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.

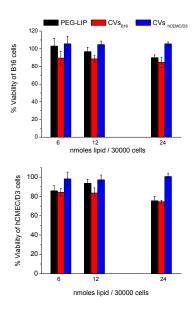


Figure S1. % Viability of B16 (top graph) and hCMEC/D3 (bottom graph) cells after 24h incubation with CVsB16, CVshCMEC/D3 and Pegylated liposomes (PEG-LIP).

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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.

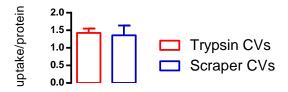


Figure S2. % Uptake of CVs_{hCMEC/D3} by hCMEC/D3 cells; CVs were produced by trypsin detached cells (Tryrsin CVs) and scraper detached cells (Scraper CVs)

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 10⁶ cells and observed under confocal microscope. All methods are reported in detail in the materials and methods section of the manuscript.

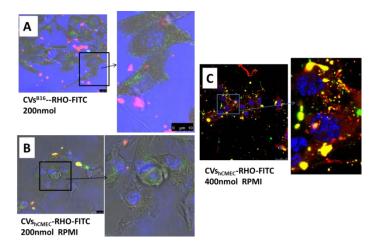


Figure S3. A] Uptake of CVs _{B16} (200nmol lipid) by B16 cells; **B and C**] Uptake of CVs _{hCMEC/D3} (produced by cells grown in RPMI) by B16 cells, after incubation of 200nmol lipid (B) and 400nmole lipid (C).

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.

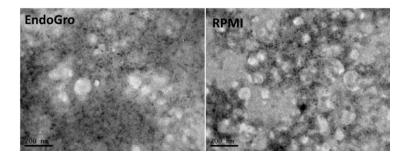


Figure S4. Representative TEM micrographs of CVshCMEC/D3, prepared form cells that were grown in EndoGro, and RPMI medium.

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;DKFZp686E075
	2
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
Wesicle-trafficking protein SEC22b	SEC22B
Protein transport protein Sec24D	SEC24D
	GLS
Glutaminase kidney isoform, mitochondrial	
Glutaminase kidney isoform, mitochondrial NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	NDUFA7

NADH-cytochrome b5 reductase 3;NADH-cytochrome b5 reductase 3 membrane-bound form;NADH-cytochrome b5 reductase 3 soluble form	CYB5R3
Catalase	CAT
Dolichyl-diphosphooligosaccharideprotein 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 Splicing factor U2AF 35 kDa subunit;Splicing factor U2AF 26 kDa subunit	CNP 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
AspartatetRNA 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
TryptophantRNA 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-14 alpha chain;HLA class I histocompatibility antigen, Cw-14 alpha chain;HLA class I histocompatibility antigen, Cw-16 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	
neterogeneous nuclear moonucleoprotein ris	HNRNPH3
Serine hydroxymethyltransferase, mitochondrial	HNRNPH3 SHMT2
Serine hydroxymethyltransferase, mitochondrial	SHMT2
Serine hydroxymethyltransferase, mitochondrial Prohibitin	SHMT2 PHB
Serine hydroxymethyltransferase, mitochondrial Prohibitin Coatomer subunit beta 60S ribosomal protein L4 Transgelin-2	SHMT2 PHB COPB2 RPL4 TAGLN2
Serine hydroxymethyltransferase, mitochondrial Prohibitin Coatomer subunit beta 60S ribosomal protein L4 Transgelin-2 V-type proton ATPase catalytic subunit A	SHMT2 PHB COPB2 RPL4 TAGLN2 ATP6V1A
Serine hydroxymethyltransferase, mitochondrial Prohibitin Coatomer subunit beta 60S ribosomal protein L4 Transgelin-2 V-type proton ATPase catalytic subunit A Stress-70 protein, mitochondrial	SHMT2 PHB COPB2 RPL4 TAGLN2 ATP6V1A HSPA9
Serine hydroxymethyltransferase, mitochondrial Prohibitin Coatomer subunit beta 60S ribosomal protein L4 Transgelin-2 V-type proton ATPase catalytic subunit A Stress-70 protein, mitochondrial 60S ribosomal protein L13a	SHMT2 PHB COPB2 RPL4 TAGLN2 ATP6V1A HSPA9 RPL13A;RPL13a
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Serine hydroxymethyltransferase, mitochondrial Prohibitin Coatomer subunit beta 60S ribosomal protein L4 Transgelin-2 V-type proton ATPase catalytic subunit A Stress-70 protein, mitochondrial 60S ribosomal protein L13a Malate dehydrogenase, mitochondrial;Malate dehydrogenase Signal transducer and activator of transcription 1-alpha/beta;Signal transducer and activator of transcription Microtubule-associated protein 1B;MAP1B heavy chain;MAP1 light chain LC1 Eukaryotic translation initiation factor 1A, X-chromosomal;Eukaryotic translation initiation factor 1A, Y-chromosomal ATP synthase subunit O, mitochondrial	SHMT2 PHB COPB2 RPL4 TAGLN2 ATP6V1A HSPA9 RPL13A;RPL13a MDH2 STAT1 MAP1B EIF1AX;EIF1AY ATP5O
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Serine hydroxymethyltransferase, mitochondrial Prohibitin Coatomer subunit beta 60S ribosomal protein L4 Transgelin-2 V-type proton ATPase catalytic subunit A Stress-70 protein, mitochondrial 60S ribosomal protein L13a Malate dehydrogenase, mitochondrial; Malate dehydrogenase Signal transducer and activator of transcription 1-alpha/beta; Signal transducer and activator of transcription Microtubule-associated protein 1B;MAP1B heavy chain;MAP1 light chain LC1 Eukaryotic translation initiation factor 1A, X-chromosomal; Eukaryotic translation initiation factor 1A, Y-chromosomal ATP synthase subunit O, mitochondrial Elongation factor Tu, mitochondrial Signal recognition particle 9 kDa protein Heterogeneous nuclear ribonucleoprotein A3 Importin subunit alpha-1 Rap1 GTPase-GDP dissociation stimulator 1 Methionine aminopeptidase 1 Coatomer subunit beta Transitional endoplasmic reticulum ATPase 40S ribosomal protein S7 26S protease regulatory subunit 8 40S ribosomal protein S8 40S ribosomal protein S8 Mall nuclear ribonucleoprotein F 40S ribosomal protein S25 Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2	SHMT2 PHB COPB2 RPL4 TAGLN2 ATP6V1A HSPA9 RPL13A;RPL13a MDH2 STAT1 MAP1B EIF1AX;EIF1AY ATP5O TUFM SRP9 HNRNPA3 KPNA2 RAP1GDS1 METAP1 COPB1 VCP RPS7 PSMC5 RPS8 RPS15A SNRPF RPS25 GNB2
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ILES	Splicing factor 3A subunit 3	SF3A3
Heat shock protein 75 kDa, mitochondrial	Aminoacyl tRNA synthase complex-interacting multifunctional protein 1;Endothelial monocyte-activating polypeptide 2	AIMP1
Cleavage stimulation factor subunit 3	Interleukin enhancer-binding factor 3	
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Rabankyrin-5 ANKFY1 V-type proton ATPase subunit H Pre-mRNA-processing factor 19 PRPF19		
V-type proton ATPase subunit H Pre-mRNA-processing factor 19 ATP6V1H PRPF19		
Pre-mRNA-processing factor 19 PRPF19		
RuyB-like 2		
	RuvB-like 2	RUVBL2
Voltage-dependent anion-selective channel protein 3 VDAC3		
tRNA-splicing ligase RtcB homolog RTCB		

Table S2.

1 able 52.				
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	

Compartments	Neighborhoods
 S1, Golgi, Endo/Lysosome S2, ER, Peroxisome S3, ER, Mito.Ribosome S4, Plasma Membrane 	Secretory

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.

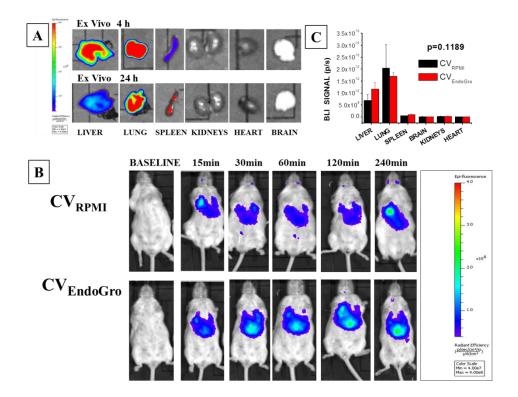


Figure S5. A] *Ex vivo* imaging of extracted organs after 4 and 24h of injection of 100ug lipid/mouse of CVRPMI. All images are in the same scale B] Representative bio fluorescence signals (photons/sec) of CV-associated DiR after injection of 200ug lipid/mouse CV_{RPMI} (upper panel) or CV_{EndoGro} (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.