Supporting Information for:

Extracellular Vesicles from Mesenchymal Stem Cells and Red Blood Cells have different Biomolecular Corona Dynamics

Experimental Section

Reagents

Solvents were purchased from Merck (Darmstadt, DE) and Carlo Erba (Cornaredo, Milan, IT) and used as received without further purification. PBS, DMEM, FBS, and Penicillin/Streptomycin were purchased from Corning (Mediatech Inc., Manassas, VA, USA). NaN_3 was purchased from Sigma Aldrich (St. Louis, MO, USA). Sulfo-Cyanine 5 NHS ester was purchased from Lumiprobe GmbH (Germany). DBCO-STP Ester, Azido-PEG_4-NHS Ester, and Cy3 Azide were purchased from ClickChemistryTools, (Scottsdale, AZz, US). Calcium ionophore (Calcimycin, A23187) was purchased from Sigma Aldrich (St. Louis, MO, USA).

Instrumentation

Centrifugation was performed with a 5804R Eppendorf Centrifuge, A-4-44 rotor, 15 mL tubes. Ultracentrifugation for large volumes (up to 50 mL) was performed with Optima XPN-100, TY45 Ti rotor (Beckman Coulter, USA) while for small volumes (up to 1.5 mL) we used an Optima MAX-XP, TLA-55 rotor, and MLS-50 (Beckman Coulter, USA). Images from western blot and gel fluorescence were acquired with Syngene G:BOX Chemi XX9 (SYNGENE, UK) and were analyzed and quantified with the software Genesys and GeneTools (SYNGENE, UK). NanoDrop™ OneC (ThermoFisher, Rockford, USA) was used to characterize mCTX. Zeiss LSM 880 with

a Zeiss C-Apochromat 40x water immersion objective (Germany) was used to perform FCS. All the VivaSpin columns used in this work were purchased from Sartorius Stedim Lab Lid (Sperry Way, Stonehouse, UK). NanoSight NS300 system and Malvern ZetaSizer Ultra were used to determinate the size distribution and the particle concentration of REV samples (Malvern Technologies, Malvern, UK).

Human amniotic mesenchymal stromal cell isolation and conditioned medium preparation

Mesenchymal stromal cells from the amniotic membrane (hAMSC) were obtained as previously reported (1). The study adhered to the principles outlined in the Declaration of Helsinki, and informed consent was obtained by the mothers following the guidelines established by the local ethical committee "Comitato Etico Provinciale di Brescia," Italy (approval number NP 2243, January 19, 2016). The isolated hAMSCs met the minimal criteria for MSCs, demonstrating positivity for mesenchymal markers CD13 (97.7 ± 1.6% mean ± SD), CD73 (88.3 ± 6.4%), CD90 (94.8 ± 7.4%), while lacking the expression of hematopoietic markers such as CD45 (1.8 ± 1.0%), CD66b (0%), and the epithelial marker CD324 (1.7 ± 1.0%) (10.1186/s13287-021-02607-z). After in vitro expansion to passage 1, hAMSCs were cultured for 5 days in 24-well plates (Corning, NY, USA) at a density of 5 × 105 cells/well in 0.5 mL of DMEM-F12 medium (Sigma-Aldrich) without serum, supplemented with 2 mM L-glutamine (Sigma-Aldrich) and 1% P/S, as previously described (10.1186/s13287-021-02607-z). Following culture, the cell-conditioned medium was collected, centrifuged, filtered through a 0.2-μm sterile filter (Sartorius Stedim, Florence, Italy), and stored at −80 °C. Two pools of conditioned medium were generated, each derived from a minimum of three different placenta donors.

Red Blood Cells EV (REV) and Mesenchymal Stromal Cell EV (MEV) collection (isolation\separation)

REVs were isolated according to the protocol outlined by Usman et al.31. In brief, upon the collection of 100 mL of blood, red blood cells (RBCs, RBCs are obtained from

anonymized healthy volunteers under written consent and provided by the A. O. Spedali Civili di Brescia, ethical approval "EritrEV NP5705") were separated by centrifugation at 1000 × g for 8 minutes at 4 °C and underwent three washes in PBS without calcium and magnesium. Following this, RBCs were subjected to two additional washes with CPBS (PBS + 0.1 g/L calcium chloride) and then transferred into a 75 mm2 tissue culture flask. Calcium ionophore was added into the flask at ha final concentration of 10 mM, and the mixture was incubated overnight at 37 °C. Subsequently, 75 mL of RBCs were gently collected from the flask, and any cellular debris was eliminated through a series of differential centrifugation steps (600 × g for 20 min, 1600 × g for 15 min, 3260 × g for 15 min, and 10,000 × g for 30 min at 4 °C). At each step, the pellet was discarded, and the supernatant was transferred into a fresh tube. The resulting supernatants were filtered through 0.45 μm nylon syringe filters. EVs were obtained through ultracentrifugation at 100,000 × g for 70 min at 4 °C. The EV pellets were then resuspended in cold PBS, layered above a 2 mL frozen 60% sucrose cushion, and centrifuged at 100,000 × g for 16 h at 4 °C, with the deceleration speed set to 0. The red layer of EVs was collected, washed twice with cold PBS, and spun at 100,000 × g for 70 min at 4 °C. Finally, the EVs were resuspended in 1 mL of cold PBS.

For MEVs, EVs were prepared from 100 ml of conditioned medium (10e+6 cells/mL) using differential centrifugation steps. All preparation and centrifugation steps were performed at 4°C. Briefly, collected cell-media were subjected to a first centrifugation at 2,000×g for 30 min to remove apoptotic bodies, and a second centrifugation at 16,000×g for 20 min to remove larger vesicles. EVs were then pelleted from the purified supernatant by centrifugation at 120,000×g for 70 min in 38 mL polycarbonate tubes. This EV-enriched pellet was resuspended in PBS, and the centrifugation was repeated as above. The final EV pellets were resuspended in PBS and stored at -80°C until their use.

Cetuximab (CTX) functionalization

CTX was kindly supplied by Professor Fabio Corsi from the University of Milan in a storage buffer containing sterile PBS and 0.1 mM NaN_3. The storage buffer was

subsequently replaced with a 0.1 mM $NaHCO_3$ solution at pH 8.4 using a VivaSpin2000 column with a 50 kDa cut-off. The CTX solutions were then subjected to centrifugation at 2000 × *g* for 15 minutes. This buffer exchange process was repeated three times, with 2 mL of 0.1 mM $NaHCO_3$ at pH 8.4 added at each step. The concentration of CTX after the buffer exchange was determined using a NanoDrop™ OneC. For antibody functionalization, 200 µL of CTX at a concentration of 0.016 mM was incubated overnight with under continuous mixing at 4 °C, along with 12 equivalents of Sulfo Cyanine 5 NHS ester (0.2 mM in DMSO) and 6 equivalents of DBCO STF ester (0.1 mM in DMSO). This resulted in the modified CTX (mCTX), which was characterized by UV-Vis spectroscopy to determine the yield of ligation of Sulfo Cyanine 5 and DBCO.

Bicinchoninic acid (BCA) assay

Protein concentrations of MEVs and REVs samples were determined with Pierce™ BCA Protein Assay Kit (ThermoFisher, Rockford, USA), following the manufacturer's instructions.

SDS-PAGE and Western Blotting

To conduct SDS-PAGE and Western Blotting, samples were subjected to boiling in reducing SDS sample buffer (containing 80 mM Tris, pH 6.8, 2% SDS, 7.5% glycerol, and 0.01% bromophenol blue) with the addition of 2% 2-mercaptoethanol. This process was carried out for 5 minutes at 95 °C, and the samples were then separated using SDS-PAGE on a 10% acrylamide/bisacrylamide gel. Densitometric analysis was performed using Image Lab software from Biorad, located in Hercules, CA, US. For the quantification of CTX, following the electrophoresis run, the gel was imaged using a Syngene G:BOX Chemi XX9 with an acquisition time of 2 minutes and a wavelength of 667 nm. For Western Blot analysis, the samples were transferred onto a PVDF membrane and subsequently blocked overnight with a solution of 5% fat-free milk in PBS-0.05% Tween-20 (PBST). The PVDF membranes were then incubated with the following antibodies, each at a 1:1000 dilution, for 90 minutes in PBST with 1% fat-free

milk: mouse anti-GM130 (clone 35/GM130, BD Biosciences, GermanyDE), mouse anti-Alix (2H12, Santa Cruz Biotechnology, USA), rabbit anti-LAMP1 (polyclonal, GeneTex, USA), mouse anti-CD63 (Santa Cruz Biotechnology, USA), mouse anti-BAND3 (Santa Cruz Biotechnology, USA), rabbit anti-EGFR (clone 15F8, Cell Signalling Technology Inc., Danvers, MA, US), mouse anti-TSG101 (Santa Cruz Biotechnology, USA). Following this, the membranes were washed three times for 10 minutes with PBST and incubated for 1 hour with rabbit anti-mouse or goat anti-rabbit secondary antibodies (Zymed, San Francisco, CA, US) at a dilution of 1:3000. The blots were then detected and revealed using Luminata Classic HRP western substrate from Merck-Millipore, and the images were acquired using a G: Box Chemi XT Imaging system, as previously described in Alvisi et al. (1)

UV-vis spectroscopy

UV-Vis spectra were measured with a JASCO V530 UV-Vis spectrophotometer, using standards 10 mm x 10 mm, 1 ml disposable plastic cuvettes. Water or PBS were used for baseline subtraction. Data acquisition was set on “fast” and Abs points were collected at 1 nm steps.

Colorimetric NANoplasmonic (CONAN) assay

To assess the purity of REV preparations and detect soluble contaminants, the CONAN assay was employed. This colorimetric test relies on the aggregation of citrate-capped gold NPs (AuNPs) onto the EV membrane and the formation of the protein corona on the AuNP surface to identify soluble proteins in EV preparations. The CONAN assay was performed in accordance with the procedure described by Zendrini et al. (2)

Transmission Electron Microscopy (TEM)

Carbon-coated copper grids (Ted Pella, Inc.) were hydrophilized, and 1.5 µL of the sample with 0.04% of paraformaldehyde was applied and left to incubate on the grid for 3 minutes. The excess solution was removed with filter paper and 1.5 µL of 1.5% uranyl acetate solution for positive staining was added to the grid and left for 3 minutes,

followed by two washing steps. Imaging was conducted with a LaB6-TEM of type JEOL JEM-1400PLUS (40kV - 120kV, HC pole piece) equipped with a GATAN US1000 CCD camera (2k x 2k).

Nanoparticle Tracking Analysis (NTA)

Nanoparticle Tracking Analysis (NTA) was conducted following the manufacturer's instructions, employing a NanoSight NS300 system equipped with a 532 nm laser. To achieve an optimal particle per frame value (ranging from 20 to 100 particles per frame), samples were diluted at a 1:1000 ratio in filtered PBS with a 0.22 µm filter, resulting in a final volume of 1 mL. A syringe pump was used for constant flow injection at 20 µl/min, while the temperature was maintained at a constant 25 °C. The particles were detected with a camera level set at 10, and three videos, each lasting 60 seconds, were captured and subsequently analyzed using NTA software version 3.2. The mean, mode, and median sizes of the EVs from each video were utilized to calculate the sample concentration, which was expressed in particles per milliliter (particles/mL).

REV and MEV chemisorption and physisorption

Chemisorption and physisorption of REVs and MEVs were accomplished through a two-step labeling procedure. Initially, 200 µL of REVs or MEVs with a concentration of $8x10^{11}$ particles/mL were subjected to a reaction with 200 equivalents of PEG_4-NHS-ester (diluted in PBS) overnight, with continuous mixing at 4°C. Excess PEG was subsequently removed using a Vivaspin500 column with a 10 kDa cut-off, involving five washes with 500 µL of PBS. The pegylated REVs were recovered from the column with 200 µL of PBS. Next, 200 µL of pegylated REVs or MEVs were subjected to an overnight reaction with 200 equivalents of mCTX (in PBS at pH 7.4) with continuous mixing at 4°C. Any unreacted antibody was removed by repeating two times ultracentrifugation at 100,000 × g for 2 hours. The resulting pellet was resuspended in 200 µL of PBS and stored at 4 °C for later use.

Dynamic Light Scattering (DLS) and ζ-potential measurement

Dynamic light scattering measurements were carried out with a Malvern ZetaSizer Ultra instrument in backscattering mode. All studies were performed at a 173° scattering angle, and temperature was kept at 25 °C in 1 ml polystyrene cuvettes. After initial equilibration, the measurements consisted of at least 15 runs. ζ-potential measurements were performed in auto-mode at 25 ◦C, with three consecutive measurements per sample in a folded capillary cell. All samples were measured three times, and the standard deviation was represented with error bars.

Fluorescence Correlation Spectroscopy (FCS)

FCS measurements were recorded with a Zeiss LSM 880 confocal microscope and analysed with Zen black software. A HeNe laser was used for excitation at 633 nm. Emission was recorded using the PMT (650 – 715 nm) detector. Measurements were conducted with a Zeiss C-Apochromat 40x, numerical aperture 1.2 water immersion objective. 100 µL of the particle dispersion was filled up to 200 µL final sample volume and transferred to a chambered polymer coverslip (Ibidi) for FCS measurement. Prior to sample measurement, the confocal volume was calibrated with a 100 nM solution of Rhodamine 6G in UHPLC-grade water using a diffusion coefficient of 430.00 $\mu m^2/s$ (Absolute Diffusion Coefficients: Compilation of Reference Data for FCS Calibration, picoquant.com). One measurement consisted of 20 runs of 20 seconds and each sample was recorded three times. The data fitting was done with QuickFit 3.0 software using the global fit of 1, 2 and 3-components and 3D normal diffusion model. Specifically, FCS measurements were performed on all our fluorescent building blocks to validate our protocol and determine the diffusion coefficients first of Cy5, and second of mCTX, which were analyzed as two components preparation (free Cy5 and mCTX). Indeed, this information is crucial for rigorously and accurately analyzing the data related to the chemisorbed and physisorbed EVs, which were considered a three-component preparation (free Cy5, mCTX, and EVs). The resulting diffusion coefficients were used to calculate the hydrodynamic diameter (HD) with the Stokes-Einstein equation.

$$D = \frac{k_B T}{6\eta\pi r^h} \qquad (1)$$

Stoichiometric evaluation of BC corona formation

A stoichiometric model was developed to estimate the BC volume as a function of EV and protein diameters. The model also allows for a rough estimation of the number of proteins populating each BC layer and their total weight, provided some assumptions are made. The model is based on simple geometrical calculations, and aspects such as membrane roughness, curvature, protein-protein interactions, etc., are not considered in the evaluation of BC. The equations shown in the following have been collected in an editable spreadsheet provided as SI. Its use, improvement, and dissemination are permitted and encouraged to the scientific community members appealed to the topic previous citation of this work.

As a first geometric approximation, the volume V_{BC} occupied by a single layer of proteins in a BC on the surface of an EV having volume V_{EV} can be defined as follows:

$$V_{BC} = (V_{tot} - V_{EV}) \cdot p \qquad (2)$$

Being V_{tot} the sum of the EV and BC volumes and p the packing factor defining the packing density of the proteins forming the layer.

Both EVs and proteins have various shapes but in solution are often modelized as perfect spheres or spheroids (e.g., when calculating their size/concentration by DLS, NTA, etc.). For spherical proteins and EVs having diameter d_p and d_{EV} respectively, eq. 1 can be reformulated:

$$V_{BC} = \frac{4}{3}\pi\left[\left(d_p + \frac{d_{EV}}{2}\right)^3 - \left(\frac{d_{EV}}{2}\right)^3\right] \cdot p \qquad (3)$$

In a lattice of packed non-overlapping spheres, p describes the fraction of the volume occupied by the spheres in relation to the total volume of the containing space. p is linked to spheres' spatial arrangement within the lattice; in a system comprising only spheres

of identical size, p usually ranges from 0.05 to ~ 0.74. Other p values often found in physical systems are, i.e., ~ 0.34, ~ 0.52, or ~ 0.63.

It is also possible to estimate the number of proteins (n) occupying V_{BC}:

$$n = \frac{V_{BC}}{Vp} \quad (4)$$

Being Vp the protein volume.

Again, for spherical particles, eq. 3 is reformulated and factored out as follows:

$$n = \frac{\left[\left(d_p + \frac{d_{EV}}{2}\right)^3 - \left(\frac{d_{EV}}{2}\right)^3\right] \cdot p}{\left(\frac{d_p}{2}\right)^3} \quad (5)$$

Furthermore, after calculating n, it is in principle possible to approximate the total protein mass T_m transported by a defined coronal layer by assuming all the carried proteins have similar molecular weight a_{MW}:

$$T_m = n \cdot a_{MW} \cdot 1.66 \cdot 10^{-24} \quad (6)$$

With $1.66 \cdot 10^{-24}$ being the approximated conversion factor from Da to grams ($1\,Da = \frac{1}{6.022 \cdot 10^{23}}\,g = \sim 1.660 \cdot 10^{-24}\,g$).

If needed, the equations can be further extended for the calculation of multiple stacked layers of spherical proteins with given d and p.

The presented equations were applied to estimate BC topology on MEVs and REVs, starting from the data collected through FCS analysis (average d_{MEV} and d_{REV}). All the calculations were performed assuming $d_p = 7 \cdot 10^{-9}\,m$ and $a_{MW} = 67000\,Da = 1.11 \cdot 10^{-19}$ g, that are respectively the hydrodynamic diameter and

the mass of human serum albumin, here used as a model protein. *p* was set to 0.63, which corresponds to a random close-pack sphere geometry. (3)

Supporting Figures

Cetuximab functionalization

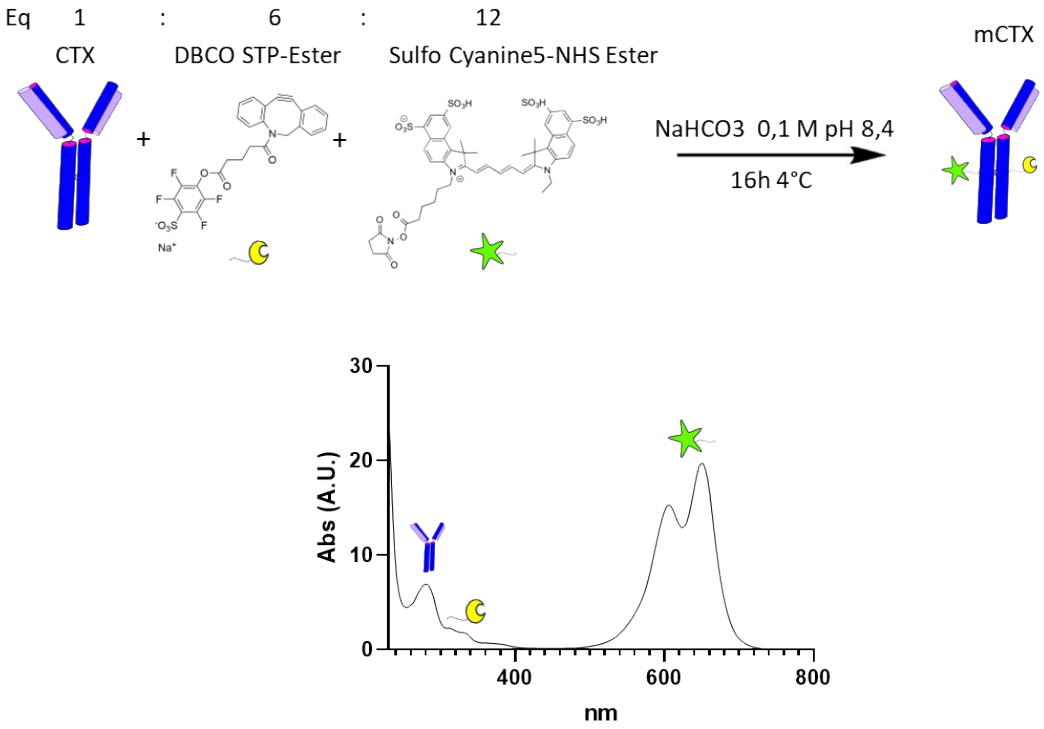
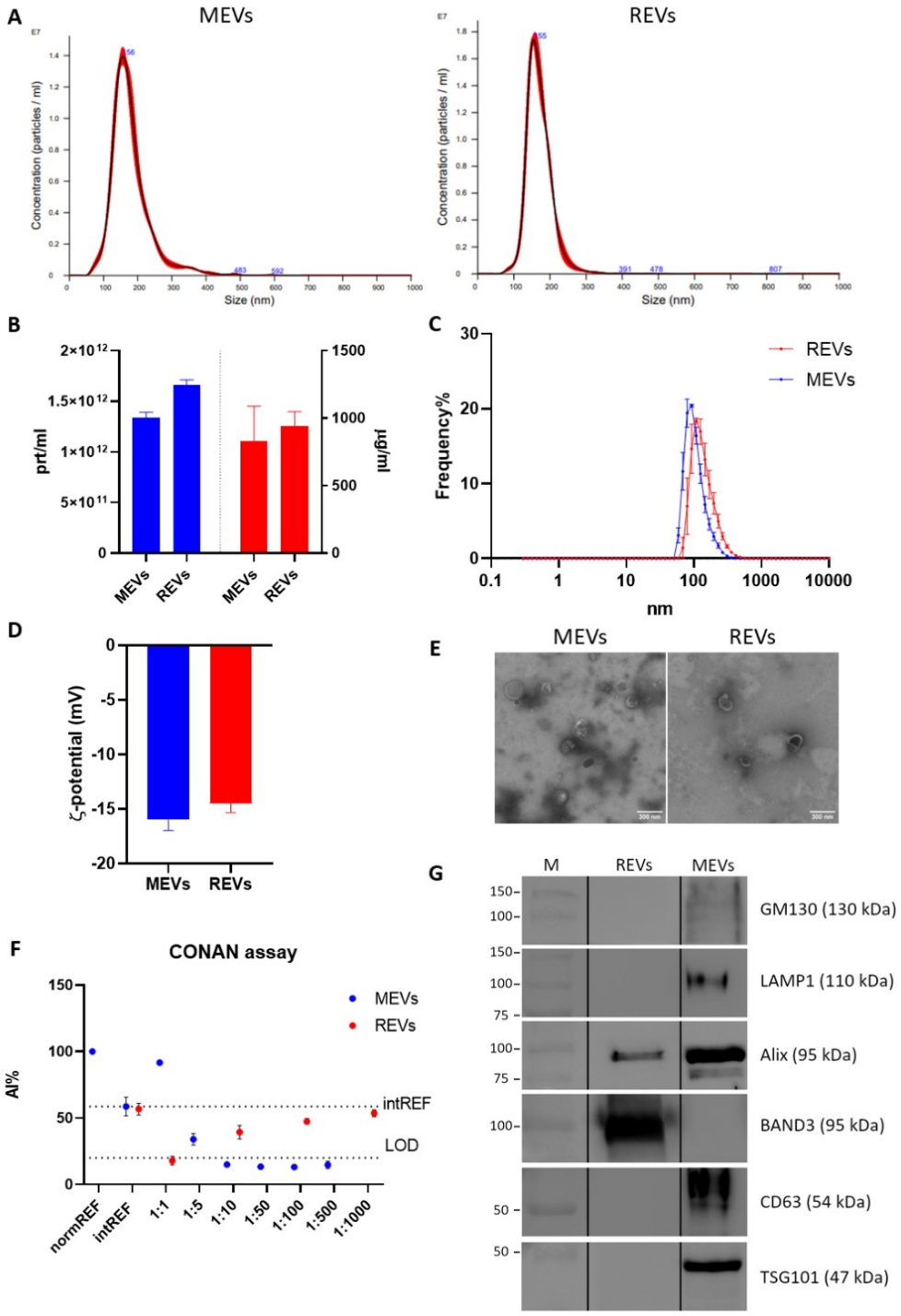


Figure S1. CTX modification and characterization. To obtain mCTX we reacted the monoclonal antibody with respectively 6 and 12 equivalent of DBCO-STP-Ester and Sulfo Cyanine5-NHS-Ester, in a single pot reaction as reported in the figure. UV-Vis spectrum of mCTX is reported, highlighting with symbols the correspondence of a peak with functional groups.



REV and MEV biophysical and biochemical characterization S13

Figure S2. MEVs and REVs were characterized following MISEV2023 guidelines. **(A)** MEVs (left) and REVs (right) size distribution profiles obtained by the average of 3 NTA measurements are reported. **(B)** Particle concentration and protein content of MEV and REV preparations. Blue bars indicate the particle concentration (expressed as prt/ml) of MEV and REV preparations obtained by NTA. Red bars indicate the protein concentration (expressed as µg/ml of proteins) of REV and MEV preparations obtained by BCA assay. Mean of 3 replicates ± SD is reported. **(C)** MEVs (blue) and REVs (red) size distribution profiles obtained by DLS**. (D)** ζ-potential measurement performed on MEVs (blue bar) and REVs (red bar). Mean of 3 replicates ± SD is reported. **(E)** TEM images of MEVs (left panel) and REVs (right panel). Scalebar = 300 nm. **(F)** CONAN assay performed to determine MEV and REV preparations purity from exogenous contaminants. The Int-REF AI ratio (AI%) defines the threshold below which the spectral redshift is only due to the interaction between the AuNPs and the EVs. The dotted line represents the CONAN assay threshold for soluble protein detection (<20% AI ratio means that the soluble protein content is ≤0.05 µg/µL). In both MEVs (blue dots) and REVs (red dots) the AI% is lower than 20%, indicating the preparations contain less than 0.05 µg/µL soluble proteins. Mean of 3 technical replicates ± SD is reported. **(G)** Western blot of MEVs and REVs highlights the different origins of these two EV subtypes. REVs and MEVs have in common general EV markers such as LAMP1 and Alix. REVs specifically express BAND3 (a marker for red blood cells), while MEVs express TSG101, and CD63 (both EV-specific markers). In both EV subtypes the presence of GM130 (a Golgi-matrix protein acting as a contaminant) is negligible.

Chemisorbed and physisorbed EV characterization

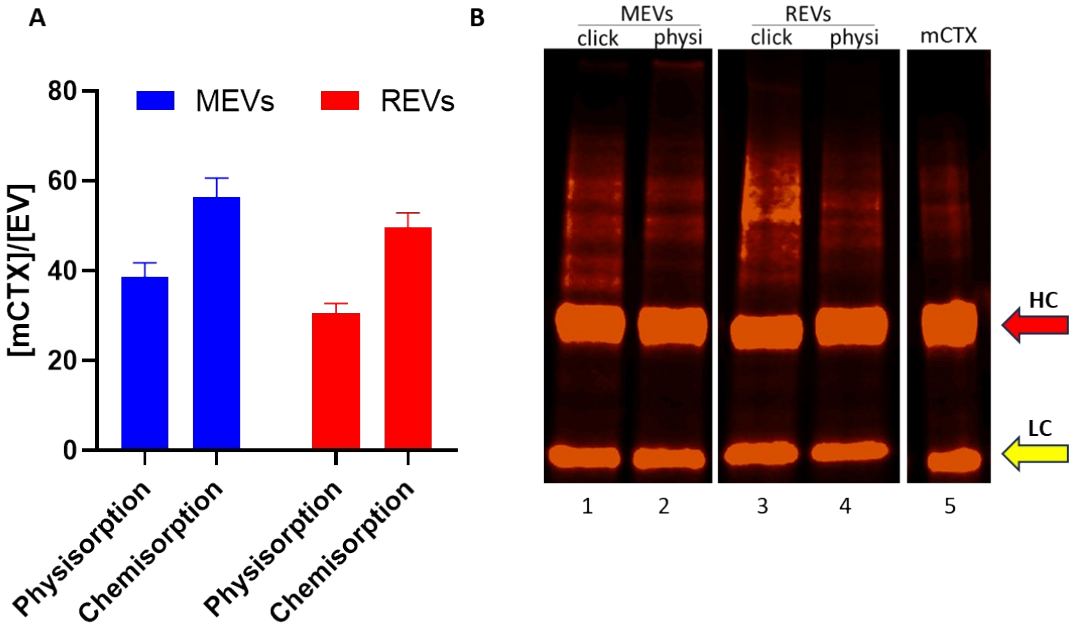


Figure S3. **(A)** MEVs and REVs showed a similar mCTX yield in both chemisorption and physisorption. The chemisorption strategy gave in both EV subtypes a higher mCTX molecules-per-EV ratio, compared to physisorption. **(B)** Fluorescent electrophoretic profile of mCTX physisorbed and chemisorbed MEVs and REVs, in both EV subtypes the lane regarding the chemisorption strategy (lane 1 for MEVs, lane 3 for

REVs) shows the presence of smeared signals, indicating the covalent bonding of mCTX with EV membrane proteins.

Building block characterization for FCS

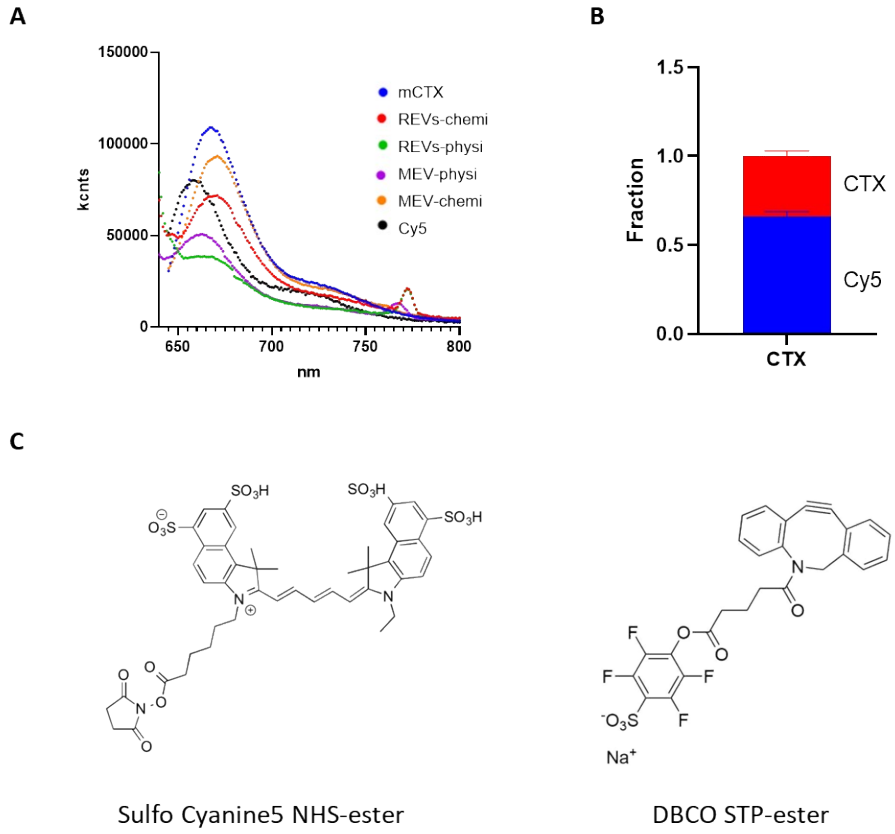


Figure S4. (A) Emission spectra of Cy5 (black), mCTX (blue), and chemisorbed (MEVs in orange and REVs in red) and physisorbed (MEVs in violet and REVs in green) EVs. Notably there is a shift of 9 nm of the peak of the Cy5 when functionalized on the antibody, but such a shift is not significant for the detector setup. mCTX and chemisorbed and physisorbed EVs emission spectra have the peak at the same wavelength. **(B)** Fraction composition of mCTX preparation obtained by FCS. In blue, is the Cy5 fraction, while in red mCTX fraction. **(C)** Drawn of the two moieties used to functionalize the CTX. Notably, both molecules present hydrophilic groups (especially Cy5) which we expected to cause the increase in the hydrodynamic diameter of mCTX.

Chemisorbed and physisorbed EV FCS characterization

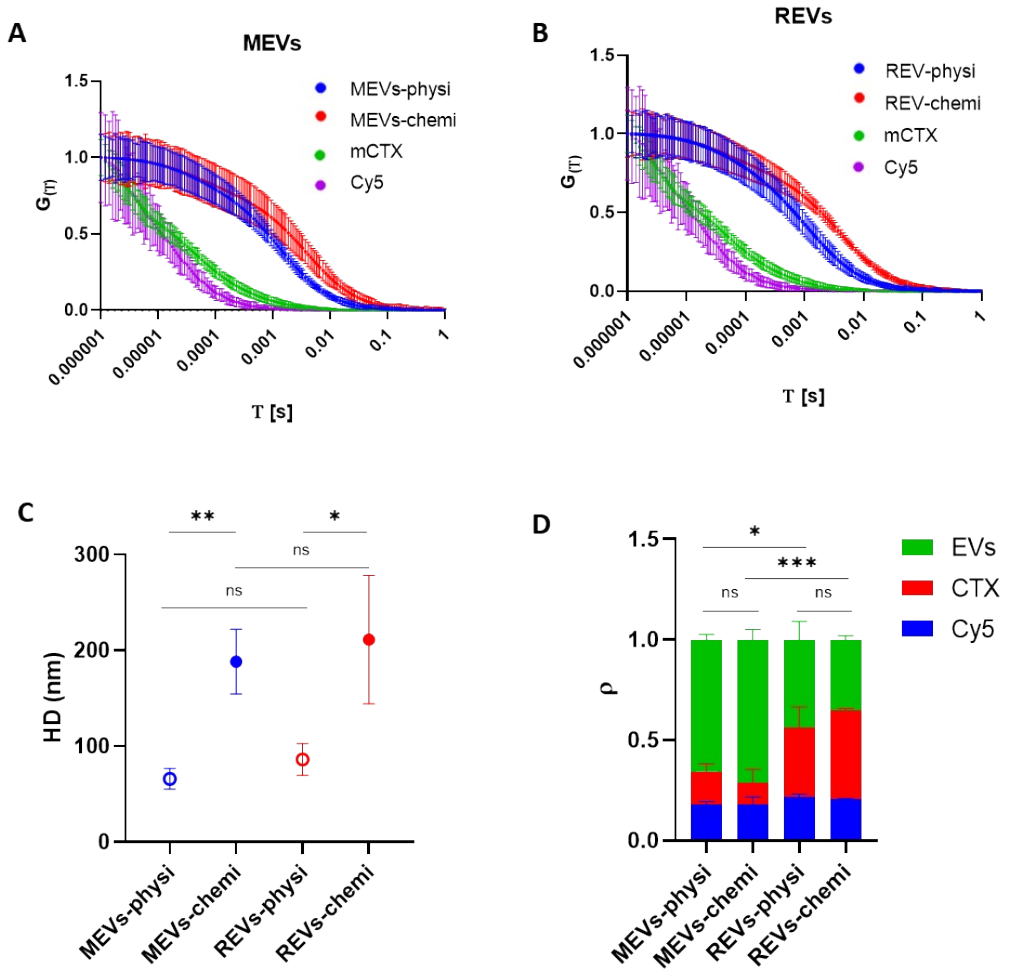
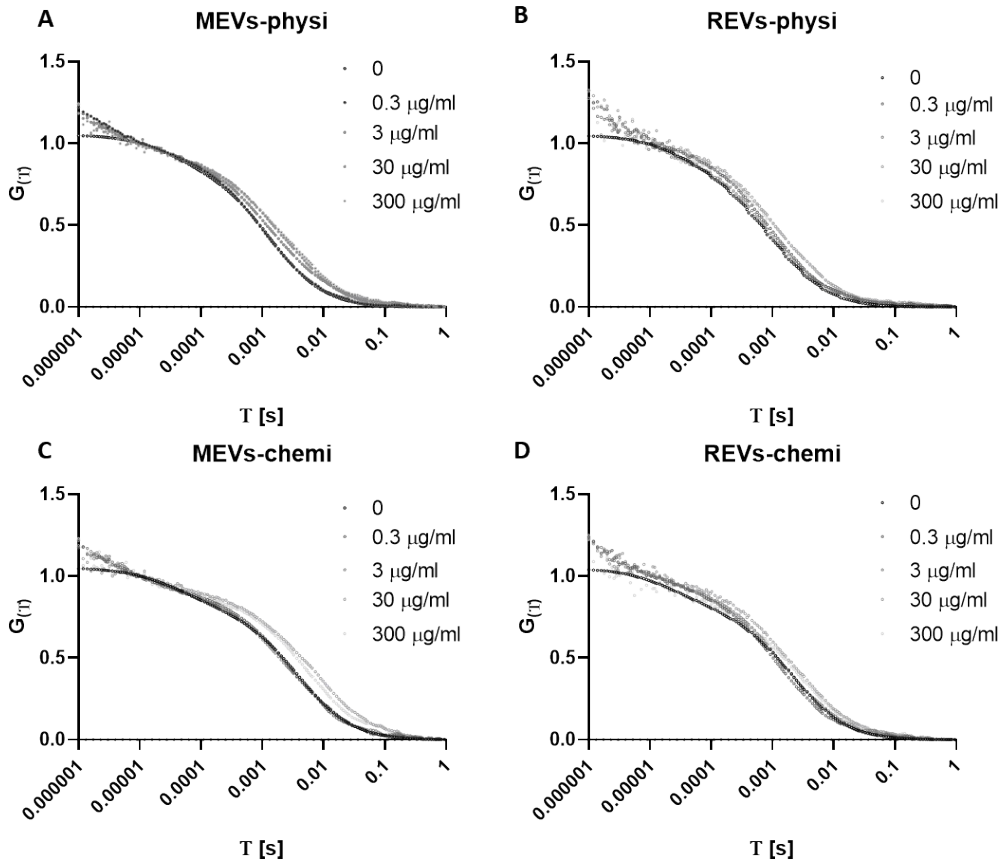


Figure S5. (A) Normalized fluorescence correlation graphs of chemisorbed (blue) and physisorbed (red) MEVs, mCTX (green), and Cy5 (violet). **(B)** Normalized fluorescence correlation graphs of chemisorbed (blue) and physisorbed (red) REVs, mCTX (green), and Cy5 (violet). **(C)** Plotting of the hydrodynamic diameter extrapolated from autocorrelation functions of chemisorbed and physisorbed MEVs and REVs. * = p value < 0.05; ** = p value < 0.01**. (D)** Fractions of fluorescent components in each preparation. P indicates the fraction of each component, namely Cy5 (blue), mCTX (red), and EVs (green). * = p value < 0.05; *** = p value < 0.001.

Dynamic BC titration

The covalent binding of mCTX to EV surface seems to bestow new surface properties to EVs, which seems strictly dependent on the modification and not on the composition of their membrane. Indeed, chemisorbed MEVs and REVs have a very similar trend in HD fluctuation, thus indicating that this parameter is normalized by the mCTX chemisorption on the two EV subtypes (Figure S6F). Furthermore, the HD of chemisorbed EVs increased only at a spike of 30 µg/ml, indicating the formation of a BC, despite the plasma protein physisorption could occur also at lower concentrations of plasma proteins, but cannot be detected with this protocol.



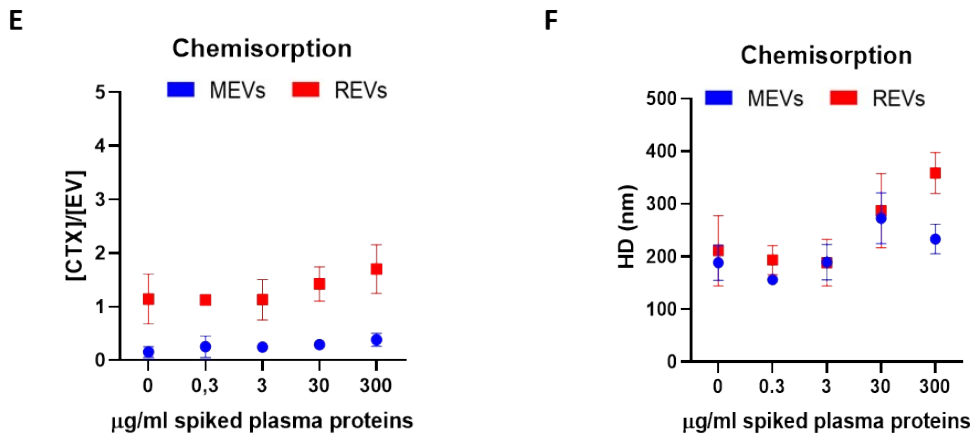


Figure S6. Representative autocorrelation functions of physisorbed**: (A)** MEVs and **(B)** REVs after each spike of plasma proteins. Representative autocorrelation functions of chemisorbed: (C) MEVs after each and **(D)** REVs after each spike of plasma proteins**. (E)** Fluctuation of the ratio between the molar concentration of mCTX and chemisorbed EVs after each spike of plasma proteins. An increase in this value indicates a detachment of mCTX from the EV surface. MEVs and REVs data are reported respectively in blue and red**. (F)** Fluctuation of the hydrodynamic diameter of chemisorbed EVs after each spike of plasma proteins. An increase in this value indicates the physisorption of macromolecules onto the EV surface. MEVs and REVs data are reported respectively in blue and red.

References

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3. Song C, Wang P, Makse HA. A phase diagram for jammed matter. Nature. 2008 May 29;453(7195):629–32.