Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Solid-phase extraction of exosomes from diverse matrices via a polyester capillary-channeled polymer (C-CP) fiber stationary phase in a spin-down tip format

Kaylan K. Jackson, Rhonda R. Powell, Terri F. Bruce, R. Kenneth Marcus

Electron microscopy

Scanning electron microscopy (SEM) was performed using a Hitachi S-4800 to confirm the capture of intact exosomes on the C-CP fiber surface. The fiber-bound vesicles were fixed with 1% osmium tetroxide for 1 hour, then washed in microcentrifuge tubes on a shaker (3 times, 5 minutes each). The samples were then dehydrated in an ethanol-distilled water gradient from 0% to 100% ethanol, followed by 3 washes of 100% ethanol for 3 minutes each. The sample was then washed in a 50–50 hexamethyldisilazane (HMDS)-ethanol solution for 3 minutes and allowed to dry in a fume hood in 100% HMDS overnight. The dehydrated samples were sputter-coated with platinum at 70 mTorr argon for 2 minutes.

Transmission electron microscopy (TEM) was performed using a Hitachi HT7830 (Chiyoda, Tokyo, Japan) to confirm the release of intact exosomes from the C-CP fiber surface. In preparation, eluted exosomes were fixed with 1 mL of 2% paraformaldehyde (PFA) for 5 min. An aliquot of 7 µL of the fixed exosome suspension was placed on a thin formvar/carbon film-coated 200 mesh copper EM grid and incubated for 1 minute. The grids were then stained using 20 drops of filtered 1% uranyl acetate. The excess uranyl acetate solution was removed, and the sample grids were allowed to dry for 10 minutes before imaging at 100 kV.

C-CP Fiber Tip Assembly and Centrifuge Adapter

An eight-rotation loop of the PET fibers (corresponding to a total of 450 fibers, ~241 µm each in circumference) were pulled collinearly through approximately 30 cm lengths of 0.8 mm inner diameter fluorinated ethylene polypropylene (FEP) polymer

tubing (Cole Parmer, Vernon Hills, IL, USA), yielding an interstitial fraction of ~0.6. The fibers were pulled out of the column to create a 3 mm open space at the opposite end for attachment to a 200 µL micropipette tip (Molecular BioProducts, San Diego, CA, USA). The capillary was cut to create a 1 cm length of fiber-packed tubing. This procedure was repeated for the entire length of the capillary, yielding 10-15 PET C-CP fiber tips. The C-CP fiber tips were prepared for application by washing with five successive, 100 µL aliquots of 40% ACN in DI water at 300 x g for 1 minute each, to ensure the entire aliquot had spun down through the tip.

For centrifugation processing, a means of holding the tips within the centrifuge tube had to be created. A utility knife was used to remove the center portion of the cap of a 15 mL centrifuge tube (Nalge Nunc International, Rochester, NY, USA), with the bottom portion of a 1.5 mL microcentrifuge tube (Fisher Scientific, Pittsburgh, PA, USA), placed into the center of the cap, to act as the micropipette tip receptacle. The C-CP fiber tips could then be inserted in the microcentrifuge tube and placed in the rotor of a table-top centrifuge. A VWR® Symphony™ 4417/R table-top centrifuge (Radnor, PA, USA) was used for spin-down processing.

Confocal Microscopy

After the induction of exosome adsorption to the fiber surface under the high salt condition (1:1000 exosome standards in 2M NH_3SO_4), the captured vesicles were rinsed with DI water (50 µL each, 300 x g, 1 minute, three times), and the elution of contaminant-free proteins was induced by rinsing the fiber surface with 25% glycerol in PBS (50 µL each, 300 x g, 1 minute, three times) to leave cleanly isolated exosomes on

the fiber surface. To prevent non-specific binding of subsequently used antibodies, the free fiber surface was blocked using a blocking solution of 1% bovine serum albumin (2 hours at room temperature on a rotator). For the immuno-identification of the captured EVs, the fibers were exposed (spin-down) then incubated with a rabbit primary antibody to the CD81 protein on the exosome surface (1:1000, overnight incubation at 4° C on a rotator). Following primary antibody incubation, the fibers were washed using 6 cycles of spin-down (50 μL), then incubation with washing buffer (PBS) for 5 minutes each. To prevent non-specific binding of the secondary antibody, the fiber surface was again blocked using a blocking solution of 1% bovine serum albumin (2 hours at room temperature on a rotator). The fibers were exposed to the secondary antibody solution containing an Alexa Fluor 647 goat anti-rabbit secondary antibody by spin-down, then incubated (1:1000, 1 hour at room temperature) with the solution to obtain a visualized fluorescence response. After incubation with the secondary antibody, the fibers were washed with PBS to remove unbound secondary antibody in preparation for imaging using the Leica SP8 Confocal Microscope.

ELISA

The mouse CD81 capture antibody was diluted 1:250 in coating buffer (0.2 M sodium carbonate/carbonate, pH=9.4), and 50 μL of the coating antibody solution was added to each well and incubated overnight at 4°C, then at 37°C for 2 hours on a shaking platform. The solution was removed, and the plate was washed using 200 μL of wash buffer (PBS) per well for 3 x 5 minutes each on a shaking platform. To prevent non-specific binding, the wells were incubated with 200 μL of blocking buffer (1% BSA)

at room temperature for 1 hour. The samples were then applied to the 96-well plate in 50 μL aliquots and incubated overnight at 4°C, then at 37°C for 2 hours on a shaking platform. The solution was removed, and the plate was washed using 200 μL of wash buffer (PBS) per well for 3 x 5 minutes each. A rabbit CD81 primary antibody of 1:1000 concentration was added to each well and incubated at 4°C, then at 37°C for 2 hours on a shaking platform. The solution was removed, and the plate was washed using 200 μL of wash buffer (PBS) per well for 3 x 5 minutes each. Then, the wells were incubated with 200 μL of blocking buffer (1% BSA) at room temperature for 1 hour. Next, the wells were incubated with 50 μL of a goat anti-rabbit horseradish peroxidase (HRP) enzyme conjugate of 1:1000 concentration for 1 hour at room temperature. The solution was removed, and the plate was washed using 200 μL of wash buffer (PBS) per well for 6 x 5 minutes each. Finally, the well was incubated with the 1-Step Ultra TMB Substrate for 30 minutes at room temperature, and the resulting UV-Vis absorbance at 450 nm was measured using the Synergy H1 Hybrid Multi-Mode Reader (BioTek). The ELISA was run in triplicate (n=3) with each obtained absorbance value an average of 8 measurements. The results were quantified based on the comparison of the absorbance values to a standard curve of linear response ($y = 3E\text{-}10x + 0.2093$) using commercial exosome standards.

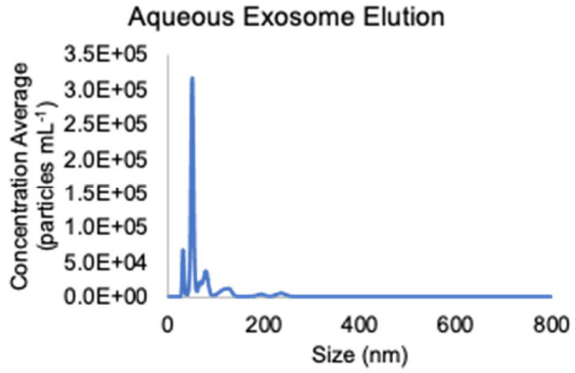
Dot Blot

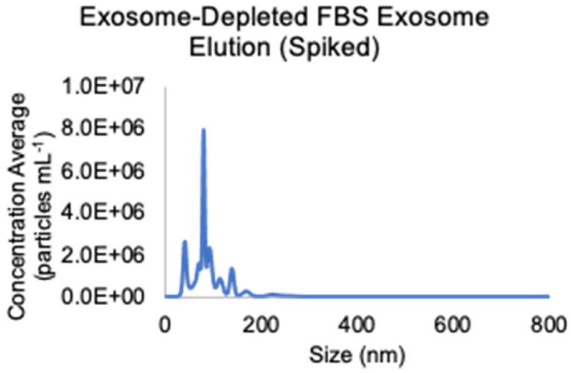
In preparation for the experiment, a piece of polyvinylidene fluoride (PVDF) membrane was cut into three 1 cm by 4 cm strips. Next, the micropipette-attachment side of a 10 μL micropipette tip was used to create 3 small indentations on the PVDF

strips to prevent sample run-off from the region of interest. The indentations on the PVDF strip were then labeled for the samples applied as followed: + (positive control, s (sample), and – (negative control). The membranes were then wet in methanol for 15 seconds, then equilibrated in PBS buffer for 5 minutes. Next, a transfer stack assembly was set up to promote sample adsorption while keeping the membrane hydrated. Dry paper towels were placed on the lab bench surface, followed by a dry piece Whatman® 3MM filter paper, then a piece of Whatman® 3MM paper wet with PBS buffer, and finally, the pre-wet PVDF membrane. A 1 μL dot of 1:1000 secondary antibody, sample, and PBS were placed on their labeled regions on the membranes and allowed to wick into the membrane. The blots were then placed in a 1% BSA blocking solution and incubated for 1 hour at room temperature on a shaking platform. Next, each dot blot was placed in a conical tube containing rabbit primary antibody solutions of 1:1000 concentration to CD9, CD81, and CD63 proteins, and incubated overnight at 4°C on a rotator. Next, the dot blots were washed with PBS for 30 minutes total with 5 buffer changes. The dot blots were then incubated with a goat anti-rabbit silver nanoparticle conjugate for 30 minutes at room temperature on a shaking platform. Finally, the resulting dot blot signal was enhanced using the silver enhancement kit for membranes (Cytodiagnostics), by incubating the dot blots in 2 mL of equal parts solution A and solution B for 45 minutes. The dot blots were finally washed for 5 minutes in PBS to stop the enhancement reaction.

Nanoparticle Tracking Analysis (NTA)

Averaged Nanosight NTA distribution profiles for eluted exosomes from the spiked aqueous and spiked exosome-depleted FBS samples. Tabulated statistics are presented below each.





Aqueous Exosome Recovery Statistics:

Mean	95.9
Mode	77.7
RSD	5.1%
D10	71.2
D50	82.2
D90	130.1

FBS Exosome Recovery Statistics:

Mean	95.9
Mode	74.3
RSD	7.2%
D10	51.2
D50	74.3
D90	155.4