Published in final edited form as:

J Am Chem Soc. 2018 January 31; 140(4): 1215–1218. doi:10.1021/jacs.7b11582.

Enzymatic Cleavage of Branched Peptides for Targeting Mitochondria

Hongjian He[†], Jiaqing Wang[†], Huaimin Wang[†], Ning Zhou[†], Dongsik Yang[†], Douglas R. Green[§], and Bing Xu^{*,†}

[†]Department of Chemistry, Brandeis University, 415 South St., Waltham, MA 02454, USA

[§]Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA

Abstract

Most of the reported mitochondria targeting molecules are lipophilic and cationic, which may become cytotoxic with accumulation. Here we show enzymatic cleavage of branched peptides that carry negative charges for targeting mitochondria. Conjugating a well-established protein tag (i.e., FLAG-tag) to self-assembling motifs affords the precursors that form micelles. Enzymatic cleavage of the hydrophilic FLAG motif (DDDDK) by enterokinase (ENTK) turns the micelles to nanofibers. After being taken up by cells, the micelles, upon the action of intracellular ENTK, turns into nanofibers to locate mainly at mitochondria. The micelles of the precursors are able to deliver cargos (either small molecules or proteins) into cells, largely to mitochondria and within two hours. Preventing ENTK proteolysis diminishes mitochondria targeting. As the first report of using enzymatic self-assembly for targeting mitochondria and delivery cargos to mitochondria, this work illustrates a fundamentally new way to target subcellular organelles for biomedicine.

Graphical abstract

As a subcellular organelle, mitochondria plays essential roles in many cellular processes, such as apoptosis and metabolism.¹ Intensive research efforts have focused on developing

Corresponding Author: bxu@brandeis.edu.

Supporting Information

This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

The authors declare no competing financial interests.

therapeutics that target mitochondria.² In fact, some of the therapeutics have already entered clinical trials.³ Except gramicidin S derivatives developed by Wipf et. al.,^{2a} most of the molecules reported for targeting mitochondria are lipophilic and cationic.⁴ Although those lipophilic cations are able to prevent mitochondrial damage in vitro, they have a major drawback due the toxicity resulted from the accumulation in the mitochondrial matrix.⁵ Thus, it is necessary to develop new molecules or processes for targeting mitochondria. We have been exploring enzyme-instructed self-assembly (EISA),⁵ a multistep molecular process that is finding increasing number of applications,⁶ including selective inhibiting cancer cells.⁷ During our study on enzymatic supramolecular hydrogelation of branched peptides⁶, we serendipitously found that EISA of branched peptides is able to target mitochondria.

Here we show enzymatic cleavage of branched peptides that carry negative charges for targeting mitochondria. Conjugating a well-established protein tag (i.e., FLAG-tag)⁷ to self-assembling motifs⁸ affords the precursors that are the substrates of enterokinase (ENTK).⁹ The precursors form micelles, which turn into nanofibers upon enzymatic cleavage of the hydrophilic branch that contains FLAG motif (DDDDK).⁷ Fluorescent microscopy of live cells reveals that, after being taken up by cells, the branched peptides and their enzyme cleaved products, mainly localize at mitochondria. Moreover, the micelles of the precursors are able to deliver cargos (either small molecules (e.g., doxorubicin (Dox)) or proteins (e.g., red phycoerythrin (RPE)¹⁰) into cells, largely to mitochondria and within two hours. Western blot indicates ENTK on the isolated mitochondria of cancer cells. Transmission electron microscopy (TEM) of the isolated mitochondria confirms the conversion of the micelles to nanofibers at the mitochondria. Preventing ENTK proteolysis diminishes the mitochondria targeting. These results confirm EISA as the process for targeting mitochondria. As the first report of using EISA for targeting mitochondria and delivery molecular cargos to mitochondria (Scheme 1), this work illustrates a fundamentally new molecular motif and process for targeting mitochondria and exploring the applications of protease-instructed assembly for biomedicine.

Scheme 1 shows the structure of the designed substrate of ENTK. We use the symbol “” to represent the branching so that the substrate is denoted as D-**1FLAG**. The branched peptides consist of (i) the FLAG-tag (DYKDDDDK)⁷ as the substrate of ENTK for enzymatic recognition and cleavage, (ii) a self-assembling peptide sequence D-**1** composed of a 2-acetylnaphthyl group (Nap), a D-tripeptide (D-Phe-D-Phe-D-Lys (ffk)), and a nitrobenzoxadiazoly-ethylenediamino moiety (NBD-EA, for enhanced fluorescence upon self-assembly¹¹) at the C-terminal of the D-peptide, and (iii) a glycine as the spacer for (i) and (ii). Using Fmoc-based solid-phase peptide synthesis,¹² we first produce the peptide segments (i.e., FLAG-Gly and the Nap-ffk, Scheme S1). After the synthesis, we generate D-**1** by conjugating NBD-EA the Nap-ffk via C-terminal activation. Subsequently, we connect the C-terminal of FLAG-Gly to the side chain of lysine in D-**1** using the same activation method. Finally, the removal of all protecting groups and the purification by high pressure liquid chromatography (HPLC) produce the designed branch peptide (i.e., D-**1FLAG**).

D-1FLAG forms nanoparticles (46 ± 11 nm in diameter) at 200 μ M (critical micelle concentration (CMC), Fig. S1), which become nanofibers (18 ± 3 nm in diameter) upon the addition of ENTK (Figure 1A, S2 and S3). Circular dichroism (CD) measurement indicates the cleavage of the branch results in largely β -sheet (**D-1**) and random-coil (**FLAG-tag**) like secondary structures for the case of **D-1FLAG** (Fig. S4), as well as for those of **D-** and **L-2FLAGs**.⁶ After the incubation of HeLa or U87MG cells with **D-1FLAG** for two hours, fluorescent imaging (Figure 1B) of these the cells exhibits strong fluorescence, indicating a rapid cellular uptake of **D-1FLAG**. Our preliminary endocytosis study suggests that the uptake of the precursors by HeLa cells likely involves macropinocytosis and clathrin-dependent pathway (Fig. S5). Cell viability assay reveals that **D-1FLAG** exhibits little cytotoxicity to HeLa and U87MG cells (Fig. S6), excluding the possibility that the rapid internalization of **D-1FLAG** originates from the increased membrane permeability caused by cell death.¹³

The pattern of the distribution of **D-1FLAG** in cytosol resembles to that of mitochondria (Figures 1B and S7). Co-staining **D-1FLAG** with MitoTracker or Lyso-tracker in HeLa cells shows that the yellow fluorescence from **D-1FLAG** mostly overlaps with the red fluorescence of MitoTracker (Figure 1C), but co-localizes poorly with the fluorescence from Lyso-tracker (Fig. S8). These results suggest that **D-1FLAG**, escaping lysosomes after endocytosis, specifically localizes in mitochondria. However, HeLa cells treated by **D-1** exhibit fluorescence both outside the cells (including membrane) and in cytosol nonspecifically (Fig. S9). Thus, we infer that the mitochondria-targeting ability of **D-1FLAG** likely depends on its unique branching architecture, as well as the electrostatic interaction between the FLAG-tag moiety (negative potential, due to carboxylic groups) and the intermembrane space of mitochondria (positive potential, resulted from abundant H^+),¹⁴ as illustrated in Scheme 1. Notably, **D-1FLAG** appears to accumulate in the lysosomes of a normal cell line (HS-5) (Fig. S10). Such cell selectivity worth further investigation. Moreover, after mixing R-phycoerythrin (RPE), a protein, with **D-1FLAG**, we incubate them with HeLa cells for two hours. While the control cells (without micelles) show little fluorescence (Fig. S11), RPE and **D-1FLAG** localize in the mitochondria (Figure 1D). TEM of the mixture of RPE and **D-1FLAG** support the encapsulation of RPE by the micelles (Fig. S12). These results indicate that the micelles formed by **D-1FLAG** are able to deliver cargo molecules to mitochondria.

Mitochondria targeting of **D-1FLAG** implies that the micelles formed by other FLAG-tagged precursors made of branched peptides would localize and deliver cargo molecules to mitochondria. Thus, we examine whether precursors **D-2FLAG** (an analog of **D-1FLAG** without NBD, Scheme S2) and **L-2FLAG** (a diastereomer of **D-2FLAG**) are able to deliver cargo molecules to mitochondria. The similar CMC values of **D-1FLAG** and **D-2FLAG** (Fig. S1) support the interchangeability between them. After mixing RPE, a protein, or doxorubicin (Dox), a drug molecule, with **L-2FLAG** or **D-2FLAG**, we incubate them with HeLa or U87MG cells for two hours. While the control cells (without the micelles formed by the precursors) show little fluorescence (Fig. S11), the cells incubated with the micelles and the cargo molecules exhibit strong fluorescence (Figure 2A and B), indicating that the

intracellular delivery of RPE and Dox by L-**2FLAG** or D-**2FLAG**. Similar to D-**1FLAG**, the patterns of the distribution of RPE (or Dox) inside the cells are akin to that of mitochondria.

To further verify whether the FLAG-tagged precursors deliver cargo molecules to mitochondria specifically, we incubate the HeLa cells expressing GFP-Cyt c¹⁵ or Omi-mcherry¹⁶ (in mitochondria) with RPE or Dox in the presence of D-**2FLAG**. Fluorescent images confirm that the fluorescence of RPE or Dox extensively overlaps with that from GFP-cyt c and Omi-mcherry (Figures 2C and S13). Quantification of the overlap of the fluorescence¹⁷ confirm that RPE or Dox alone hardly co-localize with mitochondria, but D-**2FLAG** significantly boost the localization of RPE or Dox in mitochondria (Figure 2D). Moreover, Dox, after mixing with D-**2FLAG** or L-**2FLAG**, exhibits significantly increased cytotoxicity against HeLa cells compared to Dox only. That is, the branched peptide precursors decrease the IC₅₀ of Dox from 3.0 μ M to 400 nM (Fig. S14D). In addition, D-**2FLAG** significantly boosts the activity of Dox against Dox-resistant cancer cell line (MESA/Dx5,¹⁸ Fig. S14D). The synergistic effect (Fig. S14E) of Dox and the FLAG-tagged precursors against cancer cells likely originates from that the micelles enhance the uptake of Dox and Dox interfering with mitochondrial DNA. These results confirm that the generality of the FLAG-tagged branch peptide precursors serve as carriers for delivering cargo molecules to mitochondria.

To confirm that the nanofibers form at mitochondria after ENTK cleaves the branch of the branched peptides, we isolated mitochondria from HeLa cells incubated with D-**2FLAG** (200 μ M) for different time (Figure 3 and S15). The cells treated by D-**2FLAG** for 2 h and 24 h produce mitochondria surrounded by nanoparticles and nanofibers, respectively (Figure 3), while the mitochondria from the HeLa cells incubated with only culture medium exhibit a surface without either nanoparticles or nanofibers (Fig. S15). Western blot analysis of the mitochondria isolated from HeLa and U87MG cells showed two ENTK bands (Fig. S16, the major band is the light chain¹⁹), suggesting the presence of ENTK on mitochondria. Moreover, LC/MS analysis of the isolated mitochondria confirmed that D-**2** is the main component of the nanofibers (Fig. S17), indicating that D-**2FLAG**, after reaching mitochondria, is cleaved by ENTK. The in-situ formation of nanofibers results in the localization of the nanofibers at mitochondria. To verify whether the proteolysis is necessary for mitochondria targeting, we made the D-peptide control (D-**1flag**) by connecting D-**1** to the D-enantiomer of FLAG tag (flag), which is resistant to ENTK. The fluorescent images of HeLa cells incubated with D-**1flag** (200 μ M, 2h) failed to exhibit a mitochondria-specific distribution (Fig. S18), suggesting that the prevention of proteolysis (the conversion to nanofibers) catalyzed by ENTK abolishes the mitochondria targeting.

In conclusion, we demonstrate the branched peptides as a novel type of substrates for enzymatic self-assembly to target mitochondria. Although carrying multiple negative charges, the FLAG-tagged precursors undergo rapid endocytosis and specifically accumulate in mitochondria. The deposition of the nanofibers affect little on the cellular activity, likely due to that the nanofibers impact less than cationic molecules on the membrane potential of mitochondria. The branch architecture is uniquely important for targeting mitochondria since the linear peptide, L-**2-FLAG**, is unable to deliver cargo molecule to mitochondria (Fig. S19). This work highlights the importance of reactions, in addition to

nanoarchitectonics,²⁰ for controlling biological systems. Contrasting to most of the reported mitochondria targeting molecules that usually are lipophilic and cationic,⁷ this observation illustrates a new mechanism, the integration of enzymatic reaction and self-assembly (i.e., EISA), for targeting mitochondria, which would complement other approaches (e.g., triphenyl phosphonium (TPP)²¹). Although the targeting of mitochondria relies on ENTK in this work, the strategy demonstrated here should allow the development of the substrate of other enzymes on mitochondria for mitochondria targeting. Among numerous approaches for mitochondria-specific drug delivery,²² the delivery of cargo molecules to target mitochondria by the FLAG-tagged precursors is particularly promising and worth further exploration for potential applications in biomedicine because of the cell compatibility of the FLAG-tagged precursors (Fig. S9A) and the biological importance of mitochondria.²³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was partially supported by NIH (R01CA142746) and NSF (MRSEC-1420382).

References

1. (a) Green DR, Kroemer G. *Science*. 2004; 305(5684):626. [PubMed: 15286356] (b) McBride HM, Neuspiel M, Wasiak S. *Curr Biol*. 2006; 16(14):R551. [PubMed: 16860735] (c) Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, Hofer T, Seo AY, Sullivan R, Jobling WA, Morrow JD, Van Remmen H, Sedivy JM, Yamasoba T, Tanokura M, Weindrich R, Leeuwenburgh C, Prolla TA. *Science*. 2005; 309(5733):481. [PubMed: 16020738] (d) Balaban RS, Nemoto S, Finkel T. *Cell*. 2005; 120(4):483. [PubMed: 15734681]
2. (a) Hoye AT, Davoren JE, Wipf P, Fink MP, Kagan VE. *Acc Chem Res*. 2008; 41(1):87. [PubMed: 18193822] (b) Murphy MP, Smith RAJ. Targeting antioxidants to mitochondria by conjugation to lipophilic cations. *Annual Review of Pharmacology and Toxicology*. 2007; 47:629. (c) Yousif LF, Stewart KM, Kelley SO. *Chembiochem*. 2009; 10(12):1939. [PubMed: 19637148]
3. (a) Thomas DA, Stauffer C, Zhao K, Yang H, Sharma VK, Szeto HH, Suthanthiran M. *J Am Soc Nephrol*. 2007; 18(1):213. [PubMed: 17151329] (b) Birk AV, Liu S, Soong Y, Mills W, Singh P, Warren JD, Seshan SV, Pardee JD, Szeto HH. *J Am Soc Nephrol*. 2013; 24(8):1250. [PubMed: 23813215] (c) Trachootham D, Alexandre J, Huang P. *Nat Rev Drug Discov*. 2009; 8(7):579. [PubMed: 19478820]
4. (a) Yousif LF, Stewart KM, Horton KL, Kelley SO. *Chembiochem*. 2009; 10(12):2081. [PubMed: 19670199] (b) Mahon KP, Potocky TB, Blair D, Roy MD, Stewart KM, Chiles TC, Kelley SO. *Chem & Bio*. 2007; 14(8):923. [PubMed: 17719491] (c) Zhao KS, Luo GX, Giannelli S, Szeto HH. *Biochem Pharmacol*. 2005; 70(12):1796. [PubMed: 16216225] (d) Smith RAJ, Porteous CM, Coulter CV, Murphy MP. *Eur J Biochem*. 1999; 263(3):709. [PubMed: 10469134] (e) Murphy MP, Smith RA. *Annu Rev Pharmacol Toxicol*. 2007; 47:629. [PubMed: 17014364] (f) Murphy MP. *Trends Biotechnol*. 1997; 15(8):326. [PubMed: 9263481] (g) Hurt EC, Muller U, Schatz G. *Embo Journal*. 1985; 4(13A):3509. [PubMed: 3004964] (h) Horton KL, Stewart KM, Fonseca SB, Guo Q, Kelley SO. *Chem Biol*. 2008; 15(4):375. [PubMed: 18420144] (i) Hoye AT, Davoren JE, Wipf P, Fink MP, Kagan VE. *Acc Chem Res*. 2008; 41(1):87. [PubMed: 18193822]
5. Murphy MP. *Trends Biotechnol*. 1997; 15(8):326. [PubMed: 9263481]
6. He H, Wang H, Zhou N, Yang D, Xu B. *Chem Commun*. 2018; 54:86.
7. Hopp TP, Prickett KS, Price VL, Libby RT, March CJ, Cerretti DP, Urdal DL, Conlon PJ. *Nat Biotechnol*. 1988; 6(10):1204.

8. (a) Reches M, Gazit E. *Science*. 2003; 300(5619):625. [PubMed: 12714741] (b) Burattini S, Greenland BW, Merino DH, Weng W, Seppala J, Colquhoun HM, Hayes W, Mackay ME, Hamley IW, Rowan SJ. *J Am Chem Soc*. 2010; 132(34):12051. [PubMed: 20698543] (c) Zhang Y, Kuang Y, Gao Y, Xu B. *Langmuir*. 2010; 27(2):529. [PubMed: 20608718]
9. Pavlov, IP., Thompson, WH. *The work of the digestive glands*. Charles Griffin; 1902.
10. Teale F, Dale R. *Biochem J*. 1970; 116(2):161. [PubMed: 5414092]
11. Gao Y, Shi J, Yuan D, Xu B. *Nat Commun*. 2012; 3:1033. [PubMed: 22929790]
12. Chan, WC., White, PD. *Fmoc solid phase peptide synthesis*. Oxford University Press; 2000.
13. Elmore S. *Toxicol Pathol*. 2007; 35(4):495. [PubMed: 17562483]
14. Wikstrom MK. *Nature*. 1977; 266(5599):271. [PubMed: 15223]
15. Goldstein JC, Waterhouse NJ, Juin P, Evan GI, Green DR. *Nat Cell Biol*. 2000; 2(3):156. [PubMed: 10707086]
16. Tait SW, Parsons MJ, Llambi F, Bouchier-Hayes L, Connell S, Muñoz-Pinedo C, Green DR. *Dev Cell*. 2010; 18(5):802. [PubMed: 20493813]
17. Manders E, Verbeek F, Aten J. *J Microsc*. 1993; 169(3):375.
18. Harker WG, Sikic BI. *Cancer Res*. 1985; 45(9):4091. [PubMed: 4028002]
19. Lu D, Fütterer K, Korolev S, Zheng X, Tan K, Waksman G, Sadler JE. *J Mol Biol*. 1999; 292(2):361. [PubMed: 10493881]
20. Komiyama M, Yoshimoto K, Sisido M, Ariga K. *Bull Chem Soc Jpn*. 2017; 90(9):967.
21. Zielonka J, Joseph J, Sikora A, Hardy M, Ouari O, Vasquez-Vivar J, Cheng G, Lopez M, Kalyanaraman B. *Chem Rev*. 2017; 117(15):10043. [PubMed: 28654243]
22. (a) Yamada Y, Akita H, Kogure K, Kamiya H, Harashima H. *Mitochondrion*. 2007; 7(1–20):63. [PubMed: 17296332] (b) Schatz G. *J Biol Chem*. 1996; 271(50):31763. [PubMed: 8943210]
23. Green DR, Galluzzi L, Kroemer G. *Science*. 2014; 345(6203):1466.

Figure 1.

(A) TEM images of D-1FLAG before (left) and after (right) adding ENTK (24 h), scale bar = 100 nm. (B) Fluorescent images of HeLa and U87MG cells incubated with D-1FLAG for 2 h. (C) The fluorescence images of D-1FLAG and MitoTracker in HeLa cells. (D) HeLa cells treated by the mixture of RPE (1 $\mu\text{g/mL}$) and D-1FLAG for 2 h. Scale bar = 30 μm in (B, C). The concentrations of D-1FLAG are 200 μM for A-D.

Figure 2.

(A, B) HeLa cells incubated with (A) RPE (1 $\mu\text{g/mL}$) and (B) Dox (2 μM) mixing with D-2FLAG and L-2FLAG (200 μM) for 2 h. (C) GFP-cyt c HeLa cells treated by the mixture of RPE (1 $\mu\text{g/mL}$) and D-2FLAG (200 μM) for 2 h. Scale bar = 30 μm . (D) Co-localization analysis of GFP-cyt c and Omi-mcherry expressing HeLa cells treated by RPE or Dox in the presence of D-2FLAG. R=1 indicates perfect co-localization.

Figure 3. TEM images of mitochondria isolated from HeLa cells being incubated with D-2FLAG (200 μ M) for (A) 2h and (B) 24 h. Scale bar equals 100 nm.

Scheme 1.

Structure of a representative branched peptide and ENTK cleaving the branch to convert micelles to nanofibers on mitochondria.