Supplementary files

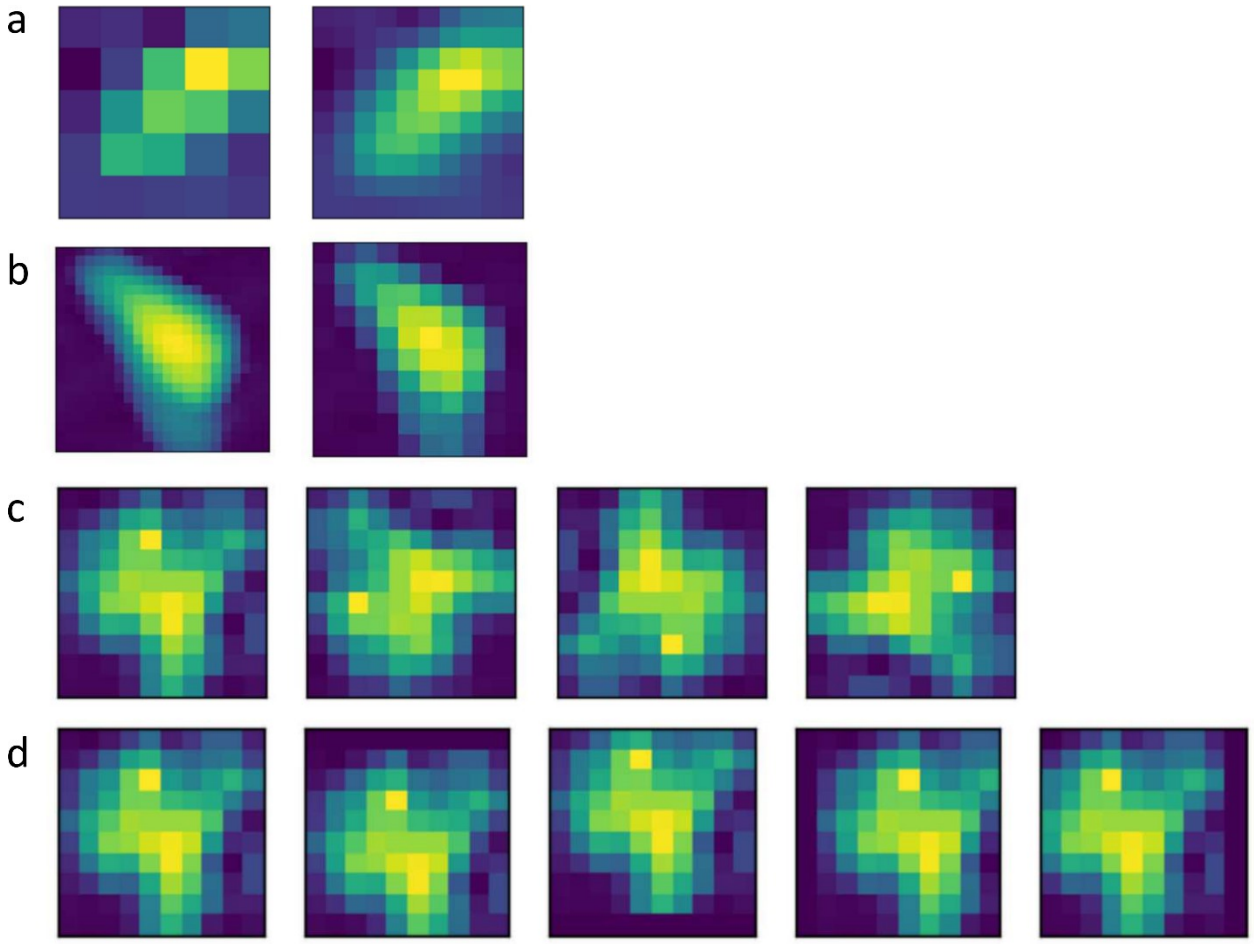

Figure S1 Illustration of image rescaling and augmentation in pre-processing steps for machine learning classification. Upscaling (a) a 5×5 pixel image to 10×10 pixels, downscaling (b) a 22×22 pixel image to 10×10 pixels, rotating (c) the original image (90°, 180°, 270°), and translating (d) the original image by 1 pixel (down, up, right, left).

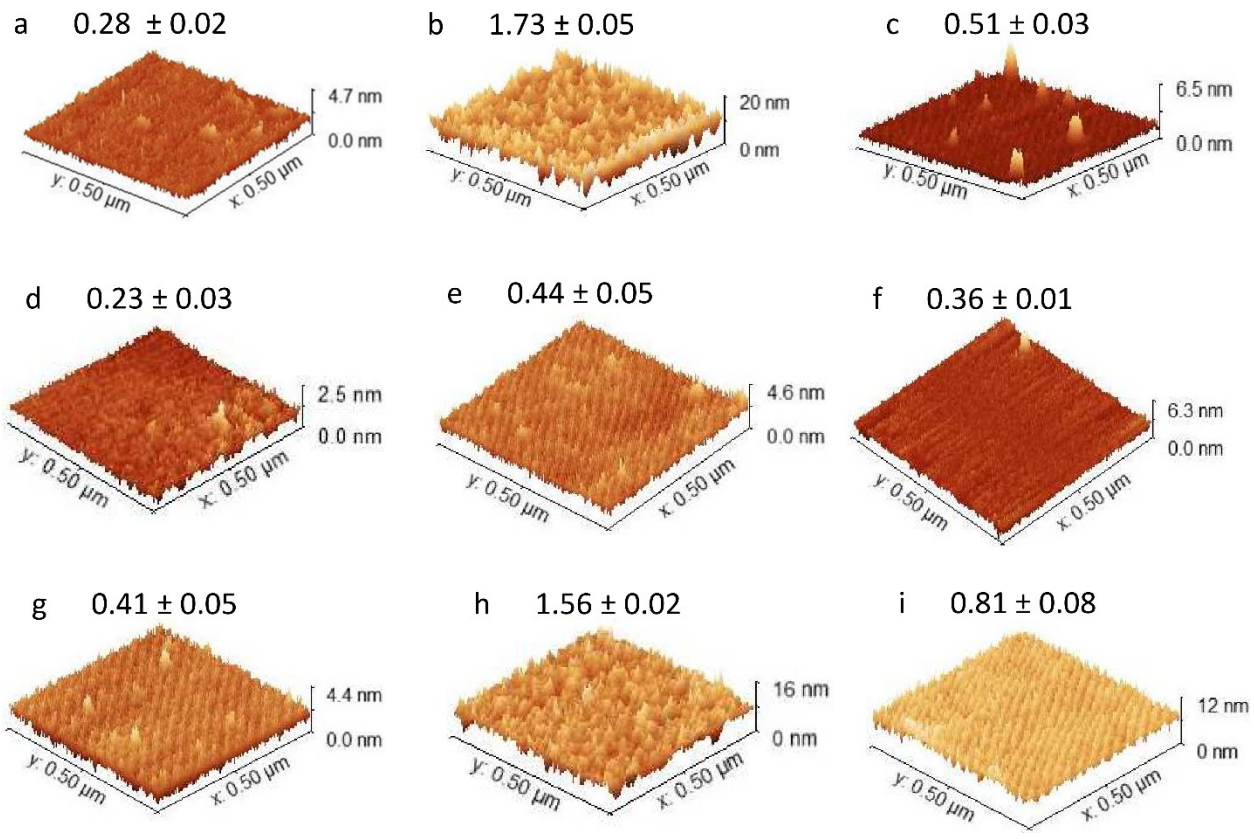

Figure S2 3D roughness (Sq, nm) analysis of mica substrates functionalised with various methods. (a) Bare mica, (b) mica functionalised with $NiCl_2$, (c) $NiCl_2$ functionalised mica with PFA/GA (3%:1.5%), (d) mica functionalised with PLL, (e) PLL functionalised mica with PFA/GA (3%:1.5%), (f) mica functionalised with APTES using vapor deposition, (g) APTES vapor functionalised mica with PFA/GA (3%:1.5%), (h) mica functionalised with APTES using liquid deposition, (i) APTES liquid functionalized mica with PFA/GA (3%:1.5%). Sq values calculated in Gwyddion software.

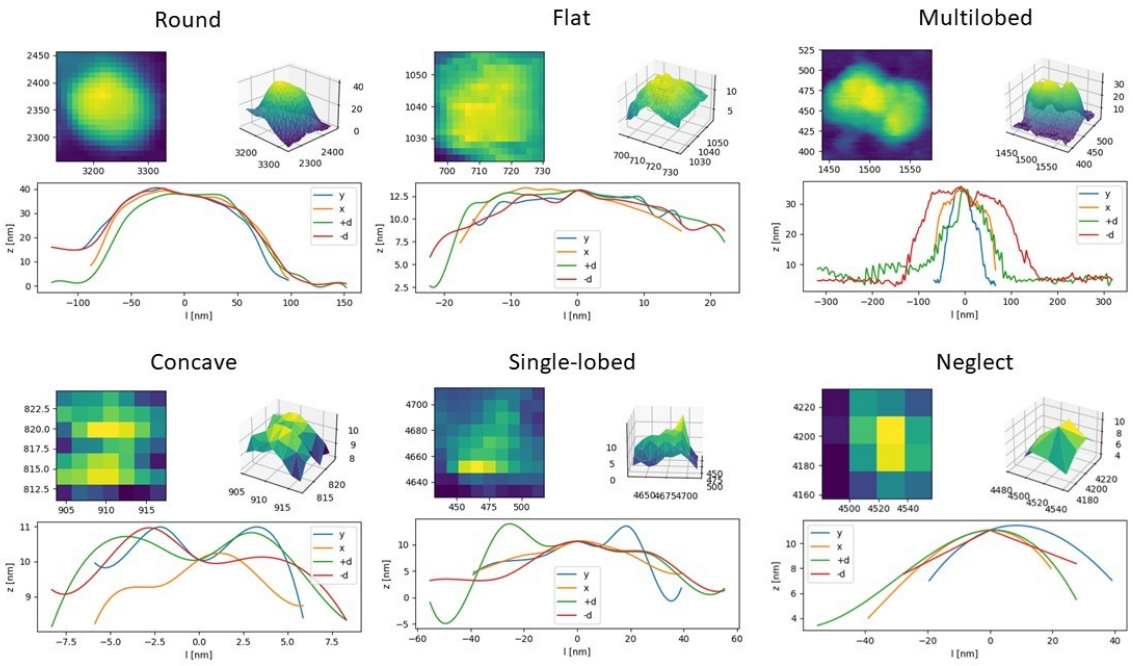

Figure S3 Example images and Z-profiles for extracellular vesicles of different morphology/shape classes.

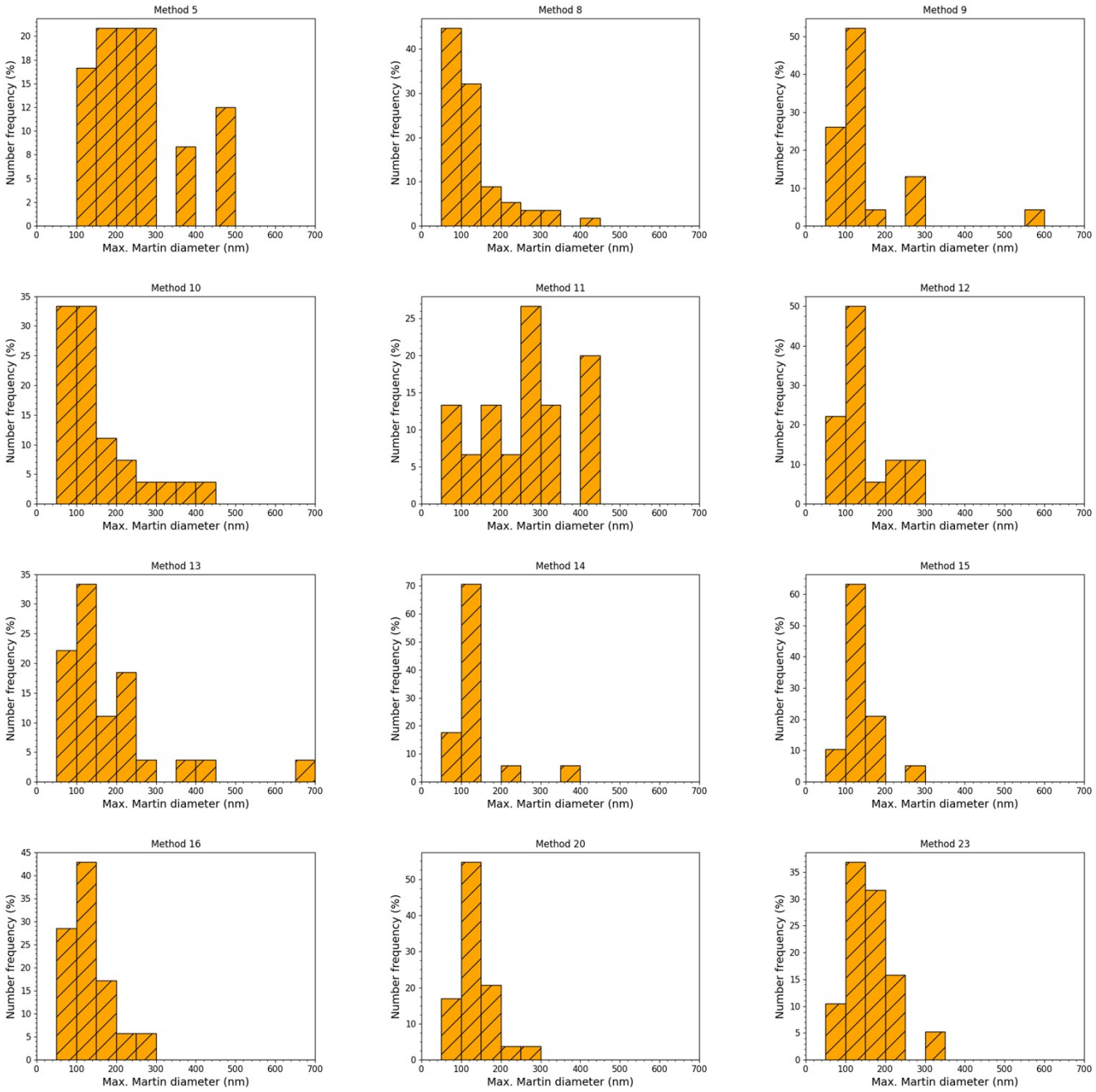

Figure S4 Histograms of maximum Martin diameters for EVs based on AFM images after different methods of sample dehydration and drying (5, 8-16, 20 and 23) that yielded more than 15 non-neglected particles altogether (based on CNN model classification). Legend for methods: (5 – $NiCl_2$, 8 – APTES-l) fixed+air-dried; (9 – $NiCl_2$, 10 – PLL, 11 – APTES-v, 12 –APTES-l) fixed+EtOH+CPD; (13 – $NiCl_2$, 14 – PLL, 15 – APTES-v, 16 – APTES-l) fixed+DMP+CPD; 20 – APTES-l+fixed+EtOH+HMDS; 23 – APTES-v+fixed+DMP+HMDS.

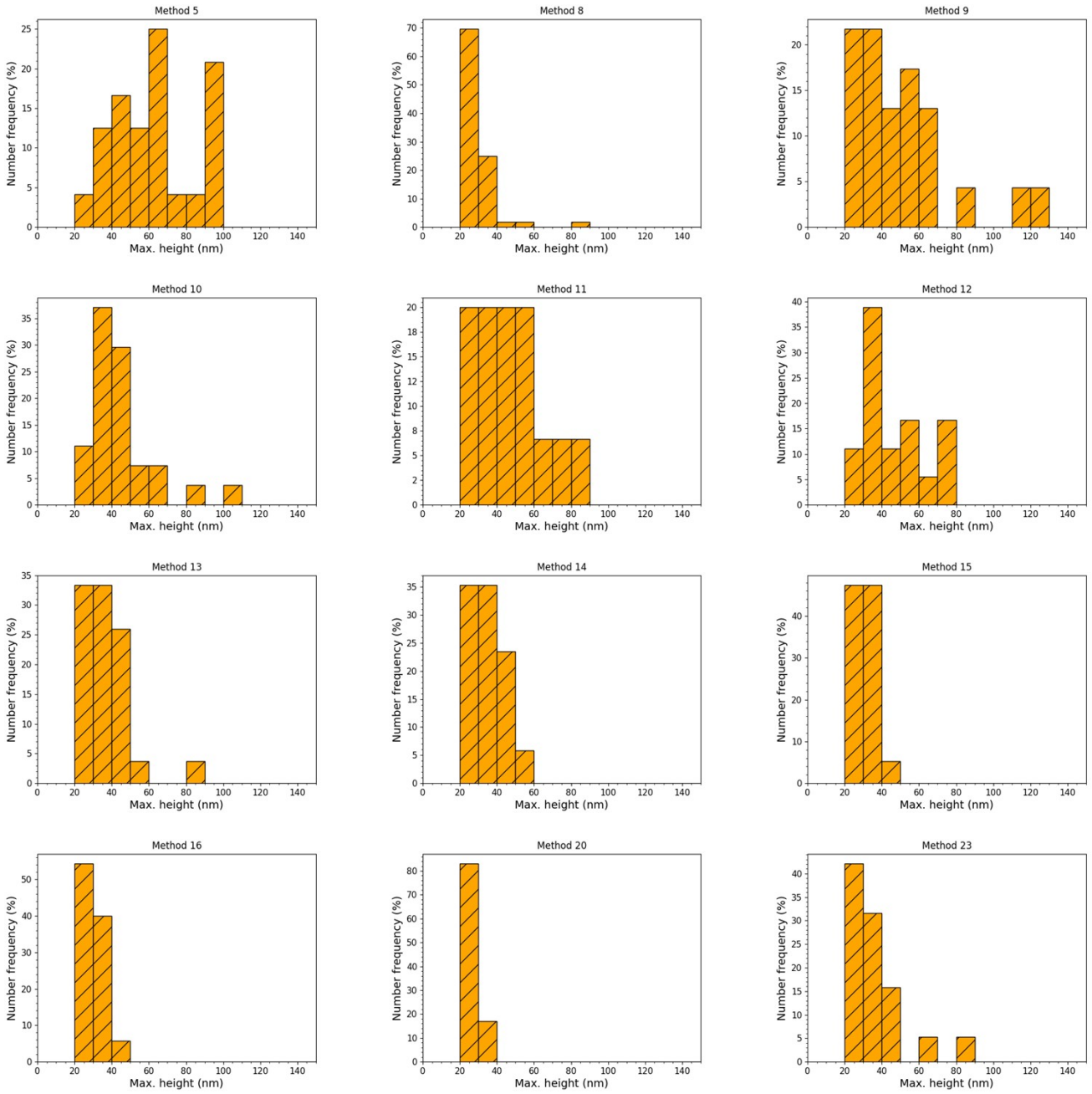

Figure S5 Histograms of maximum heights for EVs based on AFM images after different methods of sample dehydration and drying (5, 8-16, 20 and 23) that yielded more than 15 non-neglected particles altogether (based on CNN model classification). Legend for methods: (5 – $NiCl_2$, 8 – APTES-l) fixed+air-dried; (9 – $NiCl_2$, 10 – PLL, 11 – APTES-v, 12 –APTES-l) fixed+EtOH+CPD; (13 – $NiCl_2$, 14 – PLL, 15 – APTES-v, 16 – APTES-l) fixed+DMP+CPD; 20 – APTES-l+fixed+EtOH+HMDS; 23 – APTES-v+fixed+DMP+HMDS.

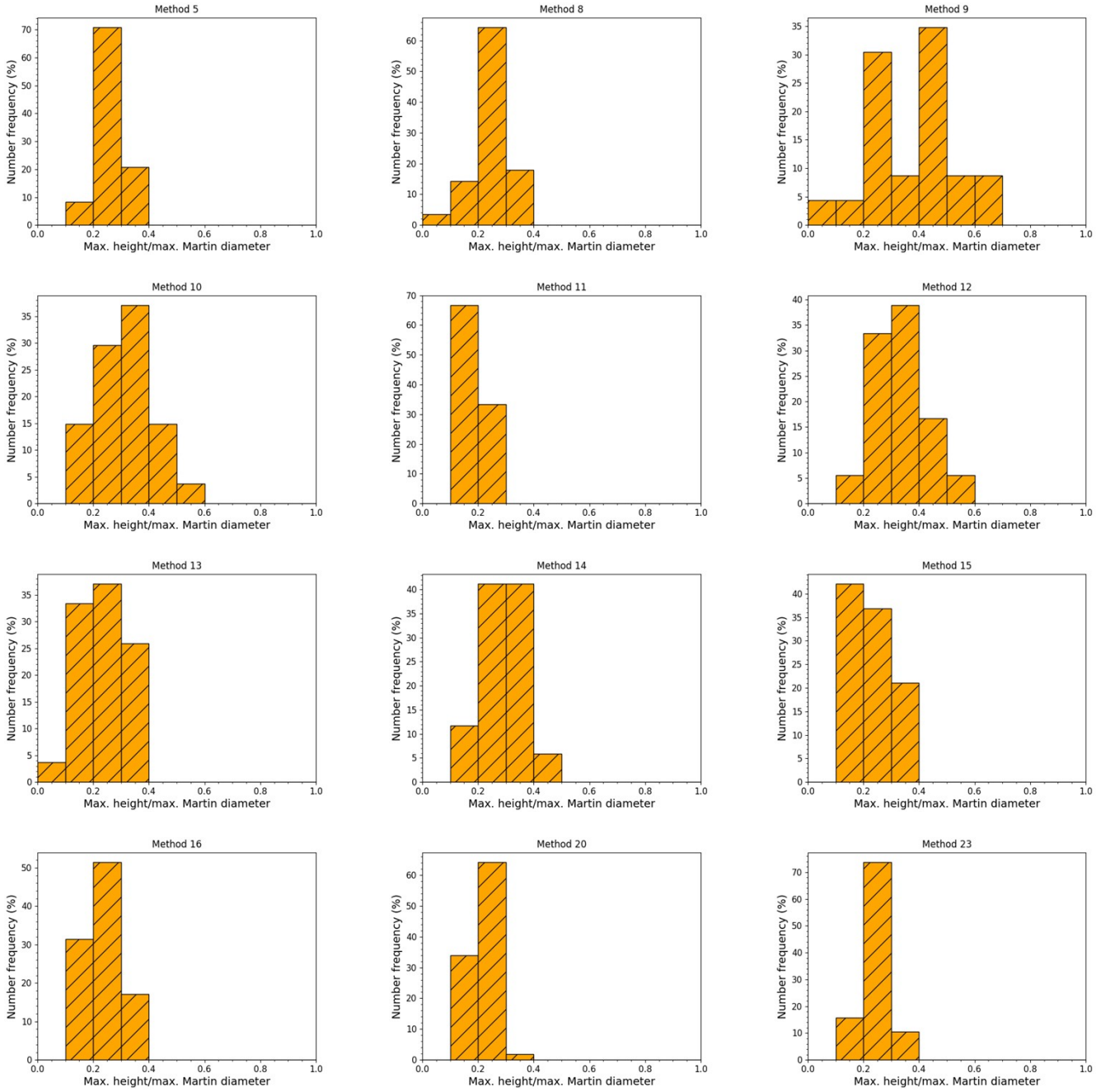

Figure S6 Histograms of aspect ratios (maximum height/maximum Martin diameter) for EVs based on AFM images after different methods of sample dehydration and drying (5, 8-16, 20 and 23) that yielded more than 15 non-neglected particles altogether (based on CNN model classification). Legend for methods: (5 – $NiCl_2$, 8 – APTES-l) fixed+air-dried; (9 – $NiCl_2$, 10 – PLL, 11 – APTES-v, 12 –APTES-l) fixed+EtOH+CPD; (13 – $NiCl_2$, 14 – PLL, 15 – APTES-v, 16 – APTES-l) fixed+DMP+CPD; 20 – APTES-l+fixed+EtOH+HMDS; 23 – APTES-v+fixed+DMP+HMDS.

Supplementary section 1. Instructions for EVIAN installation and manual for AFM images processing

1. Install Python3, scipy, numpy, matplotlib and gwyfile. Here's a link for Python3 installation with numpy, scipy and matplotlib on Windows:
   https://solarianprogrammer.com/2017/02/25/install-numpy-scipy-matplotlib-python-3-windows/
   You then add the gwyfile package in a similar way by typing:
   `pip3 install gwyfile`
   in the command prompt.
2. Open Gwyddion, open an AFM image in it and process it for EV determination according to the following steps:

   a. Align the imaging plane to match the laboratory XY plane by removing the tilt in the substrate from the scan data. To accomplish this task, select Data Process, Level and choose Plane Level (or click the icon you see here on the left).

   b. Align rows of the image by selecting Data Process, Correct Data and then choose Align Rows. Several alignment options are available. For example, Median is an algorithm that finds an average height of each scan line and subtracts it from the data.

   c. Go to Data Process, Correct Data and choose Remove Scars, which removes common scanning errors known as scars.

   d. Align the mica surface at the zero height, Z = 0, by selecting Flatten Base in Level drop-down menu accessible from Data Process. Optionally, you can check Z after this step and stretch color range to only part of data. You can also see the resolution of the image there (size in pixels), which is important for step 7.

   e. Shift Z values to have minimum at Z = 0 (to avoid negative Z values).

   f. Identify EVs on the scanned surface by using Mark by Threshold in Grains drop-down menu. This algorithm identifies surface-immobilized EVs as particles protruding from the zero-surface substrate by the height above the user-selected threshold. Select a threshold of 20 nm, which will eliminate most of the background interference.

   g. Add another threshold in the xy plane by going to Data Process, Grains and Filter (or icon Filter grains by their properties), then selecting "Keep grains satisfying A ^ B" and setting Maximum value as Condition A and Projected area as Condition B. Set minimum projected area to 1250 nm^2.

   h. Remove grains touching the edge of the image.

   i. Optionally manually adjust the mask.

   j. After AFM image processing and grain determination in Gwyddion, save the file as .gwy type.

   k. Go to "Grains —> Distributions …" and tick "Position - Center x position, Center y position", "Value - Maximum value, Mean value" and "Boundary - Maximum Martin diameter". Select "Export raw data" and "Add informational comment header". Then click OK and save the file with the same name as in the previous step (j.) but with .dat extension.
3. Make sure that EVIAN.py is in the same folder as .gwy and .dat files and that export without categorisation is set off. You can check (and modify) this by opening EVIAN.py in Notepad or similar program. Shape categorisation is enabled if line 35 says "directOutput=False".
4. By default, EVIAN reads the measured Z values from Gwyddion file as Zsensor, but different AFM machines name these values differently which causes problems in reading the .gwy files. To check the name, go to Info and Show data browser in Gwyddion. Then modify it in line 289 of EVIAN.py if necessary.
5. Open command prompt (run cmd) or Terminal (Mac users) and go to your folder with files (type "cd" and then the folder path), then type "python3 EVIAN.py". A window with 2D, 3D image and zx, zy, zx-diagonal and zy-diagonal profiles/height curves for the first grain should appear.
6. Check each grain (vesicle) and decide about its shape (click either "Round", "Single-lobed, "Concave", "Flat" or "Multi-lobed" or "Neglect" if the curves or the shape looks weird and unlike an extracellular vesicle).
7. The program distributes the images and the data of each grain into folders according to your decisions and counts grains of each type. It also stores all these data and decisions into a .json and a .tsv file and outputs .txt files with max. heights and Feret diameters for each shape and .gwy file, as well as for all shapes together without the neglected. Once you categorise all grains or you press Exit, EVIAN gives out in the command prompt window the number of each shape for all the files in the folder.

8. If you have done part of the analysis in EVIAN, pressed Exit and want to continue categorising later, write the number of the last grain that you analysed in line 16 of EVIAN.py, so that the program will continue from that grain on.