SUPPLEMENTARY DATA TO:

Cellular Vesicles: New insights in engineering methods, interaction with cells and potential for brain targeting

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1. Cytotoxicity of CVs (24h)

The viability (% compared to control) of CVshCMEC/D3 and CVsB16 towards hCMEC/D3 and B16 cells following 24h incubation of CVs at doses of 6, 12 and 24 nmoles (lipid)/3x104 cells was evaluated and results are shown in Figure S1.

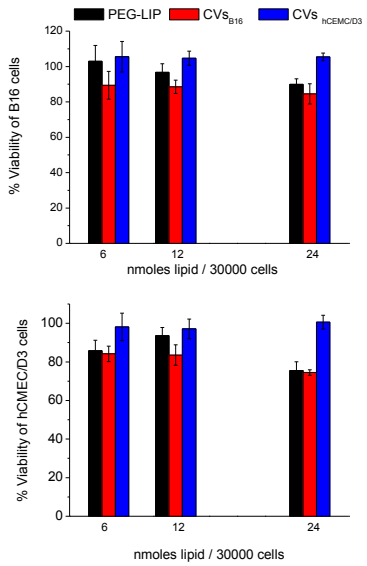


Figure S1. % Viability of B16 (top graph) and hCMEC/D3 (bottom graph) cells after 24h incubation with CVsB16, CVshCMEC/D3 and Pegylated liposomes (PEG-LIP).

2. Effect of using Trypsin during CV isolation on CV uptake by cells

CV uptake by hCMEC/D3 cells was evaluated with two sets of $CVs_{hCMEC/D3}$, in order to evaluate if the use of trypsin as a cell detaching agent (during CV preparation) has any effect on the uptake of the CVs by the cells.

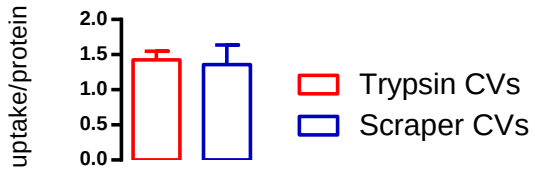


Figure S2. % Uptake of $CVs_{hCMEC/D3}$ by hCMEC/D3 cells; CVs were produced by trypsin detached cells (Tryrsin CVs) and scraper detached cells (Scraper CVs)

It is proven (Fig. S2) that the use of trypsin (as a cell detaching method) during $CVs_{hCMEC/D3}$ preparation did not decrease their uptake by hCMEC/D3 cells compared to CVs prepared without using trypsin

3. Confocal microscopy Studies

Confocal microscopy micrographs for the uptake of CVs from different cell types by B16 cells are presented. CVs (200nmol or 400nmol lipid) were incubated for 4 h with 10^6 cells and observed under confocal microscope. All methods are reported in detail in the materials and methods section of the manuscript.

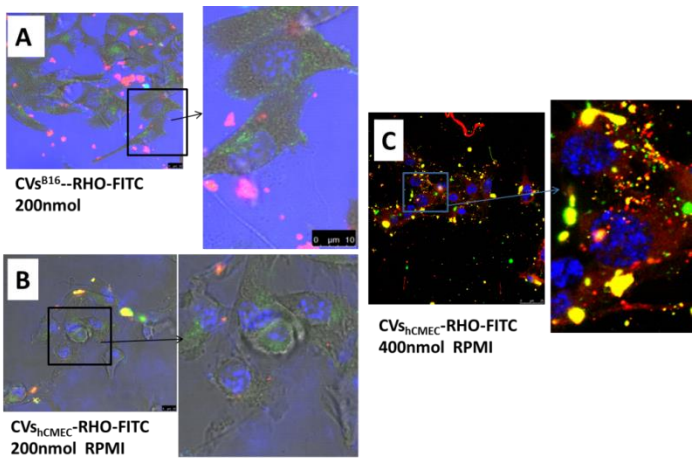


Figure S3. A] Uptake of CVs_{B16} (200nmol lipid) by B16 cells; **B and C**] Uptake of $CVs_{hCMEC/D3}$ (produced by cells grown in RPMI) by B16 cells, after incubation of 200nmol lipid (B) and 400nmole lipid (C).

4. TEM study of $CV_{hCMEC/D3}$ morphology

Representative TEM micrographs of $CVs_{hCMEC/D3}$, prepared from cells that were grown in EndoGro and RPMI medium. Negative staining was performed with ammonium molybdate as mentioned in detail in the methods section of the manuscript. As seen, the two types of CVs are morphologically similar.

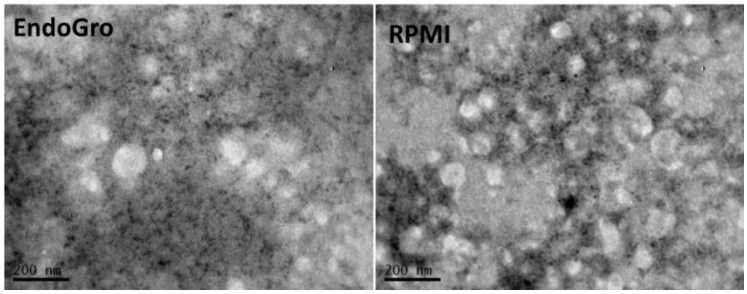


Figure S4. Representative TEM micrographs of CVshCMEC/D3, prepared form cells that were grown in EndoGro, and RPMI medium.

5. Supplementary data of Proteomic Analysis study

Table S1. Proteins identified in $CVs_{hCMEC/D3}$

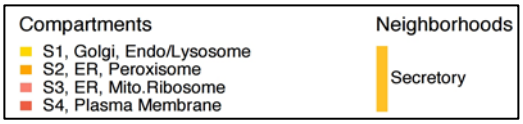
T: Protein names	**T: Gene names**
Talin-1	TLN1
40S ribosomal protein S9	RPS9
Clathrin heavy chain;Clathrin heavy chain 1	CLTC
Extended synaptotagmin-2	ESYT2
Tumor protein D54	TPD52L2
Four and a half LIM domains protein 2	FHL2
Vacuolar protein sorting-associated protein 33A	VPS33A
Small nuclear ribonucleoprotein E	SNRPE
Structural maintenance of chromosomes flexible hinge domain-containing protein 1	SMCHD1
60S ribosomal protein L23a	RPL23A
Eukaryotic translation initiation factor 3 subunit L	EIF3L
GTP-binding nuclear protein Ran	RAN
10 kDa heat shock protein, mitochondrial	HSPE1;HSPE1-MOB4
Protein transport protein Sec31A	SEC31A
High mobility group protein B3	HMGB3
Dynactin subunit 1	DCTN1;DKFZp686E0752
Heterogeneous nuclear ribonucleoprotein H;Heterogeneous nuclear ribonucleoprotein H, N-terminally processed	HNRNPH1
Unconventional myosin-Ib	MYO1B
Nuclear pore complex protein Nup155	NUP155
Prohibitin-2	PHB2
Actin-related protein 2/3 complex subunit 4	ARPC4-TTLL3;ARPC4
Translocating chain-associated membrane protein 1	TRAM1
Tumor protein D52;28S ribosomal protein S28, mitochondrial	TPD52;MRPS28
ATP-binding cassette sub-family F member 1	ABCF1
RNA-binding protein FUS	FUS
Protein disulfide-isomerase	P4HB
Mitochondrial import inner membrane translocase subunit TIM44	TIMM44
Very-long-chain enoyl-CoA reductase	TECR
Syntaxin-binding protein 3	STXBP3
Importin-5	IPO5
Histone deacetylase complex subunit SAP18	SAP18
Secretory carrier-associated membrane protein 3	SCAMP3
Actin-related protein 2/3 complex subunit 1B	ARPC1B
Membrane-associated progesterone receptor component 2	PGRMC2
26S proteasome non-ATPase regulatory subunit 3	PSMD3
TBC1 domain family member 4	TBC1D4
Perilipin-3	PLIN3
UDP-glucose 6-dehydrogenase	UGDH
General vesicular transport factor p115	USO1
NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	NDUFS6
Vesicle-trafficking protein SEC22b	SEC22B
Protein transport protein Sec24D	SEC24D
Glutaminase kidney isoform, mitochondrial	GLS
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	NDUFA7
Polypyrimidine tract-binding protein 3	PTBP3

NADH-cytochrome b5 reductase 3;NADH-cytochrome b5 reductase 3 membrane-bound form;NADH-cytochrome b5 reductase 3 soluble form	CYB5R3
Catalase	CAT
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	RPN2
60S acidic ribosomal protein P0;60S acidic ribosomal protein P0-like	RPLP0;RPLP0P6
Keratin, type I cytoskeletal 18	KRT18
ATP synthase subunit beta, mitochondrial;ATP synthase subunit beta	ATP5B
2,3-cyclic-nucleotide 3-phosphodiesterase	CNP
Splicing factor U2AF 35 kDa subunit;Splicing factor U2AF 26 kDa subunit	U2AF1;U2AF1L4
Heat shock cognate 71 kDa protein	HSPA8
ADP/ATP translocase 3;ADP/ATP translocase 3, N-terminally processed	SLC25A6
Inosine-5-monophosphate dehydrogenase 2	IMPDH2
Protein disulfide-isomerase A4	PDIA4
Heterogeneous nuclear ribonucleoprotein L	HNRNPL
Aspartate--tRNA ligase, cytoplasmic	DARS
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	ATP2A2
High mobility group protein HMG-I/HMG-Y	HMGA1
Integrin alpha-2	ITGA2
T-complex protein 1 subunit alpha	TCP1
Nucleolin	NCL
Interferon-induced GTP-binding protein Mx1;Interferon-induced GTP-binding protein Mx1, N-terminally processed	MX1
Proteasome subunit beta type-1	PSMB1
Amine oxidase [flavin-containing] A	MAOA
5-nucleotidase	NT5E
Protein-glutamine gamma-glutamyltransferase 2	TGM2
Tryptophan--tRNA ligase, cytoplasmic;T1-TrpRS;T2-TrpRS	WARS
Plasma membrane calcium-transporting ATPase 4	ATP2B4
ATP synthase F(0) complex subunit B1, mitochondrial	ATP5F1
Low molecular weight phosphotyrosine protein phosphatase	ACP1
Proteasome subunit alpha type-1;Proteasome subunit alpha type	PSMA1
Erythrocyte band 7 integral membrane protein	STOM
Proteasome subunit beta type-5	PSMB5
Ephrin type-A receptor 2	EPHA2
Transketolase	TKT
HLA class I histocompatibility antigen, Cw-12 alpha chain;HLA class I histocompatibility antigen, Cw-15 alpha chain;HLA class I histocompatibility antigen, Cw-8 alpha chain;HLA class I histocompatibility antigen, Cw-14 alpha chain;HLA class I histocompatibility antigen, Cw-4 alpha chain;HLA class I histocompatibility antigen, Cw-16 alpha chain;HLA class I histocompatibility antigen, Cw-5 alpha chain	HLA-C
Aldehyde dehydrogenase X, mitochondrial	ALDH1B1
DnaJ homolog subfamily A member 1	DNAJA1
Cytochrome b-c1 complex subunit 1, mitochondrial	UQCRC1
Heterogeneous nuclear ribonucleoprotein H3	HNRNPH3
Serine hydroxymethyltransferase, mitochondrial	SHMT2
Prohibitin	PHB
Coatomer subunit beta	COPB2
60S ribosomal protein L4	RPL4
Transgelin-2	TAGLN2
V-type proton ATPase catalytic subunit A	ATP6V1A
Stress-70 protein, mitochondrial	HSPA9
60S ribosomal protein L13a	RPL13A;RPL13a
Malate dehydrogenase, mitochondrial;Malate dehydrogenase	MDH2
Signal transducer and activator of transcription 1-alpha/beta;Signal transducer and activator of transcription	STAT1
Microtubule-associated protein 1B;MAP1B heavy chain;MAP1 light chain LC1	MAP1B
Eukaryotic translation initiation factor 1A, X-chromosomal;Eukaryotic translation initiation factor 1A, Y-chromosomal	EIF1AX;EIF1AY
ATP synthase subunit O, mitochondrial	ATP5O
Elongation factor Tu, mitochondrial	TUFM
Signal recognition particle 9 kDa protein	SRP9
Heterogeneous nuclear ribonucleoprotein A3	HNRNPA3
Importin subunit alpha-1	KPNA2
Rap1 GTPase-GDP dissociation stimulator 1	RAP1GDS1
Methionine aminopeptidase 1	METAP1
Coatomer subunit beta	COPB1
Transitional endoplasmic reticulum ATPase	VCP
40S ribosomal protein S7	RPS7
26S protease regulatory subunit 8	PSMC5
40S ribosomal protein S8	RPS8
40S ribosomal protein S15a	RPS15A
Small nuclear ribonucleoprotein F	SNRPF
40S ribosomal protein S25	RPS25
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	GNB2
60S ribosomal protein L10a	RPL10A
60S ribosomal protein L11	RPL11

Ubiquitin-40S ribosomal protein S27a;Ubiquitin;40S ribosomal protein S27a;Polyubiquitin-B;Ubiquitin;Polyubiquitin-C;Ubiquitin	RPS27A;UBB;UBC
AP-2 complex subunit beta	AP2B1
Guanine nucleotide-binding protein subunit beta-2-like 1;Guanine nucleotide-binding protein subunit beta-2-like 1, N-terminally processed	GNB2L1
General transcription factor II-I	GTF2I
DNA-dependent protein kinase catalytic subunit	PRKDC
HLA class I histocompatibility antigen, B-40 alpha chain;HLA class I histocompatibility antigen, B-47 alpha chain;HLA class I histocompatibility antigen, B-27 alpha chain;HLA class I histocompatibility antigen, B-13 alpha chain;HLA class I histocompatibility antigen, B-44 alpha chain	HLA-B
Glutamine--fructose-6-phosphate aminotransferase [isomerizing] 1	GFPT1
Peroxiredoxin-1	PRDX1
Cytoskeleton-associated protein 4	CKAP4
Quinone oxidoreductase	CRYZ
Splicing factor 3A subunit 3	SF3A3
Aminoacyl tRNA synthase complex-interacting multifunctional protein 1;Endothelial monocyte-activating polypeptide 2	AIMP1
Interleukin enhancer-binding factor 3	ILF3
Heat shock protein 75 kDa, mitochondrial	TRAP1
Cleavage stimulation factor subunit 3	CSTF3
Chromobox protein homolog 3	CBX3
Serine/arginine-rich splicing factor 6	SRSF6
Transcription intermediary factor 1-beta	TRIM28
Peptidyl-prolyl cis-trans isomerase FKBP5;Peptidyl-prolyl cis-trans isomerase FKBP5, N-terminally processed	FKBP5
Sequestosome-1	SQSTM1
CD166 antigen	ALCAM
DNA replication licensing factor MCM6	MCM6
Major vault protein	MVP
Poly(rC)-binding protein 1	PCBP1
Microtubule-associated protein RP/EB family member 1	MAPRE1
Pre-rRNA-processing protein TSR1 homolog	TSR1
MAP7 domain-containing protein 1	MAP7D1
E3 ubiquitin-protein ligase BRE1A	RNF20
Golgi-associated plant pathogenesis-related protein 1	GLIPR2
Atlastin-3	ATL3
Pre-mRNA-processing-splicing factor 8	PRPF8
Inhibitor of nuclear factor kappa-B kinase-interacting protein	IKBIP
Staphylococcal nuclease domain-containing protein 1	SND1
Zinc finger CCCH-type antiviral protein 1	ZC3HAV1
Protein PAT1 homolog 1	PATL1
Dipeptidyl peptidase 9	DPP9
Aldehyde dehydrogenase family 16 member A1	ALDH16A1
Leucine-rich repeat-containing protein 47	LRRC47
Interferon-induced protein 44	IFI44
Gem-associated protein 5	GEMIN5
Rho guanine nucleotide exchange factor 2	ARHGEF2
Ribosomal RNA small subunit methyltransferase NEP1	EMG1
FAS-associated factor 2	FAF2
RNA-binding protein 14	RBM14
Niban-like protein 1	FAM129B
Synaptic vesicle membrane protein VAT-1 homolog	VAT1
Rab3 GTPase-activating protein non-catalytic subunit	RAB3GAP2
Thioredoxin-related transmembrane protein 1	TMX1
Nucleolar RNA helicase 2	DDX21
ATP-dependent RNA helicase DDX18	DDX18
U3 small nucleolar RNA-associated protein 6 homolog	UTP6
Rabankyrin-5	ANKFY1
V-type proton ATPase subunit H	ATP6V1H
Pre-mRNA-processing factor 19	PRPF19
RuvB-like 2	RUVBL2
Voltage-dependent anion-selective channel protein 3	VDAC3
tRNA-splicing ligase RtcB homolog	RTCB

Table S2.

Gene name	Neighborhoods	Compartments	
ATL3	Secretory	S1	Up in RPMI
RPL23A	Secretory	S1	Up in RPMI
SCAMP3	Secretory	S1	Up in RPMI
ZC3HAV1	Secretory	S1	Up in RPMI
CKAP4	Secretory	S2	Up in RPMI
CYB5R3	Secretory	S2	Up in RPMI
FAF2	Secretory	S2	Up in RPMI
GLIPR2	Secretory	S2	**Up in EndoGro**
HLA-C	Secretory	S2	Up in RPMI
IKBIP	Secretory	S2	**Up in EndoGro**
NT5E	Secretory	S2	Up in RPMI
PGRMC2	Secretory	S2	Up in RPMI
RPN2	Secretory	S2	Up in RPMI
TECR	Secretory	S2	**Up in EndoGro**
TMX1	Secretory	S2	**Up in EndoGro**
TMX1	Secretory	S2	**Up in EndoGro**
CAT	Secretory	S3	**Up in EndoGro**
CNP	Secretory	S3	**Up in EndoGro**
EPHA2	Secretory	S4	Up in RPMI
GNB2	Secretory	S4	Up in RPMI
ATP2A2	Secretory	Unclassified	**Up in EndoGro**
ESYT2	Secretory	Unclassified	**Up in EndoGro**
HLA-B	Secretory	Unclassified	Up in RPMI
ITGA2	Secretory	Unclassified	Up in RPMI
MYO1B	Secretory	Unclassified	Up in RPMI
RPS15A	Secretory	Unclassified	Up in RPMI
STOM	Secretory	Unclassified	Up in RPMI
STXBP3	Secretory	Unclassified	**Up in EndoGro**



6. Supplementary Data of *in vivo* studies

Figure S5. Results of *in vivo* live animal Imaging study, comparing CVs from hCMEC/D3 cells grown in RPMI or EndGro medium.

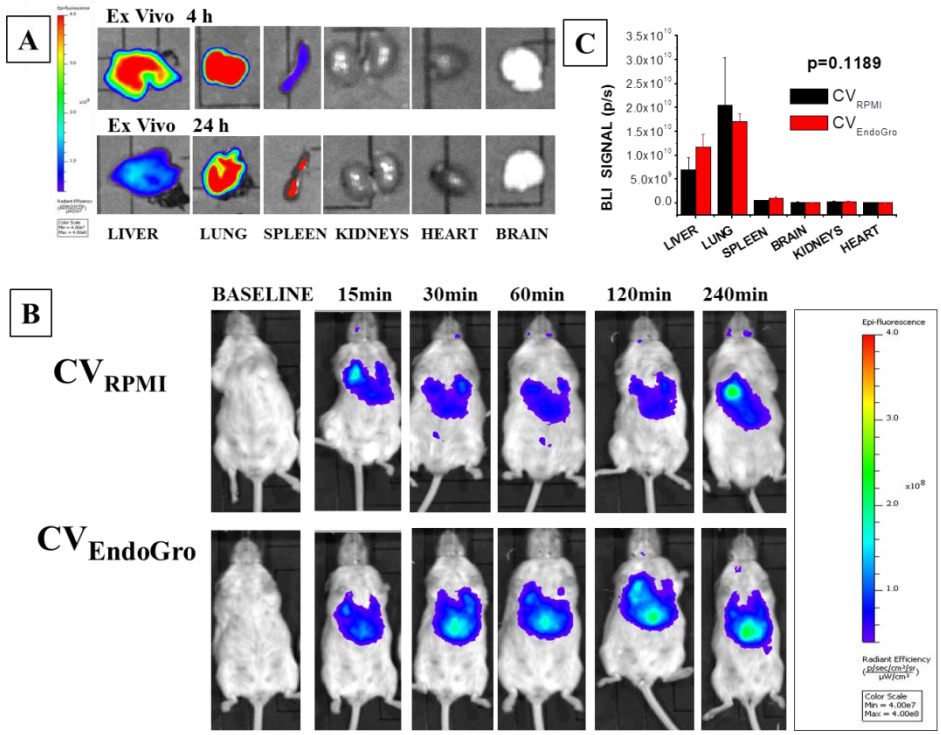


Figure S5. A] *Ex vivo* imaging of extracted organs after 4 and 24h of injection of 100ug lipid/mouse of CVRPMI. All images are in the same scale B] Representative bio fluorescence signals (photons/sec) of CV-associated DiR after injection of 200ug lipid/mouse CV_{RPMI} (upper panel) or $CV_{EndoGro}$ (lower panel). All images are in the same scale. C] *Ex vivo* DiR Signal values of organs extracted from the animals of the previous experiment, 4 h post-injection. Each value is the mean from 5 animals and SDs are presented as error bars.