ADVANCED HEALTHCARE MATERIALS

# Supporting Information

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A Comparison of Cellular Uptake Mechanisms, Delivery Efficacy, and Intracellular Fate between Liposomes and Extracellular Vesicles

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**Supplementary Table 2.** Uptake of liposomes and EVs via clathrin-mediated endocytosis in various recipient cells.

| Inhibitors | Targets | Drug delivery systems | Recipient cells | Inhibitor concentrations | Incubation times of drug delivery systems with cells | Serum supplemen- tation | Inhibition efficiencies | Intracellular fate of drug delivery systems | Key results | Ref |
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| Chlorpromazine | Amphipathic pharmacological inhibitor that depletes the plasma membrane of clathrin and adaptor protein AP2 complex by their assembly at the site of endosomal compartments thus hindering internalization via clathrin-mediated endocytosis. | Gold-encapsulated liposomes (lipid composition not mentioned) | UROtsa cells | 14-28 μM | 1 h | n.r. | ~30-95% inhibition | n.r. | • Gold-encapsulated liposomes entered UROtsa cells predominantly in a clathrin-dependent manner. | [1] |
| | | DOPE:DC-Cholesterol lipoplexes | A549 cells | 20 μg $mL^{-1}$ | 4 h | + | 34% inhibition<br>95% silencing | Endosomes | • Clathrin-mediated endocytosis was one of the main internalization mechanisms of DOPE:DC-Cholesterol lipoplexes, along with macropinocytosis.<br>• The transfection efficiency of lipoplexes was almost completely abolished following inhibition of clathrin-dependent endocytosis. | [2] |
| | | DOPE:CHEMS liposomes | COS-7 cells<br>HUVECs | 28 μM | 15 minutes | n.r. | 30% inhibition | Endosomes | • DOPE:CHEMS liposomes were partly internalized through clathrin-mediated endocytosis in both HUVECs and COS-7 cells. | [3] |
| | | DOTAP:DOPC:Cholesterol lipoplexes | A549 cells | 10 μg $mL^{-1}$ | 4 h | n.r. | ~35% inhibition | n.r. | • Cationic lipoplex formulations, DOTAP:DOPC:Cholesterol and Lipofectamine 2000, were partly | [4] |

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| | | Lipofectamine 2000 lipoplexes | HUVECs | | | | ~45% inhibition | | internalized through clathrin-associated pathways in HUVECs and A549 cells<br>● Uptake of the negatively charged DOPC:SM:Cholesterol:DOPS:DOPE EV-mimicking lipoplexes was not clathrin-mediated.<br>● DOTAP:DOPC:Cholesterol and Lipofectamine 2000 lipoplexes had a greater transfection efficiency than those of EV-mimicking lipoplexes. | |
| | | DOPC:SM:Cholesterol:DOPS:DOPE EV-mimicking lipoplexes | | | | | - (based on transfection) | | | |
| | | | HUVECs | | | | ~25% inhibition | | | |
| | | | | | | | ~30% inhibition | | | |
| | | | | | | | - (based on transfection) | | | |
| | | PC-98T:Cholesterol-enveloped plasmid-laden chitosan nanoparticles | Human conjunctival epithelial cells | 5 μg $mL^{-1}$ | 2 h | n.r. | ~63% inhibition | Endo-lysosomal compartments | ● Both plasmid-laden chitosan nanoparticle formulations were predominantly taken up via clathrin-mediated internalization in conjunctival epithelial cells.<br>● The DOTAP based formulation had a slightly higher uptake than plasmid-laden chitosan nanoparticles and more than two- | [5] |
| | | | | | | | ~58% inhibition | | | |

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| | | PC-98T:Cholesterol:DOTAP-enveloped plasmid-laden chitosan nanoparticles | | | | | | | fold increase in internalization than those lacking DOTAP.<br>● DOTAP insertion facilitated lysosomal escape, which in turn greatly enhanced their transfection ability both in vitro and in vivo. | |
| | | DOTAP:DOPE lipoplexes<br>DOPE:Cholesterol lipoplexes | COS-7 cells | 8 μg $mL^{-1}$ | 1 h | - | ~60% uptake inhibition<br>~95% silencing<br>~60% uptake and silencing | n.r.<br>Early endosomes and lysosomes | ● Both DOTAP:DOPE and DOPE:Cholesterol lipoplexes were internalized predominantly via clathrin-mediated endocytosis in COS-7 cells. | [6] |
| | | DOPC:Cholesterol liposomes<br>DOPG:Cholesterol liposomes | HeLa cells | 10 μg mL-1 | 5 h | + | -<br>55% inhibition | Lysosomes | ● Clathrin-mediated endocytosis was one of the two prevailing internalization mechanisms for DOPG:Cholesterol liposomes in HeLa and TRP3 cells, but not in A549 cells. Meanwhile, clathrin-mediated endocytosis had no contribution in the internalization of DOPC:Cholesterol liposomes in any of the recipient cell types tested.<br>● The effect of the anionic surface charge of DOPG:Cholesterol liposomes and the resulting protein corona can be attributed to the emergence of the | [7] |
| | | | A549 cells | | | | ~100% increase<br>~85% increase | n.r. | | |
| | | | TRP3 cells | | | | -<br>~65% inhibition | n.r. | | |

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| | | | | | | | | | additional clathrin-mediated endocytosis mechanism in cells. | |
| | | Amide:DOPE lipoplexes<br><br>Amide:Cholesterol lipoplexes | SK-HEP1 cells | 2.5 μg $mL^{-1}$ | 48 h | n.r. | ~40% inhibition<br><br>20% inhibition<br><br>(based on transfectio n) | n.r. | • Clathrin-mediated internalization was one of the two prevailing uptake mechanisms in the uptake of Amide:DOPE lipoplexes in SK-HEP1 cells. Meanwhile, clathrin-associated pathways had a minor role in the uptake of Amide:Cholesterol lipoplexes.<br>• Amide:DOPE lipoplexes were superior to those of Amide:Cholesterol in terms of transfection efficiency. | [8] |
| | | PE:PC:PI:PS liposomes | Huh7.5 cells | 25 μM | 1 h | - | 13% inhibition | Endoplasmic reticulum | • A minor fraction of liposomes may have been internalized through clathrin-mediated endocytosis in Huh7.5 cells.<br>• Uptake was mediated by scavenger and low density lipoprotein receptors.<br>• The lipid composition of PE:PC:PI:PS liposomes actively targeted and fused with endoplasmic reticulum. | [9] |
| | | Charge-reversal amphiphile lipoplexes | CHO-K1 cells | 10 μM | 3 h | - | ~30-35% uptake inhibition<br><br>and silencing | n.r. | • CHO-K1 cells internalized charge-reversal amphiphile lipoplexes containing GFP or β-galactosidase-encoding DNA partially through clathrin-dependent endocytosis. | [10] |

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| | | | | | | | | | • Lipoplexes did not accumulate inside lysosomes. | |
| | | DOTAP:DOPC liposomes<br><br>DOTAP:Cholesterol liposomes | HeLa cells | 50 μM | 2 h | n.r. | ~65% inhibition | Lysosomes, mitochondria, endoplasmic reticulum, trans-Golgi complex | • Both DOTAP:DOPC and DOTAP:Cholesterol liposomes were internalized through clathrin-dependent endocytosis in HeLa cells.<br>• Both lipid-based nanocarriers were transported to lysosomes, followed by their accumulation in mitochondria, endoplasmic reticulum, and trans-Golgi complex. | [11] |
| | | DPPC liposomes<br><br>Hybrid DPPC:EVs-derived from Sk-hep1 cells | Sk-hep1 cells | 30 μM | 4 h | n.r. | -<br><br>~25% inhibition | Lysosomes and endoplasmic reticulum<br><br>Trans-Golgi complex and endoplasmic reticulum | • Clathrin-mediated endocytosis was partly responsible for the internalization of DPPC:EVs in parental cell lines, whereas it did not play a role in the internalization of DPPC liposomes.<br>• DPPC:EVs circumvented lysosomal accumulation, in contrast to DPPC liposomes, and accumulated mainly in the endoplasmic reticulum and trans-Golgi complex.<br>• DPPC:EVs showed 1.7-fold increased siRNA transfection efficiency than DPPC liposomes.<br>• DPPC:EVs demonstrated enhanced antitumor efficacy in | [12] |

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| | | | | | | | | | HCC bearing mice, compared to DPPC liposomes. | |
| | | Adipose-derived regenerative cell-derived EVs | Cardiomyocytes | 10 μM | n.r. | n.r. | ~50% inhibition (in both normoxia and hypoxia conditions) | n.r. | • Clathrin-mediated endocytosis of EVs isolated from adipose-derived regenerative cells played a critical role in suppressing damage induced by hypoxia conditions in cardiomyocytes through miR-214 delivery both in vivo and in vitro.<br>• Hypoxia conditions stimulated the uptake of EVs via clathrin-dependent endocytosis in cardiomyocytes, compared to normal oxygen conditions. | [13] |
| | | Normal syncytiotrophoblast-derived EVs<br>Preeclamptic syncytiotrophoblast-derived EVs | HCAECs | 10 μg $mL^{-1}$ | 2 h | n.r. | 98% inhibition<br>95% inhibition | n.r. | • EVs were primarily taken up via clathrin-mediated endocytosis in a dynamin-dependent manner.<br>• The cellular internalization of vesicles was dependent on PI3K activity.<br>• Uptake of normal EVs down-regulated ICAM-1 protein expression, compared with preeclamptic syncytiotrophoblast-derived EVs that had no effect. | [14] |
| | | MSC-derived EVs<br>HSPC:Cholesterol liposomes | MSCs | 5 μg $mL^{-1}$ | 2 h | n.r. | 38% inhibition | n.r. | • HSPC:Cholesterol liposomes and MSC-derived EVs were taken up partly through clathrin-mediated endocytosis. | [15] |

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| | | | NIH3T3 cells | | | | 35% inhibition<br><br>(in MSCs, NIH3T3 reported to have similar values) | | • EVs exhibited a two-fold higher uptake than liposomes. | |
| | | PC12 cell-derived EVs | BMSCs | 10 μM | 3 h | n.r. | 41% inhibition | n.r. | • Uptake of PC12 cell-derived EVs proceeded partly through clathrin-mediated endocytosis in BMSCs.<br>• EVs delivered microRNAs, i.e. miR-21, through which transforming growth factor β receptor II and tropomyosin-1 expression were downregulated in recipient cells. | [16] |
| | | BMSC-derived EVs | Multiple myeloma 1S cells | 5-10 μM<br><br>20 μM | 4 h | n.r. | ~25-30% inhibition<br><br>- | n.r. | • Clathrin-mediated endocytosis may have contributed to the internalization of BMSC-derived EVs in multiple myeloma 1S cells, but not in others. Furthermore, the highest concentration of chlorpromazine did not inhibit uptake of these nanocarriers.<br>• Internalization was dependent on heparin, actin, dynamin, and PI3K activity.<br>• EV delivery promoted cell proliferation and facilitated chemotherapeutic resistance to bortezomib in multiple myeloma | [17] |

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| | | | | | | | | | cell lines, namely MM1S, RPMI 8226, and U266. | |
| | | K562 and MT4 cell-derived EVs | RAW264.7 macrophages | 50 μM | 2 h | n.r. | 24% inhibition | Phago-lysosomes | • A minor fraction of K562 and MT4-derived EVs was internalized in a clathrin-dependent manner. The majority was internalized via phagocytosis in various phagocytes, whilst in non-phagocytic cells they remained attached to the cell membrane. | [18] |
| Dynasore | Inhibits the GTPase activity of dynamin, thereby halting plasma membrane scission. | DLin-MC3-DMA:DSPC:Cholesterol:DMG-PEG lipoplexes | HeLa cells | 80 μM | 4 h | + | ~75% inhibition | Endo-lysosomal compartments | • DLin-MC3-DMA:DSPC:Cholesterol:DMG-PEG lipoplexes were internalized via clathrin-mediated endocytosis, which further stimulated uptake via macropinocytosis.<br>• Only 1-2% of siRNAs were able to escape degradation, during lipoplex translocation from early to late endosomes. | [19] |
| | | Adipose-derived regenerative cell-derived EVs | Cardiomyocytes | 50-100 μM | n.r. | n.r. | ~50% inhibition<br>(in hypoxia conditions)<br>- | n.r. | • Clathrin-mediated endocytosis of EVs played a critical role in suppressing damage induced by hypoxia conditions in cardiomyocytes through miR-214 delivery both in vivo and in vitro.<br>• Hypoxia conditions stimulated the uptake of EVs via clathrin-dependent endocytosis in | [13] |

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| | | | | | | | (in normoxia conditions) | | cardiomyocytes, compared to normal conditions. | |
| | | Normal syncytiotrophoblast-derived EVs<br><br>Preeclamptic syncytiotrophoblast-derived EVs | HCAECs | 80 μM | 2 h | n.r. | 86% inhibition<br><br>76% inhibition | n.r. | • EVs were primarily taken up via clathrin-mediated endocytosis in a dynamin-dependent manner.<br>• The cellular internalization of vesicles was dependent on PI3K activity.<br>• Uptake of normal EVs down-regulated ICAM-1 protein expression, compared with preeclamptic syncytiotrophoblast-derived EVs that had no effect. | [14] |
| FK506 | Interferes with calcineurin, subsequently affecting dynamin. | DOPC:DOPG liposomes | Hippocampal neurons | 1 μM | 30 minutes | n.r. | ¬95% inhibition | 50% of lipids recycled back to the plasma membrane, oligo-nucleotides accumulated in nuclei | • Following binding of DOPC:DOPG liposomes with low density lipoprotein receptors, the nanocarriers were taken up in a dynamin and PI3K activity-dependent manner, suggesting clathrin-mediated endocytosis.<br>• After internalization, the encapsulated oligonucleotides were located in nuclei within 1-3 h post-incubation and 50% of liposomal phospholipids were recycled back to the plasma membrane. | [20] |
| Wortmannin | Inhibits re-arrangement of actin filaments which regulate by the activity of | | | 100 nM | | | ¬80% inhibition | | | |

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| | phosphatidylinositol 3-kinase. | Normal syncytiotrophoblast-derived EVs<br><br>Preeclamptic syncytiotrophoblast-derived EVs | HCAECs | 1 μM | 2 h | n.r. | 94% inhibition<br><br>89% inhibition | n.r. | • EVs were primarily taken up via clathrin-mediated endocytosis in a dynamin-dependent manner.<br>• The cellular internalization of EVs was dependent on PI3K activity.<br>• Uptake of normal EVs down-regulated ICAM-1 protein expression, compared with preeclamptic syncytiotrophoblast-derived EVs that had no effect. | [14] |
| Amantadine | Prevents clathrin recycling to the plasma membrane. | DMPC:DMPG liposomes | HCAECs | 1 mM | 30 minutes | - | 46% inhibition without TNF-α<br><br>77% inhibition with TNF-α | n.r. | • HCAECs internalized DMPC:DMPG liposomes via clathrin-associated pathways.<br>• Inflammatory conditions, such as TNF-α stimulation, induced overexpression of clathrin proteins and subsequently enhanced liposome internalization. | [21] |
| Hyperosmolar sucrose | Prevents interaction between clathrin and adaptor proteins | DOPC:DOPG liposomes | Hippocampal neurons | 0.45 M | 30 minutes | n.r. | ¬98% inhibition | 50% of lipids recycled back to the plasma membrane, oligonucleotides accumulated in nuclei | • Following binding of DOPC:DOPG liposomes with low density lipoprotein receptors, the nanocarriers were taken up dependent on dynamin and PI3K activity, suggesting clathrin-mediated endocytosis.<br>• After internalization, the encapsulated oligonucleotides were located in nuclei within 1-3 h post-incubation and 50% of liposomal phospholipids were | [20] |

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| | | | | | | | | | recycled back to the plasma membrane. | |
| | | 0.86 mol% R8-EPC:Cholesterol or -DOPE:CHEMS lipoplexes | NIH3T3 cells | 0.4 M | 1 h | - | ~80% inhibition | High lysosomal co-localization | • Low density (0.86 mol%) R8-lipoplexes internalized mainly via clathrin-mediated endocytosis in NIH3T3 cells, ultimately accumulating inside lysosomes. | [22] |
| | | 5.2 mol% R8-EPC:Cholesterol or -DOPE:CHEMS lipoplexes | | | | | ~35% inhibition | Partial lysosomal co-localization | • High density (5.2 mol%) R8-lipoplexes were taken up partly via clathrin-associated pathways, which led to partial accumulation inside lysosomes. Hence, high density R8-lipoplexes presented higher transfection efficiency. | |
| | | DOTAP:DOPE lipoplexes | COS-7 cells | 800 μM | 1 h | - | ~35% inhibition | - | • Both DOTAP:DOPE and DOPE:Cholesterol lipoplexes were internalized predominantly via clathrin-mediated endocytosis in COS-7 cells. | [6] |
| | | DOPE:Cholesterol lipoplexes | | | | | ~50% inhibition (based on transfection) | Early endosomes and lysosomes | | |

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| | | DLPC:Cholesterol:Cholesteryl:PEG liposomes | Zebrafish hepatocytes | 300 mM | 15 minutes | n.r. | 15% inhibition | Endo-lysosomal compartments | • Hepatocytes internalized DLPC:Cholesterol:Cholesteryl:PEG liposomes partially in a clathrin-dependent manner, ultimately accumulating inside lysosomes.<br>• Lipopolysaccharide-dsRNA cocktails encapsulated in liposomes were able to stimulate both pro-inflammatory and antiviral responses in cells. | [23] |
| | | | Trout macrophages | 150 mM | 30 minutes | | - | | | |

n.r. = not reported

– = no inhibition

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