Dual Peptide Conjugation Strategy for Improved Cellular Uptake and Mitochondria Targeting

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Supporting Information

ABSTRACT: Mitochondria are critical regulators of cellular function and survival. Delivery of therapeutic and diagnostic agents into mitochondria is a challenging task in modern pharmacology because the molecule to be delivered needs to first overcome the cell membrane barrier and then be able to actively target the intracellular organelle. Current strategy of conjugating either a cell penetrating peptide (CPP) or a subcellular targeting sequence to the molecule of interest only has limited success. We report here a dual peptide conjugation strategy to achieve effective delivery of a non-membrane-penetrating dye 5-carboxyfluorescein (5-FAM) into mitochondria through the incorporation of both a mitochondrial targeting sequence (MTS) and a CPP into one conjugated molecule. Notably, circular dichroism studies reveal that the combined use of α-helix and PPII-like secondary structures has an unexpected, synergistic contribution to the internalization of the conjugate. Our results suggest that although the use of positively charged MTS peptide allows for improved targeting of mitochondria, with MTS alone it showed poor cellular uptake. With further covalent linkage of the MTS-5-FAM conjugate to a CPP sequence (R₈), the dually conjugated molecule was found to show both improved cellular uptake and effective mitochondria targeting. We believe these results offer important insight into the rational design of peptide conjugates for intracellular delivery.

INTRODUCTION

Mitochondria exhibit vital and lethal functions in both physiological and pathological conditions because they provide the most cellular energy and also function as a regulator of intrinsic pathway of apoptosis. Recently, changes in mitochondria and their interaction with many proteins were found to be responsible for many diseases including neurodegeneration and cancer. Dysfunctional mitochondria are also known to have a profound impact on the development of a host of chronic and aging related conditions. Despite the great potential as a therapeutic target, only a few drugs are able to actually accumulate in mitochondria. Drug delivery systems that could transport therapeutic agents specifically into mitochondria are therefore of critical importance to fulfill this goal.

Current approaches for a successful mitochondrial targeting have been developed based on various mechanisms including lipophilic cations such as triphenyl phosphonium (TPP), mitochondrial targeting signal peptide (MTS), protein transduction domain peptide (PTD), and DQsomes-DNA complexes. Chemical conjugation of the therapeutic agent to a molecule with the ability to target mitochondria provides an effective strategy and has been used to deliver a great diversity of cargo into mitochondria, including small molecule drugs, oligodeoxynucleotides, macromolecules, liposomes, and proteins. Among various targeting agents, mitochondria targeting sequence (MTS), the protein-sorting peptide consisting of typically 20–40 residues (a.k.a. protein tags), could arguably represent the best conjugation platform for targeted delivery of molecules into mitochondria because MTS has a well-proven role in guiding proteins into mitochondria and also because MTS can be specifically and precisely processed by mitochondrial processing proteinases (MPP) to release the delivered molecular cargo in the mitochondria. Despite the difference in amino acid sequence, all the mitochondria targeting sequences share the common amphipathic helical feature, and can be recognized by the same receptors and apparatus on the mitochondrial surface. Although precursor sequences derived from natural mitochondrial proteins exhibit high specificity in mitochondrial targeting, several reports have shown that naturally derived MTS peptides have lower levels of cellular uptake despite their amphipathic and positively charged nature. Therefore, an additional advantage of using MTS is that it can be chemically modified to deliver a variety of therapeutics, including hydrophilic molecules.
A feature must be included in the design to improve the intracellular access of MTS conjugates.

One widely used strategy to achieve effective intracellular access is to conjugate the therapeutic cargo with a cell-penetrating peptide (CPP) such as octaarginine (R8). Futaki et al. have shown that the secondary structure of the conjugated octaarginine (R8) is more favorable to enter cells primarily through adsorption-mediated endocytosis.

MTS-(5-FAM)-H3R8 was conjugated to this mitochondrial targeting system only. Cell penetrating peptide (CPP)25 possesses cell membrane-penetrating capability, but attaching to the cell membrane via nonspecific electrostatic interactions. To achieve improved mitochondrial targeting specificity and efficacy, we employed a dual peptide conjugation strategy that incorporates both CPP and MTS into one molecule. Notably, we found that the secondary structure of the conjugated peptides (both α-helices and polyproline II, PPII-like conformation) exhibits a synergistic effect on the cellular uptake of the resulting conjugate. Scheme 1 shows the molecular design and peptide sequences of the three reported conjugates: MTS-5-FAM, 5-FAM-H3R8, and MTS-(5-FAM)-H3R8. MTS-5-FAM possesses an α-helical secondary structure and showed no cell membrane-penetrating capability, but attaching to the cell membrane only. Cell penetrating peptide 5-FAM-H3R8 exhibited limited cellular uptake. Significant endocytosis was only observed for MTS-(5-FAM)-H3R8 containing both MTS and H3R8.

Scheme 1. Schematic Illustrations of (A) the Three Peptide Conjugates: MTS-5-FAM, 5-FAM-H3R8, and MTS-(5-FAM)-H3R8, and (B) Different Cellular Uptake of the Three Conjugates

MTS-5-FAM possessed α-helical secondary structure and showed no cell membrane-penetrating capability, but attaching to the cell membrane only. Cell penetrating peptide 5-FAM-H3R8 exhibited limited cellular uptake. Significant endocytosis was only observed for MTS-(5-FAM)-H3R8 containing both MTS and H3R8.

molecular design and peptide sequences of the three reported conjugates. The MTS peptide, MLRAALSTARGPRLSLRLL, a well-studied natural precursor from the mitochondrial-oriented protein aldehyde dehydrogenase (ALDH), was selected as the mitochondrial targeting entity.42,43 The CPP R8 was conjugated to this mitochondrial targeting system because of its remarkable ability to assist in cellular internalization. Three histidines (H3) were introduced in the molecular design to offer the buffering effect upon protonation of the imidazole ring inside endosomes or lysosomes, which has been reported to induce the rupture of the endosomal/lysosomal membrane for the effective release of the entrapped conjugates into cytosols.44-46 5-FAM was used as the fluorescent tracking agent due to its high quantum efficiency.

RESULTS AND DISCUSSION

Conjugate Characterization. The purities of the four studied conjugates were all above 90% according to analytical HPLC analysis (Supporting Information Figures S1–S4). The observed multiply charged ions of MTS-5-FAM, 5-FAM-H3R8, MTS-(5-FAM)-H3R8, and 5-FAM-(RLL)3R8 suggest that the masses of the four conjugates were 2263.0, 2722.2, 4381.6, and 2616.6 Da, respectively, by ESI mass spectrometry, in agreement with the expected exact masses calculated for C12H17N6O20 (2263.5 Da), C12H16N6O2S (2723.2 Da), C12H13N7O2S (4381.5 Da), and C12H14O4S (2615.6 Da). Since the imidazole side-chain of histidine has an acid dissociation constant (pKₐ) around 6, histidines under physiological conditions are not expected to be highly charged. Thus, 5-FAM-H3R8 carries more positive net charges at its arginine guanidinium groups (pKₐ around 12) when compared with that of MTS-5-FAM. MTS-(5-FAM)-H3R8 and 5-FAM-(RLL)3R8 are expected to carry the most positive charges.

Cellular Uptake. We first used flow cytometry to investigate the cellular uptake efficacy of the three designed conjugates: 5-FAM-H3R8, MTS-5-FAM, and MTS-(5-FAM)-H3R8. In these experiments, cells were trypsinized to avoid false positive signals resulting from conjugates associated with the cell membrane via nonspecific electrostatic interactions.47 Figure 1A reveals clearly that MTS-(5-FAM)-H3R8 shows superior cellular entry capability in comparison to both MTS-5-FAM and 5-FAM-H3R8, as evidenced by its strongest intensity (Supporting Information Figure S5). It is very surprising to see that MTS-5-FAM actually showed greater fluorescence intensity than 5-FAM-H3R8. Because increased positive charge would likely lead to improved cellular uptake against various cell lines via electrostatic interaction with negatively charged cell membranes,48 one would expect a lower level of cellular uptake of MTS-5-FAM relative to that of 5-FAM-H3R8. The counterintuitive observation of higher fluorescence signal for MTS-5-FAM might be related to a difference in secondary
structures of MTS peptide versus R$_p$, which is also known to play a role in cellular uptake. Schepartz et al. reported that cellular uptake of arginine-rich peptides was heavily dependent on the positive charge density, and the cellular uptake was enhanced when the arginines were clustered onto the same α-helical face.49

We therefore performed circular dichroism experiments to investigate the secondary structures for all the synthesized conjugates. In order to mimic the hydrophobic environment in which peptide conjugates interact with cell membranes, peptide conjugates were dissolved in 20 mM sodium phosphate buffer with 20 mM sodium dodecyl sulfate (SDS) aqueous solution to obtain a 50 μM solution (pH 7.4).50,51 The CD spectrum of 5-FAM-H$_3$R$_8$ was characterized by a minimum at 202 nm (Figure 1B), suggesting a PPII-like structure of H$_3$R$_8$ in a solution in the presence of SDS.52 The CD spectrum of MTS-5-FAM exhibited two minima at 208 and 222 nm (Figure 1C), in agreement with a typical CD absorption for peptides assuming α-helical conformation. This result is consistent with a previous report that the chosen MTS peptide could adopt two helices located at the N-terminal segment and C-terminal segment.42 As shown in Figure 1C inset, positive charges derived from arginine residues are clustered onto the same face within the α-helical arrangement, which may contribute to the greater fluorescence signal in flow cytometry for MTS-5-FAM. The CD spectrum of MTS-(5-FAM)-H$_3$R$_8$ reveals a similar α-helical structure that clearly originates from the MTS segment. The PPII-like structure from the CPP segment is likely to be overwhelmed by the signal of MTS due to its relatively lower CD absorption intensity (Figure 1D). To prove our assumption, we did a linear combination of the spectra collected from MTS-5-FAM and 5-FAM-H$_3$R$_8$ (Supporting Information Figure S6). As expected, the obtained spectrum predicts the spectrum from MTS-(5-FAM)-H$_3$R$_8$ well under the same conditions. It is also possible that higher cellular uptake of MTS-5-FAM compared with 5-FAM-H$_3$R$_8$ is due to the amphiphilic nature of the former molecule.

**Intracellular Distribution.** Given that flow cytometry only provides the overall fluorescence signals, we performed confocal imaging to study further the intracellular distribution of the endocytosed conjugates. As shown in Figure 2, cells treated with MTS-(5-FAM)-H$_3$R$_8$ showed intense green fluorescence throughout the cytosol, suggesting efficient intracellular access and also successful endosomal/lysosomal escape. It is noteworthy that the intense green fluorescence observed from MTS-5-FAM treated cells does not stem from endocytosed conjugates but from molecules accumulated within cell membranes. It is very likely that the amphiphilic nature of MTS-5-FAM α-helices imparts the conjugate with a high propensity to insert into the cell membrane.53–55 These membrane entrapped conjugates cannot be simply removed by trypsinization, thus offering a very high fluorescent signal in the flow cytometry measurement. The tendency to stay within cell membrane may also explain the poor intracellular accumulation of MTS-5-FAM within MCF-7 cells. In contrast, the green fluorescence for cells treated with 5-FAM-H$_3$R$_8$ albeit dim, arises primarily from within cells (Figure 2), implying both a low membrane accumulation tendency and poor cellular uptake efficiency. The difference in subcellular distribution of MTS-5-FAM and MTS-(5-FAM)-H$_3$R$_8$ leads us to speculate that effective entry into cytosols actually benefited from the combined use of an α-helical segment with a PPII-like unit in the MTS-(5-FAM)-H$_3$R$_8$ design, in which α-helical structures afford great binding affinity toward cell membrane while cell-penetrating segment H$_3$R$_8$ facilitates the entrance to the cytosols.

In order to verify our assumption that α-helical structures and PPII-like structures have synergistic effects in cellular uptake when used together, we designed and synthesized a new conjugate molecule 5-FAM-(RLL)$_2$R$_8$, in which the MTS peptide was replaced with a short α-helix forming peptide. Previous reports have suggested that a minimum of four α-helical arginines was required for efficient cell uptake.49 The -RLLRLLR$_p$ sequence was therefore chosen for the study, with arginines concentrated on the same face to resemble the secondary conformation of MTS-(5-FAM)-H$_3$R$_8$. The secondary structure and cellular uptake of 5-FAM-(RLL)$_2$R$_8$ were investigated using the same conditions as the previous three conjugates.

The CD spectrum of 5-FAM-(RLL)$_2$R$_8$ confirmed the characteristic α-helical secondary structures, with two minima at around 208 and 222 nm, similar to that of MTS-(5-FAM)-H$_3$R$_8$. The weak PPII-like structure signal was also overwhelmed by the negative absorption of α-helix at 208 as expected, resulting in a negative signal at ~200 nm. In order to compare intracellular uptake affected by secondary structures incorporated into the newly developed conjugate, flow cytometry was again performed to acquire quantitative results at the same conditions in previous study (Supporting Information Figure S8). As shown in Figure 3B, 5-FAM-(RLL)$_2$R$_8$ showed remarkably increased cellular uptake on MCF-7 breast cancer cells compared with that of 5-FAM-H$_3$R$_8$ (616 vs 24 in terms of geo mean fluorescence intensity). Since the only difference between the two studied molecules is the use of α-helix forming peptide in 5-FAM-(RLL)$_2$R$_8$, these results clearly support our assumption that α-helical secondary structures exerted a synergistic effect on cellular uptake when used together with PPII-like structures.

**Subcellular Co localization.** In order to evaluate if the dual-conjugated molecules are still capable of targeting mitochondria, we performed subcellular colocalization experiments. As shown in Figure 4, cells treated with MTS-5-FAM again showed intense 5-FAM green fluorescence only on the cell membrane, not within cells, that rarely overlaps with the Mitotracker signal. The overlap colocalization coefficients (5-
To exclude the possibility that the observed colocalization originated from the superposition of mitochondria with other conjugate-containing organelles such as lysosomes, we carefully analyzed our colocalization results and found that most of the conjugates were out of the lysosomes after 2 h incubation (Supporting Information Figure S11). We further performed the colocalization experiment on HeLa cells (human cervical cancer cell line) that are known to have a stretched morphology and less superposition between different subcellular organelles when cultured on a Petri dish. Obvious colocalization between MTS-(5-FAM)-H₃R₈ and mitochondria was noticed (Supporting Information Figure S12), which confirms the mitochondria targeting ability of the conjugate. Clearly, the MTS-(5-FAM)-H₃R₈ shows the highest targeting efficiency to mitochondria. However, as mentioned earlier, a significant amount of MTS-(5-FAM)-H₃R₈ was still out of mitochondria, which might be either trapped in endosomes/lysosomes (Figure 2) or en route to mitochondria. The relatively low colocalization efficiency could be due to the limited amount and capacity of translocase of the outer or inner mitochondrial membrane (TOM or TIM) complex that is primarily responsible for actively transporting cargos with MTS signal into mitochondria. A possibility for high cytosol retention might be the partial degradation of MTS-(5-FAM)-H₃R₈ in lysosomes during the intracellular transportation process, which could be potentially addressed in the future by use of MTS peptides of D-amino acids.

The mechanism of mitochondria accumulation of MTS-(5-FAM)-H₃R₈ is quite different from the Mitotracker used here or other triphenyl phosphonium (TPP) based targeting strategies which accumulate into mitochondria passively (therefore not saturable) due to their characteristic membrane potential. The MTS-(5-FAM)-H₃R₈ conjugate reported herein was expected to be transported into mitochondria through TOM/TIM complex. However, the resolution of fluorescent microscopy hinders the direct observation of their suborganelle distribution. Therefore, MitoBlock-6, an inhibitor of TOM/TIM complex, was used in the colocalization experiment to explore its effect on mitochondria transportation. Unexpectedly, we found that the use of MitoBlock-6 significantly limited the intracellular accumulation of MTS-(5-FAM)-H₃R₈ (Supporting Information Figure S13), which might be a result of inhibition in ATP production since MitoBlock-6 has been reported to affect the cytochrome C that is critical for ATP production.

Charged molecules or nanoparticles are known to disrupt cell membranes, thereby leading to increased intercellular uptake and possible cytotoxicity. We therefore performed experiments to evaluate the potential cytotoxicity of all four conjugates against MCF-7 cell line. As shown in Figure 5, the four conjugates studied in this work did not reveal any noticeable toxicity behavior at 5 μM for a period of 4 h incubation, a concentration that was much higher than the concentration used for previous confocal imaging and flow cytometry experiments (1 μM).

**CONCLUSION**

In summary, we reported here a dual conjugate strategy to link a mitochondria targeting sequence, a cell penetrating peptide, and a non-membrane-penetrating dye into a conjugated molecule, and studied its cellular uptake and mitochondrial targeting behavior. Our results suggest that both α-helix and polyproline II-like structures are critical for facilitating the
MATERIALS AND METHODS

Materials. All amino acids were purchased from Advanced Automated Peptide Protein Technologies (AAPPTEC, Louisville, KY) and Rink Amide MBHA Resin was purchased from NovaBiochem (San Diego, KY). 5-FAM was obtained from AnaSpec, Inc. (Fremont, CA), and all other reagents and solvents were sourced through VWR.

Cell Culture. MCF-7 cells were kindly provided by the Wirtz Lab (Department of Chemical and Biomolecular Engineering, Johns Hopkins University). Cells were cultured according to providers’ protocols. MCF-7 human breast cancer cells were grown in DMEM with 10% fetal bovine serum (FBS, Invitrogen) and 1% antibiotics (Invitrogen). Cells were cultured at 37 °C and 5% CO2 atmosphere.

Conjugate Synthesis and Characterization. All peptide conjugates were synthesized using standard 9-fluorenylmethoxycarbonyl (Fmoc) solid phase synthesis techniques. The peptides Fmoc-K(Mtt)G3H3R3-Kink, Fmoc-MLRA,LSTAR,GPRLSL,K(3mt)G3H3R3-Kink, Fmoc-MLRA,LSTAR,GPRLSL,K(Mtt)G3H3R3-Kink, and Fmoc-K-(Mtt)GRL,R2L2Kink were synthesized on a 0.25 mmol scale on the Focus XC automated peptide synthesizer (AAPPTEC, Louisville, KY). For all peptide conjugates, 5-FAM was manually coupled at the peptide N- or C-terminus (after Mtt removal) with 5-FAM/HBTU/DIEA at a ratio of 4:4:10 (for Mtt) and 5-FAM-(RLL)R2 at a three different concentrations (1, 2, and 5 μM). The results exhibited near nontoxicity of four conjugates at all three concentrations.

efficient cellular uptake of the conjugate, and when combined exert a synergistic effect. Although there are several parameters that require more rigorous evaluation and detailed studies, these results clearly demonstrate the great potential of using rationally designed peptide conjugates for intracellular delivery.

Circular Dichroism (CD) Measurement. To determine the peptide conformation, the CD spectra of all the conjugates (50 μM in 20 mM sodium phosphate buffer with 20 mM SDS) were recorded on a J-710 spectropolarimeter (JASCO, Easton, MD) from 190 to 260 nm, and the signal was converted from ellipticity (mdeg) to mean molar ellipticity per residue (deg cm2 dmol−1 residue−1).

Cellular Uptake of Peptide Conjugates. To investigate the cellular uptake efficacy of the four designed conjugates, MCF-7 cells were seeded onto a 24-well plate at 1 × 105 cells/well, and allowed to attach overnight. The medium was replaced with fresh medium containing the appropriate conjugate at 1 μM, and incubated with the cells for 4 h. The cells were then washed with DPBS twice, trypsinized, collected, washed twice with ice cold DPBS, and finally resuspended in 200 μL DPBS. The fluorescence intensity from endocytosed 5-FAM was analyzed using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA) using the FL1 (530/30) channel.

Cytotoxicity. MCF-7 cells were seeded onto 96-well plate at 5 × 103 cells/well, and allowed to attach overnight. The medium was replaced with fresh medium containing various concentrations of MTS-5-FAM, 5-FAM-H3R8, MTS-(5-FAM)-H3R8, or 5-FAM-(RLL)R2, and incubated for 48 h. The cell viability was determined using the SRB method according to the manufacturer’s protocol (TOX-6, Sigma, USA).

Subcellular Colocalization. The subcellular location of the endocytosed conjugates was investigated using confocal microscopy. Briefly, MCF-7 or HeLa cells were seeded onto an 8-well glass bottom plate (Labtek, Scott’s Valley, CA) pretreated with type I rat tail collagen (Invitrogen) at 4 × 104 cells/well, and allowed to attach overnight. For TIM/TOM complex inhibition study, cells were pretreated with 50 μM MitoBlock-6 for 15 min before conjugate addition. The cells were incubated with 5 μM peptide conjugates for 2 h. Thirty minutes before the washing step, 100 nM Lysotracker Red (Invitrogen) or Mitotracker Red (Invitrogen) was added with 10 μg/mL Hoechst 33342 (Invitrogen). The cells were then incubated in the phenol red free DMEM (Corning, Tewksbury, MA) supplemented with 10% FBS and imaged using a Zeiss S10 confocal laser scanning fluorescent microscope (Frankfurt, Germany).
associated content

Supporting Information
Additional characterization (HPLC, ESI-MS of the conjugates), cellular uptake of all the conjugates in MCF-7 cells, enlarged Figure 3 (intracellular distribution of MTS-5-FAM, 5-FAM-H$_2$R$_8$, MTS-(5-FAM)-H$_2$R$_8$, MTS-(5-FAM)-H$_2$R$_8$ in MCF-7 cells) and Figure 5 (subcellular colocalization of MTS-5-FAM, 5-FAM-H$_2$R$_8$, MTS-(5-FAM)-H$_2$R$_8$ in MCF-7 cells), flow cytometry spectra, subcellular colocalization results in HeLa cells, and results of MCF-7 cells with MitoBlock-6 treatment can be found in online supporting material. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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Abbreviations
5-FAM, 5-carboxyfluorescein; ALDH, aldehyde dehydrogenase; CD, circular dichroism; CPPs, cell penetrating peptides; DCM, dichloromethane; DIIEA, diisopropylethylamine; DMEM, Dulbecco’s modified eagle medium; DMF, N,N-dimethylformamide; DPBS, Dulbecco’s phosphate buffered saline; FBS, fetal bovine serum; Fmoc, 9-fluorenylmethylcarbonyl; HBTO, O-benzotrazole-N,N,N,N’-tetramethyluronium hexafluorophosphate; MeCN, acetonitrile; MTS, mitochondria targeting sequence; Mtt, 4-methyltryrithione; ODN, oligodeoxynucleotide; PNA, peptide nucleic acid; PPI, Polyproline II; R$_8$, octaarginine; SDS, sodium dodecyl sulfate; SRB, Sulforhodamine B; Tat, transactivator of transcription; TFA, trifluoroacetic acid; TIS, triisopropylsilane

References
(1) Kroemer, G., Galluzzi, L., and Brenner, C. (2007) Mitochondria within cells by conjugation to lipophilic cations: implications for mitochondrial DNA replication, expression and disease. Nucleic Acids Res. 29, 1852–1863.
(6) Mayer, A., Neupert, W., and Lill, R. (1995) Mitochondrial protein import - reversible binding of the presequence at the trans side of the outer-membrane drives partial translocation and unfolding. Cell 80, 127–137.
(7) Schatz, G. (1996) The protein import system of mitochondria. J. Biol. Chem. 271, 31763–31766.
(8) Schwarze, S. R., Hruska, K. A., and Dowdy, S. F. (2000) Protein transduction: unrestricted delivery into all cells? Trends Cell Biol. 10, 290–295.
(9) Futaki, S. (2002) Arginine-rich peptides: potential for intracellular delivery of macromolecules and the mystery of the translocation mechanisms. Int. J. Pharm. 245, 1–7.
(10) Weissig, V., D’Souza, G. M., and Torchilin, V. P. (2001) DQAsome/DNA complexes release DNA upon contact with isolated mouse liver mitochondria. J. Controlled Release 75, 401–408.
(11) Yamada, Y., Akita, H., Kogure, K., Kamiya, H., and Harashima, H. (2007) Mitochondrial drug delivery and mitochondrial disease therapy - An approach to liposome-based delivery targeted to mitochondria. Mitochondrion 7, 63–71.
(12) Jean, S. R., Tulumello, D. V., Wisnovsky, S. P., Lei, E. K., Pereira, M. P., and Kelley, S. O. (2014) Molecular vehicles for mitochondrial chemical biology and drug delivery. ACS Chem. Biol. 9, 323–333.
(13) Kelley, S. O., Stewart, K. M., and Mourtada, R. (2011) Development of Novel Peptides for Mitochondrial Drug Delivery: Amino Acids Featuring Delocalized Lipophilic Cations. Pharm. Res. 28, 2808–2819.
(14) Lei, E. K., Pereira, M. P., and Kelley, S. O. (2013) Tuning the intracellular bacterial targeting of peptideic vectors. Angew. Chem., Int. Ed. 52, 9660–9663.
(15) Smith, R. A. J., Porteous, C. M., Coulter, C. V., and Murphy, M. P. (1999) Selective targeting of an antioxidant to mitochondria. Eur. J. Biochem. 263, 709–716.
(16) Chamberlain, G. R., Tulumello, D. V., and Kelley, S. O. (2013) Targeted delivery of doxorubicin to mitochondria. ACS Chem. Biol. 8, 1389–1395.
(17) Flierl, A., Jackson, C., Cottrell, B., Murdock, D., Seibel, P., and Wallace, D. C. (2003) Targeted delivery of DNA to the mitochondrial compartment via import sequence-conjugated peptide nucleic acid. Mol. Ther. 7, 550–557.
(18) Del Gaizo, V., MacKenzie, J. A., and Payne, R. M. (2003) Targeting proteins to mitochondria using Tat. Mol. Genet. Metab. 80, 170–180.
(19) Patel, N. R., Hatziantoniou, S., Georgopoulos, A., Demetzos, C., Torchilin, V. P., Weissig, V., and D’Souza, G. G. M. (2010) Mitochondria-targeted liposomes improve the apoptotic and cytotoxic action of sclaroel. J. Liposome Res. 20, 244–249.
(20) Torchilin, V. P. (2006) Recent approaches to intracellular delivery of drugs and DNA and organelle targeting. Annu. Rev. Biomed. Eng. 8, 343–375.
(21) Yousif, L. F., Stewart, K. M., Horton, K. L., and Kelley, S. O. (2009) Mitochondria-penetrating peptides: sequence effects and model cargo transport. ChemBioChem 10, 2081–2088.
(22) Vonheijne, G. (1986) Mitochondrial targeting sequences may form amphiphilic helices. EMBO J. 5, 1335–1342.
(23) Karslake, C., Piotto, M. E., Pak, Y. K., Weiner, H., and Gorenstein, D. G. (1990) 2D NMR and structural model for a mitochondrial signal peptide bound to a micelle. Biochemistry 29, 9872–9878.
(24) Pak, Y. K., and Weiner, H. (1990) Import of chemically synthesized signal peptides into rat-liver mitochondria. J. Biol. Chem. 265, 14298–14307.
(25) Bode, S. A., Wallbrecher, R., Brock, R., van Hest, J. C. M., and Lowik, D. (2014) Activation of cell-penetrating peptides by disulfide bridge formation of truncated precursors. Chem. Commun. 50, 415–417.
Bioconjugate Chemistry

(26) Hansen, M. B., van Gaal, E., Minten, I., Storm, G., van Hest, J. C. M., and Lovik, D. (2012) Constrained and UV-activatable cell-penetrating peptides for intracellular delivery of liposomes. J. Controlled Release 164, 87–94.

(27) Temming, R. P., Eggermont, L., van Eldijk, M. B., van Hest, J. C. M., and van Delft, F. L. (2013) N-terminal dual protein functionalization by strain-promoted alkyne-nitrene cycloaddition. Org. Biomol. Chem. 11, 2772–2779.

(28) Frankel, A. D., and Pabo, C. O. (1988) Cellular uptake of the tat protein from human immunodeficiency virus. Cell 55, 1189–1193.

(29) Zhang, P. C., Cheetham, A. G., Lin, Y. A., and Cui, H. (2013) Self-assembled Tat nanofibers as effective drug carrier and transporter. ACS Nano 7, 5965–5977.

(30) Zhang, P. C., Cheetham, A. G., Lock, L. L., and Cui, H. G. (2013) Cellular uptake and cytotoxicity of drug-peptide conjugates regulated by conjugation site. Bioconjugate Chem. 24, 604–613.

(31) Zhang, P. C., Lock, L. L., Cheetham, A. G., and Cui, H. G. (2014) Enhanced cellular entry and efficacy of Tat conjugates by rational design of the auxiliary segment. Mol. Pharmaceutics 11, 964–973.

(32) Zhang, K., Fang, H. F., Chen, Z. Y., Taylor, J. S. A., and Wooley, K. L. (2008) Shape effects of nanoparticles conjugated with cell-penetrating peptides (HV Tat PTD) on CHO cell uptake. Bioconjugate Chem. 19, 1880–1887.

(33) Derossi, D., Chassaing, G., and Prochiantz, A. (1998) Trojan peptides: the penetratin system for intracellular delivery. Trends Cell Biol. 8, 84–87.

(34) Mitchell, D. J., Kim, D. T., Steinman, L., Fathman, C. G., and Rothbard, J. B. (2000) Polyarginine enters cells more efficiently than other polycationic homopolymers. J. Pept. Res. 56, 318–325.

(35) Lindgren, M., Hallbrink, M., Prochiantz, A., and Langel, U. (2000) Cell-penetrating peptides. Trends Pharmacol. Sci. 21, 99–103.

(36) Copolovici, D. M., Langel, K., Eriste, E., and Langel, U. (2014) Cell-penetrating peptides: design, synthesis, and applications. ACS Nano 8, 1972–1994.

(37) Ryser, H. J., and Hancock, R. (1965) Histones and basic polyanion acids stimulate the uptake of albumin by tumour cells in culture. Science 150, 501–3.

(38) Wadia, J. S., Stan, R. V., and Dowdy, S. F. (2004) Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat. Med. 10, 310–315.

(39) Green, M., and Loewenstein, P. M. (1988) Autonomous functional domains of chemically synthesized human immunodeficiency virus Tat trans-activator protein. Cell 55, 1179–1188.

(40) Futaki, S., Suzuki, T., Ohashi, W., Yagami, T., Tanaka, S., Ueda, K., and Sugiyama, Y. (2001) Arginine-rich peptides - An abundant protein from human immunodeficiency virus. J. Biol. Chem. 276, 3836–3840.

(41) Wang, Y., and Weiner, H. (1993) The presequence of rat-liver aldehyde dehydrogenase requires the presence of an alpha-helix at its N-terminal region which is stabilized by the helix at its C-termini. J. Biol. Chem. 268, 4759–4765.

(42) Takayama, K., Nakase, I., Michiue, H., Takeuchi, T., Tomizawa, K., Matsu, H., and Futaki, S. (2009) Enhanced intracellular delivery using arginine-rich peptides by the addition of penetration accelerating sequences (Pas). J. Controlled Release 138, 128–133.

(43) Varkouhi, A. K., Scholte, M., Storm, G., and Haisma, H. J. (2011) Endosomal escape pathways for delivery of biologics. J. Controlled Release 151, 220–228.

(44) Murphy, R. F., Powers, S., and Cantor, C. R. (1984) Endosome pH measured in single cells by dual fluorescence flow-cytometry - rapid acidification of insulin to pH-6. J. Cell Biol. 98, 1757–1762.

(45) Fernandez-Suarez, M., and Ting, A. Y. (2008) Fluorescent probes for super-resolution imaging in living cells. Nat. Rev. Mol. Cell Biol. 9, 929–943.