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Enzymatic noncovalent synthesis of peptide assemblies generates multimolecular crowding in cells for biomedical applications†

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Enzymatic noncovalent synthesis enables the spatiotemporal control of multimolecular crowding in cells, thus offering a unique opportunity for modulating cellular functions. This article introduces some representative enzymes and molecular building blocks for generating peptide assemblies as multimolecular crowding in cells, highlights the relevant biomedical applications, such as anticancer therapy, molecular imaging, trafficking proteins, genetic engineering, artificial intracellular filaments, cell morphogenesis, and antibacterial, and briefly discusses the promises of ENS as a multistep molecular process in biology and medicine.

Introduction

Multimolecular crowding is an essential feature of all living cells because biomacromolecules occupy 20–30% of the total volume of cells. It has been well-recognized that multimolecular crowding plays a role in all biological processes.[1] The recent progresses on liquid–liquid phase separation (LLPS) or membraneless condensates in cell biology have highlighted the importance and generality of multimolecular crowding for modulating functions in cells.[2] Although multimolecular crowding or membraneless condensates is an easily comprehensible biophysical concept, the processes that lead to multimolecular crowding are rather complex and tightly regulated by multiple biochemical reactions. In particular, interdependent enzymatic reactions (*e.g.*, posttranslational modifications (PTMs)) control the intermolecular interactions to form high-order and dynamic multimolecular superstructures for functions.[3] A prominent example of such processes would be the formation of actin filaments, which are maintained by ATP hydrolysis catalyzed by actins.[4] Despite the well-established facts that cells use enzymatic reactions to control the intermolecular interactions for generating multimolecular crowding or assemblies, a process that is termed as enzymatic noncovalent synthesis (ENS),[5] the development of ENS of synthetic molecules to control cell behaviors is only at its beginning.

As shown in Scheme 1A, ENS integrates two fundamental non-genetic attributes of life, self-assembly, and enzymatic reactions. The essence of ENS is that enzymatic reactions

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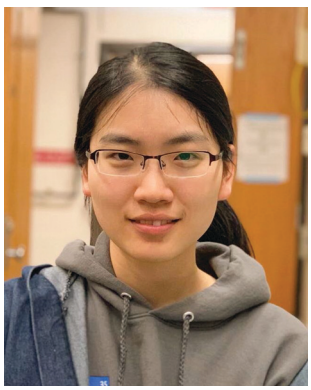

Meihui Yi

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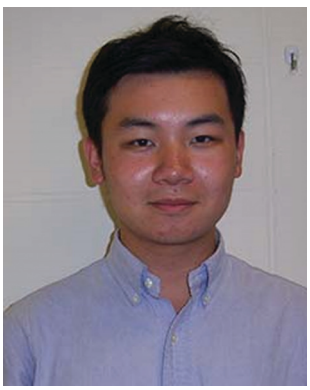

Weiyi Tan

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control intermolecular noncovalent interactions to form higher-order assemblies of new molecules (*i.e.*, multimolecular crowding) for emergent properties and functions. Generally, the molecular assemblies formed by ENS are less diffusive and/or possess different morphology from the precursors of ENS. To illustrate the promises of ENS of synthetic molecules, as opposed to endogenous molecules used by cells, this article focuses on the assemblies of small peptides (or self-assembling peptides) generated by the ENS process. Because (i) the precursors for ENS are normally soluble molecules and diffuse relatively freely in the aqueous phases; and (ii) the assemblies formed by ENS are non-diffusive, it is straightforward for chemists to use ENS for creating multimolecular crowding at the location at which the enzymes reside. When the concentration of the assemblies reaches a certain value, it would lead self-assembly and/or phase transition (*e.g.*, hydrogelation[6]). Moreover, the states of assemblies depend on the enzyme concentration and the time of the reaction, thus leading to a dynamic continuum that is context-dependent and particularly useful for achieving selectivity at the nanoscale. These properties of ENS allow spatiotemporal control of the multimolecular crowding at subcellular locations (Scheme 1B), thus offering a versatile and context-dependent approach to control the cell behavior by the emergent properties of supramolecular assemblies.

To highlight the unique features of ENS, we have arranged this article in the following way. After briefly introducing some representative enzymes and molecular building blocks for generating peptide assemblies as multimolecular crowding in cells, we highlight the biomedical applications of ENS, such as anticancer therapy, molecular imaging, protein trafficking, genetic engineering, artificial cytoskeletons, morphogenesis, and antibacterial. Then, we discuss the outlooks of ENS for exploring multimolecular crowding in biomedicine. We also apologize that we are unable to include all the exciting development on the ENS of peptide assemblies due to the limited space, and like to point out a few excellent recent reviews[7,8] related to this topic.

Enzymes

Because the key step of ENS is the enzymatic reaction(s), the enzyme is the centerpiece to connect biological functions with the self-assembly of biological or synthetic molecules. Although nature has evolved a large pool of enzymes for controlling the assemblies of biomacromolecules, the enzymes, being explored in ENS of peptide assemblies, have largely centered on the phosphatases, esterases, and proteases.

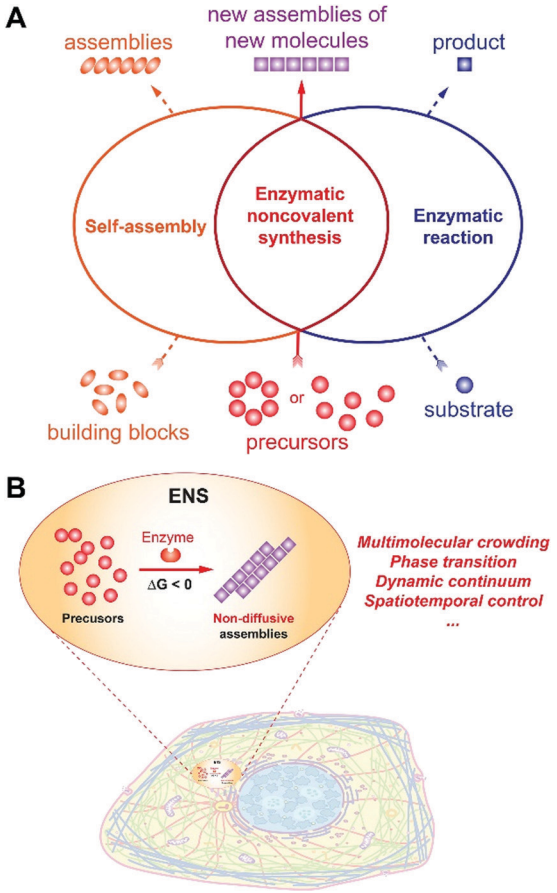
Phosphatases. Being the functional opposite to protein kinases, phosphatases remove the phosphate groups from proteins. As an essential part of the enzyme switch made of phosphatase/kinase, phosphatases regulate all protein dephosphorylation and act as a key regulator for the formation and dissociation of multimolecular crowding in cells. For example, recent advances have shown that phosphatases regulate protein assembly in the formation of inflammasomes.[9] Stimulated by the success of kinase inhibitors for treating a variety of diseases, a majority of research interests in the biology and biochemistry of phosphatases have focused on the development of the inhibitors of phosphatases as potential drug candidates.[10]

Jiaqi Guo

Jiaqi Guo received her BS degree in chemical biology from Xiamen University in 2018. In the same year, she joined the laboratory of Prof. Bing Xu at Brandeis University, where she is now a third-year graduate student focusing on peptide assemblies and their emergent properties in cells.

Bing Xu

Bing Xu, after receiving his BS and MS from Nanjing University in 1987 and 1990, respectively, obtained his PhD, under the supervison of Professor Timothy Swager, in 1996 from the University of Pennsylvania. Before starting his independent research at the Hong Kong University of Science and Technology from Aug 2000, he was an NIH postdoctoral fellow in the Whitesides lab at Harvard University. He was tenured as an associate professor in Jan 2006 and became a full professor in Jul 2008 at HKUST. Based on his works in HKUST and Brandeis University, Bing Xu features on the Thomson Reuters "Highly-Cited Researchers 2014–2017 in Chemistry" lists. He is currently a professor at the Department of Chemistry, Brandeis University. He has made contributions to metallogels, multifunctional magnetic nanoparticles, self-delivery drugs, and supramolecular hydrogels. His current research focuses on the applications of enzymatic noncovalent synthesis in materials, biology, and medicine.



Scheme 1 Schematic illustration of (A) definition of ENS and (B) ENS in cells and the representative features of ENS of small molecules.

While the rapid enzyme kinetics and promiscuity of phosphatases make the clinical translation of phosphatase inhibitors challenging,[11] phosphatases, especially alkaline phosphatases (ALP), become the most explored enzyme for the ENS of peptide assemblies[8,12] because ALP is readily available, exhibits high catalytic activity, and is applicable to a wide range of phosphate-bearing substrates from peptides to nucleotides. The high efficiency of phosphatases is the most important feature for exploring multimolecular crowding regulated by ENS because a very low concentration of phosphatase is needed for dephosphorylation. This key feature warrants that the substrates/products of the phosphatases, not the phosphatase molecules themselves, are the main components of the multimolecular crowding. Protein phosphatases of nine species have been recently classified into a hierarchy of 10 protein folds, 21 families, and 178 subfamilies.[13] It is likely that increasing the exploration will reveal more substrates[14] of different phosphatases[15] for multimolecular crowding. Undoubtedly, more insights will emerge once the focus shifts from the inhibitors of phosphatases back to the substrates of phosphatases,[16] especially with the perspective of the functions (*vide infra*) of multimolecular crowding.

Esterases. Esterases, mainly referring to carboxylesterases (CES) in this article, are a type of enzymes commonly used for activating prodrugs. Being members of the serine hydrolase superfamily, CES catalyzes the hydrolysis of a variety of ester-, amide-, and carbamate-containing molecules to their respective free acids. While the endogenous functions of CES is to facilitate the elimination of toxic xenobiotics by making them more soluble molecules, several clinically used drugs[17] (*e.g.*, capecitabine, dipivefrin, lovastatin, and oseltamivir), paradoxically, rely on CES for activation. After the early demonstration of ENS of intracellular peptide assemblies catalyzed by CES,[18] the use of CES to catalyze the formation of multimolecular crowding is less explored[19] than the case of ALP. The recent report on the esterification of proteins that enhances the cellular uptake of proteins[20] may imply a new way for generating high-order protein assemblies based on ENS catalyzed by CES.

Proteases. Being the catalysts that speed up the breakdown of proteins into smaller polypeptides or single amino acids, proteases also are indispensable enzymes for regulating the multicellular crowding for many cell signalling processes, such as the formation of apoptosomes and inflammasomes.[21] Although the first demonstration of using proteases to trigger the formation of multimolecular crowding of peptide assemblies was achieved by reverse hydrolysis catalyzed by thermolysin,[22] the addition of a protease (*e.g.*, MMP-9) to catalyze the proteolysis of a small nonapeptide (FFFFCGLDD) results in the sol–gel transition after 45 min, which agrees with the self-assembly of FFFFCG after MMP9 cleaves LDD from the nonapeptide.[23] While it is rather common to cleave peptide segments from a polymer for enabling the self-assembly of nanoparticles,[24] it is relatively recent for using proteases to catalyze the formation of the assemblies of peptides.[25,26] Considering the large pool of proteases and their substrates and the cellular distribution of proteases, it is likely that more reports of protease-catalyzed multimolecular crowding in cells will emerge.

Obviously, many other enzymes (*e.g.*, SIRT5[27]) are useful for the ENS of synthetic molecules. The list of the enzymes for ENS certainly will expand in the future with the increasing research interest on this simple yet effective strategy that has been evolved and extensively utilized by nature.

Molecular building blocks

The essential ingredients of ENS are substrates of enzymes. As a precursor of the final assemblies resulting from the enzymatic reaction, the substrates also may aggregate as long as the morphologies or shapes of the final assemblies differ from those of the precursors. Obviously, the structure and the concentration of the precursor determine its state of aggregation or assemblies. Quantitative description, such as critical micelle concentration, is necessary for revealing the self-assembling abilities of the precursors and the products of the enzyme reactions for exploring ENS.[28] Fig. 1 shows some representative molecular building blocks used for the ENS of peptide assemblies and are discussed in this article. They share a few common features: (i) all the precursors are more soluble in water than the products, as shown by their Log *P* values (Table S1, ESI†) estimated by the aggregator advisor.[29] (ii) Most of the peptides bear the well-known self-assembling motif,

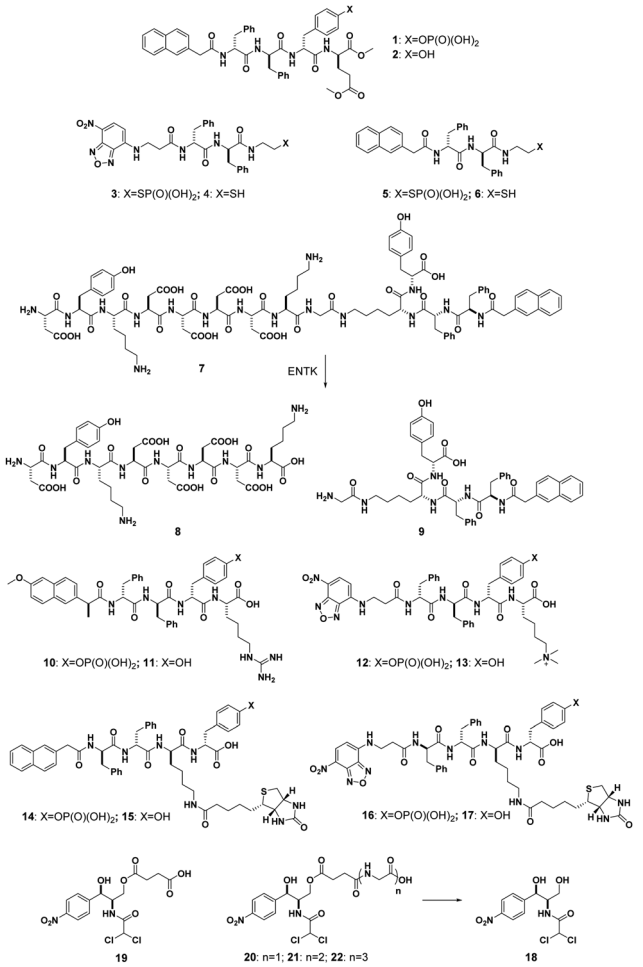


Fig. 1 The molecular structures of molecular building blocks for ENS.

diphenylalanine.[30] (iii) The morphologies of the final assemblies made by ENS drastically differs from the assemblies of the precursors. For example, the TEM of the precursors usually shows the formation of nanoparticles at the physiological pH, and the TEM of the products formed by ENS usually exhibits nanofibers or nanoribbons. In essence, ENS not only converts the precursors to the products but also results in morphological transformation (or shape-shifting) of the assemblies. This feature is critical for designing ENS and controlling the emergent properties of molecular assemblies for functions.

Representative applications

The objective to form assemblies or multimolecular crowding by cells is to regulate the functions, which usually result from or associated with enzymatic reactions. Because the easiest observable regulated cellular function is regulated cell death,[31] it is not surprising that the most explored applications of the ENS of peptide assemblies up-to-date is to selectively kill cancer cells for anticancer therapy.[32] With the increased numbers of works on ENS, other applications are emerging. The following subsections discuss a few recent and representative examples of ENS of peptides in the cellular environment for functions.

Anticancer therapy. Anticancer chemotherapy, though the most important adjuvant therapy to surgery, unavoidably becomes ineffective during the course of treatment because of adaptive drug resistance caused by genomic instability and complex tumor microenvironment.[33] Current cancer drugs are largely based on tight binding to achieve high specificity and inhibitory activity against a target. Paradoxically, tight binding also leads to multidrug resistance (MDR) in cancer therapy when the target mutates. On the other hand, multimolecular crowding, such as the formation of amyloids, is associated with selective cell death in neurodegenerative diseases.[34] Considering that the cytotoxicity of amyloids likely originates from the polymorphism of amyloids and multiple mechanisms,[35] it is difficult for the cells to evolve acquired drug resistance to amyloids. Thus, it is reasonable to generate peptide assemblies, as an mimic of amyloids, for selectively killing cancer cells[36,37] and minimizing acquired drug resistance.[38] There are increased number of reports that peptide assemblies or the assemblies of the conjugates of peptide and clinically-used drug kill cancer cells or inhibit tumor growth.[39] The report on tumor inhibition by only the ENS of the peptide assemblies, however, is rather recent, as demonstrated by the inhibition of osteosarcoma tumor in an orthotropic mice model.[40]

As shown in Fig. 2, ALP, being identified as a cancer marker over half a century ago,[41] also converts extracellular ATP into immunosuppressive adenosine[42] in tumor microenvironment, thus leading to unresponsiveness toward immunotherapy.[43] However, ALP is regarded as "undruggable"[44] due to the critical role of ALP in embryogenesis, bone metabolism, and neuron functions.[44,45] Thus, the ENS of the assemblies of the peptide (**2**) catalyzed by ALP becomes an unprecedented approach that inhibits tumor growth without inhibiting ALP. Specifically, ALP overexpressed on and inside the Saos2 cancer cells, an osteosarcoma cell line, cleaves the phosphate from the precursor (**1**)

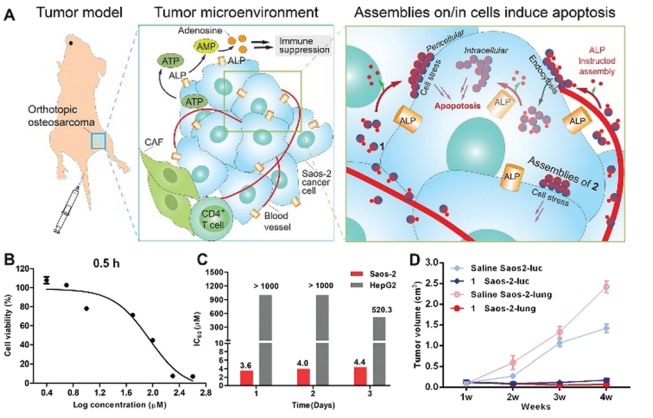


Fig. 2 (A) Schematic representation of ALP-instructed assembly for inhibiting metastatic osteosarcoma within an immunosuppressive tumor. (B) Cell viability of Saos-2 cells treated with **1** for 0.5 h. (C) IC_{50} values of **1** (24 h, 48 h, and 72 h) against Saos-2 or HepG2 cells. (D) Tumor growth curves for orthotopic osteosarcoma model established by Saos2-luc and Saos2-lung cells at weeks 1–4 after **1** or saline treatment. The tumor volumes were calculated with measured average width and length of the tumor (adapted with permission from ref. 40, Copyright 2019 Elsevier Inc).

and triggers the self-assembly of the peptide (**2**). The peptides assemblies, being selectively formed on and inside the Saos2 cells (Fig. 2A),[40] likely activate extrinsic cell death receptors[46] and/or generating intracellular cell stresses[47,48] for killing the cancer cells and inhibiting tumor growth. In particular, **1** exhibits high activity for inhibiting Saos2 cells, with the IC_{50} of 4 μM at 24 h and IC_{90} of about 216 μM at 0.5 h (Fig. 2B). The fast action of **1** indicates rapid ENS processes catalyzed by the high expression level of ALP in the Saos2 cells. Moreover, bearing an esterase detoxification motif (dimethyl-D-glutamate (e_{Me2})),[49] the IC_{50} of **1** on the hepatocyte cells (HepG2) is over two orders of magnitude higher than the IC_{50} on Saos2 (Fig. 2C). This fact warrants that **1** selectively inhibits the osteosarcoma cells without harming the liver cells. After treating the tumor bearing mice with **1** and saline for four weeks, compared to saline, the treatment of **1** results in 8-fold and 25-fold reduction in the tumor volume of Saos2-luc and Saos2-lung tumors, respectively (Fig. 2D), and significantly prolongs the survival time of the osteosarcoma-bearing nude mice. This result confirms that the ENS of the assemblies of **2** effectively inhibits the tumor. This study establishes the use of ENS to generate tumor-targeting multimolecular crowding for cancer therapy. The ability of ENS for targeting ALP-expressing and immunosuppressive cancer cells may be particularly important for developing therapeutics against metastatic tumors, which usually are ALP-overexpressing and immunosuppressive. Moreover, ENS, generating nanostructures *in situ* or on site,[18,50] is an emerging approach for developing nanomedicines.

Golgi imaging and targeting. The use of various imaging modalities (fluorescent, radioactive, and magnetic) to report the biological event *in vivo* promises new strategies for disease diagnosis and monitoring.[51] The use of ENS of peptide assemblies has received considerable exploration for theranostic applications.[52] Despite the considerable progress in molecular imaging, selectively imaging a subcellular organelle remains a challenge, the non-diffusive feature of ENS provides a unique advantage for imaging subcellular organelles,[26,53] especially for imaging and targeting Golgi apparatus (GA).[53]

GA, a stack of dynamically regulated membrane-enclosed disks in mammalian cells, is a key signaling hub of cells and an important target for cancer therapy but there are few approaches for targeting Golgi and selectively killing cancer cells. A recent study unexpectedly shows that changing an oxygen atom of the phosphoester bond in the phosphopeptides by a sulfur atom enables instantly targeted GA and selectively killing cancer cells by ENS. Specifically, the conjugation of cysteamine S-phosphate to the C-terminal of a self-assembling peptide generates a thiophosphopeptide (**3**). As shown in Fig. 3A, **3** being a substrate of ALP, undergoes rapid ALP-catalyzed dephosphorylation to form the thiopeptide (**4**) that self-assembles. This ENS process quickly transforms the nanoparticles of **3** to the nanoribbons of **4** (Fig. 3B). After **3** enters the cells *via* caveolin-mediated endocytosis and micropinocytosis, **4** accumulates in GA because of dephosphorylation and formation of disulfide bonds in Golgi by themselves and with Golgi proteins. In fact, incubating HeLa cells with **3** shows

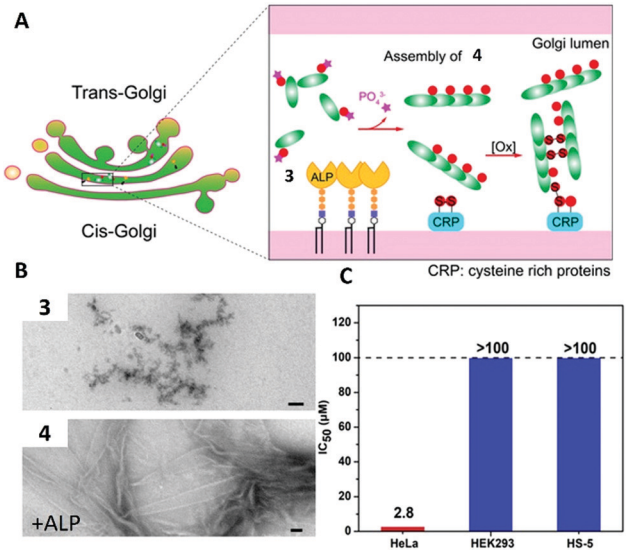


Fig. 3 (A) Illustration of thiophosphopeptides instantly targeting the Golgi apparatus by enzymatic assembling and forming disulfide bonds. (B) The TEM images of **3** (5 μM) before and after the addition of ALP (0.1 U mL^{-1}) for 24 h. Scale bars = 100 nm. (C) IC_{50} of **5** against HeLa cells, HEK293 cells, and HS-5 cells (adapted with permission from ref. 47, Copyright 2021 Wiley).

that **4** instantly accumulates at the GA of the HeLa cells at a concentration as low as 500 nM. Similar rapid ENS-based accumulation of **4** also takes places in the GA of several other cancer cells (*e.g.*, Saos2, SJSA1, OVSAHO, and HCC1937). On the contrary, the parent phosphopeptide requires hours for cellular uptake and largely remains in the endosomes. Moreover, thiophosphopeptide **5**, the analog of **3**, potently and selectively inhibits cancer cells (*e.g.*, HeLa) with the IC_{50} of about 3 μM (Fig. 3C), which is an order of magnitude more potent than that of the parent phosphopeptide. As the first report of thiophosphopeptide for targeting GA, this work illustrates the promise of combining ENS with other intracellular reactions, such as disulfide redox dynamics for functions.

Protein trafficking. Highly dynamic protein trafficking between subcellular organelles (*e.g.*, cytosol, endoplasmic reticulum (ER), mitochondria, and nucleus) is an important way for regulating the cellular functions.[54] While such inter-organelle communications are well-regulated in normal cells, little information exists on how the inter-organelle crosstalk impacts the cancer cells due to the lack of approaches that manipulate inter-organelle communication in the cancer cells. An unexpected observation indicates that the ENS of peptide assemblies is able to traffic the histone protein (H2B), a nuclear protein, to the mitochondria in the cancer cells.[55] As shown in Fig. 4A, precursor (**7**), which contains an aspartic acid (D) repeat, is able to traffic H2B to the mitochondria in the cancer cells (HeLa and HepG2) but not in normal cells (HEK293). This selectivity originates from entereokinase (ENTK) cleaving the aspartic acid repeat off the side chain of **7**. This enzymatic cleavage converts the nanoparticles of **7** to the nanofibers of peptide **9**. Because ENTK localizes at the mitochondria of the

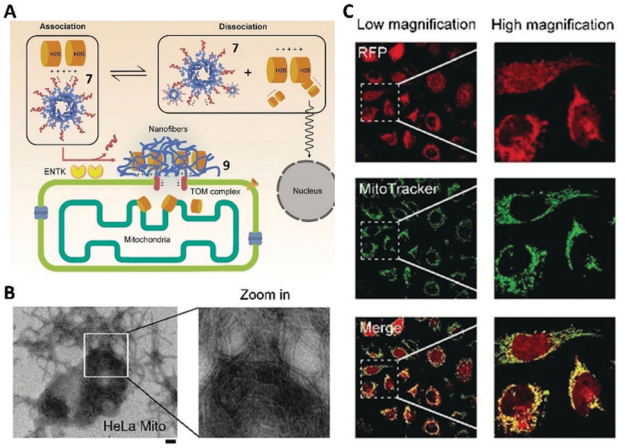


Fig. 4 (A) Illustration of ENS trafficking histone H2B to mitochondria. (B) TEM images of mitochondria isolated from the HeLa cells incubated with 200 μM **7** for 24 h. Scale bar = 100 nm. (C) Fluorescent images of HeLa_H2B-RFP cells incubated with **7** (200 μM, 24 h) (adapted with permission from ref. 49, Copyright 2020 Wiley).

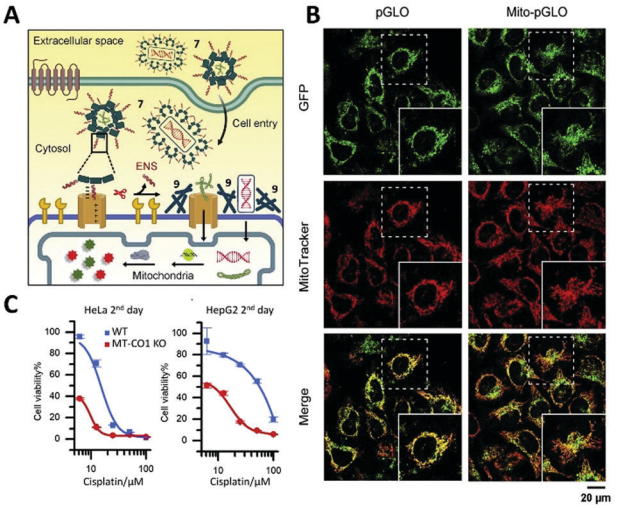


Fig. 5 (A) Schematic illustration of mitochondrial ENS delivering carious cargos for genetic engineering of cancer mitochondria. (B) Fluorescent images of the HeLa cells incubated with pGLO and Mito-pGLO plasmid in the presence of 7. (C) The KO of MT-CO1 sensitizes the HeLa dn HepG2 cells to cisplatin (adapted with permission from ref. 52).

cancer cells (*e.g.*, HeLa or HepG2 cells), this ENS process not only occurs in the cell-free condition but also takes place on the mitochondria of the cancer cells, as shown by the TEM images (Fig. 4B), that **7** transforms into nanofibers after **7** approaches to the mitochondria of the HeLa cells.

The baculovirus encoding the RFP-labelled histone H2B (H2B-RFP)[56] is able to transfect the HeLa cells, and generated the HeLa cells expressing H2B-RFP (labelled as HeLa_H2B-RFP). Without the addition of **7**, the HeLa_H2B-RFP cells themselves exhibit red fluorescence exclusively in the nucleus, which is the normal location of H2B. But the incubation of **7** with the HeLa_H2B-RFP cells results in the fluorescence of H2B-RFP at the mitochondria (Fig. 4C). Immunofluorescence staining and western blot analysis also support that the ENS of **7** traffics H2B-RFP and endogenous H2B to mitochondria. Presumably, ENS enables the nanoparticle-to-nanofiber transformation to increase the local viscosity for retaining H2B on the mitochondria of the cancer cells. A subsequent study suggests that the ENS of the linear peptides that contain aspartate or glutamate repeats also traffics H2B to mitochondria.[57] These works suggest that the ENS of negative charged peptides may be able to mimic the role of protein chaperones for manipulating the inter-organelle communication in cancer cells.

Genetic engineering of mitochondria. Despite the great promise of genetic engineering, it remains a challenge to transfect the genes for cancer therapy because of the difficulty in differentiating cancerous and normal cells for selective transfection. Recent advances reveal that mitochondria, acting as the metabolic center of the cells, participate in multiple cellular signalling processes. Since human mitochondria carry their own DNA, one rational strategy is to deliver the nucleic acids or gene vectors directly to the mitochondria of the cancer cells for cancer therapy. Similar to the proteolytic cleavage that changes the state of viral surface proteins to facilitate the viral entry of specific host cells, the use of perimitochondrial ENS of the peptides (Fig. 5A) provides a new approach for cancer-selective mitochondrial genetic engineering.[58] Specifically, after the micelles made of **7** enter cells mainly *via* clathrin-mediated endocytosis, this multiple-step ENS process likely consists of two critical steps: (i) micelles of **7** bind to receptors on the mitochondrial membrane; (ii) ENTK cleaves the aspartate repeats from the side chain of **7** to convert the micelles/nanoparticles of **7** to the nanofibers of **9**, which likely increases the perimitochondrial viscosity. This perimitochondrial morphology/phase transition enables gene vectors carried by the micelles to be locally positioned at the mitochondria for transfection. When the plasmid of green fluorescent protein (pGLO) is mutated with mitochondria-specific codons[59] to generate a mitochondrial specific plasmid of GFP (Mito-pGLO), this perimitochondrial ENS process still transfects Mito-pGLO in the mitochondria (Fig. 5B) to give green fluorescence. This expression of Mito-pGLO confirms the perimitochondrial ENS for genetic engineering illustrated in Fig. 5A.

Moreover, the use of **7** is able to transfect the gene vectors encoding CRISPR/Cas9[60] into the mitochondria of the cancer cells, which knockouts the MT-CO1 gene, depletes oxidative phosphorylation (OXPHOS), and re-sensitizes the cancer cells to cisplatin (Fig. 5C). In addition, **7** also facilitates the gene expression of FUNDC1 and GFP-tagged p53 proteins in the mitochondria of cancer cells for mitophagy and apoptosis, respectively.[58] Preliminary mechanistic study reveals that the electrostatic interaction between **7** and voltage-dependent anion channel (VDAC) on the mitochondrial surface favors the mitochondria-specific attachment of the micelles of **7**. For normal cells having a low level or the HeLa cell with the knockdown of mitochondria ENTK, perimitochondrial ENS is likely too slow to produce nanofibers of **9** on the mitochondria; thus, there is little transfection of the gene vectors in the mitochondria. This work, illustrating perimitochondrial ENS

for assisting the genetic engineering of cancer mitochondria, may lead to a versatile strategy for selectively targeting the mitochondria of the cancer cells.

Enzyme sequestration. A new insight from the study of membraneless condensates[61] in the cells is that multimolecular crowding is a fundamental mechanism of cellular signalling.[62] This perspective has inspired the design of ENS of synthetic mimics to form membraneless condensates for enzyme sequestration[63] because ENS easily results in sol-to-gel transition, a type of liquid–liquid phase transition. For example, the conjugation of an NSAID drug (naproxen), the self-assembling D-diphenylalanine (D-Phe–D-Phe), an enzyme trigger (D-phosphotyrosine), and a positively charged homoarginine residue at the C-terminal of the peptide creates an ENS precursor, **10**. As shown in Fig. 6, this straightforward design enables **10** to interact selectively with COX-2[64] and to serve as a substrate for PTP1B[65] simultaneously. Moreover, homoarginine favors the assemblies of **11** to form on ER *via* ENS (Fig. 6).[47] Being partially dephosphorylated by the phosphatases, precursor (**10**) and its corresponding hydrogelator (**11**) likely co-assemble to form supramolecular assemblies that promote the association of COX-2 and PTP1B on ER, as revealed by immunofluorescence staining (Fig. 6). Further structure–activity relationship studies also confirm that the COX-2 binding NSAID motif and the phosphatase substrate are essential for the association of the enzymes. This work represents the first example of using ENS to generate a type of synthetic membraneless condensates for enzyme sequestration. Further exploration along this direction may provide useful insights for understanding intracellular liquid condensates and offer a new strategy for modulating protein–protein interactions by multimolecular crowding.

Artificial intracellular filaments. Intracellular protein filaments, such as actin filaments and microtubules, are essential for cellular functions but forming *bona fide* biomimetic intracellular filaments of small molecules in the living cells has remained elusive until the recent report of artificial intracellular filaments formed by the ENS.[66] The precursor (**12**) consists of a fluorescent dye (nitrobenzoxadiazole (NBD)), D-Phe–D-Phe, D-phosphotyrosine, and a C-terminal trimethyl-L-lysine. As the substrate of ALP, the nanoparticles of **12** turn into the filaments of the NBD-conjugated peptide (**13**) (Fig. 7) upon the dephosphorylation catalyzed by ALP in the cell-free condition and inside the cells. It was unexpected that the trimethylation of L-lysine is necessary for the formation of intracellular filaments, which are confirmed by electron tomography.[66] The filaments made of **13**, exhibiting monodispersed diameters, pack as twist bundles and extend from the plasma membrane to the nuclear membrane inside the cells. Moreover, the cryo-EM structural determination by helical reconstructions using IHRSR[67] reveals that **13** self-assembles into two distinct types of cross-β structures that possess either C7 or C2 symmetries. MD simulations suggest that water and ions are likely present in the central pore of the filament to stabilize the filament structure. The intracellular filaments made of **13**, though exhibiting the appearance of microtubules, are orthogonal to endogenous cytoskeletons. Although these artificial filaments impede cell migration, they remain cell compatible. As the first definitive confirmation of artificial intracellular filaments since the demonstration of intracellular hydrogelation,[36] this work established the use of ENS to generate the regulated multimolecular crowding of small molecules in a highly dynamic and crowded intracellular environment, which may provide valuable insights for understanding the pathogenic filaments of protein or peptides.

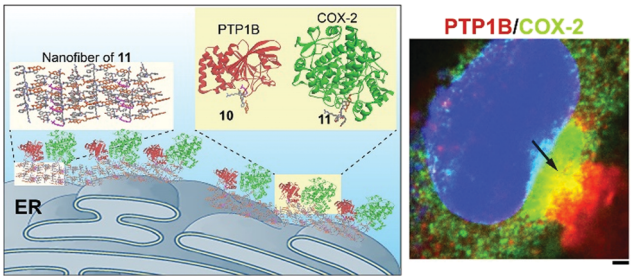


Fig. 6 Illustration of the ENS of peptide assemblies for the intracellular sequestration of PTP1B and COX-2 at the ER region (CLSM images of Saos-2 cells treated with **10** (12.5 μM) for 1 h and then stained with antibodies of PTP1B (red) and COX-2 (green)) (adapted with permission from ref. 63, Copyright 2018, American Chemical Society).

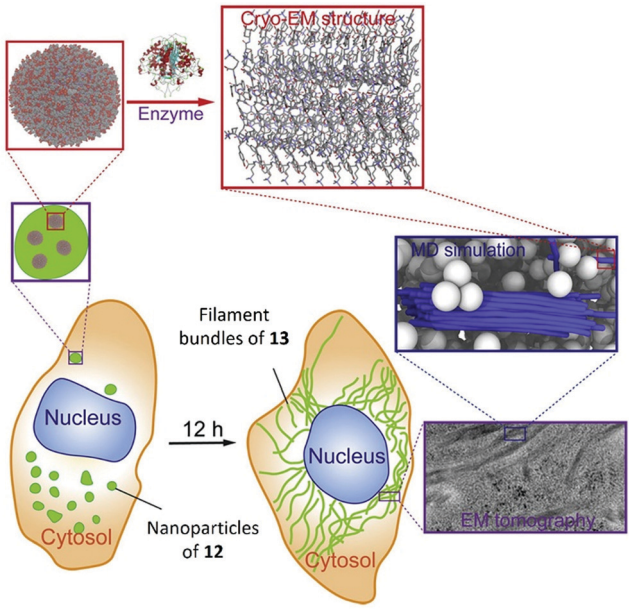


Fig. 7 Enzymatic morphological transition 12 to 13 leads to the *in situ* formation of self-limiting intracellular peptide filaments in the live cells (adapted with permission from ref.62).

Cell morphogenesis. Extracellular matrix (ECM) plays multifarious roles in cellular processes.[68] Considerable efforts have centered on designing biomaterials to mimic ECM for guiding the behavior of cells.[69] Most synthetic ECM materials, however, lack the dynamic features exhibited by ECM proteins.[70] For example, ECM undergoes cell-mediated remodelling, which plays essential physiological roles in regulating tissue architecture, morphogenesis, and homoeostasis.[71] A well-studied example of cell-mediated ECM remodelling is the unfolding of fibronectin (FN), which exhibits globular-to-fibrillar protein

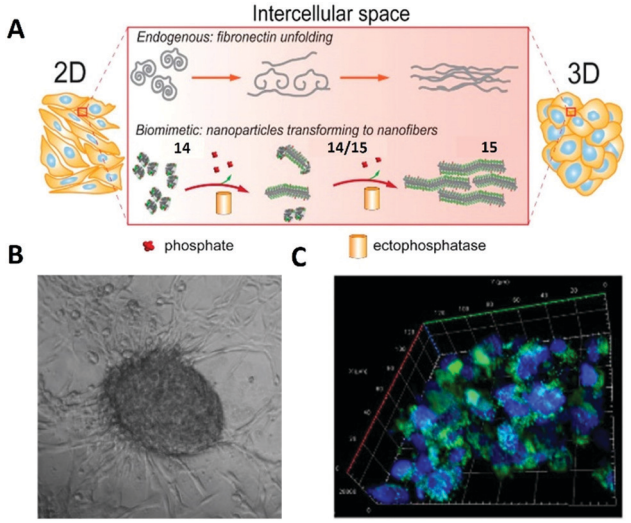


Fig. 8 (A) Intercellular ENS to mimic the essence of the dynamic of an ECM protein (*e.g.*, fibronectin unfolding). (B) Optical images of HS-5 cells in the culture medium and coincubation with 14. (C) Three-dimensional construction of HS-5 cells treated with 16 (200 μM) for 24 h (adapted with permission from ref.,69 Copyright 2019, American Chemical Society).

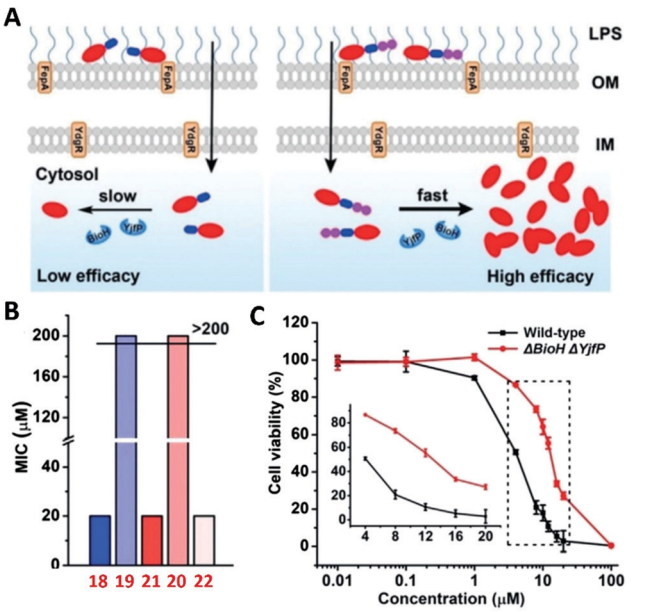


Fig. 9 (A) and (B) The minimum inhibitory concentrations (MIC) of CL, CLsu, **19**, **18**, and **20** against a wild-type *E. coli* strain (K12). (C) The antibacterial activity of **19** against a double esterase (BioH and YjfP) deletion mutant of *E. coli* (inset: magnified image in the dashed square) (adapted with permission from ref. 71, Copyright **201**9 Wiley).

transformation. Several recent reports show that ENS is able to mimic the essence of such remodelling by generating a dynamic continuum of peptide assemblies to promote spheroid formation,[72,73] the simplest form of cell morphogenesis. For example, a biotinylated D-phosphopeptide (**14**), as the substrate for ENS catalyzed by phosphatase, is able to induce cell spheroids, *i.e.*, being partially dephosphorylated by phosphatases, the nanoparticles of **14** turn into the nanofibers of the biotin-conjugated peptide (**15**) at the intercellular space (Fig. 8A). This ENS process transforms a 2D cell sheet of the HS-5 cells to 3D cell spheroids (Fig. 8B). The fluorescent analogue (**16**) of **14** also confirms that the intercellular assemblies made of the biotin-conjugated fluorescent peptide (**17**) (Fig. 8C) interact with multiple ECM components (*e.g.*, laminin, and collagens III and IV) within the cell spheroids.[72] This rather simple result demonstrates that the morphological transition of multimolecular crowding controlled by ENS is able to modulate intercellular mechanical forces, which may serve as localized biophysical determinants for cellular signal transductions.

Antibacterial prodrugs. While the exploration of the ENS of peptide assemblies has been centered on mammalian cells, a similar study is less frequent on bacteria, although the assemblies of antibiotics exhibit high potency against drug-resistant bacteria.[74] The recent reports related to antibacterial ENS are on the hydrolysis of the conjugates of chloramphenicol (CL (**18**)) and peptides.[75,76] CL, being a broad-spectrum antibiotic, inhibits protein production in both Gram-negative and Gram-positive bacteria.[77] Because of the adverse effects[78] of CL, its prodrug, **19**, was developed. Hydrolyzing too slow and existing body too fast, **19**, however, is less effective than even CL when **19** is administrated intravenously.[79] The conjugation of the simplest dipeptide, diglycine (GG), with **19** creates a new conjugate, **21**, which can be rapidly hydrolyzed by intrabacterial esterases (*e.g.*, BioH[80] and YjfP[81]) to regenerate CL inside the bacteria (Fig. 9A). As shown in Fig. 9B, while the conjugates of **19** with peptides differ only in the number of glycine residues, these conjugates (**20**, **21**, and **22**) exhibit drastically different antibacterial activity. For example, their MIC value against *E. coli* follows the order of **21**, **22** (20 μM) < **19**, **20** (higher than 200 μM). The conjugates **21** and **22** not only exhibit about 10 times higher inhibitory activity than **19** against *E. coli* (Fig. 9B) but also show lower cytotoxicity than **18** toward bone marrow stromal cells.[75] Moreover, the double deletion of BioH and YjfP (two bacterial esterase genes) significantly reduces the antibacterial activity of **21**, down to 50% of the activity against the wild type *E. coli* (Fig. 9C). This result suggests that, after **21** enters the *E. coli*, various esterases in the bacterial cytoplasm rapidly convert **21** to the active antibiotic agent (**18**) and likely accumulate inside the bacteria. As the first example of increasing the rate of intrabacterial activation of antibiotic prodrugs, this work indicates that the intracellular ENS of the peptide assemblies[82] may lead to a new approach that increases the intracellular accumulation of antibiotics for combating antimicrobial drug resistance, which remains a threat to public health.

Outlooks

The above examples of the ENS of the peptide assemblies have highlighted that ENS regulates spatially organized higher-order molecular assemblies to modulate the cellular processes. There are several directions of ENS likely worth exploration

for multimolecular crowding in the cells. (i) New secondary structures. Most of the peptide assemblies formed by ENS result from the self-assembly of the β-sheets. The large pool of the peptides that form assemblies made of α-helices,[83] in fact, may be a treasure trove for exploring ENS. It is likely that expanding the peptide building blocks to α-helices will lead to new applications of ENS, as recently shown in the selective elimination of human-induced pluripotent stem cells.[84] (ii) New substrates. While the immense diversity offered by peptides warrants further applications of ENS of peptides, molecular building blocks other than peptides, such as carbohydrate,[85] nucleobase,[86] or other organic molecules,[87] should be useful to serve as the precursors or products of ENS. (iii) New interactions. Most of the current activities have focused on designing the substrates of ENS for generating supramolecular assemblies, the interactions of the assemblies with endogenous cellular components remain less explored. It is conceivable that the precise interactions between the cellular targets and the assemblies would lead to new applications. (iv) Structure determination. One prerequisite for designing the assemblies for the functions is to determine the structures of the assemblies without and with the ENS. While the recent structural determination of the structure of the artificial intracellular filaments[66] highlights the feasibility for determining the structure of the assemblies resulting from ENS, it also underscores the need and challenge for the structural determination of the multimolecular crowding/assemblies *in situ* inside the cells. (v) New applications. The applications of ENS for multimolecular crowding are certainly beyond the examples highlighted in this article. Undoubtedly, the use of enzymatic reactions to regulate the intermolecular interactions of synthetic molecules from the perspective of multimolecular crowding will lead to new discoveries in science and practical applications for addressing societal needs.

Conflicts of interest

There are no conflicts to declare.

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