Encapsulation of Hydrophilic Compounds in Small Extracellular Vesicles: Loading Capacity and Impact on Vesicle Functions

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Supporting Information

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Figure S1. HS-5 cell-derived small EV characterization following isolation by differential ultracentrifugation. (A-C) Small EV were isolated from HS-5 cells by differential ultracentrifugation. The size distribution profile was determined by NTA (A), and the vesicle morphology was analyzed by TEM (B). Expression levels of small EV protein markers (CD73, TSG101, CD9, CD63) and the contaminating endoplasmic reticulum protein marker calregulin were determined by Western blotting, using GAPDH as a loading control (C). Data (A) represent mean ± SD, n=3. Representative images are shown from triplicates (B, C).

Figure S2. Liposome characterization. (A, B) Liposomes were characterized by their modal diameter (A) and zeta potential (B) by NTA. The x-axis indicates the liposomal composition expressed in mol%. Data represent mean ± SD, n = 3.
Figure S3. The osmotic shock approach efficiently encapsulates pyranine into small EV.

(A-H) Pyranine was loaded into small EV by osmotic (osm) shock induction at 4 °C for overnight (o/n) (A) or at RT for either overnight (B) or 4 h (C), freeze-thawing (D), sonication (E), saponin permeabilization at isotonic conditions (F) or by fusion with pyranine-loaded liposomes (DOPC/DOTAP/DOPE 70:10:20 mol%) (G). Coincubation served as loading control (H). For qualitative assessment, microscope images were recorded in the fluorescence mode at an excitation and emission wavelength of 460 and 530 nm, respectively. Representative images from triplicates are shown, scale bars = 10 µm.
Figure S4. The loading methods can affect the size distribution of small EV. (A) The size distribution of native HS-5 cell-derived small EV was determined by NTA. The green arrow indicates the main vesicle population. (B-H) Small EV were subjected to the indicated loading methods and their size profile was measured by NTA. Green arrows mark the main
vesicle population, red arrows indicate additional vesicle populations. All data represent mean ± SD, n = 3.
Figure S5. Morphology of small EV post-loading. (A-E) Native HS-5 cell-derived small EV (A), HS-5 cell-derived small EV subjected to either osmotic (osm) shock at RT for 4 h (B) or fusion with liposomes (DOPC/DOTAP/DOPE 70:10:20 mol%) (C) were analyzed by cryo-TEM. Freeze-thawed small EV (D) and freeze-thawed liposomes (DOPC/DOTAP/DOPE 70:10:20 mol%) (E) served as controls. Yellow arrows indicate potentially damaged membranes. Representative images are shown, scale bars = 100 nm.

Table S1. Estimated pyranine transfer efficiency with different encapsulation processes. Small EV were subjected to the different loading processes, and the estimated transfer efficiency was calculated as the percent ratio of the internal small EV fluorophore concentration and the initial fluorophore concentration in the bulk medium. Data represent mean ± SD, n = 3.

| Loading method    | Estimated transfer efficiency [%] |
|-------------------|----------------------------------|
| Coincubation      | 35 ± 10                          |
| Osm shock 4 °C o/n| 87 ± 18                          |
| Osm shock RT o/n  | 100 ± 20                         |
| Osm shock RT 4 h  | 92 ± 12                          |
| Freeze-thawing    | 76 ± 8                           |
| Sonication        | 10 ± 3                           |
| Saponin           | 3 ± 1                            |
| Fusion            | 42 ± 19                          |

Note: These values should be interpreted with caution as a number of assumptions was made in the calculations (i.e. no specific EV-dye interactions, identical vesicle sizes, vesicles completely filled with the pyranine solution).