Electronic Supplementary Material (ESI) for Nanoscale.

Electronic supplementary information

Monitoring the dynamics of cell-derived extracellular vesicles at the nanoscale by liquid-cell transmission electron microscopy

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Experimental details

Cell culture.

Human umbilical vein endothelial cells (HUVEC), purchased from Lonza inc., were cultured at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 U/mL streptomycin. Porcine Mesenchymal Stem Cells (MSC) were obtained from adipose tissue-derived stromal cells isolated from the abdominal fat of 6 month-old female pig (ABCell-bio Society, Paris, France). Cells were cultured at 37°C and 5% CO2 with Alpha minimum essential medium (ABCell-bio Society, Paris, France) including 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin).

Production and loading of Extracellular Vesicles (EVs)

EVs were produced from HUVEC and MSC. Briefly, cells were cultured in serum-free DMEM for 3 days. Media was then harvested and EVs were isolated from the conditioned culture medium with a differential (ultra)centrifugation method as previously described by Théry et al.[1] First cell debris were eliminated by 2,000 ×g centrifugation for 10 minutes. From the supernatant, microvesicles and exosomes were isolated after the 100,000 ×g ultracentrifugation step for 1h. EVs produced by HUVEC were re-suspended in phosphate-buffered saline (PBS), HEPES buffer or hyperosmolar HEPES media (2.5x) while EVs produced by MSC were re-suspended in Roswell Park Memorial Institute medium (RPMI). The mean purity ratio of the samples, defined as the number of particles measured by NTA analysis per gram of total protein content measured by mini Bradford assay, was 2×10^{10} particle/microgram of protein, corresponding to a high purity sample.[2]

Preparation of liposomes

Multi-lamellar vesicles were prepared according to the thin layer evaporation technique. [3] In brief, the lipid phase was dissolved in chloroform which was then removed under argon gas followed by reduced pressure in a rotary evaporator, thus obtaining a thin film of dry lipid on the tube. Evaporation was continued for 2 h after the dry residue appeared, to completely remove all the traces of the organic solvent. The film was then hydrated by adding 1 mL of PBS to obtain a final lipid concentration of 1 mM under vigorous stirring in order to favor the vesicle formation. Multi-

lamellar vesicles were then frozen and thawed. The suspensions were placed in a falcon tube and dipped in a nitrogen bath for 30 s. The tubes were then removed and placed in a water bath at 40 °C until defreeze. The sequence was repeated 7 times. Liposomes composed of 70% POPC and 30 % DOPE, were prepared using the extrusion method. 1 mL of the suspension were placed in a mini-extruder (Avanti Polar Lipids, Birmingham, AL) at room temperature and manually extruded through a 0.1 μm polycarbonate membrane for 19 times.

Cryo-TEM

Annexin-A5 conjugated to Au nanoparticles (Anx5-Au-NPs) were synthesized according previously published protocol.[4] Anx5-Au-NPs consist of colloidal Au particles with a size ranging from 10 to 12 nm covalently grafted with a PEG-based spacer linked to the Anx5 protein in a stereo-specific manner. HEPES annexin binding buffer was bought from Roche (annexin V fluo staining kit). 1μL of Anx5-Au-NP solution at 1–3 x 10^{16} NPs/L and 1 μL of 100 mM $CaCl_2$ solution were mixed with 8μL HUVEC derived EVs and incubated for 15 minutes at ambient temperature, and then processed for cryo-EM. Briefly, 4 μL sample aliquot was deposited onto an EM grid coated with a perforated carbon film (Ted Pella, Redding, CA, USA), the excess liquid was blotted off with a filter paper, and the grid was then quickly plunged into liquid ethane using a Leica EMCPC cryo-chamber. EM grids were stored in cryo-boxes under liquid nitrogen until use, then mounted in a Gatan 626 cryo-holder and transferred in a Tecnai F20 microscope operated at 200 kV. Images were recorded with an USC1000-SSCCD Gatan camera.

Nanoparticle tracking analysis

EV size distribution and concentration were determined with nanoparticle tracking analysis (NTA) using a Nanosight LM10-HS (NanoSight, UK) with a 532 nm laser. A monochromatic laser beam at 532 nm was applied to the dilute suspension of vesicles before measurements, EVs were diluted to an appropriate concentration with sterile PBS (confirmed to be particle-free). 9 movies of 30 s were recorded using camera level 16. Data was analyzed with NTA Analytical Software. Brownian movement of vesicles was tracked and measured from frame to frame. The velocity of particle movement was used to calculate particle size by applying the two-dimensional Stokes–Einstein equation. NTA post-acquisition settings were optimized and kept constant between replicate

analysis, and each video was then analyzed to give the mean and mode vesicle size as well as an estimate of the concentration.

Statistics

Statistical data are presented as standard deviation from the mean. Student t-tests were carried out to determine a significant difference between groups using Prism 3.0 version of GraphPad software. A minimum of 95% confidence level was considered significant.

Measurement of the liquid thickness in the observation area.

Knowing the liquid thickness within the liquid-cell is crucial for the interpretation of the dynamical phenomena analyzed by liquid-cell transmission electron microscopy (LCTEM). When observing extra-cellular vesicles (EVs), it is essential to compare the liquid thickness and the size of the vesicles to determine if the biomaterials are squeezed between the two SiN films. First, we qualitatively checked before and after the EV analyses if the liquid-cell was full of liquid by analyzing the contrast of low magnification TEM images. As illustrated in figure S5b, the contrast of the window was always characteristic of a fully filled liquid-cell with an increasing liquid thickness (i. e. the contrast decreases) from the corners to the center of the viewing window, due to an outward bowing of the SiN membranes under vacuum.

Then, energy-filtered TEM (EFTEM) was used to quantitatively evaluate the liquid thickness. These measurements were performed on a test liquid-cell filled with water, over 12.5 μm^2 areas near the corners of the liquid cell. EFTEM (or Electron-Energy-loss spectroscopy) is a very convenient technique to estimate the thickness of the sample (t) crossed by the electron beam.[5] We acquired an unfiltered image and a zero-loss filtered image from the same region with the same optical condition. Then, these two images were used to compute a relative thickness map (figure S5c) by using the Poisson statistic of inelastic scattering:

$$\frac{t}{\lambda} = -\ln\left(\frac{I_0}{I_t}\right)$$

with λ the inelastic mean free path of electron in the sample, I_0 and I_t the intensity of the filtered and unfiltered images, respectively. On this thickness map we can measure the t / λ profile that

show the variability of the liquid thickness in the liquid-cell because of the bowing effect (black curve in figure S5c). As expected the lowest t / λ values are found at the corners and this ratio increase linearly along the diagonal of the viewing window. It is worth noting that this signal is affected by both the water layer and the two SiN membranes that encapsulate the liquid. Nevertheless, given that:

$$\left(\frac{t}{\lambda}\right)_{sample} = \left(\frac{t}{\lambda}\right)_{water} + \left(\frac{t}{\lambda}\right)_{SiN\ membranes}$$

we can easily deduce the contribution of the SiN films by measuring the t / λ value of a single membrane. The relative thickness map of a SiN film acquired with the same optical conditions has a mean t / λ ratio of 0.36 ± 0.02. Assuming that the two membranes have the same thickness, we can extract the t / λ profile of water in the liquid-cell (red curve in figure S5c). Importantly, we note that the t / λ profiles were very similar in all corners of the liquid-cell.

Previously reported EELS measurements of liquid-cell thickness showed that in the microscope each membrane bows out and takes a parabolic shape that are orthogonal to each other. Because of this liquid-cell geometry, most likely due to the cross configuration of the two rectangular windows, the water layer is thinner at the corners where its thickness was found to roughly correspond to the thickness of the gold spacers separating the two Echips.[6, 7] The parabolic intensity profile measured along the diagonal of the viewing window on the low-magnification image of the liquid-cell (figure S5b) indicates that during our experiments, the liquid cell takes this very common geometry. If we assume that the thickness of liquid at the corner is 150 nm (thickness of the gold spacers), we can conclude that the liquid thickness in the 12.5 μm^2 areas near the corners varies linearly from 150 nm to 315 nm along the diagonal of the viewing window. All the images and videos of the present work were recorded in 12.5 μm^2 areas located 1 µm away from the liquid-cell corners (figure S5b). By extrapolating the t / λ curves 1 µm further along the x axis, we estimate that the water thickness in the observation areas to vary from 185 nm to 350 nm. These observation areas were chosen because then the majority of EVs cannot be squeezed between the membranes and their contrast is significantly higher than in the middle of the liquid-cell.

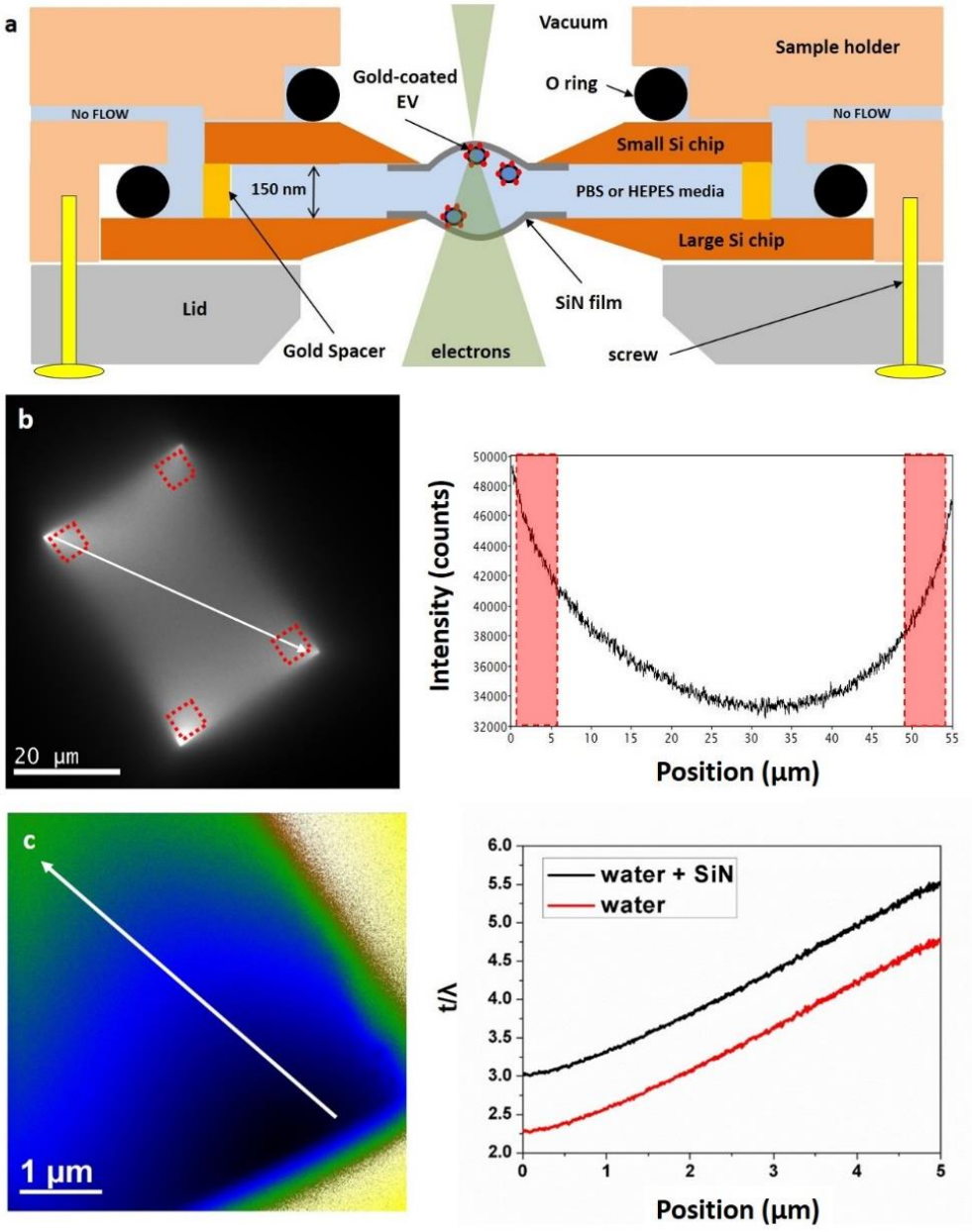

Figure S1. (a) Schematic cross section of the sealed liquid cell in the JEOL ARM microscope. (b) Left: Low-magnification TEM image of the viewing window after EV imaging showing the characteristic contrast of a filled liquid cell. The 12.5 μm^2 observation areas are indicated by red squares. Right: intensity profile measured along the white arrow indicated on the low magnification image (c) Left: Relative thickness map a corner area of the liquid cell filled with water. Right: t/λ profile measured along the white arrow indicated on the thickness map (black curve). The red curve corresponds to the t/λ profile from which the contributions of the two SiN membranes (0.36 per film) has been subtracted.

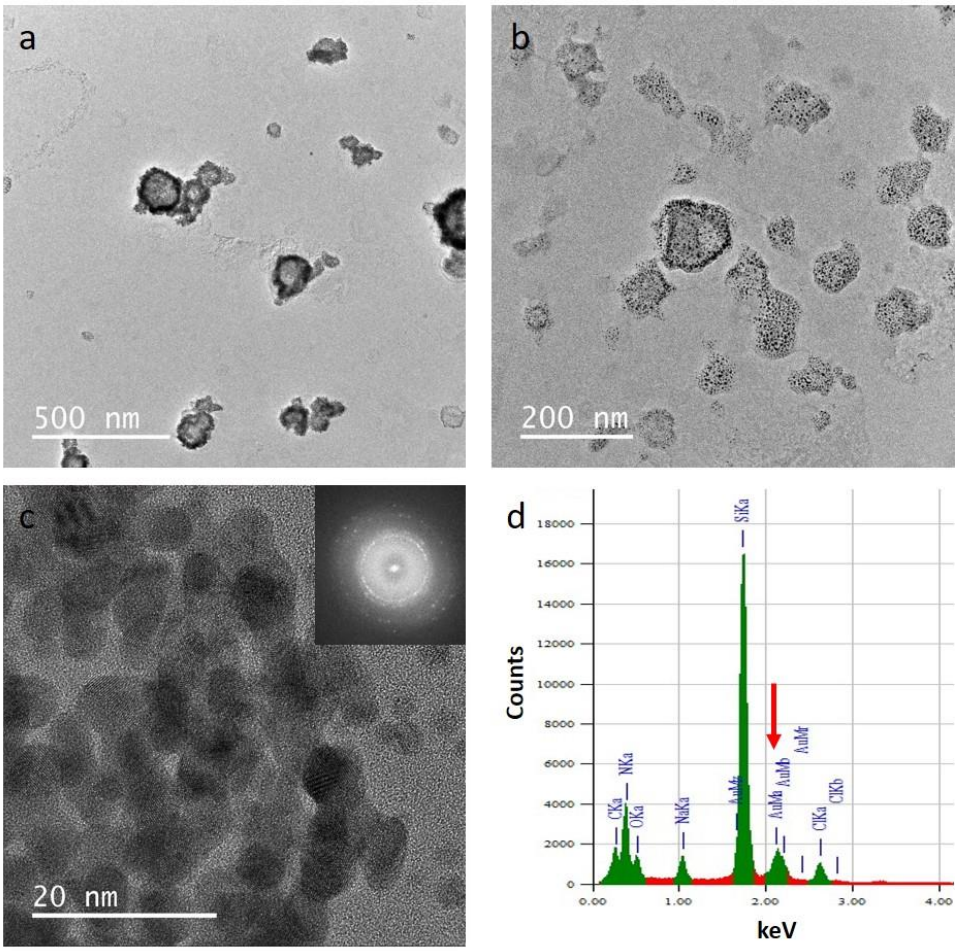

Figure S2: Ex situ analysis of the gold nanoparticles formed on the EVs during LCTEM experiments. After unsealing the liquid-cell, the small Echip was placed in conventional sample holder for TEM observations (under vacuum). (a-b) Low magnification images of gold layers with various thicknesses over dried EVs. Note that the spherical shapes of the EVs is probably maintained by the gold layers during drying processes. (c) High resolution image of a gold layer made of percolated nanoparticles. The Fourier transform of the image (in insert) shows the characteristic inter-reticular distances of the face-centered cubic structure of gold. (d) EDX spectrum acquired on the same area showing the presence of gold, together with silicon nitride (substrate), carbon (bilayer membrane), and salt residues (NaCl) present in the cell medium.

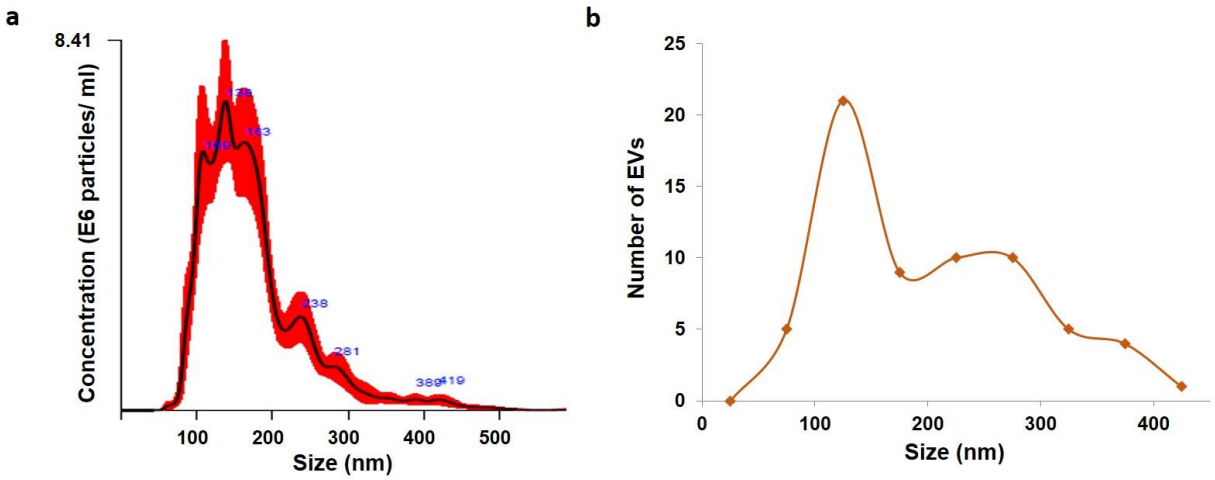

Figure S3. Size distribution of EVs produced by MSC cells in RPMI media. (a) Measured by NTA. (b) Measured by LCTEM (n = 65).

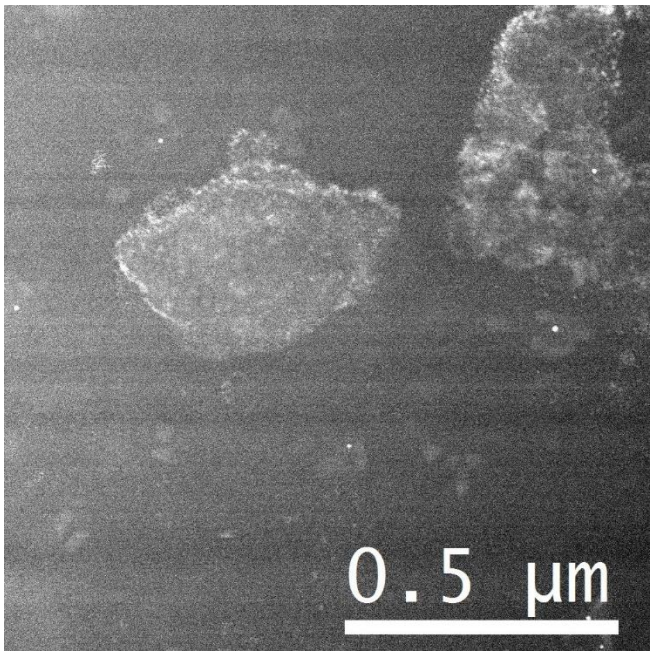

Figure S4. LCTEM image of a large EV (> 300 nm)

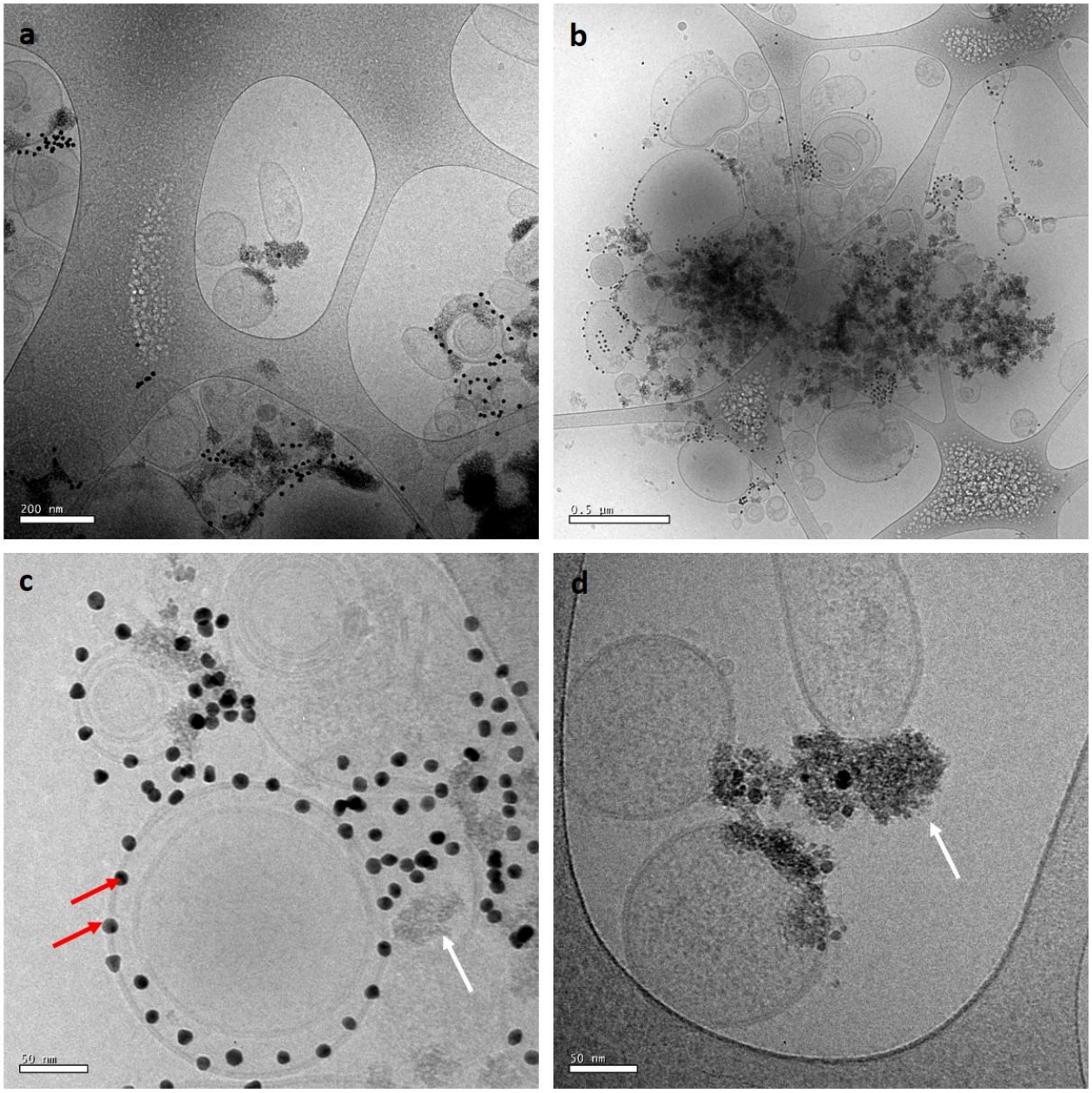

Figure S5: (a-d) Bright field Cryo-TEM images of EVs in frozen HEPES acquired at different magnification. White and red arrows in c) and d) indicate calcium-phosphate nanocrystals and gold nanoparticles, respectively. In line with LCTEM observations, round and elongated (oblate / prolate) EVs are observed and the distribution of gold nanoparticles over the EVs is uneven. In contrast with LCTEM experiments, the encapsulation of EVs in larger EVs is frequently observed.

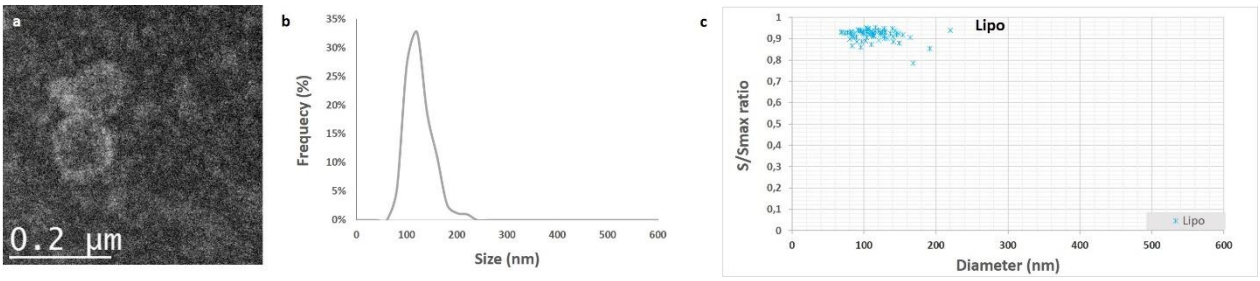

Figure S6: Structural characterization of liposomes in physiological media. (a) Representative LCTEM pictures of liposomes in PBS (300 mOsmol/L). (b) Size distribution of liposomes measured by LCTEM (n = 72). (c) S/Smax ratio plotted as a function of the diameter of liposomes measured by LCTEM in PBS.

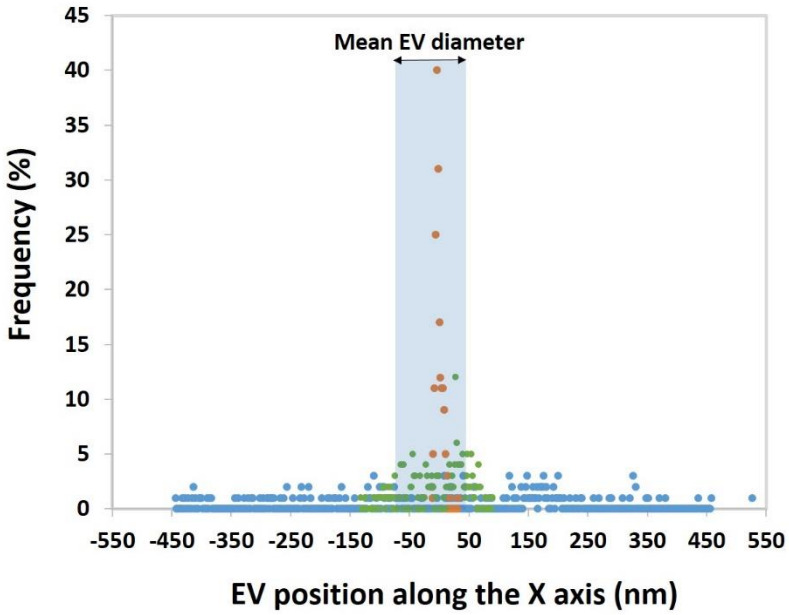

Figure S7. Dispersion of the position of 3 EVs along the X axis measured on 180 images with a frame rate of 2 frame per second. These dispersions is compared to the mean EV diameter (blue rectangle). We have intentionally selected three EVs (green, orange and blue data points) with very different mobility.

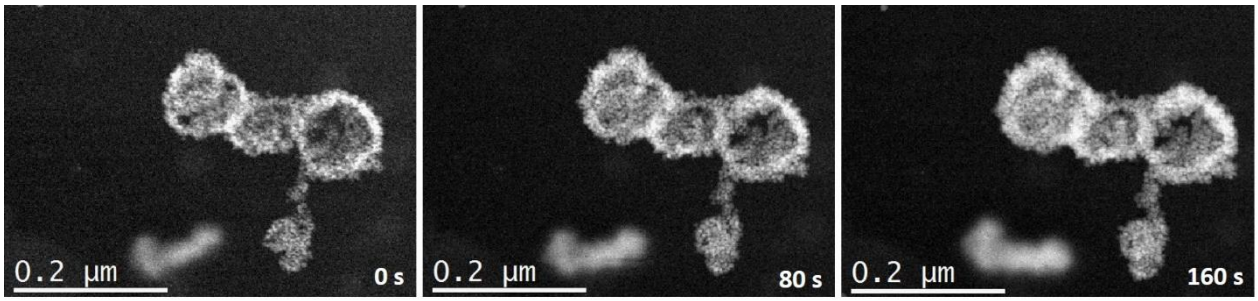

Figure S8. LCTEM images of EVs extracted from a video file (the observation time is indicated in the bottom-right corner of each image) showing the increase of the gold-layer thickness over time.

Video files

Videos 1, 2 and 3 were used to make the figures 4, 5b and 5c, respectively. Video 1 is not accelerated while video 2 and video 3 are accelerated four and eight times, respectively.

References

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