Electronic Supplementary Material (ESI) for Nanoscale.
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Electronic Supporting Information

A positron-emission tomography (PET)/magnetic resonance imaging (MRI) platform to track *in vivo* small extracellular vesicles

Materials and Methods

1. Live Subject statement

All experiments involving laboratory animals were performed in compliance with the animal protocol approved by the Ethics committee of Animal welfare of the institute ICNAS (Known as ORBEA). This animal protocol (Ref No of the ORBEA protocol 2-1018) was written by following the rules of the Portuguese legislation (Decree-Law 113/2013 of 7 August) and the institutional guideline (ICNAS). All human umbilical cord blood samples were collected from patients of Daniel de Matos and Bissaya Barreto maternities in Coimbra. All donors signed an informed consent form according with the Portuguese legislation and approved by the respective hospital committee.

2. Cell Cultures

2.1. Isolation of mononuclear cells from human umbilical cord blood

All human umbilical cord blood samples were collected from patients of Daniel de Matos and Bissaya Barreto maternities in Coimbra. The samples were stored in single blood bag systems containing anticoagulant citrate phosphate adenine solution. Mononuclear cells were isolated within 48 h after collection using established protocols.[1, 2]

2.2. SEV isolation from MNCs

For exosome isolation from MNCs, cells (2 million cells per mL) were seeded into each well of a 6-well plate (Costar) in X-VIVO15™ (Lonza, Switzerland) medium supplemented with stem cell factor (50 ng/mL, SCF, Petrotech), fms like tyrosine kinase 3 (50 ng/mL, FLT-3, Petrotech) and DNAse I (50 µg/mL, Sigma Aldrich Co) and incubated in a hypoxia chamber (BioSpherix culture chamber, BCA Scientific) under humidified atmosphere with 0.5% O_2 and 5% CO_2, at 37ºC. After 18 h, cell culture medium was collected and SEVs isolated by differential ultracentrifugation.[3] First, conditioned medium was centrifuged at 300 g for 10 min at 4ºC to pellet cells and supernatant was again centrifuged at 2000 g for 20 min, also at 4ºC to pellet cell debris. Using an Optima™ XPN 100K ultracentrifuge with a swinging bucket rotor SW 32 Ti (Beckham Coulter), supernatant was two times centrifuged at 10,000 g for 30 min at 4 ºC in order to pellet microvesicles. SEVs were then pelleted with a 1,00,000 g centrifugation for 120 min at 4ºC. Pelleted SEVs were washed with cold PBS, recentrifuged at the same speed, resuspended in 150-200 µL of cold sterile PBS and conserved at -80ºC.

2.3. Size exclusion purification

The SEV suspension collected by ultracentrifugation (100-150 µL; see point 1.2) was loaded on qEV size exclusion columns (Izon) followed by elution with PBS. We collect 200 µL in each fraction. After loading the sample first 1 mL was the void volume of the column. SEV started coming out of the column at the first fraction after the void volume. The first 2

fractions, i.e. 400 µL after the void volume, were collected. We characterized the fractions collected from the column by NTA, microBCA protein quantification and flow cytometry using CD9 magnetic beads.

2.4. HUVECs survival

The bioactivity of SEVs and SEV-DOTA-Cu (cold Cu^{2+} was used) was evaluated on HUVECs subjected to oxygen-glucose deprivation (OGD). HUVECs at passage 4 were cultured in a 96 well plate at a density of 10,000 cells per well in EGM-2 medium and allowed to grow for 24 h before OGD. Cells were then placed in the anaerobic chamber (OGD chamber, Thermo Forma 1029, Thermo Fisher Scientific, Waltham, MA), in a glucose free deoxygenated buffer medium (10 mM HEPES, 116 mM NaCl, 5.4 mM KCl, 0.8 mM NaH_2PO_4, 25 mM sodium bicarbonate, 25 mM sucrose, 1.8 mM $CaCl_2$, 0.04% phenol red, pH 7.1) for 2 h.

2.5. Evaluation of uptake of SEVs by confocal microscopy

HUVEC (1×10^4) were seeded in a IBIDI™ µ-slide angiogenesis plate, left to adhere for 24 h and then incubated with SEV and SEV-DOTA-Cu (4.5×10^9 particles/mL; cold Cu^{2+} was used) labelled with DyLight 650 for 4 h in EGM-2 medium. After incubation, cells were washed with PBS to remove non-internalized SEVs and fixed with 4% (w/v) paraformaldehyde. In order to stain the cell membrane, samples were first blocked with 1% (w/v) BSA for 1 h and then incubated with primary antibody mouse anti human CD31 (1:50, Dako) for 1 h, followed by incubation with secondary antibody Alexa fluor-555 anti-mouse (1:800, Invitrogen). Finally, nuclei were stained with DAPI and cells were imaged using a Zeiss LSM 710 confocal scanning equipment, with the excitation wavelengths of, 405, 561 and 633 and a Plan-Apochromat 40×/1.4 oil immersion objective. Z-stacks were acquired to confirm the intracellular localization of DYLight 650 labelled SEVs. For the quantification of SEV internalization by HUVEC, cells were imaged using InCell 2200 Analyzer (GE Healthcare™) with a 20× objective. For each replicate, a total of nine images were acquired. Fluorescence quantification was done using INCell Developer software. Cells were segmented using a nuclear segmentation protocol and DyLight 650 fluorescence in each cell was determined.

3. Characterization of SEVs

3.1. SEV protein quantification

Micro BCA™ protein assay kit (Thermo Fisher Scientific) was used to quantify the total protein content of SEVs. SEVs were diluted in 2% sodium dodecyl sulphate (SDS) in order to disrupt SEVs membrane. The protein quantification was done against a standard BSA curve. Dilutions were prepared following manufacturer's instructions. Absorbance values were read in a microplate reader at 562 nm after 2 h incubation at 37° C.

3.2. Dynamic light scattering (DLS) and zeta potential

Particle size and surface charge of SEVs (non-modified SEVs and SEV-DOTA-Cu; cold copper has been used) were determined using light scattering via Zeta PALS potential analyzer and ZetaPlus particle sizing analyzer software, v2.27 (Brookhaven Instruments Corporation). SEV suspension (10 µL of SEVs diluted in 240 µL of biological grade ultrapure water (Fisher Scientific)) was added to a quartz cuvette and average particle size obtained at 25ºC with a detector angle of 90º and equilibration time of 5 min. A total of 5 individual runs of 60 sec were performed in each analysis. For the surface charge measurements, SEVs used in the last step were diluted in 1 mL of ultrapure water and placed

in contact with zeta potential electrode. Five runs were done for each sample, at 25ºC with a relative residual value (measure of data fit quality) of 0.03.[4]

3.3. Nanoparticle tracking analysis

The concentration and particle distribution of SEVs (non-modified SEVs and SEV-DOTA-Cu; cold copper has been used) were measured by nanoparticle tracking analysis - NTA (NanoSight NS300, Malvern instrument Ltd.). For that purpose, SEVs (1.5 µg) were diluted in MilliQ water (1 mL), in order to achieve a final concentration between 5×10^8 and 1×10^9 particles/mL, which represents between 30 and 60 particles per frame. Temperature was monitored across all samples. All samples were recorded 5 times (60 sec each) using a syringe pump speed of 10 µL to maintain a slow fluid. Camera level was set at 11 or 12 and detection threshold at 2. Videos were processed using NTA 3.2 analytical software.

3.4. Transmission electronic microscopy (TEM)

TEM was performed in SEV-DOTA-Cu (with cold copper) isolated from MNCs and as previously described.[3, 5] Briefly, SEVs (10 µg or 4.5×10^9 particles) were fixed with 2% paraformaldehyde (PFA) and deposited on Formvar-carbon coated grids (TAAB Technologies) for 20 min at room temperature. After washing 4 times with PBS, grids were placed on a drop of 1% glutaraldehyde for 5 min, followed of 7 water washes. In a dark environment, grids were incubated with uranyl-oxalate solution pH 7 for 5 min, and then placed on ice in contact with a solution of methyl cellulose (9:1) for 10 min. SEVs imaging were performed using a Tecnai G2 Spirit BioTWIN electron microscope (FEI) at 80 kV.

3.5. Flow cytometry

For the analysis of SEV surface markers by flow cytometry, 2.5×10^{10} SEV particles (SEV and SEV-DOTA-Cu; cold copper has been used) were incubated with latex beads (0.3 µL, average: 3.8 µm, Life Technologies). After 15 min, PBS was added to achieve a final volume of 60 µL and the mixture was incubated in a rotate shaker overnight at 4ºC. This step is required for SEV adsorption to the beads. On the next day, a solution of glycine (110 µL; 1 M) was added for 30 min to saturate all the free binding sites on the beads. Next, SEV and beads were centrifuged at 1,300 g for 3 min. Pelleted beads were resuspended in 1 mL of 0.5% BSA/PBS and centrifugation step was repeated 3 times. After the final centrifugation, beads were resuspended in 0.5 mL of 0.5% BSA/PBS. Then, SEV-coated beads (10 µL) were mixed with an antibody of interest (50 µL, diluted 5 times in 0.5% BSA/PBS) for 30 min at 4ºC. Labelled SEVs were washed three times with 0.5% BSA/PBS (150 µL) and suspended in 0.5% BSA/PBS (200 µL). SEV analyses were performed in a BD Accuri 6 (BD Bioscience) cytometer and plotted using FlowJo™ (v10, FlowJo, LLC). Commercial antibodies used for flow cytometry were: CD9-FITC (RD Systems, FAB1880F), CD63-PE (Invitrogen, 12-0639-42) and CD45-PE (BD Pharmingen, 555483).

3.6. Determination of thiol groups in SEVs

The amount of free surface thiol was quantifying by using thiol fluorescent probe IV which was purchase from Merck Millipore. In brief, a calibration curve was made by using cysteine standards in PBS at pH 7.4. The thiol fluorescent probe IV was mixed with the samples and incubate for 15 min. After that the fluorescence of the samples were measured at 465 nm (λ_{ex} 400nm). 1.0×10^{10}- 2.5×10^{10} number of SEV particles from three different donors were used for this measurement.

3.7. Quantify the number of DOTA-maleimide attached to SEVs

The number of free –SH on the SEV surface before and after the conjugation of DOTA-

maleimide was calculated by using thiol fluorescent probe IV. The number of DOTA-maleimide was measured by the difference in the number of the free –SH before and after the conjugation.

3.8. Quantify the copper in SEV-DOTA-Cu

The purified SEV-DOTA-Cu (number of particles 1.0×10^{10}, cold copper has been used) samples were freeze dried. After that the concentration of copper in those freeze-dried samples were analysis using ICP-MS. An Agilent 7700x ICP-MS was used to determine copper concentration. Samples were introduced via a concentric glass nebulizer with a free aspiration rate of 0.3 rps, a Peltier-cooled double pass glass spray chamber, and a quartz torch. A peristaltic pump carried samples from an Integrated Autosampler I-AS (Agilent) to the nebulizer.

3.9. Stability tests of SEV-DOTA-Cu

SEV-DOTA-Cu (1×10^{10} SEV particles in 100 μL PBS; here $^{64}Cu^{2+}$ has been used) were incubated with 1 mL PBS and 1 mL human serum (1: 1; serum: PBS) for 24 h. The radiochemical purity of those mixtures were tested in several time points (0,1,2,3,4,6, and 24 hr) using ITLC. Lowering the pH of the suspension of SEV-DOTA-Cu to pH 1.5-2.0 by using Hydrochloric Acid-Potassium Chloride Buffer (0.1 M) showed release of copper upto ~65% and ~55 % in PBS and human serum which suggest our system had enough sensitivity to detect the release of copper from SEV-DOTA-Cu. Similarly, the stability of SEV-DOTA-Cu in blood circulation of mice was measured. The animal was sacrificed after 1 h and 4 h of administration. The serum was isolated from the blood of those animal and the purity of SEV-DOTA-Cu in the serum was tested by ITLC.

4. Surface modification and radiolabelling

The radiolabelling of SEVs was done in two steps. In the first step, SEV surface was modified with DOTA-maleimide (MD, Chematech). MD (1 μM) was added to SEV solution (0.3-0.7 mg/mL) in PBS and incubated at 37° C for 2 h. The unreacted MD was removed from the SEV by using exosome spin column (MW 3000, Invitrogen). For that purpose, columns were initially hydrated using PBS (650 μL, 10-15 min), excess interstitial fluid was removed by spinning the column at 750 g for 2 min. SEVs were then loaded on top of the column which was then centrifuged at 750 g for 2 min at room temperature to isolate SEVs from free MD. In the second step, $^{64}CuCl_2$ in 0.1 M sodium acetate (pH ~ 5.5-6.0, 4.0-12.0 mCi) was added to SEV-DOTAs (in PBS, pH 7.4) and reacted for 1 h with continuous shaking at room temperature. The free $^{64}CuCl_2$ was removed by using exosome spin column (MW 3000, Invitrogen™). At the end of the procedure, we had 1-1.9 mCi activity which was used to inject 2-8 animals (180 and 665 μCi for PET/MRI scan and 100-150 μCi for biodistribution). Our labelling process had 16-25 % radiochemical yield (RCY).

4.1. Purity of SEV-DOTA-Cu

The radiochemical purity of the SEV-DOTA-Cu (here $^{64}Cu^{2+}$ has been used) was measured by thin-layer chromatography (TLC) using Whatman no.1 paper and citrate buffer (0.1 M)/ MeOH (25%) solution as eluent.

5. Total RNA quantification and miR-150 expression

SEV (1×10^{11} particles) and SEV-DOTA-Cu (1×10^{11} particles, cold copper has been used) were lysed with QIAzol lysis reagent (350 μL). Total RNA was isolated from samples using miRNeasy Micro kit from Qiagen. RNA was quantified using the Qubit RNA HS assay kit

from Life Technologies. The cDNA synthesis of samples was accomplished using Mir-X miRNA First-Strand Synthesis kit (Clontech; Takara) according to the manufacture's instructions. The amplification occurred in CFX Connect Real-Time system (Bio Rad), under the following conditions: 1 h at 37°C and 5 min at 85°C. Afterwards, real time PCR was performed using Mir-X SYBR qRT-PCR kit (Clontech; Takara) with miR-150-5p (5' TCTCCCAACCCTTGTACC; from Sigma) using the CFX Connect Real-Time system (Bio Rad). Each sample was analysed in triplicate and the reference used was the level of U6 snRNA. Final concentration of all primers was 10 μM and qPCR conditions were: 10 sec at 95°C, 40 cycles at 95°C for 5 sec and 60°C for 20 sec. In all qPCR the melt curve was evaluated for 60 sec at 95°C, 30 sec at 55°C and 30 sec at 95°C.

6. Biodistribution and PET/MRI imaging

All PET scans were performed using a prototype of a high-acceptance small-animal PET based on resistive plate chambers (RPC-PET).[6] All mice underwent the PET acquisition with the radiopharmaceutical SEV-DOTA-Cu or DOTA-Cu (here $^{64}Cu^{2+}$ has been used). The PET acquisition lasted for 3h 40 minutes post injection. Images were reconstructed using OSEM algorithm and cubic voxel of 0.5 mm width. Height fiducial markers that can be viewed both in the PET and MRI imaging were placed in the mice bed and MRI imaging was done after PET without moving the mice from the bed, under anesthesia. Mice were kept anesthetized by isoflurane (1-2%) with 100% O_2 with body temperature and respiration monitoring (SA Instruments SA, Stony Brook, USA). All MR experiments were performed in a BioSpec 9.4T MRI scanner (Bruker Biospin, Ettlingen, Germany) with a volume resonator (transmitter/receiver). Morphological images were acquired with localizer-multi-slice in axial, saggital and coronal orientations, and a 2D T2-weighted turbo RARE sequence in axial orientation. The localizer sequence had the following parameters: TE/TR=1.801/4.0 ms, FOV=40.0*40.0 mm, acquisition matrix=256*256, averages=6, segments=4, flip angle=8.0°, 100 axial continuous slices with 1.0 mm thick and acquisition time of 10m15s. The 2D T2-weighted turbo RARE sequence had the following parameters: TE/TR=33/10451 ms, FOV=50.0*50.0 mm, acquisition matrix=250*250, averages=2, rare factor=8, echo spacing=11 ms, 100 axial continuous slices with 1.0 mm thick and acquisition time of 10 m 48 s.

6.1. ^{64}Cu-DOTA uptake quantification

First, based on the fiducial marker, the images obtained from the PET were manually registered by the MRI images using the 3D Slicer 4.4 software. Then, the MRI images were manually segmented using the ITK-SNAP 2.2 software. Then, the segmentations obtained were applied to the registered PET images. Finally, the mean counts per mm^3 in each region of interest were computed and normalized by the activity injected per gram.

6.2. Quantification by scintillation counting

For biodistribution study, 100-150 μCi of SEV-DOTA-Cu (here $^{64}Cu^{2+}$ has been used) was injected into the tail vein of the mice. Mice were sacrificed after 1, 1.5, 2.0 and 3.0 h. Heart, lung, muscle, liver, spleen, stomach, intestine, kidneys, brain, bone and urine samples were taken and weighed. The radioactivity of organs was determined by scintillation counting. The results were expressed as %ID/g.

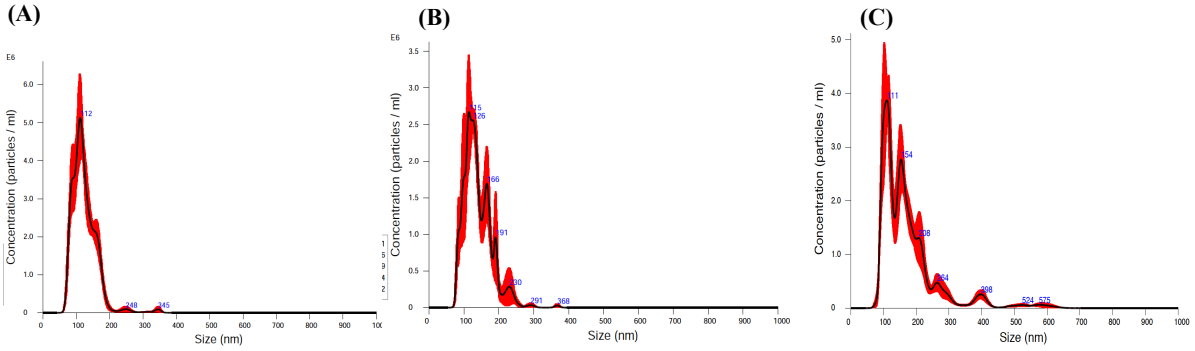

Figure S1. NTA analyses of SEVs. (A) NTA profile of SEVs before chemical modification. (B) NTA profile of SEVs after chemical modification (SEV-DOTA-Cu). (C) NTA profile of SEV-DOTA-Cu stored at room temperature for 24 h in PBS. The results indicate that no significant SEV aggregation was observed.

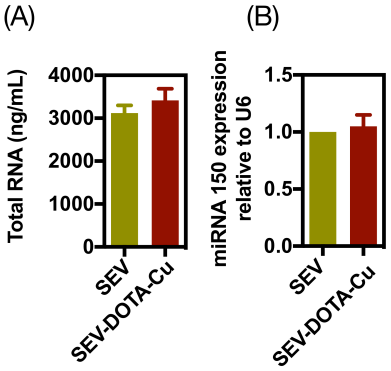

Figure S2. Integrity of SEVs after labelling with copper. (A) Quantification of total RNA in SEV and SEV-DOTA-Cu (n=3). (B) Quantification of miR150 by qRT-PCR analyses. Results are average ± SEM, n=3.

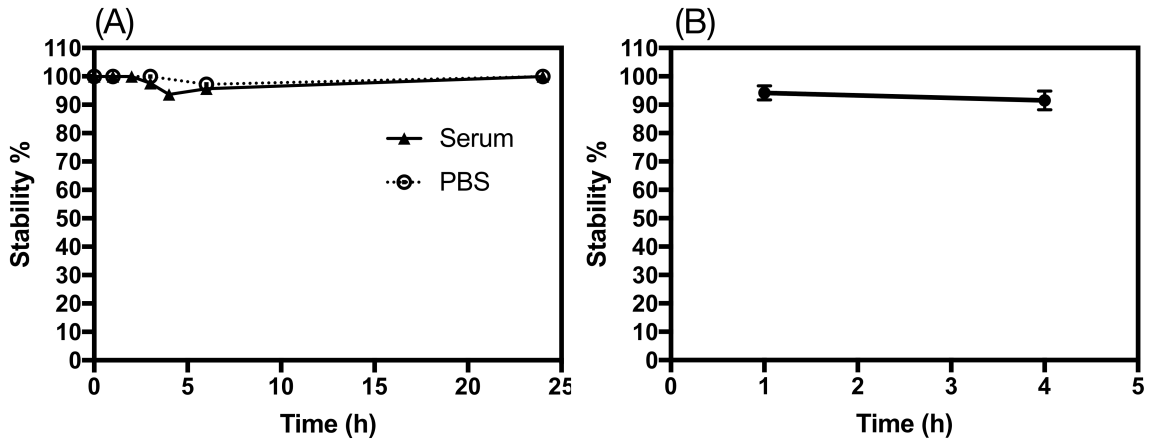

Figure S3. Stability of SEV-DOTA-Cu. (A) Stability of SEV-DOTA-Cu in PBS and human serum at different time points. The stability of SEV-DOTA-Cu (1×10^{10} SEV particles in 100 μL PBS pH 7.4) in PBS (1 mL) and human serum (1 mL; human serum : PBS 1:1) at room temperature was evaluated by ITLC. Lowering the pH of the suspension of SEV-DOTA-Cu to pH 1.5-2.0 by using hydrochloric acid-potassium chloride buffer (0.1 M) showed release of copper up to ~65% and ~55 % in PBS and human serum respectively which suggest our system had sensitivity to detect the release of copper from SEV-DOTA-Cu. Results are average ± SEM, n=3. (B) Stability of SEV-DOTA-Cu in the blood stream after injection in mice. After sacrifice of the animals, the stability of the SEV-DOTA-Cu was monitored in the blood plasma by ITLC. Results are average ± SEM, n=2.

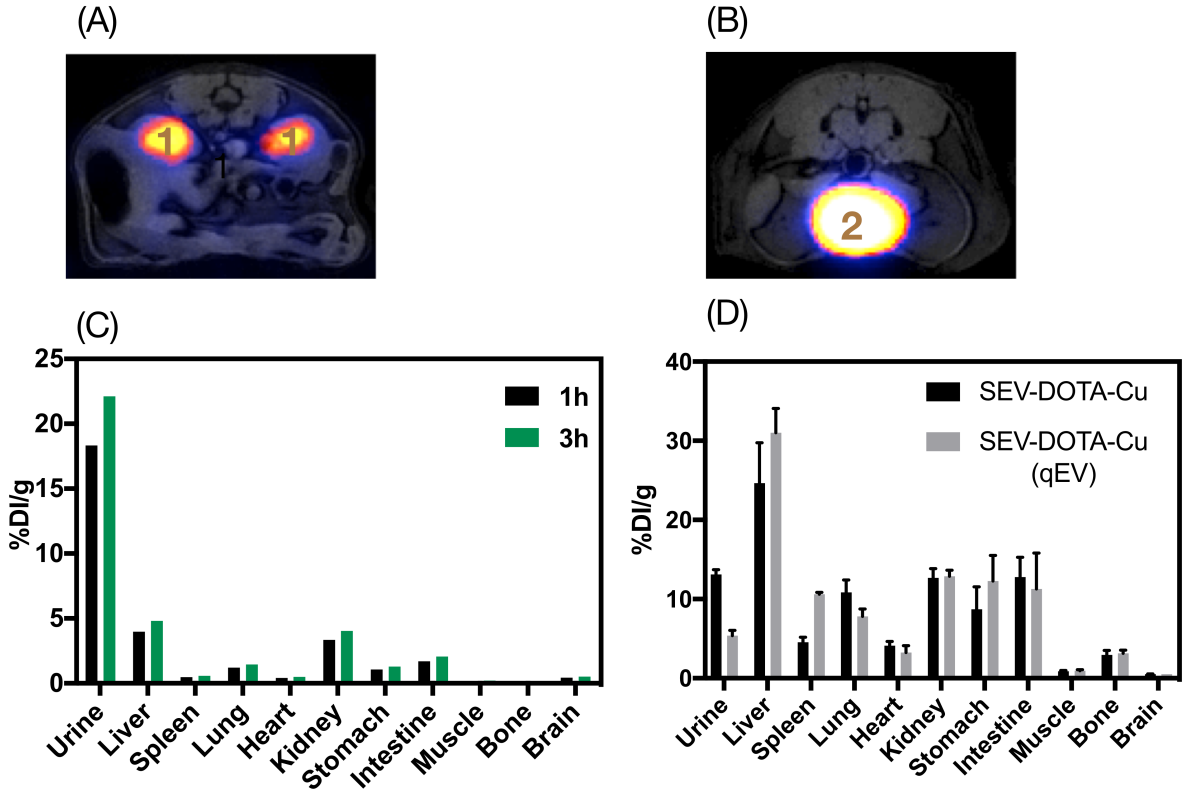

Figure S4. Biodistribution of SEVs. Uptake of DOTA-Cu in kidneys (A) and bladder (B) after 20 min and 100 min post- radiotracer injection, respectively. Axial orientation slices overlapping the MRI and PET imaging. **(**C) Biodistribution of DOTA-Cu. Mice were sacrificed at 1 h and 3 h after intravenous injection of DOTA-Cu and radioactivity was measured in several organs by scintillation (n=1 for time point). (D) Comparison of biodistribution of the SEV-DOTA-Cu and SEV-DOTA-Cu (qEV) purified SEVs. All the animals were sacrificed after 1.5 h. No significant change in biodistribution was observed. Results are average ± SEM, n=4.

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