Enzyme-Instructed Self-Assembly for Subcellular Targeting

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ABSTRACT: Subcellular compartmentalization is a key feature of eukaryotic cells. Selectively targeting subcellular compartments, though holding many exciting opportunities for biomedicine, remains rather underdeveloped. Self-assembly provides a new way for subcellular targeting. In this mini-review, we briefly introduce the development of supramolecular self-assemblies for targeting the nucleus, mitochondria, endoplasmic reticulum, and cell membranes. We mainly focus on the use of enzyme-instructed self-assembly (EISA), which spatiotemporally controls the formation of supramolecular assemblies for subcellular targeting and its applications, such as developing cancer therapeutics.

Self-assembly, as a process for generating orders,1 enables biomacromolecules to form the structural basis of living organisms. The understanding of self-assembly in cells also offers useful guides for chemists to design various molecules that self-assemble into different nanostructures by noncovalent bonds (i.e., supramolecular interactions) under different physiological conditions. Enzyme-instructed self-assembly (EISA) is a dynamic and integrated molecular process that selectively generates assemblies of both macromolecules and small molecules under the catalysis of enzymes.2 Because of its unique ability to spatiotemporally control assemblies in the cellular environment, EISA exhibits emergent properties and offers (almost) unlimited opportunities for biological applications by targeting subcellular compartments in eukaryotic cells. Divided by membranes, subcellular compartments provide a special environment for specific functions. Since enzymes localize at certain subcellular compartments, the supramolecular assemblies from EISA are able to locate and to accumulate at the location of enzymes. This subtle feature allows the use of EISA for subcellular targeting. Subcellular targeting is emerging as a promising strategy for drug design and drug delivery3 because subcellular targeting, accumulating drugs in special compartments of cells, offers a promising strategy that improves efficacy and decreases the side effects of drugs.4

In this mini-review, we briefly introduce the development of supramolecular self-assemblies to target the nucleus, mitochondria, endoplasmic reticulum, and cell membranes of cancer cells. We chose cancer cells because there are convenient and reliable assays to report cell proliferation and considerable knowledge of many immortalized cancer cell lines. Thus, it is easy to verify that the phenotypes resulted from subcellular targeting and to warrant the reproducibility of the observations. Moreover, subcellular targeting should facilitate the accumulation of anticancer drugs inside cancer cells to reach higher concentration than a simple diffusion. The accumulation of assemblies of the drugs inside cells may minimize the development of drug resistance and activate multiple cell death pathways by increasing the stress of subcellular organelles of the cancer cells. These promising features lead to the development of the application of subcellular targeting by molecular assemblies via EISA, especially for developing potential cancer therapeutics.

EISA-ENHANCED TARGETING OF THE NUCLEUS

The nucleus is the largest membrane-bound organelle found in eukaryotic cells. Because many drug targets are located in the nucleus, nuclear targeting would be a highly effective strategy for cancer chemotherapy. Cells, however, have evolved sophisticated machinery to regulate molecular trafficking in and out of the nucleus. One prominent component of such machinery is the nuclear pore complexes (NPCs), having diameters of about 9 nm, for regulating the diffusion of macromolecules into the nucleus.5 The study of protein trafficking has revealed that a type of specific peptide sequences, nuclear location sequences (NLS), is a common feature of the proteins that assist nucleic acids to go through NPCs6 to reach the nucleus. For example, NLS, which contain repeats of cationic amino acid residues, interact with negatively charged DNA/RNA to traffic the nucleic acid into the cell...
nucleus. Thus, creating the assemblies to carry positive charges becomes a useful approach for targeting the nucleus, as shown by the study reported by Yang et al. (Figure 1).6

![Figure 1](image)

**Figure 1.** (A) Chemical structures of HCPT and 1. (B) Schematic illustration for the preparation of dual-drug assemblies and the nuclear drug delivery. (A, B) Adapted with permission from ref 8. Copyright 2017, American Chemical Society.

In that study,6 the authors designed 1 (Figure 1A), a molecule that consists of (i) 10-hydroxycamptothecine (HCPT), a DNA-topoisomerase I inhibitor; (ii) diphenylalanine, a self-assembly moiety; and (iii) peptide sequence ERGD, of which the carboxyl can chelate with cisplatin. Molecules of 1 coassemble with 1 equiv of cisplatin to form nanoparticles, named as complex 1-1, and with 1.5 equiv of cisplatin to form nanofibers, named as complex 1-2. With the positive charge provided by cisplatin, those complexes can enter the nucleus of cells to achieve the nuclear delivery and release of both HCPT and cisplatin (Figure 1B). A549 cells, being treated by the complexes, show green fluorescence of HCPT in the nucleus. The related experiments confirm that the formation of the complexes increases the uptake of those two drugs. Those complexes show significantly higher cytotoxicity to cancer cells and higher antitumor efficiency than HCPT or cisplatin only, suggesting local accumulation. This research illustrates an approach for dual-drug nuclear delivery that significantly inhibits cancer cells and tumors. It may ultimately help develop a new nuclear delivery system. However, one of the concerns about 1 is that it contains an L-peptide motif, which is susceptible to proteolysis. One way to address this concern is to utilize D-peptides, as it was shown in a recent report that positively charged D-peptide interacts with RNA for targeting the cell nucleus. The membraneless condensates formed in the nucleus result in DNA damage and eventually induce cell death. The concentration of the D-peptides in that study is, however, too high to be considered as clinically useful. Nevertheless, it establishes the feasibility of using D-peptides for targeting the nucleus and underscores the need of more research on the EISA of D-peptides.

### TARGETING MITOCHONDRIA BY COMBINING EISA AND A CATION OR BY THE PERIMITOCHONDRIAL EISA

Mitochondria, playing key roles in metabolism, are essential for eukaryotic life. Mitochondria also are an essential organelle for cell death and are associated with apoptosis, necroptosis, pyroptosis, and ferroptosis. For example, the permeabilization of the mitochondrial outer membrane can release activators for cell death so that mitochondrial-related cell death is an effective way to kill cancer cells. To induce mitochondrial-related cell death, the first step is to target mitochondria. The usual methods for mitochondria targeting include inducing mitochondrial permeability transition (MPT), promoting mitochondrial outer membrane permeabilization (MOMP), and disrupting mitochondrial metabolism. Besides, HSP90 inhibitors and some natural products such as betulinic acid and resveratrol can also target mitochondria in cancer cells.12

A well-established small molecular motif for targeting mitochondria is triphenyl phosphinium (TPP), which is also suitable for enabling supramolecular assemblies for targeting the mitochondria of cancer cells, as shown by a study that combines EISA and TPP. Specifically, a TPP-modified molecule, 2P, targets mitochondria and selectively kills cancer cells without causing acquired drug resistance.13 Besides incorporating TPP, 2P contains a tetrapeptide (FFYK or fffy) as the self-assembling motif, phosphorylated tyrosine as an alkaline phosphatase (ALP) substrate, and 7-nitro-1,2,3-benzoazadiazole (NBD) as a fluorophore (Figure 2A). On the membrane of cancer cells, dephosphorylation of 2P by overexpressed ALP provides 2, which self-assembles. The assemblies of 2, thus, enter the cells via endocytosis. The assemblies can escape from lysosomes (while the mechanism remains to be elucidated), target mitochondria, and finally result in cell apoptosis (Figure 2B). Confocal laser scanning microscopy (CLSM) shows the trafficking of assemblies in cells: after 1 h incubation with 2P, the fluorescence of NBD appears in the lysosome, and 4 h incubation results in fluorescence localizing in mitochondria. During the cell apoptosis, ELISA analysis shows the expression level of phosphorylated Bad, one of the key signaling proteins in apoptotic pathways, increases first and then decreases quickly, confirming that Bad is activated by the assemblies of 2. This process also leads to the release of cyt c from mitochondria, as confirmed by time-dependent Western blot analysis. After the Saos2 cells are repeatedly stimulated with 2P for 5 weeks, 2P shows almost the same cytotoxicity to the stimulated cells and the unstimulated cells. This result confirms that Saos2 cells are unable to evolve acquired drug resistance to 2P. This research illustrates that the combination of EISA and mitochondria-targeting motif is a promising strategy for cancer treatment without inducing acquired drug resistance.

Most of the reported mitochondria-targeting motifs are lipophilic and cationic, and TPP is one of them. However, lipophilic and cationic molecules may be toxic to normal cells because their accumulation in the mitochondrial matrices lacks
selectivity. A recent study shows that it is feasible to target mitochondria of cancer cells using negatively charged molecules. As shown in Figure 3, a negatively charged amphipathic peptide (3) is able to target the surface of mitochondria via electrostatic interaction (between the negatively charged carboxylic and the intermembrane of mitochondria (positive potential)) and enzymatic proteolysis catalyzed by the proteases on the membrane of mitochondria. The peptide, 3, consists of (i) a self-assembling peptide sequence (Nap-flk); (ii) FLAG-tag (DYKDDDDK), which is not only a substrate of enterokinase (ENTK) but also carries negative charge; and (iii) fluorescent NBD. 3 self-assembles to form nanoparticles, which turn into nanofibers after ENTK cleaves the FLAG-tag off 3. The circular dichroism (CD) spectrum agrees with this transition as well, showing the CD signal of largely β-sheet from the random coil upon the enzymatic cleavage. Being incubated with cells, the micelles of 3 enter cells via endocytosis, escape from the lysosome, traffic to the mitochondria, and then, upon the proteolytic cleavage catalyzed by ENTK, turn into nanofibers on the mitochondria. The cell viability assay has confirmed that 3 exhibits little cytotoxicity to both HeLa and U87MG cells. The cells exhibit strong fluorescence after incubation with 3 for 2 h, which indicates a rapid cellular uptake. The fluorescence of 3 colocalizes well with the fluorescence of the MitoTracker but poorly with the fluorescence of the LysoTracker, suggesting the lysosomal escape. The mitochondria-targeting ability of FLAG-tag enables the potential applications of FLAG-based assemblies to deliver cargos to mitochondria. Replacing the NBD in 3 by tyrosine, the authors obtained an analogue without fluorescence and showed that the analogue could deliver R-phycocerythrin (RPE) and doxorubicin (Dox) into the mitochondria of cancer cells. The delivery of Dox significantly increases the efficacy of Dox against cancer cells. Besides delivering cargos, the FLAG-based sequence is also able to traffic Histone H2B to mitochondria in cancer cells. H2B, being a core histone protein, enters the nucleus to form nucleosomes after being synthesized in the cytoplasm. The FLAG-based sequence traffics H2B to the mitochondria in cancer cells (HeLa and HepG2) but not in normal cells (HEK293). These results illustrate a new way for mitochondria targeting, which may lead to many other applications.

**EISA TO INDUCE ENDOPLASMIC RETICULUM STRESS**

The endoplasmic reticulum (ER) is a membrane-enclosed tubule and sac. As the largest membrane-delimited cellular organelle, it plays many roles in the cells, including calcium storage, protein synthesis, and lipid metabolism. The disturbance in the folding capacity of the ER prompts a cellular stress condition known as ER stress, which can induce apoptosis and cell death. Therefore, the ER stress-related cell death has been considered as a promising anticancer strategy. Since an ER-targeting motif is usually positively charged and lipophilic, it is feasible to design proper substrates of EISA for ER targeting, as shown in a recent study. As shown in Figure 4, a small-molecule 4P consists of (i) a 2-naphthylacetyl group coupled with d-diphenylalanine (Nap-d-Phe-d-Phe), as a self-assembling motif that provides strong aromatic–aromatic interactions and excellent biostability; (ii) a d-phosphotyrosine, as a substrate of ALP; and (iii) a positively charged i-homoarginine residue, as a membrane-interacting motif. In water, 4P self-assembles to form uneven nanoparticles, which converts into crescent-shaped assemblies of 4 and 4P after ALP dephosphorylates some of 4P. On the surface of cells, the crescent-shaped aggregates interact with the lipid membrane to disrupt the membrane. After getting into the cells, the assemblies accumulate at the ER, increase the ER stress, and then lead to the cell death (Figure 4B). Related experiments show that 4P exhibits significantly higher cytotoxicity to ALP-expressing cancer cells than normal cells, indicating the selectivity for the cancer cells that overexpress ALP. While being incubated with 4P, HeLa cells release lactate.
dehydrogenase (LDH) into the culture medium, confirming the disruption of cell membrane. Time-lapse microscopy also shows membrane curvature and tabulation on 4P-treated HeLa cells. To study the localization of 4P in cells, the authors synthesized the fluorescent analogue of 4P, in which NBD replaces the naphthyl group. With the fluorescent analogue, time-lapse microscopy shows that the fluorescence of NBD first appears on the cell surface and then in the cytoplasm after 40 min. The fluorescence of NBD overlaps well with the ER tracker after 1 h incubation. Accumulation of assemblies induces ER dysfunction, as evidenced by the expression of ER-stress markers (Bip, CHOP). The increased expression of XBP-1 and phospho-JNK indicates the activation of the IRE1\(\alpha\) pathway, and the phosphorylation of PERK and eIF2\(\alpha\) reveals the activation of PERK signaling. These results suggest that the EISA of 4P activates IRE1\(\alpha\) and PERK to induce ER stress. This research validates an EISA-based ER targeting for selectively killing cancer cells, which may lead to new cancer therapeutics.

## EISA-TARGETING MEMBRANES

The plasma membrane, mainly consisting of phospholipids, is the outermost subcellular location, which stands between the cytoplasm and external environment. Membranes, containing various proteins, lipids, and small molecules with unique chemical signatures, control the transportation of materials and information. Membranes also form microdomains called lipid rafts, which are enriched cholesterols. This feature makes it feasible to design cholesterol-based assemblies to target lipid rafts of the membrane. For example, a small molecule SP, consisting of (i) a cholesterol, as the motif for membrane targeting, (ii) a phosphorylserine, as the substrate of alkaline phosphatase (ALPL), and (iii) a fluorophore (NBD), is able to target lipid rafts. The targeting largely relies on that ALPL, an ectoenzyme presumably colocalized with lipid rafts, catalyzes the dephosphorylation of SP to provide S, which self-assembles to form the assemblies with strong fluorescence (Figure 5). The antibody to GPI-anchored ALPL colocalizes well with the fluorescence of S, confirming that the assemblies of S localize the lipid raft well. With this probe, the authors studied the dynamic movement of lipid rafts on the membrane of Saos2 cells and observed the lateral mobility of membrane domains and dynamics. Contrasting to other commercial cholesterol-containing probes that are less suitable to show the dynamics of the cell membrane, SP is a robust fluorescent probe for dynamic imaging of the cell membrane. An analogue of SP, without fluorescent NBD, is able to inhibit drug-resistant ovarian cancer cells. This analogue inhibits A2780cis (an ovarian cancer cell line) with a much lower dosage than cisplatin but hardly inhibits HS-5 (a normal cell line). Related experiments proved that the analogue increases the microheterogeneity of the cell membrane. The analogue also activates both extrinsic and intrinsic cell death signaling and induces apoptosis and regulated necrosis to kill the cancer cells. Those two studies illustrate combining EISA and cholesterol for membrane targeting. The cholesterol-rich assemblies from EISA not only act as the probes for imaging of membrane dynamics but also inhibit cancer cells. This work may lead to a new approach for cancer diagnosis and treatment, which would be an alternative to the usual membrane-targeting methods, such as targeting membrane proteins and targeting membrane lipids.

## OUTLOOKS

The distribution of enzymes at different subcellular locations provides a cellular basis for developing EISA as a new approach for developing molecular therapeutics. Because EISA allows the spatiotemporal control of self-assembly in the cellular environment, the chemotherapy agents based on EISA show higher selectivity and efficacy but with less side effects and drug resistance. Moreover, the assemblies, being able to activate multiple cellular pathways, may serve as the candidates of multispecific drugs. As discussed above, EISA has shown promises to target subcellular compartments, including the nucleus, mitochondria, endoplasmic reticulum, and membrane, and to selectively inhibit cancer cells. Further development of EISA may provide fundamentally new approaches for cancer therapeutics.
theranostics. However, several challenges remain to be met. First, the mechanism of cell death in the context of molecular assemblies is less understood. For example, what are the proteins that interact with the molecular assemblies generated by EISA in subcellular organelles? What are the cell pathways being modulated by EISA processes? How does EISA affect the metabolism of molecular assemblies? To address these questions, more detailed studies that combine cell biology, chemical biology, and EISA are necessary. Second, EISA mainly focuses on inhibition of cancer cells, and more animal experiments are needed to prove its antitumor efficacy. Third, it is highly desirable to use EISA to target subcellular organelles for inducing other phenotypes to treat other diseases. For example, how can EISA prevent neurodegenerative diseases or stimulate immune response to boost vaccination? Fourth, how can one reduce the dosages of EISA substrates for treating diseases? Undoubtedly, with increased activities and advancements of the research of EISA for targeting subcellular organelles, more innovations will be developed to address these challenges.

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Notes
The authors declare no competing financial interest.

Biographies

Shuang Liu received his BS and PhD degrees from the University of Science and Technology of China in 2011 and 2016 and then worked as a lecturer at Wuhan University of Technology. After working in Kyoto University as a JSPS postdoctoral researcher under the supervision of Prof. Itaru Hamachi during May 2017 to April 2019, he moved to Brandeis University as a visiting scholar in the group of Prof. Bing Xu. His current work focuses on enzyme-instructed self-assembly to modulate cellular responses.

Bing Xu received his BS and MS degrees from Nanjing University in 1987 and 1990 and obtained his PhD in 1996 from the University of Pennsylvania. Before starting his independent research at the Hong Kong University of Science and Technology (HKUST) in August 2000, he was an NIH postdoctoral fellow at Harvard University. He was tenured as an associate professor in Jan. 2006 and became a full professor in July 2008 at HKUST. Bing Xu currently is a professor in the Department of Chemistry, Brandeis University. His research focuses on the applications of molecular engineering in materials, biology, and medicine.

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