ChemComm

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Journal:	*ChemComm*
Manuscript ID	CC-FEA-10-2021-005565.R1
Article Type:	Feature Article

ARTICLE

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

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Enzymatic noncovalent synthesis enables 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 for biology and medicine.

Introduction

Multimolecular crowding is an essential feature of all living cells because biomacromolecules occupying 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 the liquid-liquid phase separation (LLPS) or membraneless condensates in cell biology has 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. Particularly, 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 the ATP hydrolysis catalysed by actins.[4] Despite the well-established facts that cells use enzymatic reactions to control 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 behaviours 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 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 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 certain value, it would lead self-assembly and/or phase transition (e.g., hydrogelation[6]). Moreover, the states of the assemblies depend on the enzyme concentration and time of reaction, thus lead to a dynamic continuum that is context dependent and particularly useful for achieving selectivity at nanoscales. 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 cell behaviour by the emergent properties of supramolecular assemblies.

To highlight the unique features of ENS, we arrange 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 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.

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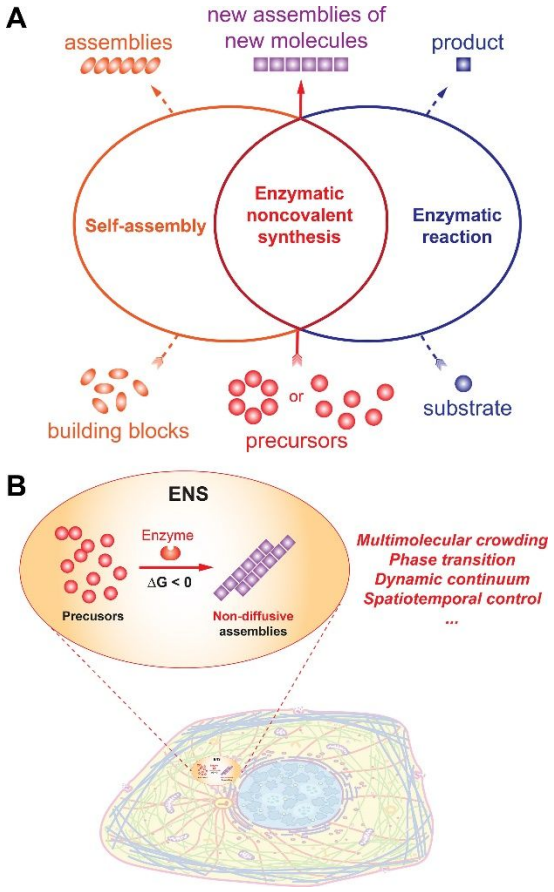
Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x



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

Enzymes

Because the key step of ENS is the enzymatic reaction(s), enzyme is the centrepiece 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 centred on the phosphatases, esterases, and proteases.

Phosphatases. Being the functional opposite to protein kinases, phosphatases remove 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 various of diseases, the 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] 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, exhibit 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 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 catalyses 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 become 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 catalysed by CES,[18] the use of CES to catalyse the formation of multimolecular crowding is less explored[19] than the case of ALP. The recent report of the esterification of proteins that enhances cellular uptake of proteins[20] may imply a new way for generating high-order protein assemblies based on ENS catalysed by CES.

Proteases. Being the catalysts to 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 the reverse hydrolysis catalysed by thermolysin,[22] the addition of a protease (e.g., MMP-9) to catalyse the proteolysis of a small nonapeptide (FFFFCGLDD) is able to result in the sol-gel transition after 45 minutes, 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 self-assembly of nanoparticles,[24] it is relatively recent for using proteases to catalyse 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 catalysed multimolecular crowding in cells will emerge.

Obviously, many other enzymes (e.g., SIRT5[27]) are useful for 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.

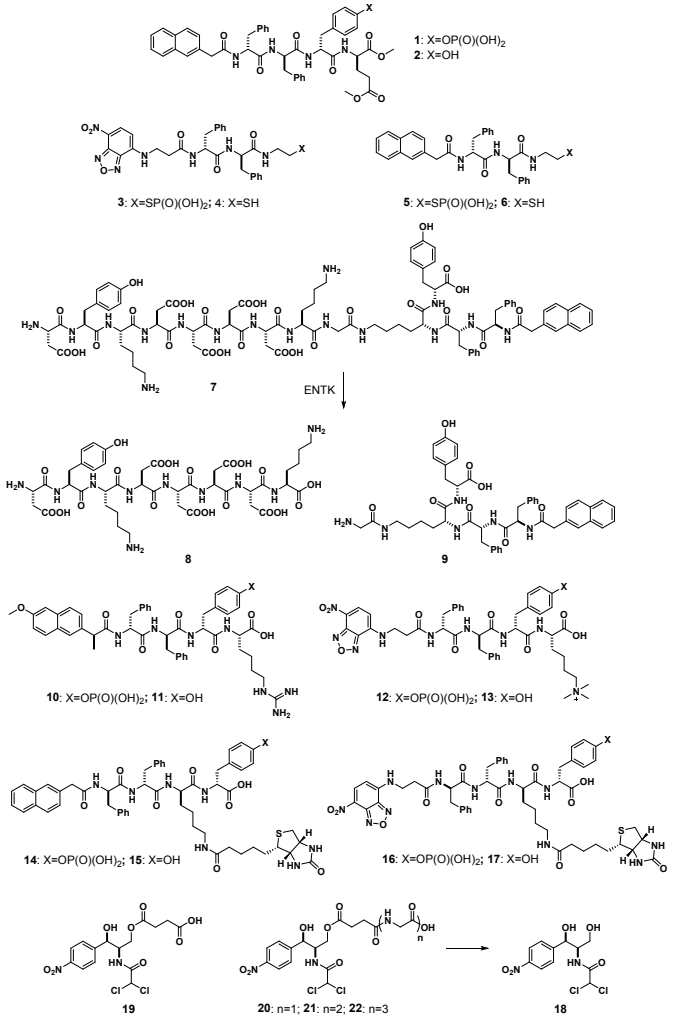


Figure 1. The molecular structures of molecular building blocks for ENS.

Molecular building blocks

The essential ingredients of ENS are substrates of enzymes. As a precursor of the final assemblies resulted 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 the 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] Figure 1 shows some representative molecular building blocks used for the ENS of peptide assemblies and 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 LogP values (Table S1) estimated by the Aggregator Advisor.[29] (ii) Most of the peptides bear the well-known self-assembling motif, diphenylalanine.[30] (iii) The morphologies of 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 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 functions, which usually are resulted 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 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 cellular environment for functions.

Anticancer therapy. Anticancer chemotherapy, though remaining to be 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, the 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 polymorphism of amyloids and multiple mechanisms,[35] it is difficult for 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 of tumor inhibition by only the ENS of peptide assemblies, however, is rather recent, as demonstrated by the inhibition of osteosarcoma tumor in an orthotropic mice model.[40]

As shown in Figure 2, ALP, being identified as a cancer marker over half-century ago,[41] also converts extracellular ATP into immunosuppressive adenosine[42] in tumor microenvironment, thus leading unresponsiveness to immunotherapy.[43] But ALP is regarded as "undruggable"[44] due to the critical role of ALP in embryogenesis, bone metabolism, and neuron functions.[44, 45] Thus, ENS of the assemblies of the peptide (**2**) catalysed by ALP becomes an approach that inhibits tumor growth without

inhibiting ALP. Specifically, ALP overexpressed on and inside Saos2 cancer cells, an osteosarcoma cell line, cleaves the phosphate from the precursor (**1**) and triggers the self-assembly of the peptide (**2**). The peptides assemblies, being selectively formed on and inside 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. Particularly, **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 catalysed by the high expression level of ALP in 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 liver cells. After treating the tumor bearing mice with **1** and saline for four weeks, comparing to saline, the treatment of **1** results in the 8-fold and 25-fold reduction in 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 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 develop nanomedicines.

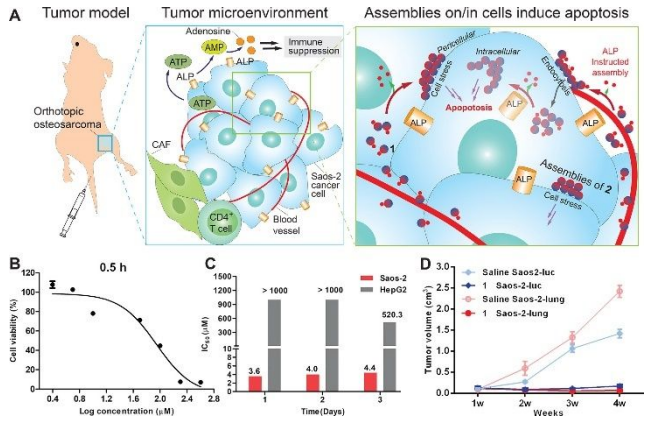


Figure 2. (A) Schematic representation of ALP-instructed assembly for inhibiting metastatic osteosarcoma within an immunosuppressive tumor. (B) Cell viability of Saos-2 cells treated with **1** for 0.5 h. (C) IC_{50} values of **1** (24 h, 48 h, and 72 h) against Saos-2 or HepG2 cells. (D) Tumor growth curves for orthotopic osteosarcoma model established by Saos2-luc and Saos2-lung cells at week 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)

Golgi imaging and targeting. The use of various imaging modalities (fluorescent, radioactive, and magnetic) to report 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 phosphopeptides by a sulfur atom enables instantly targeting 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 Figure 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** (Figure 3B). After **3** enters 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 that **4** instantly accumulates at GA of the HeLa cells at the 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 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 (Figure 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.

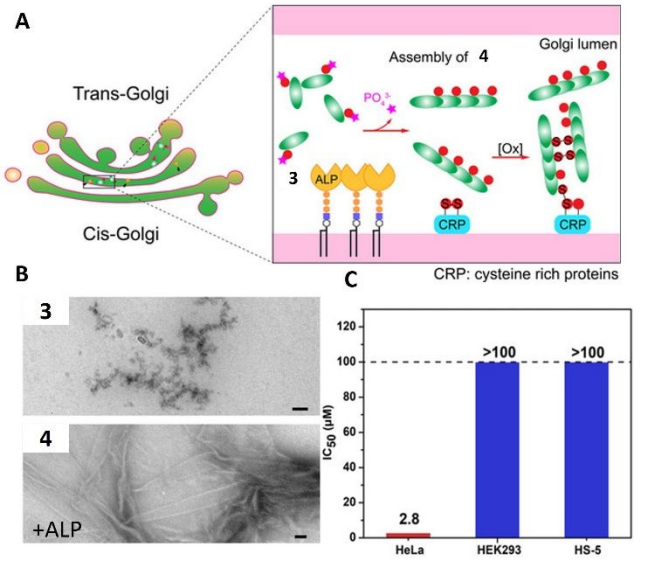


Figure 3. (A) Illustration of thiophosphopeptides instantly targeting the Golgi apparatus by enzymatic assembling and forming disulfide bonds. (B) the TEM images of **3** (5 μM) before and after the addition of ALP (0.1 U/mL) for 24 h. Scale bars = 100 nm. (C) IC_{50} of **5** against HeLa cells, HEK293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

Protein trafficking. Highly dynamic protein trafficking between subcellular organelles (e.g., cytosol, endoplasmic reticulum (ER), mitochondria, and nucleus) is an important way for regulating cellular functions.[54] While such inter-organelle communications are well-regulated in normal cells, little information exists on how the inter-organelle crosstalk impacts cancer cells due to the lack of approaches that manipulate inter-organelle communication in cancer cells. An unexpected observation indicates that ENS of peptide assemblies is able to traffic histone protein (H2B), a nuclear protein, to the mitochondria in cancer cells.[55] As shown in Figure 4A, the precursor (**7**), which contains an aspartic acid (D) repeat, is able to traffic H2B to the mitochondria in cancer cells (HeLa and HepG2), but not in normal cells (HEK293). This selectivity originates from that entereokinase (ENTK) cleaves 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 cancer cells (e.g., HeLa or HepG2 cells), this ENS process not only occurs in cell-free condition, but also takes place on the mitochondria of the cancer cells, as shown by the TEM images (Figure 4B) that **7** transforms into nanofibers after **7** approaches to the mitochondria of the HeLa cells.

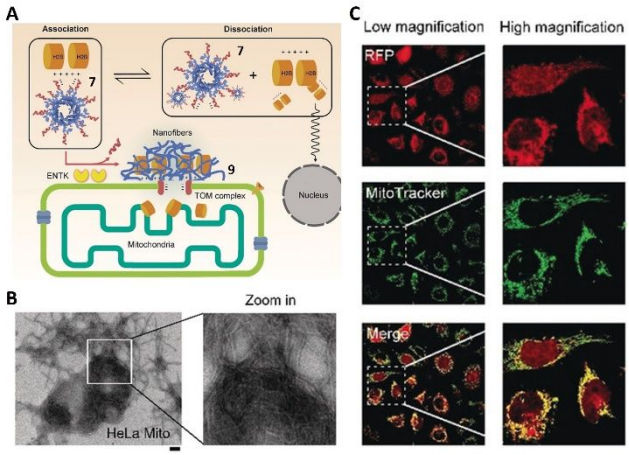


Figure 4. (A) Illustration of ENS trafficking histone H2B to mitochondria. (B) TEM images of mitochondria isolated from HeLa cells incubated with 200 μM **7** for 24 h. Scale bar = 100 nm. (C) Fluorescent images of HeLa_H2B-RFP cells incubated with **7** (200 μM, 24 h). (Adapted with permission from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

The baculovirus encoding RFP-labelled histone H2B (H2B-RFP)[56] is able to transfect 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 mitochondria (Figure 4C). Immunofluorescence staining and western blot analysis also support that ENS of **7** traffics H2B-RFP and endogenous H2B to mitochondria. Presumably, ENS enables the nanoparticles-to-nanofiber transformation to increase local viscosity for retaining H2B on mitochondria of the cancer cells. A subsequent study suggests that ENS of linear peptides that contain aspartate or glutamate repeats also traffics H2B to mitochondria.[57] These works suggest that 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 genes for cancer therapy because of the difficulty in differentiating cancerous and normal cells for selective transfection. Recent advances reveal that mitochondria, acting as metabolic centre of 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 cancer cells for cancer therapy. Being similar to that the proteolytic cleavage changes the state of viral surface proteins to facilitate viral entry of specific host cells, the use of perimitochondrial ENS of peptides (Figure 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 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 increase the perimitochondrial viscosity. This perimitochondrial morphology/phase transition enables gene vectors carried by the micelles to be locally positioned to 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 mitochondria (Figure 5B) to give green fluorescence. This expression of Mito-pGLO confirms that the perimitochondrial ENS for genetic engineering illustrated in Figure 5A.

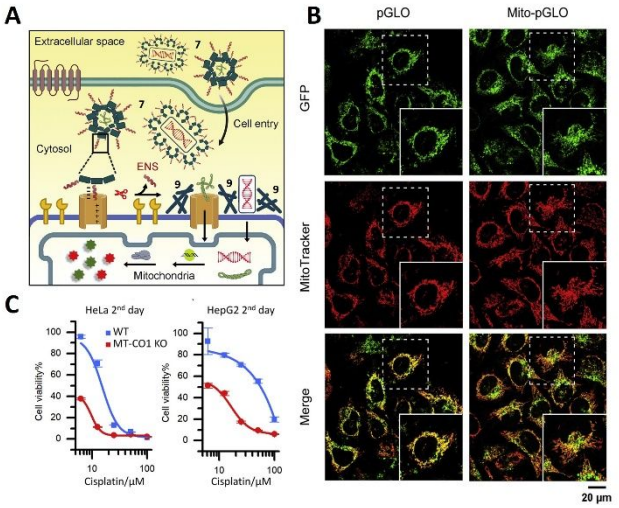


Figure 5. (A) Schematic illustration of mitochondrial ENS delivering carious cargos for genetic engineering of cancer mitochondria. (B) Fluorescent images of the HeLa cells incubated with pGLO and Mito-pGLO plasmid in the presence of **7**. (C) The KO of MT-CO1 sensitizes the HeLa dn HepG2 cells to cisplatin.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

Moreover, the use of **7** is able to transfect gene vectors encoding CRISPR/Cas9[60] into the mitochondria of cancer cells, which knockouts MT-CO1 gene, depletes oxidative

phosphorylation (OXPHOS), and re-sensitizes the cancer cells to cisplatin (Figure 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 mitochondrial surface favours the mitochondria-specific attachment of the micelles of **7**. For the normal cells having a low level or the HeLa cell with knockdown of mitochondria ENTK, perimitochondrial ENS likely is too slow to produce nanofibers of **9** on mitochondria, thus there is little transfection of the gene vectors in the mitochondria. This work, illustrating perimitochondrial ENS for assisting genetic engineering of cancer mitochondria, may lead to a versatile strategy for selectively targeting mitochondria of cancer cells.

Enzyme sequestration. A new insight from the study of membraneless condensates[61] in 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 a 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 Figure 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 favours the assemblies of **11** to form on ER via ENS (Figure 6).[47] Being partially dephosphorylated by phosphatases, the 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 (Figure 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.

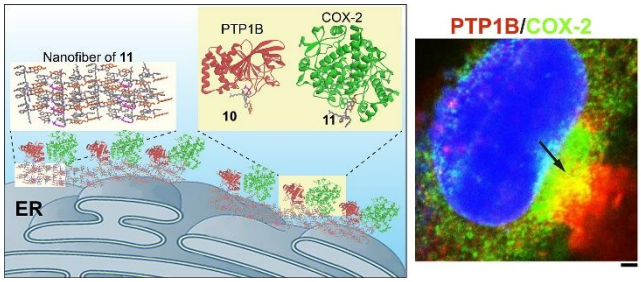


Figure 6. Illustration of ENS of peptide assemblies for intracellular sequestration of PTP1B and COX-2 at ER region (CLSM images of Saos-2 cells treated with **10** (12.5 μM) for 1 h and then stained with antibodies of PTP1B (red) and COX-2 (green)). (Adapted with permission from ref[63], Copyright 2018,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

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 living cells remains elusive until the recent report of artificial intracellular filaments formed by 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** turns into the filaments of the NBD-conjugated peptide (**13**) (Figure 7) upon the dephosphorylation catalysed by ALP in cell-free condition and inside cells. It was unexpected that 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 nuclear membrane inside 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 likely are present in the central pore of the filament to stabilize to 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 regulated multimolecular crowding of small molecules in a highly dynamic and crowded intracellular environment, which may provide valuable insights for understanding pathogenic filaments of protein or peptides.

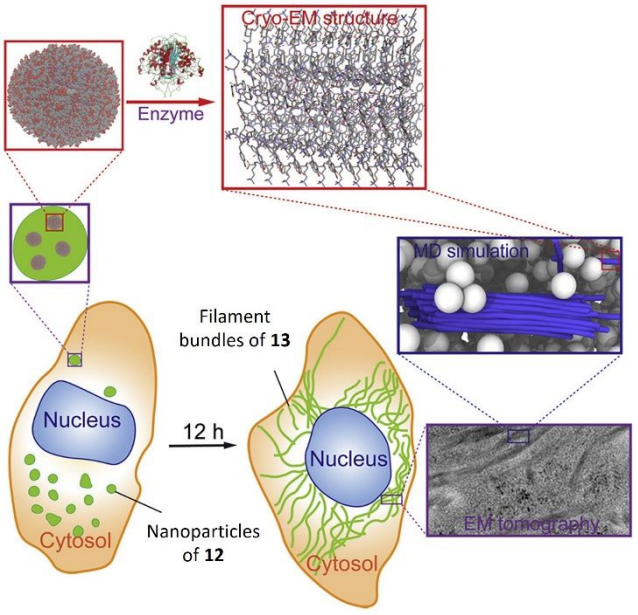


Figure 7. Enzymatic morphological transition **12** to **13** leads to the in-situ formation of self-limiting intracellular peptide filaments in live cells. (Adapted with permission from ref.[62])

Cell morphogenesis. Extracellular matrix (ECM) plays multifarious roles in cellular processes.[68] Considerable efforts have centred on designing biomaterials to mimic ECM for guiding the behaviour 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 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 catalysed by phosphatase, is able to induce cell spheroids. That is, being partially dephosphorylated by phosphatases, nanoparticles of **14** turn into nanofibers of the biotin-conjugated peptide (**15**) at the intercellular space (Figure 8A). This ENS process transforms a 2D cell sheet of HS-5 cells to 3D cell spheroids (Figure 8B). The fluorescent analogue (**16**) of **14** also confirms that the intercellular assemblies made of the biotin-conjugated fluorescent peptide (**17**) (Figure 8C) interact with multiple ECM components (e.g., laminin, collagens III and IV) within the cell spheroids.[72] This rather simple result demonstrates that 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.

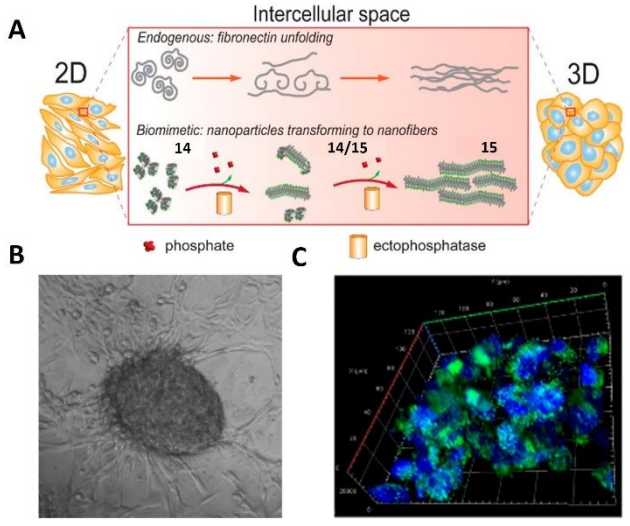


Figure 8. (A) Intercellular ENS to mimic the essence of the dynamic of an ECM protein. (e.g., fibronectin unfolding) (B) Optical images of HS-5 cells in culture medium and coincubation with **14**. (C) Three-dimensional construction of HS-5 cells treated with **16** (200 μM) for 24 h. (Adapted with permission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

Antibacterial prodrugs. While the exploration of ENS of peptide assemblies has been centred on mammalian cells, the 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 proteins production in both of Gram-negative and Gram-positive bacteria.[77] Because of the adverse effects[78] of CL, its prodrug, **19**, was developed. Hydrolysing too slow and existing body too fast, **19**, however, is less effective than CL even when **19** being administrated intravenously.[79] The conjugation of the simplest dipeptide, diglycine (GG), with **19** creates a new conjugate, **21**, which can be rapidly hydrolysed by intrabacterial esterases (e.g., BioH[80] and YjfP[81]) to regenerate CL inside bacteria (Figure 9A). As shown in Figure 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 of 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* (Figure 9B), but also show lower cytotoxicity than **18** towards 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* (Figure 9C). This result suggests that, after **21** entering E. coli, various esterases in bacterial cytoplasm rapidly convert **21** to the active antibiotic agent (**18**) and likely accumulate inside bacteria. As the first example of increasing the rate of intrabacterial activation of antibiotic prodrugs, this work indicates that intracellular ENS of peptide assemblies[82] may lead to a new approach that increases intracellular accumulation of antibiotics for combating antimicrobial drug resistance, which remains a threat to public health.

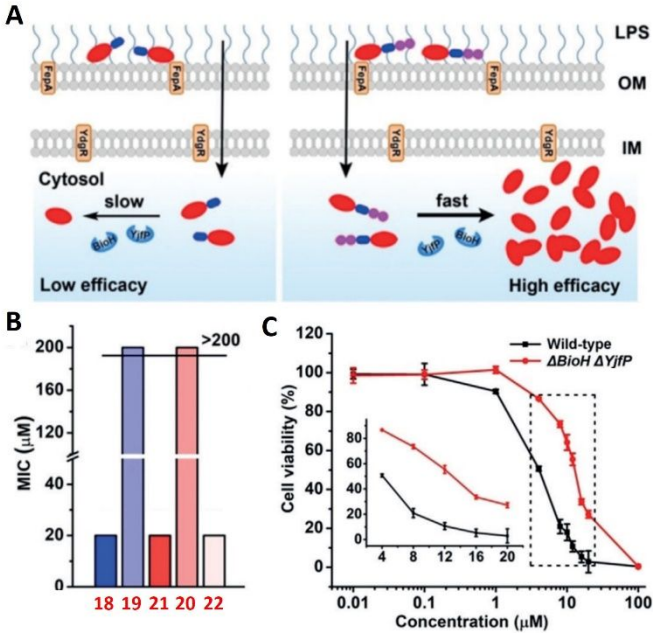


Figure 9. (A) (B) The minimum inhibitory concentrations (MIC) of CL, CLsu, **19**, **18**, and **20** against a wild-type *E. coli* strain (K12). (C) The antibacterial activity of **19** against a double esterase (BioH and YjfP) deletion mutant of *E. coli* (inset: magnified image in the dashed square). (Adapted with permission from ref[71], Copyright 2019 Wiley)

Outlooks

The above examples of ENS of peptide assemblies have highlighted that ENS regulates spatially organized higher-order molecular assemblies to modulate cellular processes. There are several directions of ENS likely worth exploration for multimolecular crowding in cells. (i) New secondary structures. Most of the peptide assemblies formed by ENS are resulted from the self-assembly of 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 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 precise interactions of between cellular targets and the assemblies would lead to new applications. (iv) Structure determination. One prerequisite to design the assemblies for 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 assemblies resulted from ENS, it also underscores the need and challenge for the structural determination of the multimolecular crowding/assemblies in-situ inside cells. (v) New applications. The applications of ENS for multimolecular crowding certainly are 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 new discoveries in science and practical applications for addressing societal needs.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The work is partially supported by This work was partially supported by the NIH (CA142746 and CA252364) and NSF (DMR-2011846).

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