10^7
ExoQuick	1.8×10^8
Our Device	7.4×10^8

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