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ARTICLE

Application of Coiled Coil Peptides in Liposomal Anticancer Drug Delivery Using a Zebrafish Xenograft Model

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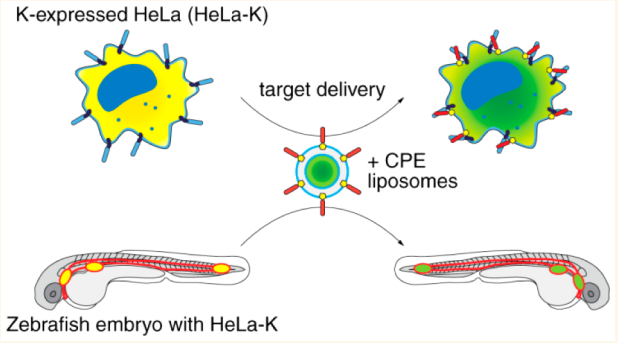
S *Supporting Information*

ABSTRACT: **The complementary coiled coil forming peptides E_4 [$(EIAALEK)_4$] and K_4 [$(KIAALKE)_4$] are known to trigger liposomal membrane fusion when tethered to lipid vesicles in the form of lipopeptides. In this study, we examined whether these coiled coil forming peptides can be used for drug delivery applications. First, we prepared E_4 peptide modified liposomes containing the far-red fluorescent dye TO-PRO-3 iodide (E_4-Lipo-TP3) and confirmed that E_4-liposomes could deliver TP3 into HeLa cells expressing K_4 peptide on the membrane (HeLa-K) under cell culture conditions in a selective manner. Next, we prepared doxorubicin-containing E_4-liposomes (E_4-Lipo-DOX) and confirmed that E_4-liposomes could also deliver DOX into HeLa-K cells. Moreover, E_4-Lipo-DOX showed enhanced cytotoxicity toward HeLa-K cells compared to free doxorubicin. To prove the suitability of E_4/K_4 coiled coil formation for *in vivo* drug delivery, we injected E_4-Lipo-TP3 or E_4-Lipo-DOX into zebrafish xenografts of HeLa-K. As a result, E_4-liposomes delivered TP3 to the implanted HeLa-K cells, and E_4-Lipo-DOX could suppress cancer proliferation in the xenograft when compared to nontargeted conditions (*i.e.*, zebrafish xenograft with free DOX injection). These data demonstrate that coiled coil formation enables drug selectivity and efficacy *in vivo*. It is envisaged that these findings are a step forward toward biorthogonal targeting systems as a tool for clinical drug delivery.**



KEYWORDS: *α-helices, DDS, lipopeptides, xenotransplantation, chemotherapeutic agent, animal testing*

The design of anticancer drug delivery systems is of great interest, as many of the drugs in the clinic cause serious toxic side effects due to nonspecific cytotoxicity, outweighing the therapeutic effect. Therefore, targeted drug delivery systems are being developed, for example, micellar-, liposomal-, and nanoparticle-based drug formulations combined with peptide or surface-antibody targeting, photochemical internalization, and ultrasound stimulation.[1] Of these, liposomes are the most clinically established nanosystems for drug delivery, *e.g.*, doxorubicin (DOX)-containing liposomes.[2,3] However, these clinically approved drugs are nontargeted and rely on the enhanced permeability and retention effect, resulting in cardiotoxicity, thereby limiting their cumulative dose in cancer patients. To overcome this problem, liposomes have been conjugated with active targeting ligands, such as antibodies and cell-penetrating peptides, for target-specific drug delivery.[4−6] However, as these targeted ligands frequently interact with natural membrane receptors, liposome internalization occurs *via* endocytosis. As a result, drug accumulation in early endosomes induces lysosomal activation, leading to rapid degradation of these drugs inside cells.[7] Furthermore, the number of these "cell-specific" receptors is limited, thereby putting restrictions on this type of drug delivery system.

Therefore, it is important for the development of new drug delivery systems to avoid lysosomal degradation in order to enhance the delivery efficiency. We decided to design an

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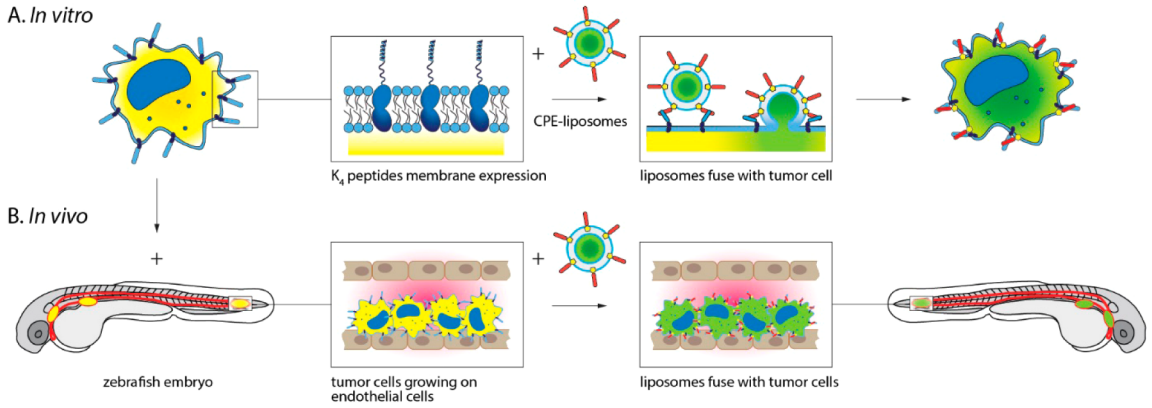
Scheme 1. Drug Delivery by E4/K4 Coiled Coil Formation in Cells (A) and Zebrafish (B)



artificial biorthogonal targeting system that would be able to target liposomes and other nanoparticles more efficiently to the tissue of interest. In addition, this targeting system should also be able to enhance cellular uptake, preferentially in a nonendocytic manner. For this, synthetic coiled coils are attractive candidates as a new targeting motif.[8,9] Coiled coil motifs are found in approximately 10% of all protein sequences in nature,[10] and many of these noncovalent peptide motifs play a vital role in the efficient transport of molecules across membranes. For example, in the process of HIV infection, viral entry into CD4-positive cells is accomplished by intramolecular coiled coil formation between helices of viral glycoprotein gp41.[11] In neuronal exocytosis, docking of transport vesicles to the target plasma membrane is mediated by the coiled coil formation of complementary SNARE protein subunits on the opposing membranes.[12] This forces the opposing membranes into close proximity, resulting ultimately in lipid mixing followed by pore formation and concomitant content transfer. These natural systems have inspired researchers to design drug delivery systems based on noncovalent binding of two macromolecules. To introduce bioactive compounds into the cytosol of the target cells, various non-natural coiled coils have been used to decorate nanoparticles,[13,14] which in turn are complexed to DNA antisense oligonucleotides,[15] short interfering RNAs,[16] proteins,[17] drugs,[18,19] or vaccines.[20] Recently coiled coil motifs have also been used in drug-free therapeutic systems.[21,22]

In our previous study, we developed a fully synthetic membrane fusion system composed of a complementary pair[23] of lipidated coiled coil peptides, denoted K and E,[18] which are covalently linked to cholesterol anchors *via* a poly(ethylene glycol) linker, yielding lipopeptides CPK and CPE.[24] We demonstrated that this complementary lipopeptide pair was able to induce efficient fusion between liposomes.[25,26] The E/K coiled coil formation is thought to be responsible for specific molecular recognition,[27,28] and recently membrane fusion between liposomes and cells without triggering endocytosis was achieved (Yang *et al.*, unpublished data). This system seems suitable for drug delivery, as the drug inside these liposomes avoids lysosomal degradation. While coiled coil formation induced membrane fusion has shown to be successful to deliver drugs *in vitro*, it has to be confirmed whether these synthetic peptide pairs are also functional in an *in vivo* environment. In this study we therefore used a human cancer cell xenograft in a zebrafish embryo to investigate whether coiled coil formation can be used to specifically deliver drugs to tumor cells *in vivo*.

RESULTS AND DISCUSSION

E_4-Liposomes Deliver TP3 to HeLa Cells That Genetically Express K_4 Peptide *in Vitro*. Our objective is to use a pair of complementary coiled coil forming peptides, E and K,[29] of which one is conjugated to a liposome to introduce chemicals into peptide-conjugated cancer cells in zebrafish (Scheme 1). The coiled coil peptide E, $(EIAALEK)_4$, was conjugated to a PEG_4 spacer (PEG = poly(ethylene glycol)) and cholesterol linker, yielding lipopeptide CPE_4, then inserted into the bilayer of liposomes containing the fluorescent dye TO-PRO-3 iodide (TP3) (denoted E_4-Lipo-TP3; Scheme 1A). TP3 is a DNA-intercalating fluorescent dye and cell membrane impermeable and stains DNA only when it is actively taken up by live cells.[30] We previously confirmed that E_4-Lipo-TP3 could deliver TP3 into the cytosol of HeLa cells that were pretreated with cholesterol-PEG_4-K_4 (CPK_4).[28] While targeted drug delivery under *in vitro* conditions was thus successfully achieved, demonstrating the *in vivo* functionality of these synthetic peptides would be a significant step forward. Due to the fast turnover of membrane components in dividing cells, CPK_4 inserted in a cell membrane is not expected to remain throughout the zebrafish xenotransplantation setup and experiments. For this reason, we created HeLa cells constitutively expressing a genetically encoded K_4 peptide on their cell membrane (denoted HeLa-K), by fusing it to the transmembrane domain of the human platelet-derived growth factor receptor (PDGFR-TMD).[31] The fusion protein is preceded by a mouse IgK-leader sequence for efficient secretion and localization on the cell membrane of the K_4-fusion protein. Functional display of the K_4 peptide on the outside of the cell membrane was verified in a binding experiment using a carboxyfluorescein-labeled E_4 peptide (fluorescent E_4; Figure S1A). Analogously to HeLa-K cells, we also created HeLa-E cells that potentially expressed E_4 peptide on their cell membrane and HeLa-ctrl cells that only expressed the transmembrane domain of PDGFR. Fluorescently labeled CPK_4-liposomes (K_4-Lipo-NBD) were bound to the cell membrane of HeLa-E cells (Figure S2); however fluorescent E_4 peptide was taken up by the cells (Figure S1C). Ono *et al.* already demonstrated that the positive charge of the K_4 peptide allowed nonspecific binding to negatively charged cell membranes.[32] Thus, we decided to continue with HeLa-K cells and E_4 peptide in the following study.

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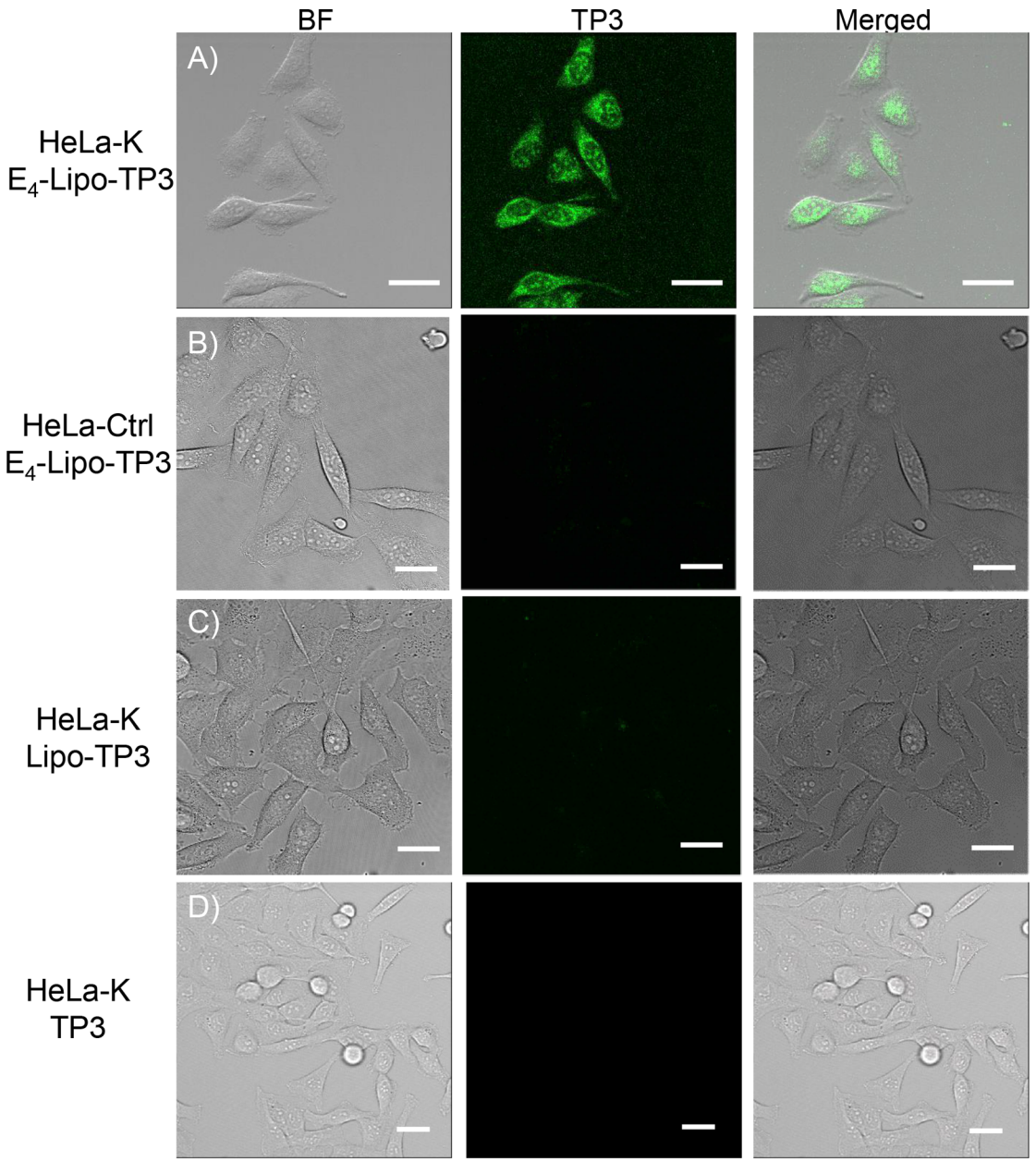

Figure 1. E_4/K_4 coiled coil formation mediated TP3 delivery into the cytosol. HeLa-K or control HeLa (HeLa-ctrl) cells were treated with 0.25 mM E_4-Lipo-TP3, Lipo-TP3 (without E_4 peptide), or 2.5 μM free TP3 for 15 min. After three washes with culture medium, the cells were imaged. E_4-Lipo-TP3 can deliver the TP3 into HeLa-K cells (A), not into HeLa-ctrl cells (B). (C) Without E_4 peptide, liposomes could not deliver the TP3 into HeLa-K cells. (D) TP3 without liposome also could not deliver TP3. Green: TP3. The scale bar represents 25 μm.

To investigate whether E_4-liposomes could deliver TP3 to HeLa-K cells, HeLa-K cells were exposed to E_4-Lipo-TP3 for 15 min. This resulted in the appearance of a bright TP3 signal in the cells (Figure 1A). Control experiments in which non-modified TP3-liposomes (Lipo-TP3; Figure 1C) or free TP3 (Figure 1D) was added to the HeLa-K cells showed less fluorescent signal in the cells. In addition, when E_4-Lipo-TP3 was added to control HeLa cells (HeLa-ctrl), which express only PDGFR-TMD, also no TP3 signal was observed (Figure 1B). These results indicate that the coiled coil formation between E_4 on the liposome and K_4 on the cell membrane leads to delivery of liposomal contents into the cell in a selective manner.

E_4-Liposomes Can Deliver TP3 in a Zebrafish Xenograft of HeLa-K Cells. Zebrafish is becoming a suitable model for characterization of nanoparticles against cancer, because of phenotypic and molecular conservation of cancer development in zebrafish and humans.[33] In addition, the transparency of zebrafish embryos enables visualizing fluorescently labeled cancer cells and nanoparticles through their body wall. To monitor the fate of the implanted cells in zebrafish, HeLa-K and HeLa-ctrl cells were labeled with a red fluorescent protein that was expressed from a transfected retroviral vector expressing tdTomato (tdTom). We injected the resulting HeLa-K-tdTom or HeLa-ctrl-tdTom cells into the blood circulation of zebrafish larvae at 48 h postfertilization (hpf), according to a previous study.[34] We used transgenic zebrafish that express vascular-specific EGFP to visualize their vasculatures. Five hours post cell injection (hpi), we confirmed that the injected cells accumulated in the caudal hematopoietic

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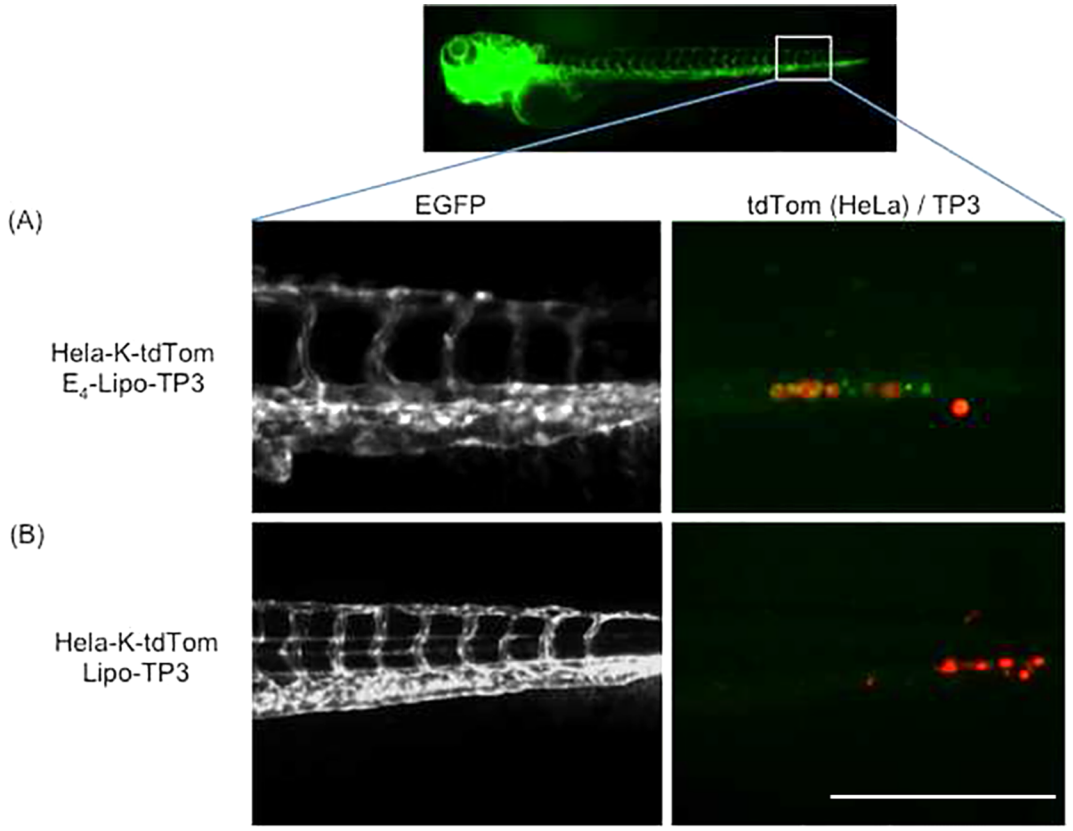

Figure 2. E_4/K_4 coiled coil formation delivers the content in the liposome to cancer cells in the xenograft zebrafish. HeLa-K cells (50−100 cells) were injected into the duct of Cuvier of 48 hpf zebrafish. Five hours after implantation (hpi), 1 nL of 1 mM E_4-Lipo-TP3 or Lipo-TP3 was injected into the CV and imaged at 72 hpf. (A) E_4-Lipo-TP3 can deliver the TP3 to HeLa-K in the xenograft zebrafish, while (B) Lipo-TP3 could not. White, red, and green indicate vasculatures, cancer cells, and TP3, respectively. The scale bar represents 200 μm.

(CHT) region. Then, E_4-Lipo-TP3 or Lipo-TP3 was injected into the circulation from the posterior cardinal vein (CV). Immediately after E_4-Lipo-TP3 injection, no TP3 signal could be detected in the xenografts. However, at 24 hpi, TP3 accumulation was observed in HeLa-K-tdTom cells in the CHT region (Figure 2A). Control experiments in which one of the two peptides was omitted (HeLa-K with Lipo-TP3 (Figure 2B) and free TP3 [Figure S2A], and HeLa-ctrl with E_4-Lipo-TP3 [Figure S2B]) showed no TP3 uptake by the HeLa cells. These results demonstrated that E_4/K_4 coiled coil formation regulates delivery of liposomal cargo to the targeted cells in an *in vivo* setting.

E_4/K_4 Coiled Coil Formation Enhances DOX Cytotoxicity. Having demonstrated the feasibility of the E_4/K_4 system to target xenografted cancer cells in zebrafish, we next investigated whether our E_4-liposomes could also deliver an anticancer drug such as doxorubicin (E_4-Lipo-DOX). DOX has been commonly used as a routine anticancer drug in combined chemotherapy against a variety of tumors.[35] DOX interacts with DNA by intercalation and disruption of topoisomerase-II-mediated DNA repair, ultimately leading to cell death.[36] Noteworthy, DOX is a popular research tool due to its inherent fluorescence associated with the central anthracycline chromophore group. This allows visualization of DOX distribution in various tissues or cells *via* fluorescence imaging.[37] Indeed, we were able to visualize DOX intake into HeLa-K cells. After 15 min incubation with 0.25 mM E_4-Lipo-DOX (1 mM DOX-containing E_4-liposome, equal to 0.25 mM free-DOX concentration), DOX fluorescent signal was already observed in the cytosol (Figure 3A). Five hours after washout of E_4-Lipo-DOX from the culture medium, DOX signal had increased (Figure 3B). Mohan *et al.* reported that the DOX fluorescence

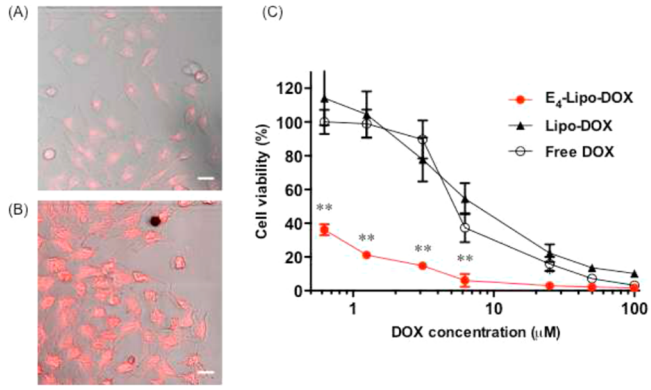

Figure 3. E_4/K_4 coiled coil formation promotes doxorubicin intake and cytotoxicity. HeLa-K cells were incubated with 0.25 mM E_4-Lipo-DOX for 15 min, and after three washes with culture medium, the cells were imaged (A). After 5 h, the cells were imaged again using the same settings (B). Red indicates DOX. The scale bar represents 25 μm. For images of negative controls, see supplementary Figure S3. (C) Cell viability after DOX delivery. HeLa-K cells were incubated with different concentrations of E_4-Lipo-DOX (red circles), Lipo-DOX (black triangles), or free-DOX (open circles) for 12 h, then washed three times. Twenty-four hours after treatment, cell viability was measured. n = 4, error bar indicates SD. *P < 0.05.

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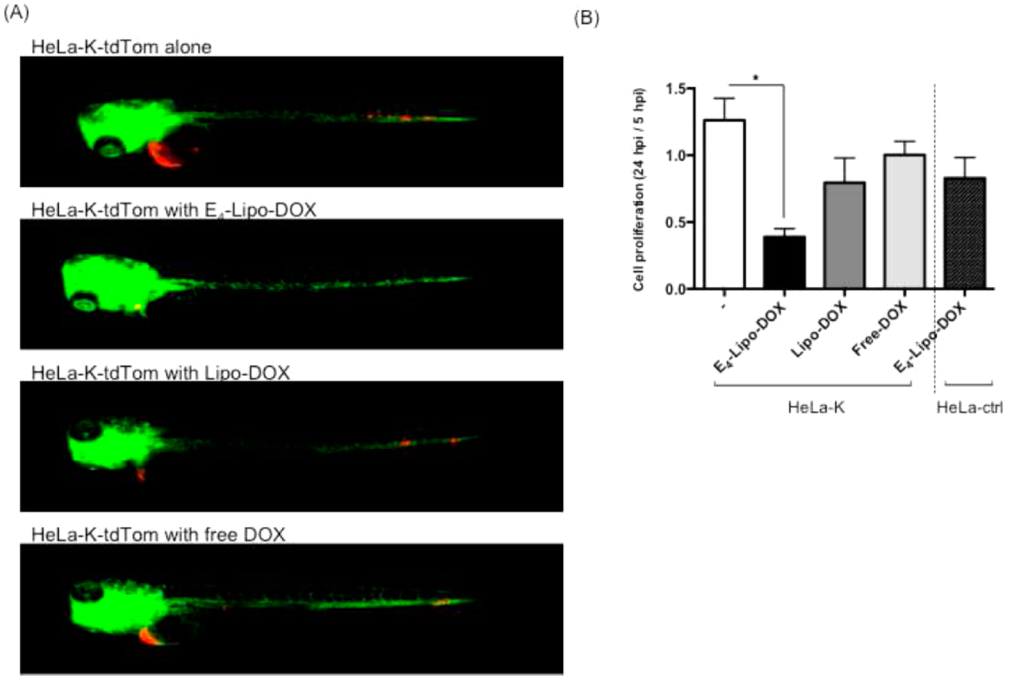

Figure 4. E_4/K_4 coiled coil formation enhances anticancer property of doxorubicin in the xenografts. (A) HeLa-K or HeLa-ctrl cells were injected into the duct of Cuvier of 48 hpf zebrafish. After 5 h, 1 nL of 1 mM E_4-Lipo-DOX, Lipo-DOX, or 0.25 mM free-DOX was injected from the CV and imaged at 72 hpf. HeLa-K xenograft with E_4-Lipo-DOX injection (left upper panel) shows the decrease of the injected cells. Green and red indicate vasculatures and cancer cells, respectively. (B) Quantification of the cancer proliferation in the xenografts. Cancer cell proliferation was calculated as the ratio of tdTomato fluorescence intensity in the tumor area relative to that at 5 hpi. n = 10−12, error bar = +SE, *P < 0.05.

inside cells increases in a time-dependent manner because of the alternation of DOX's binding partners: DNA, histones, and phospholipids.[37] Thus, we assumed that DOX-phospholipid and DOX-DNA-histone complexes would be detected at this time point. In contrast, control experiments in which E_4 peptide was omitted from the liposomes (HeLa-K with Lipo-DOX [Figure S3A] or using free DOX [Figure S3B]) showed very low cellular uptake of DOX. As expected, free DOX was not taken up by HeLa-ctrl (Figure S3C) and HeLa-E cells (Figure S3D), similarly to HeLa-K. We repeated the experiment in the presence of several well-known endocytosis inhibitors in order to study the pathway of cellular uptake (Figure S6). It was shown that uptake *via* endocytosis was the minor pathway, suggesting that fusion might be the major pathway. However, additional studies are required to confirm this finding.

To investigate whether coiled coil mediated delivery enhances the cytotoxicity of DOX, we treated HeLa-K cells with E_4-Lipo-DOX, Lipo-DOX, or free DOX for 12 h. As a result, 1 μM E_4-Lipo-DOX exhibited around 80% reduction in the number of HeLa-K cells, while free DOX had no measurable effect at the same concentration (Figure 3C). In addition, Lipo-DOX showed the same toxicity as that of free DOX to HeLa-K cells. This result indicates that E_4/K_4 coiled coil formation increases the cytotoxicity of DOX and reduces the dose required for the induction of cancer cell death. Interestingly, under nontargeting conditions with free DOX, HeLa-ctrl cells (Figure S4, open circles) seem more sensitive than HeLa-K cells (Figure 3C, open circles). Membranous expression of K_4 peptide might have some protective effects to the cells; however the IC_{50} values of DOX for HeLa-ctrl and HeLa-K cells are similar: 4.0 and 5.3 μM, respectively.

E_4/K_4 Coiled Coil Formation Enhances Anticancer Efficacy of DOX in Xenografts. To investigate the toxicity of DOX toward cancer cells *in vivo*, we conducted another zebrafish xenograft experiment similar to the TP3 study above. We injected HeLa-K-tdTom cells into the circulation from the duct of Cuvier at 48 hpf, followed by E_4-Lipo-DOX, Lipo-DOX, or free DOX injection at 5 hpi. Twenty-four hours after DOX injection, these xenografts were imaged using fluorescent microscopy (Figure 4A). As illustrated by the images in Figure 4A, the fluorescence of the Hela-K-tdTom cells was substantially more reduced by treatment with E_4-Lipo-DOX (second image) than by treatment with either Lipo-DOX (third image) or free DOX (fourth image). Quantification of the fluorescent intensities showed that injection of E_4-Lipo-DOX resulted in a significantly (P < 0.05) reduced tumor cell proliferation compared to Lipo-DOX or free DOX treatment (Figure 4B). Other nontargeting conditions (HeLa-K-tdTom with Lipo-DOX and free-DOX) did not exhibit significant reduction of cancer cell burden, similarly to the *in vitro* experiment in Figure 3C. As described in the Materials and Methods, we injected 1 nL of 1 mM liposomes containing 0.25 mM DOX. Based on a wet weight estimation of 240 μg for zebrafish larvae,[38] the injection volume of DOX was equal to 1 μmol/kgBW (kilogram per body weight). In the clinic, DOX is usually injected into the circulation of cancer patients at a concentration of 2.5 mg/kgBW, which equals 5.16 μmol/kgBW.[39] Therefore, the administered amount of DOX using E_4-liposomes was 5-fold lower than that used in a clinical setting with lesser opportunity for exhibition of cardiac toxicity, while the anticancer efficacy of E_4-Lipo-DOX is far greater than that of free-DOX.

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CONCLUSION

Targeted drug delivery systems should increase the efficacy of a drug and concomitantly reduce the toxic side effects caused by off-target reactions. Coiled coil motifs were recently used in a "drug-free therapeutic system" to specifically kill cancer cells.[40] In this study, we demonstrated that coiled coil formation between liposomes and live cells enables the *in vivo* delivery of an anticancer drug encapsulated in CPE-decorated liposomes to the targeted cells in an animal model. This is one of the first examples using a synthetic coiled coil motif for *in vivo* targeted drug delivery. Although this system currently requires K_4 peptide expression on the cell membrane of targeted cells in zebrafish xenografts, this method can be used for animal testing of a wide range of drug candidates because of the surprising degree of functional conservation in basic cell-biological processes between zebrafish and mammals.[41] Furthermore, the combination of coiled coil mediated delivery and zebrafish xenografts of human cancer has the potential to become a powerful and rapid *in vivo* drug-screening platform. For future clinical applications, however, it will be necessary to introduce K_4 peptide to the targeted cells or tissues of interest (*e.g.*, tumor). This might be achieved by injection of a PEGylated CPK that can be locally deshielded by irradation with light,[27] conjugation of K_4 peptide to cancer-specific cell membrane antibodies (*e.g.*, Her2 antibody[42]) or direct introduction of our K_4-PDGFR-TMD construct using adeno-associated virus vector injection.[43] The proposed improvements of the current method should enable spatiotemporal control of liposomal drug targeting in the near future. These approaches to introduce K_4 peptide into diseased tissues or cells combined with the enhanced drug selectivity circumventing lysosomal degradation might increase the drug efficacy and reduce toxic side effects.

MATERIALS AND METHODS

Zebrafish Strain, Husbandry, and Egg Collection. Tg (fli1:EGFP) zebrafish[44] were used in this study. Livestock were maintained and handled according to the guidelines from http://zfin.org. Fertilization was performed by natural spawning at the beginning of the light period, and eggs were raised at 28 °C. All experimental procedures were conducted in compliance with the directives of the animal welfare committee of Leiden University.

Plasmids. The insert designs for membrane expression of K_4 and E_4 peptide are described in Supplementary Methods. The insert sequences were synthesized by BaseClear (Leiden, The Netherlands), then digested with SalI and NotI and ligated into episomal plasmid pEBMulti-Hyg (Wako Pure Chemicals, Osaka, Japan) to create pEBM-K and pEBM-E. The backbone vector harbors OriP (replication origin) and EBNA-1 derived from Epstein–Barr virus, which allows the distribution of this plasmid to daughter cells by episomal replication to generate stable transformants.[45]

Cells. HeLa human cervical cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FCS; Life Technologies), 100 units/mL of penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100 μg/mL streptomycin (Sigma-Aldrich) at 37 °C with 5% CO_2. To construct HeLa-K and HeLa-E cells, HeLa cells were transfected with pEBM-K and pEBM-E plasmids, respectively, and cultured in the presence of hygromycin B (200 μg/mL; Sigma-Aldrich) for at least 2 weeks to obtain stable transformants. To create control cells (HeLa-ctrl), HeLa cells were transfected with pEBM harboring PDGFR-TMD (see Supplementary Methods). For tdTomato fluorescent protein expression, the cells were infected with tdTomato lentivirus (pLenti-tdTom-Bla; a gift from Dr. Maciej Olszewski) according to the standard protocol. The tdTomato cells were cultured in the presence of blasticidin (10 μg/mL; Life Technologies) to obtain stable transformants.

Cell Imaging. The cells were seeded in an eight-well slide (μ-Slide 8 well; Ibidi, Munich, Germany) at a density of 2.5×10^4 cells per well in DMEM–10% FCS medium without phenol red and cultured overnight. Then, the medium was refreshed and test compounds were added (TP3 or DOX-containing liposomes, which were diluted to a final concentration using DMEM [− phenol red)]. After incubation for 15 min, cells were washed three times with medium. The fluorescent images were acquired using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) and merged with Leica application suite advanced fluorescence software (Leica Microsystems) or ImageJ software (National Institutes of Health, Bethesda, MD, USA). The wavelength settings for TP3 and DOX were Ex/Em: 641/662 nm (Ex laser: 633 nm) and 480/580 nm (Ex laser: 532 nm), respectively.[46]

Zebrafish Xenografts and Compound Treatment. HeLa cell implantation was performed as previously reported.[34] In brief, dechorionized 48 hpf zebrafish were anaesthetized with 0.003% tricaine (MS222; Sigma-Aldrich). Then trypsinized HeLa cells (100–200 cells) were injected into the duct of Cuvier by using a Pneumatic Pico pump and a manipulator (World Precision Instruments, Sarasota, FL, USA). After implantation, zebrafish were maintained at 34 °C. Five hours after implantation, 1 nL of test compounds was injected into the caudal vein using the Pico pump with a fine glass needle. For live imaging, xenografts were anaesthetized and mounted in 0.6% low-melting agarose. Fluorescent image acquisition was performed using a Leica MZ16FA stereo-microscope (Leica Microsystems). Images were adjusted for brightness and contrast using ImageJ.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.6b01410.

Peptide synthesis, liposome preparation, Dox release; cell assays, Dox delivery (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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