Supplementary Information I

COMPARTMENTALIZED DRUG LOCALIZATION STUDIES IN EXTRACELLULAR VESICLES FOR ANTICANCER THERAPY

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SUPPORTING MATERIALS AND METHODS

Materials. 1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (DSPG), 1,2-Distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE),1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol) (DSPE-PEG, 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(carboxyfluorescein) (ammonium salt) were purchased from Avanti Polar Lipids (Alabama, USA). Doxorubicin hydrochloride, Cholesterol, Trehalose, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-Hydroxysuccinimide (NHS), Proteinase K and PHK67 dye were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies for CD-9, CD-81, β-actin, and horse radish peroxidase-linked mouse kappa IgG binding protein were purchased from Santa Cruz Biotechnology (SCBT). All reagents and chemicals were of analytical grade.

Cell lines. Human adipose-derived Mesenchymal stem cells (hADSC) was donated by Prof. Felipe Prosper and Dr. Beatriz Pelacho from the University of Navarra, Spain. hADSC cells were grown and continuously maintained with low passage numbers (<passage 8) in EMEM medium supplemented with β-FGF (1ng/mL) along with 10% FBS and penicillin/streptomycin (100ug ml) and maintained at 37°C in 5% CO_2 environment. Human glioblastoma cells were procured from ATCC, cultured, and maintained in EMEM culture media with 10% FBS,as per the instructions of ATCC.

DOX-DSPE (L-DOX) synthesis. The preparation of the L-DOX pro-drug involved the EDC/NHS chemical reaction between the chemotherapeutic molecule doxorubicin and the 1,2-Distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE) lipid, as reported in our earlier publication[1]. In brief, an aqueous solution at 5mg/ml of Doxorubicin was mixed with a50mg/ml sodium bicarbonate buffer for drug neutralization. Similarly, 50mg/ml of DSPE-succinic acid in ethanol was incubated with a 3:1 molar ratio of EDC/NHS under a magnetic stirrer for 1h at room temperature. The obtained DSPE mixture was added drop-wise to the neutralized doxorubicin solution and stirred overnight. Then, the resultant mixture was centrifuged at 127,000 rpm for 30min. The resulting red pellet was washed three times in distilled water and lyophilized for further use and characterization.

Isolation and purification of hADSC-derived EV. Extracellular vesicles from Human Adipose-derived mesenchymal stem cells were isolated using gold-standard serum starvation and ultracentrifugation method with some modifications (An et al, 2018). In brief, hADSC were cultured in complete EMEM media using multi-cell culture dishes. At 80% confluence, the dishes were washed with 1X PBS and cells were treated with starvation media (EMEM without FBS and growth factor) for 72h. After 72h, the conditional media was collected up to reach a total volume of 1 liter, before starting the EV extraction process. The conditional media was centrifuged at 300g for 10 min to remove dead cells and then at 2,400g for 10 min to remove smaller debris. The resultant media was membrane filtered using 0.22µm PES Membrane filters under vacuum to remove apoptotic bodies and large vesicles. The conditional media was further processed in Amicon centrifugal filter 100kDaby centrifugation at 4,000g for 20min. A concentrated pellet of EV was collected in new centrifugal vials. These concentrated solutions were then ultracentrifuged at 2,00,000g for 2h, then the EV pellets were collected by pellet dissolution in 25mM trehalose-PBS (T-PBS) buffer. The isolated EVs were quantified by protein concentration using a BCA protein quantification assay kit and stored at -80°C until further use.

Cellular uptake studies. The intracellular uptake of the hADSC EV was investigated in human glioblastoma cells (U87-MG), both qualitatively using confocal laser scanning microscopy (CLSM) and quantitatively via Flow cytometry. For CLSM, 10□□g of PKH67-labelled hADSC EV in the presence and the absence of the therapeutic loading (DOX and L-DOX) were incubated with 50,000 U87-MG cells, cultured in 8-chambered slides and incubated for 6h at 37°C. For Proteinase-K studies, hADSC EV was incubated with 25mM Proteinase-K enzyme and incubated for 1h at 37°C. After incubation, the PK-Treated EV was collected using Amicon 100kDa centrifugal filtration. For receptor-mediated endocytosis analysis, U87-MG cells grown in 6-well plates were incubated with either PK-treated hADSC EV or untreated naïve EV. After 6 or 12 h, cells were analyzed at FACS for quantitative cellular uptake.

Drug loading and release kinetics studies. EVs were associated with the therapeutic molecules, either free Doxorubicin (DOX) or DSPE-conjugated doxorubicin (L-DOX), by the passive drug loading method. For DOX and L-DOX association, different concentrations of the drug were incubated with 200µg of EV and subjected to a gentle agitation at 37°C for 2h incubation. After 2h, the samples were purified three times via Amicon centrifugal filtration using a molecular cut-off of 100kDa. The so purified DOX-loaded EV (DOX@EV) and L-DOX-loaded EV (L-DOX@EV) were stored at 4°C for further use. The percentage of drug encapsulation in DOX@EV and L-DOX@EV was determined using High-pressure liquid chromatography by analyzing the characteristic DOX signals (Agilent 1260 Infinity, Germany) as reported in our previous work (Ferreira et al, 2020; Feliciet al, 2021). The stability of the drug-loaded EV was investigated inT-PBS at 4°C and by analyzing the DOX signal associated with the EV, periodically using HPLC. Furthermore, the drug release profile of DOX from the hADSC EV was performed in 1X PBS (pH = 7.4) and acetate buffer (pH = 5.8). In brief, 50ug of drug-loaded EV were sealed in Slide-A-Lyzer MINI dialysis cups with a molecular cut-off of 10 kDa and dialyzed against 4 liters of PBS or acetate buffer (pH 5.8), with gentle shaking at 37°C. At pre-determined time points, the aliquot samples in the dialysis cup had taken and analyzed for their drug content in HPLC.

Preparation and characterization of anionic and cationic liposomes. For the NSET study, anionic liposomes were prepared as reported in our previous literature[2]. The anionic liposomes were prepared using lipids DSPG:DSPE:DSPE-PEG:Cholesterol in the molar ratio of 33: 35 : 18: 14 using the lipid hydration method. Similarly, for the serum stability test, we prepared and used cationic liposomes as a positive control with the lipids DOTAP:DOPE:cholesterol in the molar ratio of 47:44:9 [3]. For core drug loading, both free DOX and L-DOX (100ug/mL) were dispersed in 1X PBS and the solutions were used to hydrate the lipid cakes, followed by an extrusion process, for the formation of coredrug-loaded liposomes. For surface labeling of drugs, free DOX and L-DOXwere dissolved in acetonitrile and were mixed with the above lipids for the formation of a lipid cake. Then the lipids along with the drugs were hydrated with 1X PXS for the preparation of surface drug-labeled liposomes. The hydrodynamic size and the zeta potential of all liposome formulations were characterized using dynamic light scattering (DLS).

NSET analysis. To confirm the localization of the therapeutic agents within the EV, a nanoparticle surface energy transfer method (NSET) was used. Relying on the fluorescence of DOX and 40nm citrate-capped negatively charged gold nanoparticles (AuNP) (Sigma) were used to perform an NSET analysis in a Tecan Spark microplate reader. In a 96-well plate, 100µl of free DOX, free L-DOX, DOX@LIPO, L-DOX@LIPO, and LIPO-DOX (equivalent DOX concentration of 50µg/mL) were left to interact with 10µg of AuNP. The fluorescence intensity of DOX was measured in each well using a fluorescence kinetic mode (for every 10sec) with the excitation and the emission wavelength of 490nm and 580nm, respectively. Based on the time 0 sec (T0) condition, the change in fluorescent signal was quantified over time. Similarly, DOX@EV and L-DOX@EV were tested for their change in fluorescence over time, in the presence and the absence of AuNP.

Coarse-grained molecular dynamics simulations of Dox and L-Dox localization in the lipid membrane of vesicles. The umbrella sampling Molecular Dynamics (MD) simulation method was employed to estimate the partitioning and relative orientation of a pro-drug interacting with a liposome in an aqueous environment[4]. For computational efficiency, the problem was modeled at a coarse-grained resolution according to the definition of the MARTINI forcefield[5]. CG models for the 1,2-Distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE) moiety, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) lipids, ions, water (W), octanol (O) solvents, as well as the vesicle topology employed in this study, were readily available from the MARTINI website (http://cgmartini.nl). Following the MARTINI strategy, the CG topology for doxorubicin (DOX) was built by mapping CG beads with the center of mass(COM) of the atoms constituting the original molecule in a reference atomistic representation. Bead types were assigned based on the reproducibility of the DOX experimental octanol/water partition coefficient ($logP_{ow}$) that was predicted by the alchemical free energy method. PyCGTOOL was used to generate bonded terms between CG beads from a 50 ns atomistic trajectory of DOX in water, modeled using the united atom forcefield GROMOS (GROningenMOlecular Simulation) version 54a7[6,7]. The CG model was validated by comparing the bond distributions obtained from the coarse-grained model with those derived from the reference atomistic distributions.

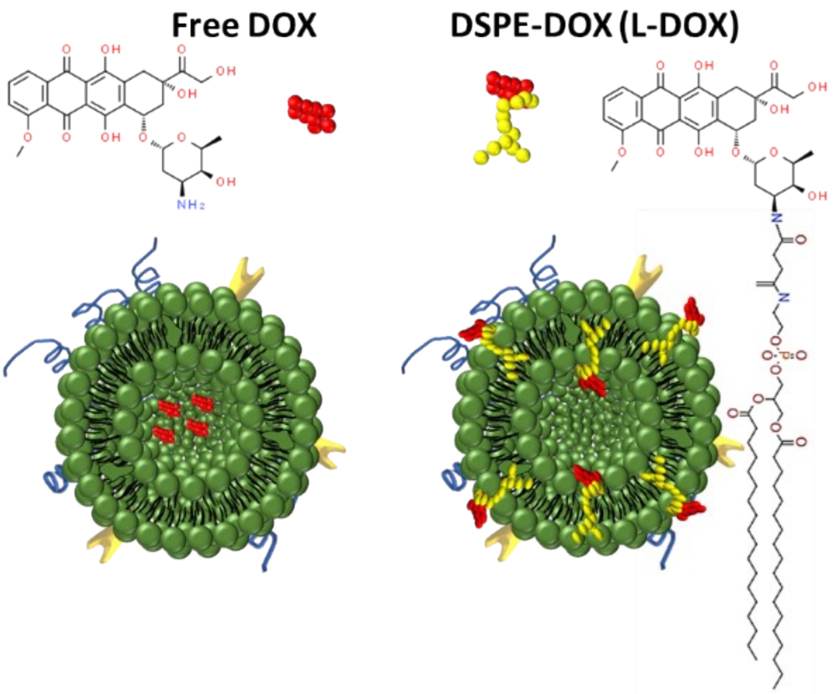
As for the alchemical simulations,the Lennard-Jones interactions of the solute with the solvent were progressively turned off using a coupling parameter lambda λ ($\lambda = 0, 0.1,...,1$)[8]. Each λ window was sampled for 20 ns at 300K using the stochastic dynamics (SD) integrator. The Multistate-Bennetts-Acceptance-Ratio (MBAR) method was employed to estimate the solvation-free energy differences between λ=0 and λ=1 and statistical errors[9].As for the US protocol, the center of mass (COM) of the pro-drug of interest was pulled from the aqueous solution towards the COM of the DPPC vesicle along the z direction. The initial structures for the umbrella simulations were extracted at 0.2 nm intervals for a total of 60 adjacent windows. Before the production run, each starting configuration was equilibrated for 100 ns with constraints applied to the compound. Each window was next sampled for 1 µs at 310 K, with the distance between the COM of the compound and that of the vesicle restrained using a biasing potential with a spring constant of 1,000 kJ/(mol·nm^2). Calculations were performed using the stochastic dynamics (SD) integrator and a timestep of 2 fs. The weighted histogram analysis method (WHAM) was employed to combine probability densities associated with sampled windows spanning the reaction coordinate and derive the free energy profile [10]. All simulation details are followed as described in our previous reports[11].

Morphological and biological characterization of the extracellular vesicles. Human adipose mesenchymal stem cells (hADSC)EV were characterized by dynamic light scattering (DLS) analyses for their hydrodynamic size and surface ζ-potential. The morphology of the individual EV was analyzed by cryo-EM by vitrification method [12].The protein expression analysis was carried out using a western blot analysis. In brief, 30 µg of protein sample was processed in PVDF membrane and were treated with the antibodies CD-9, CD-81, and β-actin, followed by HRP-conjugated anti-mouse IgG secondary antibody. The blots were then developed using SignalFire™ ECL Reagent (Cell signaling) and imaged for chemiluminescence signal under Bio-Rad ChemiDoc™ MP Imaging System (Bio-Rad, USA). For PKH67 dye labeling, EVs were labeled according to the manufacturer's instructions. For red fluorescent labeling, 100 µg of EVs in trehalose-PBS were treated with 10µg of lipid-rhodamine B (lipid-RhB) and incubated at 37°C for 2h, and purified with Amicon centrifugal filter at 4,000g for 10min. The purified RhB-labelled EVs were dispersed with PBS for further analysis. The stability of the labeled EV was investigated under PBS conditions using standard Slide-A-Lyzer MINI dialysis cups with a molecular cut-off of 10 kDa (Thermo Scientific) filter.

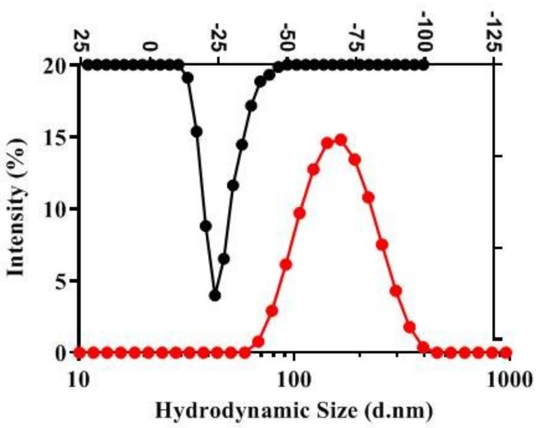
Cryo-EM analysis. For cryo-EM, vitrification of samples was performed in liquid ethane cooled at liquid nitrogen temperature using the FEI Vitrobot Mark IV semiautomatic auto-plunger. Bright-field cryo-EM was run at −176 °C in an FEI Tecnai G2 F20 transmission electron microscope, working at an acceleration voltage of 200 kV and equipped, relevant for this project, with a field emission gun and automatic cryo-box. The images have been acquired in a low dose modality with a GATAN Ultrascan 1000 2k × 2k CCD.

***In vitro* Cytotoxicity assays.** The in vitro cytotoxicity of DOX@EV and L-DOX@EV against the human glioblastoma cell line U87-MG was investigated using an MTT assay. In brief, 10,000 cultured cells/well in 96-Well plates were treated with different concentrations of DOX@EV and L-DOX@EV (equivalent DOX concentration) along with free DOX and Free L-DOX at 37°C for 24h, 48h, and 72h incubation. After treatment, the percentage of viable cells was determined by dissolving the formazan crystals and reading the resulting signal in the UV-VIS spectrophotometer as per the manufacturer's protocol.

Statistical Analysis. Based on the experimental data, One way ANOVA was used for all statistical analyses. All data were presented as mean ± Standard Deviation. Values of $p < 0.001$ (***),$p<0.01$ (**), and $p<0.05$ (*) were considered significant.

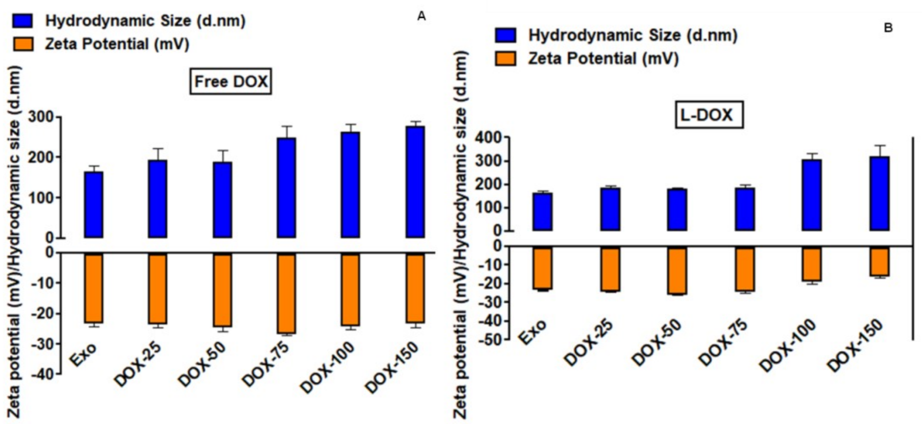


Supplementary Fig.S1: Schematic illustration and structure of DOX and L-DOX loading into hADSC EV.

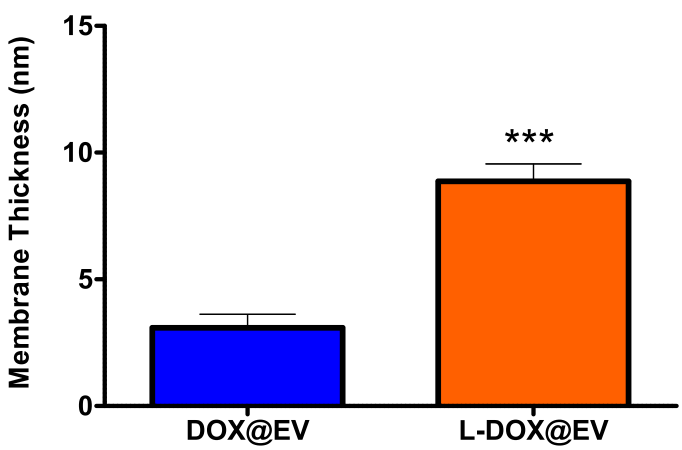


Supplementary Fig. S2: Dynamic Light Scattering analyses of the extracellular vesicles. Data show a hydrodynamic diameter of 165±12 nm and a surface ζ-potential of -23±0.8mV, respectively. (hydrodynamic diameter: red line; surface ζ-potential: black line).

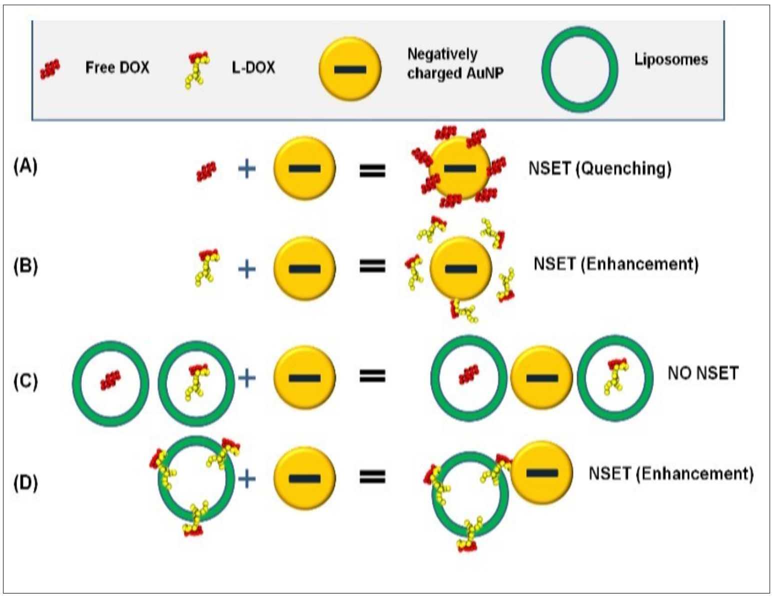
Supplementary Fig. S3: Western-Blot analysis of the extracellular vesicles. Data show the expression of the characteristic receptor proteins CD-9 and CD-81 of EV isolated from the hADSC in the presence and the absence of the Proteinase-K treatment. (EV:hADSC EV; EV-PK Proteinase-K treated hADSC EV).



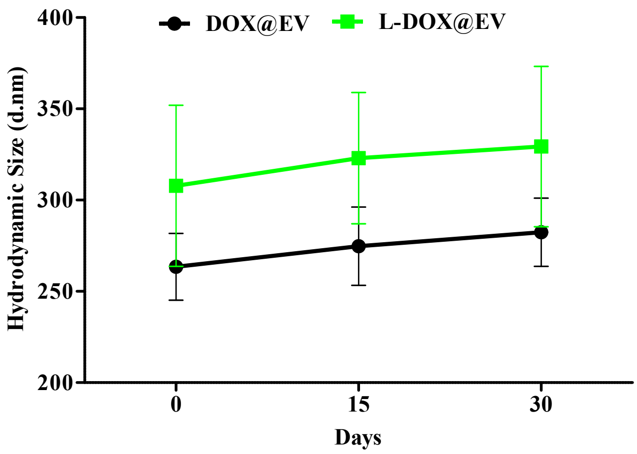
Supplementary Fig. S4: Hydrodynamic diameter and surface electrostatic potential of the extracellular vesicles. A. and **B.** Dynamic light scattering analyses for the hydrodynamic diameter and surface-potential distribution of various amounts of DOX and L-DOX loaded EVs.



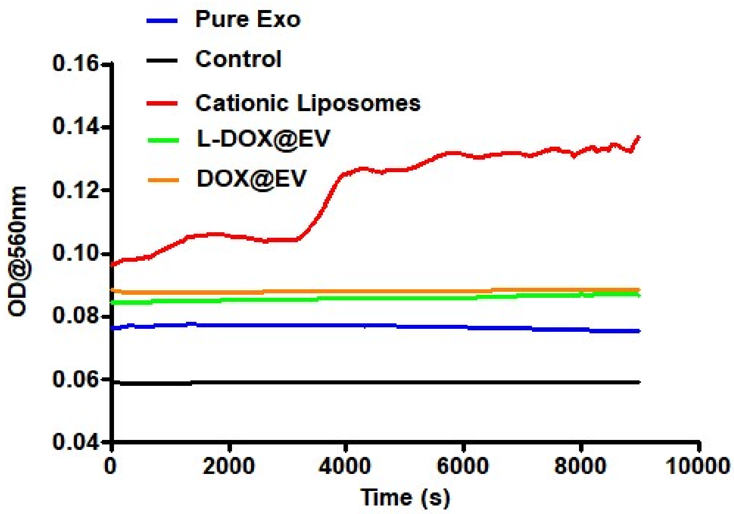
Supplementary Fig. S5: Line intensity profile of DOX and L-DOX loaded EV showing differential membrane thickness analyzed using cryo-TEM micrograph. Data presents mean ± Standard Deviation (n=6), value $p < 0.001$ (***) were considered significant.



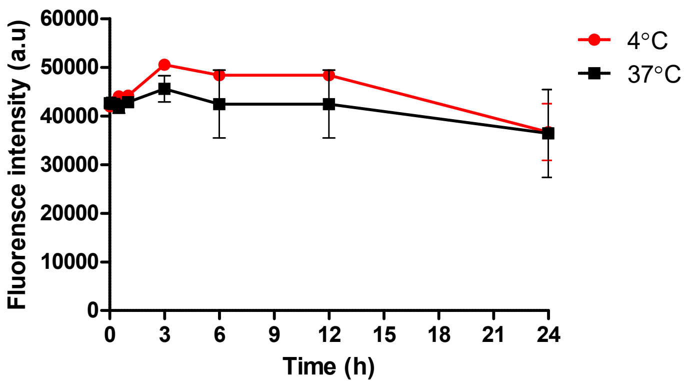
Supplementary Fig. S6: Nanoparticle surface energy transfer (NSET) characterizations depicting the interaction between citrate-capped AuNP (negatively charged) and fluorescent biomolecules (doxorubicin). A. Interaction of free DOX with AuNP results in the fluorescent quenching of the DOX, due to electrostatic interaction. **B.** interaction of free L-DOX with AuNP results in the fluorescent enhancement of the DOX, due to NSET (close proximity). **C.** Core encapsulation of DOX and L-DOX, in anionic liposomes, resulting in no NSET effect, due to large proximal distance. **D.** surface labeled or membrane exposed L-DOX in liposomes interacts with AuNP and enhances the DOX fluorescence.



Supplementary Fig. S7: DLS analysis of drug-loaded EVs(DOX@EV and the L-DOX@EV) showing storage stability at -20˚C for up to 30 days.



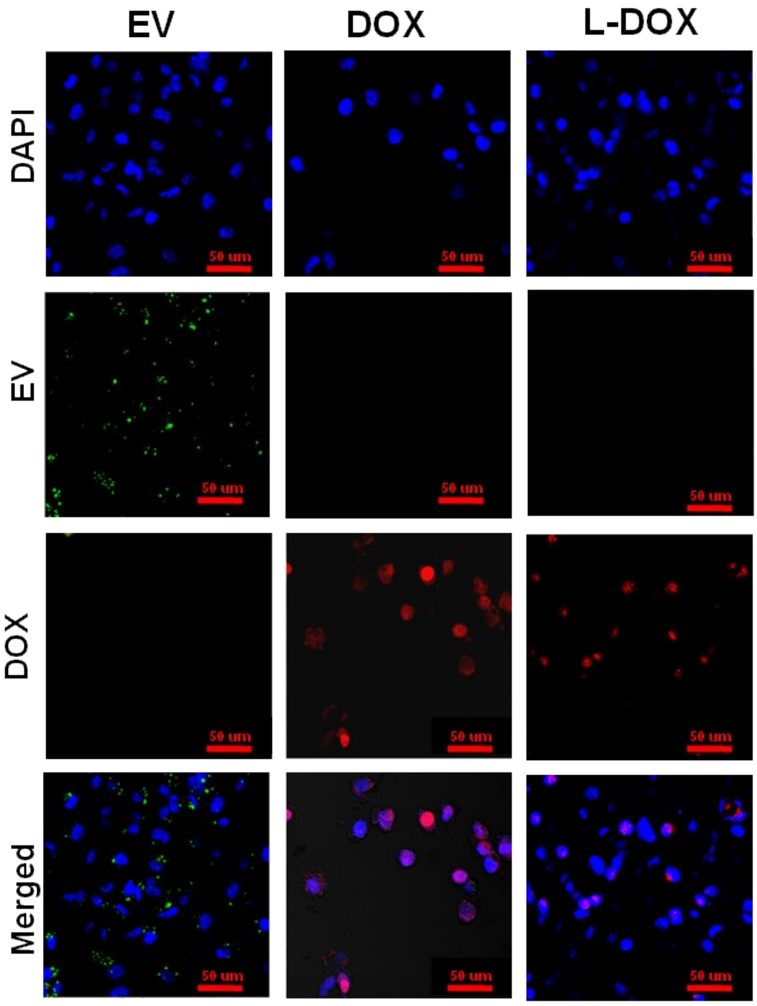
Supplementary Fig. S8: Serum stability of DOX associate extracellular vesicles. Biological serum stability of DOX@EV and L-DOX@EV, along with pure EV in 90% FBS at 37°C. (Control – OD of 90% FBS in the absence of any biological components measured at 560nm). Cationic liposomes were used as a positive control for the index of agglomeration.



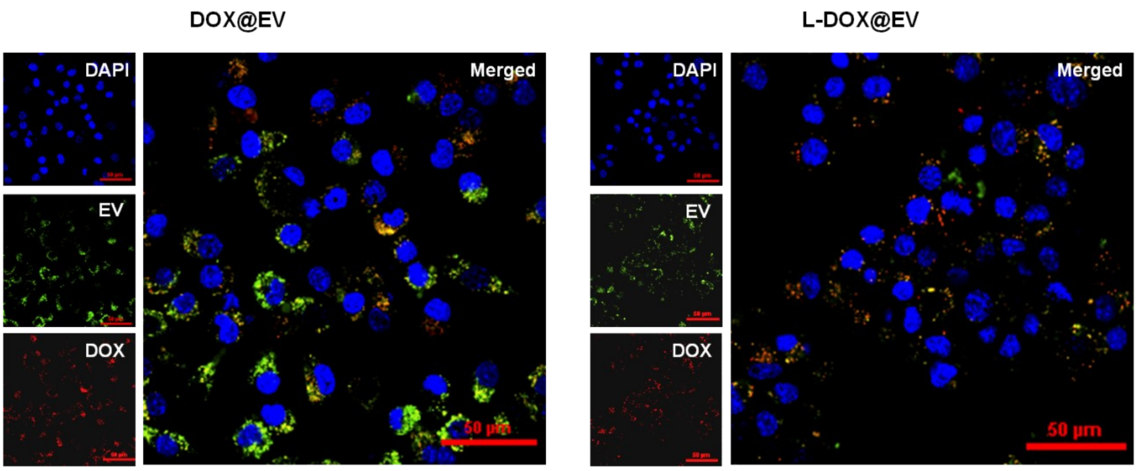
Supplementary Fig. S9: Stability of PKH67 labeled hADSC EVs showing physiological stability at 4˚C and 37˚C for up to 24h.

Liposomeformulations	hydrodynamic diameter (nm)	surface ζ-potential (mV)
Bare Liposomes	110±17	-37±7
DOX@LIPO	132±24	-34±9
L-DOX@LIPO	128±19	-32±4
LIPO-LDOX	138±29	-20±4

SupplementaryTable S1: Characterization of the doxorubicin-associated liposome formulations. Liposome formulations were used for the calibration of the NEST analysis (n=4).



Supplementary Fig. S10:Cellular uptake studies of extracellular vesicles with U87-MG. Confocal laser scanning micrograph of U87-MG cells treated with free hADSC EV (PHK67), free DOX, and free L-DOX for 6h at 37°C.



SupplementaryFig.S11: Interaction of doxorubicin-associated extracellular vesicles with U87-MG. CLSM micrograph of U87-MG cells treated with DOX@EV and L-DOX@EV shows clear de-localization of DOX signals from EV after 12h incubation at 37°C.

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