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Chapter 2

Drug Delivery via Cell Membrane Fusion Using Lipopeptide Modified Liposomes

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ABSTRACT

Efficient delivery of drugs to living cells is still a major challenge. Currently, most methods rely on the endocytotic pathway resulting in low delivery efficiency due to limited endosomal escape and/or degradation in lysosomes. Here, we report a new method for direct drug delivery into the cytosol of live cells in vitro and vivo utilizing targeted membrane fusion between liposomes and live cells. A pair of complementary coiled-coil lipopeptides was embedded in the lipid bilayer of liposomes and cell membranes respectively, resulting in targeted membrane fusion with concomitant release of liposome encapsulated cargo including fluorescent dyes and the cytotoxic drug doxorubicin. Using a wide spectrum of endocytosis inhibitors and endosome trackers we demonstrate that the major site of cargo release is at the plasma membrane. This method thus allows for the quick and efficient delivery of drugs and is expected to have many in-vitro, ex-vivo and in-vivo applications.
INTRODUCTION

The plasma membrane is the protecting interface between cells and their surrounding environment. Uptake of nutrients occurs through this interface using specialized mechanisms such as endocytosis. Nutrients, or drugs for that matter, are frequently internalized into small transport vesicles called endosomes, which are derived from the cell membrane. For many medicines to become an active drug, they have to enter the cell’s cytosol. However, the detrimental environment inside these endosomes can result in degradation of the drug. To date, intracellular delivery of macromolecules is still a major challenge in research and therapeutic applications. It is therefore highly desirable to develop new alternative delivery methods that circumvent the endocytosis pathway. So far, all attempts in drug delivery using particles as carriers have been unsuccessful in avoiding this pathway, hence current efforts to develop ways of enhancing endosomal escape. Cell penetrating peptides (CPP) have been studied extensively to achieve efficient uptake into the cytosol. However, the current view is that CPPs conjugated to large molecular weight cargo (e.g. liposomes) predominantly are internalized via endocytosis. Moreover, the positive charge of CPPs such as the Tat peptide leads to unfavorable interaction with blood components. Other transfection techniques have been devised, such as viral vectors and physical methods. These methods have their own limitations, including safety issues or their reliance to electrical fields or high pressure.

Fusion of lipid membranes is a vital process in biological systems, facilitating the efficient transport of molecules across membranes. In vivo membrane fusion shows a broad variety, from synaptic to viral and extracellular fusion and was found to be a highly regulated process, specific in time and place, which is achieved by a complex interplay of different functional proteins. As a bottom-up approach, several synthetic models systems have been developed to mimic membrane fusion events, but in general these simple systems do not always recapitulate the basic characteristics of native membrane fusion. Furthermore, all these approaches were limited to liposome-liposome fusion studies and have not shown to induce fusion events in live cells, thereby limiting their use for future drug delivery purposes.

Inspired by the SNARE protein complex, our laboratory has developed a fully artificial membrane fusion system composed of a complementary pair of lipidated coiled coil peptides enabling targeted liposome-liposome fusion. This model system possesses all the key characteristics of targeted membrane fusion similar to SNARE mediated fusion including lipid and content mixing in the absence of leakage (Figure 1A-B). In our membrane fusion system, coiled-coil forming peptides "E₃" [(EIAALEK)₃] and “K₃” [(KIAALKE)₃] were conjugated to a cholesterol moiety via a polyethylene glycol (PEG) spacer, yielding lipopeptides CPE₃ and CPK₃.
cholesterol moiety allows for the immediate insertion of the lipidated peptides into any phospholipid membrane. We demonstrated that plain membranes could become fusogenic by the spontaneous insertion of CPE₃ and CPK₃ in the bilayer. A follow-up study showed that CPK₃ modified cells and zebrafish embryos could be specifically labeled with the complementary fluorescently-labeled E₃ peptide,²³ revealing that E₃/K₃ coiled-coil formation is also functional in an in vivo environment thereby paving the way for targeted delivery using peptide modified liposomes.

Here, we report a new drug delivery method based on targeted membrane fusion between liposomes and live cells. We demonstrate that a wide range of cell lines can be specifically modified with lipopeptide CPK₄ and upon addition of CPE₄ decorated liposomes membrane fusion occurs with concomitant efficient cytosolic delivery of a variety of compounds like fluorescent dyes propidium iodide (PI), TOPRO3, and the cytotoxic drug doxorubicin (DOX). The mechanism of content uptake was studied using endocytosis inhibitors and endosome trackers in order to prove that the major site of cargo release into the cells is indeed at the plasma membrane due to liposome-cell fusion. Additionally, we show cytosolic dye (and drug) delivery in vivo using zebrafish embryos. Our method thus allows for quick and efficient delivery of drugs and bio(macromolecules) without cell damage and is expected to have many applications in vitro, ex vivo and in vivo.

Results and Discussion

Coiled-Coil Formation between CPE₄ and CPK₄

Previously, we reported docking of liposomes at cell membranes using peptides CPE₃ and CPK₃, but membrane fusion was not observed.²³ In the present study we increased the number of heptad repeats in CPE and CPK to four thereby enhancing coiled-coil stability,²⁶ expecting that this would favor liposome-cell fusion. Figure 1C shows that the cholesterol- and PEG-modified E₄ and K₄ – hereafter called lipopeptides CPE₄ and CPK₄ - when attached to liposomes, are capable of coiled-coil formation as evident from circular dichroism (CD) spectroscopy, in agreement with previous experiments using CPE₃ and CPK₃. Next, lipid mixing experiments were performed to investigate the fusogenicity of the CPE₄/CPK₄ pair in a liposome-liposome assay. In these experiments a fluorescence resonance energy transfer (FRET)-pair consisting of nitrobenzoxadiazole (NBD) and lissamine rhodamine (LR) fluorophore labeled lipids was incorporated into the membrane of CPK-decorated liposomes.²¹ Upon lipid
mixing of the latter liposomes with CPE₄-liposomes the distance between NBD and LR increased, resulting in increased NBD-fluorescence as shown in Figure 1D. Content mixing was quantified by incorporating a sulforhodamine B at a self-quenching concentration of 20 mM into CPE-decorated liposomes and mixing these with CPK-liposomes as described. The increase in sulforhodamine B fluorescence over time indicated that full fusion took place between CPE₄ and CPK₄-liposomes (Figure 1D). Control experiments verified that the increase in sulforhodamine B fluorescence was not caused by leakage during fusion (Figure S1).

Coiled-Coil Formation Triggers Liposome-Cell Fusion

Next we investigated whether CPE₄ and CPK₄ could also mediate membrane fusion between liposomes and living cells. To this end, HeLa cells were pre-incubated with a micellar solution of CPK₄ for 0.5-2 h before CPE₄-decorated liposomes (lipid composition DOPC:DOPE:CH, 50:25:25 mol%) containing the nucleic acid stain propidium iodide (PI) or TOPRO3 in their aqueous interior were added as schematically shown in Figure 1E. In order to localize the lipid bilayer, these liposomes also contained 1 mol% of green-fluorescent NBD-DOPE lipids. As expected, confocal microscopy showed that cell membranes became labeled with the green NBD-dye on their outside in line with previous studies. Strikingly, the red dye was observed in the cytosol and nucleus, indicating that membrane fusion and content release had occurred (Figure 2A & Figure S2A for TOPRO3). Control experiments in which one of the two lipopeptides was omitted showed neither uptake of PI or TOPRO3 nor NBD-labeling of the cell plasma membrane (Figure 2B, 2C, 2E, and Figure S2). We note that when CPK-treated cells were incubated with empty CPE₄-decorated liposomes in the presence of free dye only a weak fluorescent signal was observed inside cells (Figure 2C & Figure S2C). This control experiment rules out the possibility that residual non-encapsulated dye in our liposome preparation entered the cell by transient membrane destabilization during fusion events. Finally cell incubation with free dyes also did not show any signal of the dye inside the cells (Figure 2F & Figure S2F). Similar to CPE₄ decorated liposomes, we also used CPK₄ decorated liposomes containing PI and incubated these with CPE₄ pre-treated HeLa cells. However, the delivery of PI was less efficient. A reason might be the asymmetric nature of the fusion system. It was recently shown that peptide E does not interact with a membrane. In contrast, peptide K does interact with the membrane in a so-called snorkeling mode, and this peptide-membrane interaction is in equilibrium with either peptide K homocoiling or E/K coiled coil formation. These studies suggest that peptide K-membrane interactions result in increased membrane curvature supporting membrane fusion. A cell membrane is more complex in composition and therefore less susceptible to undergo fusion as compared to the fusogenic liposomes (DOPC:DOPE:CH 2:1:1) used in this study.
Figure 1. Schematic representation of (A) coiled-coil structure between peptides E and K (adapted from PDB 1UOI), (B) Targeted liposome fusion mediated by coiled-coil formation between CPE\textsubscript{E} modified liposomes and CPK\textsubscript{K} modified liposomes, (C) CD spectra of CPE\textsubscript{E} modified liposomes, and CPK\textsubscript{K} modified liposomes and a equimolar mixture thereof. The total lipid concentrations were 0.5 mM with 1mol% of lipidated peptide in PBS. (D) lipid mixing and content mixing between CPE\textsubscript{E}-liposomes and CPK\textsubscript{K}-liposomes. Fluorescence traces showing lipid mixing between E and K decorated liposomes, as measured through an increase in NBD fluorescence. Total lipid concentrations were 0.1 mM with 1mol% of lipidated peptide, in PBS; Fluorescence graphs indicating content mixing between sulphorhodamine loaded (20 mM), CPE\textsubscript{E} decorated liposomes and non-fluorescent, CPK\textsubscript{K} decorated liposomes. Total lipid concentrations were 0.25 mM with 1 mol% lipidated peptide in PBS. (E) Scheme of fusion between cell and liposomes.

Our current thought is that peptide K needs to be on the cell membrane prior to a fusion event in order to activate the complex cell membrane by inducing membrane curvature.\textsuperscript{29} However, more studies are required to support this hypothesis. To exclude the possibility that peptidemiated liposomal dye delivery was a peculiarity of HeLa cells, the membrane fusion experiments were repeated with Chinese hamster ovary (CHO) and mouse fibroblast (NIH/3T3) cell lines. Again the appearance of TOPRO3 and PI was observed inside cells suggesting that the peptide-mediated delivery of the dye is cell type independent (Figure S3 & S4). Importantly, we found that uveal melanoma cells (Mel270), which are generally hard to transfected,\textsuperscript{30, 31} could also be modified with TOPRO3 using this method (Figure S3D).
Figure 2: Delivery of PI by peptidated-liposomes is dependent on coiled-coil formation between CPK and CPE. Confocal microscopy images of Hela cells. Cells were pre-incubated with CPK (A, B, C) or medium (D, E, F) for 2 hours, followed by treatment with: CPE-decorated liposomes containing PI (a,e), liposomes containing PI (B,D), CPE-decorated liposomes plus free PI (C), or free PI(F). Green: NBD, Red: PI. Scale bar is 25 µm. Overlay is red and green channel plus bright field image.

To address the potential toxicity of CPK<sub>4</sub>, CPE<sub>4</sub>, and liposomes towards CHO, NIH/3T3 and HeLa cells cell viability assays were carried out. These assays indicated that lipopeptides CPE<sub>4</sub> and CPK<sub>4</sub> and liposomes, with or without CPE<sub>4</sub>, at the concentrations used throughout this study are well tolerated by all cell lines (Figure S5A). Higher concentrations of these lipopeptides, even up to 100 µM, did not significantly reduce cell viability when exposed for 2 hours but only did so after 24 hours of exposure (Figure S5B & S5C). Altogether, these results show that coiled-coil formation between CPK<sub>4</sub> and CPE<sub>4</sub> is critical for fusion and release of the dyes, and that these compounds are not toxic for living cells at the concentrations used allowing to investigate potential applications and their uptake mechanism.

**Delivery of Doxorubicin**

Doxorubicin (DOX) is one of the mostly used drugs for cancer treatments in the clinic today but as a free drug has serious cardiotoxicity. DOX is a cell permeable drug whose fluorescence is strongly enhanced upon binding to nucleic acids. Intercalation into DNA ultimately results in apoptosis. To test delivery of liposo-
mal DOX, HeLa cells were pre-incubated with CPK, and subsequently exposed to CPE₄-decorated liposomes containing 5 µM DOX for 15 min. As can be seen in Figure 3A and 3B this resulted in strong nuclear (and cytosolic) fluorescence. Control experiments showed that DOX delivery is highly dependent on the presence of CPE₄ and CPK₄ (Figure S6). To investigate cytotoxicity of liposomal delivered DOX, HeLa cells pre-incubated with CPK were exposed with increasing concentrations of DOX-loaded liposomes for 12 h. Cell viability was measured 24 h later. Figure 3C shows cell viability as a function of liposomal and free DOX. As expected, very low concentrations of free DOX (< 1 µM) did not affect the viability of HeLa cells as passive crossing into cells is not efficient at this concentration. Importantly, in current treatments in the clinic the DOX concentration is up to 9 µM in the serum of patients. In contrast, liposomes loaded with 1 µM DOX did showed a significant effect as the DOX uptake is significantly enhanced. Liposomally delivered DOX reduced cell viability at DOX concentrations as low as 0.1 nM with an IC₅₀ of ~0.01 µM, while free DOX did not affect cell viability at concentrations up to 1 µM (IC₅₀ ~5 µM). Control experiments in which either CPK₄ or CPE₄ was omitted showed 100-fold or higher IC₅₀ values (Figure S7). Thus, our peptide-mediated delivery of DOX can potentially reduce the dose of DOX needed for anticancer treatments thereby lowering the cardiotoxicity of DOX. The presented fusion mediated delivery approach is also promising for the delivery of other drugs or biomolecules like DNA or siRNA.

![Figure 3A](image1.png) ![Figure 3B](image2.png) ![Figure 3C](image3.png)

**Figure 3. Delivery of DOX into HeLa cells.** (A) CPE/CPK₄ mediated delivery of DOX into HeLa cells. Cells were treated with CPK₄ for 1 h followed by incubation with 0.25 mM CPE₄ liposomes containing with DOX for 15 min. Images were taken after washing. A) bright field. (B) fluorescence channel. The inset shows a magnified overlay image, revealing the presence of DOX in the nucleus. The concentration of DOX loaded into liposomes is 5 µM. Scale bar represents 25 µm. (C) Cytotoxicity of CPE/CPK₄ delivered DOX and free DOX. HeLa cells were treated with CPK₄ for 1 h and series of concentrations of CPE₄ decorated liposomes containing DOX (blue line), or the same concentrations of free DOX (red line) for 12 h. After washing and incubation with medium for 24 h, cell viability was measured by a WST-1 assay.
Liposomes and Content Only Partially Co-localize with Endosomes

Endocytosis is the most common pathway for the uptake of small particles including liposomes by cells. To investigate whether endocytosis played a role in the liposomal delivery, the endosome tracker pHrodo, a fluorescently labeled dextran, was used in combination with TOPRO3 loaded liposomes. TOPRO3 was chosen as encapsulated dye for this experiment instead of PI because its emission (Ex/Em 642/661 nm) is expected not to interfere with emission of pHrodo (Ex/Em 560/585 nm) making investigation of colocalization of dyes easier. pHrodo and CPE\textsubscript{4} decorated liposomes containing 1 mol% NBD-DOPE and TOPRO3 were simultaneously added to CPK\textsubscript{4}-modified HeLa cells. Confocal microscopy showed the presence of TOPRO3 in the cytosol and to a lesser extent in the nucleus (Figure 4B) while pHrodo was mainly observed as individual dots in the cytosol in agreement with its endosomal uptake (Figure 4C). Overlaying the fluorescent images of TOPRO3 and pHrodo revealed some overlap between TOPRO3 and endosomes (Figure 4E, pink dots) but the majority of TOPRO3 signal remains unmixed. Again, the signal from NBD-DOPE (Figure 4A, white dots) remained at the plasma membrane, although some overlap with pHrodo was observed at the plasma membrane (Figure 4F).

**Figure 4. Visualization of endosomes using an endosome tracker.** CHO cells were treated with CPK\textsubscript{4} for 2 h, followed by co-incubation with pHrodo red dextran and CPE\textsubscript{4}-decorated liposomes (0.25 mM total lipid concentration and 1 mol% CPE\textsubscript{4}) loaded with TOPRO 3. (A) White channel showing DOPE-NBD liposomes. (B) Red channel (TOPRO3). (C) Blue channel (pHrodo). (D) Overlay of panels A and B. (E) Overlay of panels B and C. (F) Overlay of panels A and C. Scale bar is 25 \(\mu\)m.
This could be the result of both liposomes and endosome tracker binding at a common spot at the plasma membrane or could mean that some liposomes are initially taken up by endocytosis but then rapidly fuse with the endosomal membrane. These results suggest that the endosomal uptake pathway only plays a minor role in CPE₄-CPK₄ mediated liposomal uptake and that liposome-cell membrane fusion is the main route for cargo delivery. This is also illustrated by performing the same experiment at 4°C, conditions under which active uptake by endocytosis is inhibited. Imaging of cells over a period of three hours showed the increasing uptake of TOPRO3 (Figure 5A, upper panels). In contrast only a faint signal of endosome tracker pHrodo was observed after three hours indicating that endocytosis was severely limited at 4°C (Figure 5A, lower panels). Quantification of the fluorescence intensity using software (Image-J) showed that after 3 h the uptake of TOPRO3 reached ~80% of the level obtained after 30 min at 37°C (Figure 5B). The slower uptake is presumably caused by the reduced rate of liposome-cell fusion events at 4°C. This is supported by the observation that liposome-liposome lipid mixing induced by CPE₄/CPK₄ is also significantly slower at 4°C than at room temperature (Figure S8).

Endocytosis and Macropinocytosis Inhibitors Marginally Affect Delivery

As independent support for our conclusion that fusion at the plasma membrane is the major pathway for our liposome-based delivery system, several well characterized inhibitors of endocytotic pathways were tested using flow cytometry measurements and confocal microscopy imaging. Wortmannin blocks PI3-kinase and inhibits macropinocytosis, Chlorpromazine interferes with clathrin-dependent endocytosis, Genistein inhibits tyrosine-phosphorylation of Cav 1 and caveola-dependent endocytosis. In addition, nocodazole, an inhibitor of microtubule formation, was used to investigate whether intracellular trafficking and internalization mechanisms are involved. Moreover, endocytosis of nanoparticles is an energy-dependent mechanism. Sodium azide was therefore used to deplete the energy needs for endocytosis and restrict metabolic activity. HeLa cells were first incubated for 1 h with each inhibitor at concentrations that have been reported by others to show optimal activity. After removal of the inhibitors, cells were treated with CPK₄ and subsequently with CPE₄-decorated liposomes containing PI dye in the presence of freshly added inhibitors. FACS analysis showed that genistein and nocodazole had no adverse effect on the delivery of PI (Figure 5C), whereas in the presence of wortmannin, chlorpromazine and sodium azide PI uptake was reduced less than 20%. These results argue against a major role of
endocytosis or pinocytosis in uptake of liposomal cargo and support that the
dominant pathway for delivery is indeed targeted membrane fusion between
liposomes with the plasma membrane of live cells.

Figure 5. Investigation into the uptake mechanism. (A) Effect of low temperature incubation of HeLa
cells on liposomal delivery of TOPRO3 and endosomal uptake of pHrodo. Cells were pre-incubated on
ice with 5 μM CPK (2h), followed by 15 min incubation with 0.25 mM CPE-decorated liposomes contain-
ing TOPRO3. After three washes confocal images were taken immediately (0 min) and after 60 min, 120
min and 180 min. Top row: TOPRO3 (red), bottom row: pHrodo (blue). (B) Graphical representation of
the percentage of TOPRO dye uptake by HeLa cells on ice. Fluorescence intensities were calculated by
Image J and plotted as a percentage relative to the fluorescence of TOPRO3 delivery at 37˚C (100%).
Scale bar is 25 μm. (C) Effect of endocytosis and macropinocytosis inhibitors on delivery of PI by
liposomes to HeLa cells. Cells were incubated with medium (Ctrl+), or medium containing 0.25 μM
wortmannin (Wor), 40 μM chlorpromazine (Chl), 200 μM genistein (Gen), 40 μM nocodazole (Noc) for
1 hour, 0.01% w/v sodium azide (NaN3), followed by 2 hour incubation with 5 μM CPK in presence of
inhibitors, and then treated for 15 min with CPE-liposomes containing PI. Final concentration of lipids
(liposomes) was 0.25 mM. Cellular uptake was measured by flow cytometry. Positive control (100%):
fluorescence of PI dye in the absence of inhibitors.

Intracellular Delivery in Vivo

As a first step towards clinical application, we used zebrafish embryos to evaluate
direct cytoplasmic delivery in vivo. We previously established coiled-coil mediated
docking of liposomes onto the zebrafish embryonic skin. During embryonic
stages, the zebrafish skin is composed of a layer of ridged, mucus-covered
enveloping layer (EVL) cells. Through interspersed gaps in the EVL layer, cells
within the underlying epidermal basal layer (EBL), including mucus-secreting cells
and ionocytes, are exposed to the external environment. To test for in vivo de-

livery to skin epithelial cells, we exposed 48h-old zebrafish embryos to CPK in
embryo medium for 30 minutes. After washing, embryos were exposed to NBD-la-
beled, CPE₄-decorated liposomes containing DOX for 30 minutes. Consistent
with previous results, we observed widespread liposome docking after 30 min of
incubation, as evidenced by NBD and DOX co-labeling. Importantly,
we identified nuclear DOX labeling within a subset of skin epithelial cells
(Figure 6) consistent with delivery into EBL-layer, but not EVL layer cells, which
appeared to be inaccessible due to mucus covering or membrane ridging. Control
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experiments established that cytoplasmic delivery was specific to coiled-coil interaction (Figure S9).

**Figure 6. In vivo delivery of DOX using CPK and CPE.** 2 dpf zebrafish were treated with CPK for 30 minutes, followed by 30 minutes incubation with CPE-decorated liposomes (0.25 mM total lipid concentration and 1 mol% CPE) loaded with DOX. (A,B) Whole-embryo imaging showing widespread DOX delivery in living zebrafish embryos (control experiments in Supplementary Figure S9). (C-E) Single zebrafish skin epithelial cell (from the indicated site of the embryo in (A,B) displaying membrane associated DOPE-NBD labeling (NBD) and predominantly nuclear DOX labeling.

We further confirmed intracellular delivery using liposomes loaded with PI, which becomes highly fluorescent only after interaction with cellular DNA or RNA (Figure S10 & S11). Together, these results indicate the potential application of coiled-coiled induced membrane fusion for direct cellular drug delivery in vivo.

**CONCLUSION**

Numerous methods exist to deliver drugs and (bio)macromolecules to living cells. Depending on the nature of these molecules they can be delivered into cells via electroporation, micro-injection, calcium phosphate co-precipitation, nanoparticles, or viral particles. However many of these methods are either not suitable for in vitro use or cannot be safely applied in in vivo applications, or are inefficient due to endosomal entrapment and degradation. The membrane fusion system described here involves the targeted fusion of liposomes with the plasma membrane of live cells. As a result, endosomal pathways are almost completely circumvented and therefore this efficient drug delivery method is suited for labile (bio)molecules. In addition, the lipopeptides and modified liposomes have a low toxicity at the used concentration - in contrast to CPP-based delivery approaches or PEG-induced liposome fusion. We anticipate that this membrane fusion strategy will spark new in vitro, ex vivo in the field of chemical biology and possibly in the long-term in vivo applications enabling new basic and applied research studies for gene therapy. Moreover any compound that can be encapsulated in liposomes like hydrophilic low molecular weight drugs or DNA/siRNA could be considered as well as many hydrophilic drugs are unable to enter...
cells effectively and are known to be degraded in a lysosomal environment thereby lowering their therapeutic efficacy.\textsuperscript{52}

Here, fusion mediated delivery could result in less degradation of sensitive molecules and might therefore find use as a new transfection agent in in vitro cell studies. Also lipid bilayer-coated nanoparticles\textsuperscript{53-57} might be delivered more efficiently when coiled coil mediated membrane fusion is applied thereby increasing the scope of molecules and nanoparticles/nanodevices that can be delivered into cells. Future \textit{in vivo} application of this technique requires cells to be pre-modified with one of the two peptides and is currently not cell-type specific due to the cholesterol-anchor, several applications are still conceivable. These include topical administration of drugs to treat e.g. pulmonary disease or combat respiratory infections like influenza. On the other hand delivery of liposomally encapsulated mRNA or DNA coding for the tumor suppressor p53 will only affect tumor cells and leave healthy cells unharmed.\textsuperscript{58} Similarly, liposomal delivery of miRNA or siRNA to upregulate tumor suppressors or downregulate oncogenes could selectively kill only tumor cells.\textsuperscript{59}

Finally, a certain degree of selectivity can be achieved using a light-induced membrane fusion system that was recently developed in our laboratory. This system makes use of photoinduced deshielding of a PEGylated CPE and thus allows potentially for spatiotemporal control of liposomal drug delivery in \textit{vivo}.\textsuperscript{60}

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**

Fmoc-protected amino acids were purchased from Novabiochem and Biosolve. Sieber Amide resin was purchased from Chem-Impex International and Agilent Technologies. DOPE, DOPC, DOPE-NBD, and DOPE-LR were purchased from Avanti Polar Lipids. cholesterol, propidium iodide (#BCBM1455V) and sulphorhodamine were obtained from Sigma-Aldrich. Topro3-Iodide (#1301286), pHrodo\textsuperscript{TM} Red dextran 10,000MW were purchased from Life Technologies. 8 wells slide Lab-tek was purchased from Thermo Scientific, USA. DMEM medium was obtained from Gibco, life technologies. N\textsubscript{3}-PEG\textsubscript{4}-COOH\textsuperscript{61} and 3-Azido-5-cholestene\textsuperscript{62} were synthesized following literature procedures.
Lipopeptide Synthesis and Purification

The peptide components of CPK₄ and CPE₄, i.e., E₄ (EIAALEK)₄ and K₄ (KIAALKE)₄ were synthesized on an automatic CEM peptide synthesizer on a 250 µmol scale using Fmoc chemistry and standard solid-phase peptide synthesis protocols as previously described. After Fmoc deprotection N₃-(ethylene glycol)₄-COOH was coupled to the peptide on the resin. After azide reduction cholesteryl-4-amino-4-oxobutanoic acid was coupled to the PEG₄ linker to yield the CPE₄ and CPK₄ peptides as described. The final products were purified by HPLC using a C4 column and their identity confirmed by LC-MS.

Liposome Preparation and Characterization

Lipids were dissolved in CHCl₃ in the molar ratio DOPC, DOPE, Cholesterol and DOPE-NBD of 49.5:24.75:24.75:1 [total lipid concentration] = 1 mM. Peptide stock solutions of 50 µM were prepared in CHCl₃/CH₃OH (1:1 v/v). Liposomes were prepared by mixing the appropriate amount of lipids and CPE₄ in a 20 mL glass vial and evaporating the solvents over air pressure to form lipid films. Traces of solvent were removed under high vacuum for 3-4 h at 25 °C. Each sample was then hydrated with 15 mM PI (SigmaAldrich #BCBM1455V) or 0.25 mM TOPRO3 (Life Technologies, #1301286, after removing DMSO by freeze drying) or FITC-dextran (35 mg/ml) in PBS buffer and sonicated for 2-3 min in a sonication bath at 55°C. Nonencapsulated dyes or FITC-dextran were removed via Sephadex G25 or G50 size-exclusion PD-10 Columns (GE-Healthcare, USA). Liposomes were characterized by Dynamic Light Scattering (DLS) at 25°C to determine the average diameter (80 – 100 nm in general). The final concentration of lipids and CPE₄ in each sample before cell treatments was 250 µM and 2.5 µM, respectively.

A CPK₄ stock solution (50 µM) was prepared in CHCl₃:CH₃OH (1:1). For a typical cell treatment the appropriate amount of CPK₄ stock solution was taken and the organic solvent was evaporated under air stream. After that it was hydrated by DMEM (+/- FCS, w/o phenol red) and sonicated at 55 °C for 1-2 min.

Doxorubicin (DOX) was entrapped as follows. The lipid film was hydrated with citrate buffer (pH 3.5), and sonicated in a sonication bath at 50°C for 30 min. The citrate buffer was replaced by PBS (pH 7.4) through Sephadex G-25 filtration, leaving the inside of liposomes acidic. Doxorubicin powder (SigmaAldrich # 44538) was added into liposomal dispersion at a drug-to-lipid molar ratio of 1:3, and subsequently rotated at 4°C overnight. Untrapped free DOX was separated from liposomes by size exclusion chromatography using a Sephadex G-25 column. The entrapment efficiency was determined using UV-vis spectrophotometry (Figure S12). Liposomes obtained were ~120 nm in diameter with a PDI of < 0.2.
Cellular Uptake Assay and Confocal Microscopy Measurements

All incubations were done in complete medium without phenol red. Cells were grown in an 8-well slide at a density of 2.5×10⁴ cells per well and incubated at 37 °C in 7% CO₂ atmosphere. After 21 h, medium was removed and a CPK₄ solution (5 µM) in medium was added and incubated for 0.5-2 h at 37 °C in 7% CO₂. After removal of CPK₄, cells were washed with medium, and incubated with CPE₄-decorated liposomes (250 µM) containing NBD, PI, TOPRO3. After 15 min. incubation, cells were washed three times with medium and fluorescent images were acquired on Leica TCS SP8 confocal laser scanning microscope. Leica application suite advanced fluorescence software (LAS AF, Leica Microsystems B.V., Rijswijk, The Netherlands) and Image-J (developed by the National Institutes of Health) were used for image analysis and liposome co-localization studies. Wavelength settings for pHrodo™ Red dextran were Ex/Em: 560/585 nm (Ex laser: 488 nm), for Topro3 Ex/Em: 641/662 nm (Ex laser: 633 nm), for Propidium iodide Ex/Em: 535/617 nm (Ex laser: 543 nm), for NBD-DOPE Ex/Em: 455 / 530 nm (Ex laser: 488 nm) and for DOX Ex/Em: 490/590 nm (Ex laser: 543 nm).

When performing cellular uptake assays on ice, an 8-well slide was placed on ice for 1 h, before adding CPK₄. After two hours on ice, CPK₄ was removed and after washing CPE-decorated liposomes loaded with TOPRO3 and endosome tracker were added simultaneously. After 15 min. incubation on ice, cells were washed three times with ice-cold medium and imaged immediately (time point 0 h). After 1, 2, and 3 hours the slide was transferred to the microscope and images were recorded. In between measurements the cells were kept on ice.

Cell Viability Assay

Cells were seeded in a 96 wells-plate at a concentration of 1×10⁴ cells per well and incubated for 24 h prior to the WST-1 assay. The medium was removed and cells were incubated with 100 µL of CPK₄ (5 µM) solution in medium (w/o phenol red) for 2 h. After 2 h CPK₄ was removed by washing three times with medium and the cells were incubated with liposomes containing 1 mol% CPE₄ decorated liposomes for 15 min. In parallel cells were incubated with liposomes in the absence of lipopeptides. After these treatments, fresh medium was added to each well and the plate was incubated at 37 °C for 24 h prior to the WST assay. After 24 h, medium was removed and 200 µl of cell proliferation reagent WST-1 (Serva, #140330 and PMS-Ome Santa Cruz Biotechnology, #D3013) in DMEM (w/o phenol red) was added to each well and the plate was incubated for 3 h at 37 °C. After 3 hours the absorbance at 450 nm was measured at room temperature using a Tecan X fluorometer and a 96 well plate, which was shaken for 60 seconds prior to measurement (2 mm linearly, 654 rpm). The values for metabolic activity (cell survival) were normalized with respect to
control (no liposomes), which was set at 100% cell survival. For the DOX cell viability assay, Hela cells were incubated with CPK$_4$ for 2 h, then treated with series of diluted CPE$_4$ decorated liposomes loaded with DOX (stock lipid concentration was 1 mM, containing 1 mol% of CPE$_4$; DOX concentration was 0.25 mM), final concentration of DOX in liposomes were from 100 µM to 0.1 nM (100 µM, 50 µM, 25 µM, 10 µM, 5 µM, 2.5 µM, 1 µM, 0.1 µM, 0.05 µM, 0.01 µM, 1 nM, 0.5 nM, 0.1 nM). In parallel cells were incubated with liposomes in the absence of lipopeptides. After 12h, all the medium was removed from the wells and cells were incubated in fresh medium for 24 h prior to the WST assay.

Flow Cytometry Measurements

HeLa cells and NIH/3T3 cells were seeded in a 24-well plate at a density of 1×10$^5$ cells per well and incubated at 37°C. After 21 h medium was removed and cells were incubated with 500 µL of nocodazole (40 µM), wortmannin (0.25 µM), chlorpromazine (40 µM), genistein (200 µM), or sodium azide 0.01% w/v in medium. After 1 h pre-incubation, inhibitors were removed and the cells were treated with 500 µl of CPK$_4$ 5 (µM) for 2 h followed by addition of 500 µl of CPE$_4$-liposomes containing PI (250 µM) in the presence of fresh inhibitors. After 15 min. liposomes and inhibitors were removed and washing steps were performed. The cells were incubated at 37 °C for 1 h. Finally the cells were detached using PBS/EDTA for 15 min, centrifuged and re-suspended in fresh medium at a concentration of 200,000 cells/ml medium. The mean fluorescence intensity of the cells was measured by flow cytometry using a Beckman Coulter Quanta SC machine.

Zebrafish Embryo Assay

Zebrafish (Danio rerio) were handled in compliance with the local animal welfare regulations and maintained according to standard protocols (http://ZFIN.org). Embryos were treated with 0.16 mM 1-phenyl-2-thiourea from 24 hours post fertilization (hpf) to prevent pigment formation. At 48 hpf, embryos were exposed in groups of 10 in 12-well plates to 5 µM CPK$_4$ at 31°C for 30 minutes; untreated embryos were used as controls. Next, embryos were washed and treated for 30 min with liposomes containing CPE$_4$, NBD-PE and DOX or PI (15 µM). Liposomes without CPE$_4$, or liposomes without PI or DOX, or with free PI or DOX added to the medium, were used as controls. After 3x washing in embryo medium, embryos were anesthetized in 0.02% tricaine methane sulfonate, mounted in 0.4% agarose and imaged by confocal microscopy.
SUPPLEMENTARY METHODS

Synthetic Procedures for Cholesteryl Amino Hemisuccinate (see Scheme S1)

3-Amino-5-cholestene(1). To a solution of 3-Azido-5-cholestene (15.8 g, 38.4 mmol) in dichloromethane, 4 equivalents of trimethylphosphine (1M solution in THF or toluene) was added and the reaction was stirred overnight. When 3-Azido-5-cholestene was consumed as evidenced by TLC (eluent: hexane), the reaction was concentrated in vacuo to yield 1 (14.8 g, 100%) as a pure compound. 1H NMR (300 MHz, CDCl₃) δ 5.27 (s, 1H), 2.60 (m, 1H), 2.17-0.85 (m, 40H), 0.64 (s, 3H); 13C NMR (75 MHz, CDCl₃) δ 141.8, 120.7, 56.8, 56.2, 52.0, 50.3, 43.4, 42.3, 39.8, 39.5, 38.2, 36.6, 36.2, 35.8, 32.7, 31.9 (× 2), 28.3, 28.0, 24.3, 23.9, 22.8, 22.6, 21.0, 19.5, 18.7, 11.9; IR (film) ν 3354, 3260, 3154, 2936, 2896, 2849, 1464, 1437, 1381 cm⁻¹; LRMS (ESI+) m/z 386.4 ([M+H]+, C₂₇H₄₈N calcd. 386.4).

Cholesteryl- 4-Amino-4- Oxobutanoic Acid (2)

To a solution of 1 (10g, 25.9mmol) in chloroform (150ml) succinic anhydride (7.8 g, 77.9 mmol) was added. Triethylamine (8ml, 57.3mmol ) was added drop-wise to the solution and the reaction mixture was stirred overnight under nitrogen atmosphere at room temperature, until the starting material was completely consumed as evidenced by TLC (eluent: hexane: ethylacetate 1:1). The reaction mixture was transferred to a separatory funnel, and the organic layer was washed with water and brine, was dried with MgSO₄ and was concentrated in vacuo to afford cholesterol amide acid (10g) as a yellow solid. The crude cholesterol amide acid was purified by recrystallization in Acetonitrile: MeOH (1:1) at 4°C and was vacuum filtered to afford 2 as an off-white solid (8.7 g, 76%) 1H NMR (400 MHz, CDCl₃) δ 5.36 (s, 1H), 3.60 (m, 1H) 2.62 (m, 2H), 2.44 (m, 2H), 2.20-1.72 (m, 7H), 1.67-1.03 (m, 20H) 1.01 (s, 3H) 0.92 (d, J = 6.5 Hz, 3H), 0.87 (dd, J = 6.6, 1.7 Hz, 6H), 0.69 (s, 3H).TLC-MS (ESI+) m/z 486.4 ([M+H]+, C₃₁H₅₁NO₃ calcd. 486.4 g/mol).

![Scheme S1. Synthetic procedure towards cholesteryl- 4-amino-4- oxobutanoic acid (compound 2).](image-url)
Synthesis of Lipopeptides (Scheme S2/3).

\(E_4\) (EIAALEK)\(_4\) and \(K_4\) (KIAALKE)\(_4\) peptides were synthesized on an automatic CEM peptide synthesizer on a scale of 250 \(\mu\)mol. Fmoc chemistry was applied for this synthesis and Sieber amide resin with a loading of 0.69 mmol/g was used. Amino acid couplings were performed with 4 eq. of the appropriate amino acid, 4 eq. of the activator HCTU and 8 eq. of the base DIPEA. Fmoc deprotection was performed with piperidine:DMF (4:6 v/v). After that \(N_3\)-PEG\(_4\)-COOH was coupled to the peptide on the resin using 4 eq. of DIPEA and 3 eq. of HOBT in DMF overnight. After that \(N_3\) was reduced to \(NH_2\) using 8 eq. of P(CH\(_3\))\(_3\) (1 M in toluene) in dioxane/H\(_2\)O 4:1 mixture. In the final step cholesteryl- 4-amino-4-oxobutanoic acid (compound 2, 2 eq) was coupled to the PEG\(_4\) linker using 5 eq. of DIPEA and 4 eq. of PyBOP in DMF:DCM (2:1) over 72 h. CPK or CPE lipopeptide was cleaved from the resin by shaking the resin with a mixture of TFA/TIS/H\(_2\)O (95:2.5:2.5 v/v) for 1.5 hour. The cleavage mixture was precipitated in cold diethyl ether. Precipitate was collected and the crude product was purified by RF-HPLC using a Vydac C4 reversed phase column (214TP1022, 22 mm diameter, 250 mm length, 10 \(\mu\)m particle size).

Scheme S2. Synthetic procedure towards lipopeptide CPK\(_4\).
Crude lipopeptides were eluted with a linear gradient from 20% to 80% of acetonitrile in water with 0.1% TFA (v/v) over 36 min with the flow of 20 mL/min. Sample elusion was detected by UV detection at 214 nm and 256 nm. The purity of the collected fractions were confirmed using LC-MS (Gemini C18 column coupled with Finningan LCQ advantage max(Thermo) ESI-MS analyze). CP₄K₄: LC-MS m/z Calcd. [1867.8 M+2H]²⁺, found 1868.2. Calcd. [1245.6 M+3H]³⁺, found 1246.2. [934.4 M+4H]⁴⁺, found 934.5. CP₄E₄: TLC-MS m/z Calcd. [1869.5 M+2H]²⁺, found 1869.9. Calcd. [1246.7 M+3H]³⁺, found 1247.1. [935.2 M+4H]⁴⁺, found 935.5.

**Cell Culture**

Hela, NIH/3T3, and CHO cells were grown as a monolayer at 37 °C in 7% CO₂ atmosphere, and were maintained in a continuous logarithmic culture in Dulbecco’s Modified Eagle Medium (DMEM) containing phenol red completed with 10% Fetal Calf Serum (FCS), penicillin/ streptomycin (100 units/ml, 0.1 mg/ml, respectively), and Glutamax (2 mM). Medium was replaced every 3 days and cells were passaged by trypsinization at 70% confluence. Uveal melanoma cells (Mel 270) were maintained in RPMI1640 medium with 10% FCS and 2 mM Glutamax.

**Liposome Preparation for Lipid Mixing Assay**

The mixing of lipids between batches of liposomes was assayed according to Struck (D. Struck, Biochemistry 1981, 20, 4093-4099). Fluorescence experiments were performed using the fluorescent resonance energy transfer (FRET) pair DOPE-
NBD (λex= 460 nm, λem= 534 nm) and DOPE-LR (λex= 550 nm, λem= 590 nm). CPE4-decorated liposomes had no fluorescent labels, while fluorescent CPK-decorated liposomes were prepared (DOPC/DOPE/CHO/DOPE-LR/DOPE-NBD 49.5:24.75:24.75:0.5:0.5 mol%). The 0% value was determined by measuring NBD emission of K liposomes to which an equal amount of PBS was added, F(t) was the fluorescence intensity measured when 100 µl of the fluorescent-labeled CPK₄-liposomes was mixed with non-labeled CPE liposomes at time t. The 100% value was determined by using liposomes which contained half the probe (NBD and LR) concentrations. The percentage of fluorescence increase (%F(t)) is calculated as %F(t)=(F(t)-F₀)/(Fmax-F₀). Lipid mixing at 4 °C was measured every 120 seconds, in between measurements the plate was kept on ice.

**Decorated Liposome Preparation for Contents Mixing Assay**

The mixing of aqueous contents between batches of liposomes was assayed according to Wilschut et al. For this assay, fluorescence time series measurements were started immediately after mixing 100 µl of the fluorescent-labeled (20 mM sulphorhodamine) liposome suspension with 100 µl of unlabeled liposome suspension in 96 well plate. The sulphorhodamine fluorescence intensity at 580 nm was monitored in a continuous fashion for 3600 seconds. After that the liposomes were lysed by the addition of 10 µl of 10 wt% Triton X-100 in PBS to obtain 100% values. The percentage of fluorescence increase (%F(t)) is calculated as %F(t)=(F(t)-F₀)/(Fmax-F₀) where F(t) is the fluorescence intensity measured at time t, F₀ is the 0% fluorescence and Fmax is the fluorescence intensity measured after addition of Triton X-100. The experiment was performed in three replicates.

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SUPPLEMENTARY FIGURES

**Figure S1. Content mixing between CPK₄ and CPE₄ liposomes.** CPE₄ decorated liposomes were loaded with 20 mM sulphorhodamine B, and mixed with CPK₄ liposomes containing PBS buffer (Red line); or mixed with CPK₄ liposomes loaded with 20 mM sulphorhodamine B as a control of leakage (black line). Total lipid concentration is 0.1 mM, with 1mol% of lipopeptides.

**Figure S2. Delivery of TOPRO by peptidated-liposomes is dependent on coiled-coil formation between CPK₄ and CPE₄.** Confocal microscopy images of Hela cells. Cells were pre-incubated with CPK (A, B, C) or medium (D, E, F) for 2 hours, followed by a 15 minute treatment with: CPE₄-decorated liposomes containing TOPRO (A, E), liposomes containing TOPRO (B), CPE₄-decorated liposomes plus free TOPRO (C), liposomes plus free TOPRO (D), or free TOPRO (F). Green: NBD, Red: TOPRO. Scale bar is 25 µm. Overlay is red and green channel plus bright field image.
Figure S3. Delivery of TOPRO3 by CPE$_x$-decorated liposomes to various CPK$_y$-functionalized cells. Cells were pre-treated with CPK$_x$ for 2 h and incubated with CPE$_x$-decorated liposomes containing TOPRO and 1 mol\% NBD-DOPE for 15 min. Images were taken after washing three times with medium. Green, NBD; Red, TOPRO3. Scale bar is 25 µm.
Figure S4. Delivery of PI by CPE$_{4}$-decorated liposomes to various CPK$_{4}$-functionalized cells. Cells were pre-treated with CPK$_{4}$ for 2 h and incubated with CPE$_{4}$-decorated liposomes containing PI dye and 1mol% NBD-DOPE for 15 min. Images were taken after washing twice with PBS. Green, NBD; Red, PI. Scale bar is 25 µm.
Figure S5. A) Cell viability assay by WST of Hela (green); NIH/3T3 (red); CHO (blue) cells exposed to CPK
subscript 4, CPE
subscript 4, liposomes with lipid composition of DOPC:DOPE:CHO (2:1:1), and combinations thereof. Metabolic activity of untreated cells (ctrl) is 100%. Concentrations: liposome (lipids) 0.25 mM; CPK
subscript 4, CPE
subscript 4: 5 µM; CPE decorated liposomes contained 1 mol% of lipopeptide. B and C) Cell viability assay by WST of Hela cells exposed to different concentration of CPE
subscript 4 (B) or CPK
subscript 4 (C) for 2 h or 24 h. Metabolic activity of untreated cells (ctrl) is 100%. Error bars are standard deviation of three independent experiments.
Figure S6. DOX delivery to HeLa cells. Cells were treated with CPK$_4$ (A, B, C) or medium (D), followed by incubation with CPE$_4$-liposomes containing with DOX (A), or plain liposomes loaded with DOX (B), or CPE$_4$ liposomes with free DOX (C), or free DOX (D). The scale bar represents 25 µm.

Figure S7. Cell toxicity of doxorubicin controls. HeLa cells were treated with CPK$_4$ for 1 h and plain liposomes containing DOX (black), or untreated HeLa cells incubated with CPE$_4$ decorated liposomes loaded with DOX (red) for 12 h, after washing and incubation with medium for 24 h, cell viability was measured by WST assay.
Figure S8. Lipid mixing between CPK₄ and CPE₄ decorated liposomes at 4°C and room temperature.

Figure S9. Control experiments for in vivo DOX delivery. Zebrafish were incubated with embryo medium (A) or embryo medium plus 5 µM CPK₄ (B) for 1 h, followed by incubation with either CPE₄ decorated liposomes containing DOPE-NBD and DOX (15 µM) (A) for 1 h or non-decorated liposomes containing DOPE-NBD and DOX (15 µM) (B).
Figure S10. Delivery of propidium iodide (PI) into the skin cells of a zebrafish embryo by membrane fusion. Confocal microscopy images of a 2-day old zebrafish. The embryo was first pre-incubated with CPK, for 30 min, followed by 30 min treatment with CPE-decorated liposomes containing PI. Green: NBD, Red: PI. Scale bar is 10 µm. Overlay is red and green channel. The full embryo image is a compilation of four overlapping confocal stacks spanning the entire embryo.

Figure S11. Control zebrafish embryos exposed to CPK (b, c) or egg water (a) for 30 min followed by treatment with: CPE-decorated liposomes containing PI (a), liposomes containing PI (b), CPE-decorated liposomes plus free PI (c). Overlay of green (NBD) and red (PI) channels. Images are compilations from 3-4 overlapping confocal stacks spanning the entire embryo.
**Figure S12.** Loading efficiency of DOX into liposomes. Absorption of series of concentrations of free DOX.

**SUPPORTING REFERENCES**