Chapter 4
Enzyme-Instructed Self-assembly of Small Peptides In Vivo for Biomedical Application

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Abstract With the development of technology, there have developed many methods to treat diseases. Among them, precision medicine is in an urgent need for public healthcare. In the past several decades, the rapid development in nanotechnology significantly improves the realization of precision nanomedicine. Comparing to well-established nanoparticle-based strategy, in this chapter, we focus on the strategy using enzyme-instructed self-assembly (EISA) in biological milieu for biomedical application. Generally speaking, the principles of designing small molecules for EISA require two aspects: (1) the substrate of enzyme of interest; (2) self-assembly potency after enzymatic conversion. This strategy has shown its irreplaceable advantages in nanomedicine, specific for cancer treatments and Vaccine Adjuvants. Up to now, all the reported examples rely on only one kind of enzyme-hydrolase. Therefore, we envision that the application of EISA strategy just begins and will lead a new paradigm in nanomedicine.

Keywords Nanotechnology · Enzyme · Self-assembly · Biomedical application

4.1 Introduction

Supramolecular chemistry focuses on the intermolecular bond and the structures and functions of the supramolecules, while the molecular chemistry is based on the covalent bond [1]. The molecular self-assembly is a branch of supramolecular chemistry [2]. The macrobehavior of molecular self-assembly is to form gels.

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H. Wang and L.-L. Li (eds.), *In Vivo Self-Assembly Nanotechnology for Biomedical Applications*, Nanomedicine and Nanotoxicology,
https://doi.org/10.1007/978-981-10-6913-0_4

Although molecular gels have been studied for over 170 years, the discovery and design of small molecules forming gel have still drawn great attention of many scientists due to their potential applications in tissue engineering drug release [3, 4] and drug release [5, 6]. As we all know, for biological applications, one of the most important problems is to find biodegradable materials to realize controlled drug release. Compared to polymers gels, the small molecule gels could resolve this problem because that they consist of biocompatible components and are held together by noncovalent force, which make them easier for the body to degrade. Therefore, the research on the small molecule gels is rapidly developing.

4.2 The Development of Small Molecular Gels

According to Flory, a gel has a continuous structure with macroscopic dimensions that is permanent on the time scale of an analytical experiment and is solid-like in its rheological behavior below a certain stress limit. In another words, the gels consist of two parts, the gelators and the solvent. Generally speaking, the gels have quite a few gelators, usually within the range of 0.1–10 wt%. When the gel formed, in the macroscope, the solvent is immobilized and can support its own weight and is not flowed. The simplest test is to turn the test tube upside down and observe it flows or not. In the microscope, the gel forms many nanofibers, which are twisted and traps the solvent by surface tension [7, 8]. To molecular gels, there already have many studies on mechanisms of gelators, [9, 10] different types of gelator, [11, 12], and their applications [13, 14]. Considering the difference of the solvents, the gels can be simply classified into organogels and hydrogels, which have different applications.

4.2.1 The Development and Characteristic of Organogels

The organogels are called "π-gelators" which are soft materials. The "π-gelators" derive from gelators with more than one aromatic π-unit, which has contributed to self-assembly and easier to from gels. Besides, duo to their delocalized π-electrons, π-systems often have some special properties such as electronic conductivity and luminescence [15, 16]. The π-systems have great applications in organic electronic devices by coating if the size and shape of aggregates can be controlled. And one possible way is to use the weak force such as hydrogen bonding and π-stacking interactions to realize self-assembly. In organogels, the solvents can almost be any common organic solvents as far as recent studies. Simply speaking, gelation is a balance between crystallization and solubilization. Therefore, to a given solvent, a molecule requires functionality that will provide both of them. Mostly, the gelators of organogels have some functional groups such as hydroxyl and carboxylic acid, which has been proved to be essential for gelation because that they are capable of

forming hydrogen bond. And an aromatic core is another characteristic of organgelators because the presence of an aromatic core can help π−π stacking. The organogels can be triggered by light, pH, temperature, ionic strength, and other factors.

Just like many scientific discoveries, we are not planned to enter into the field of organogels. It was found by Y.-C. Lin during a photochemical investigation [17]. In this experiment, the 3-b-cholesteryl-4-(2-anthryloxy) butanoate (CAB) can turn organic solution to gel at very low concentrations (less than 2 wt%). Then, the scientists focus on searching for the simpler organogelators. The molecules with only one aromatic group and one linking chain had achieved little success. And the molecules with one aromatic group and two linking chain has been proved to successfully form organogels in many organic solutions [18]. And with the developing of the investigation, some conclusion has been drawn:

(1) H-bond is not inevitable if other packing contribution dominated such as π–π interactions and London dispersion forces [19].
(2) the interactions of charge—transfer among gelators can help to stabilize gels [20].
(3) thixotropy can be induced by adding a small concentration of a second molecule with poor ability to form gel, which is similar to the gelator in structure.
(4) the temperature and solubility of the gelator in organic solvent have great influence on the fraction of the gelator within the gels [21].
(5) what determines the shape, the dimension, and the T_{gel} (the sol↔gel transition temperature) values are the properties of the liquid mixture, rather than the individual components [22].

4.2.2 The Development and Characteristic of Hydrogels

With the development of organogels, the limitation is obvious when applied to biomedical field. And water is the unique solvent to maintain life forms and is the most abundant substance in the body of life. In 1921, Hoffman reported a first small molecule hydrogelator, the compound dibenzoyl-L-cystine, which could form gel at 0.1 wt% concentration in macroscope [23]. And about 100 years later, Menger and co-workers applied modern physical methods such as X-ray crystallography and electron microscope to examine the hydrogel of dibenzoyl-L-cystine [24]. And they revealed the microstructure of hydrogels. Besides, they found that aromatic moieties have great improvement on intermolecular interaction in water. Although there are many principles of supramolecular hydrogelation, the first gel is an accidental discovery of a particular molecule that forms a gel in a solvent. Just like organogels, it usually gains hydrogels of peptides in accident when the researchers invest the oligomeric peptides [6, 25]. And it can be concluded that the small molecules self-assembly is a universal phenomenon, rather than particular process. Therefore, it will be meaningful to explore the supramolecular hydrogels. Essentially, the

hydrogels formed by the self-assembly of small organic molecules are different from organogels and polymer hydrogels. The polymeric hydrogels originated from a random cross-linked network is a strong covalent bond. As to hydrogels, the molecular self-assembly is forced by weak and noncovalent interaction among hydrogelators in water, which make the hydrogels more ordered. While simple swelling usually makes a polymeric hydrogel, a stimulus or a triggering force is necessary to bias thermodynamic equilibrium for initiating the self-assembly process or phase transition to obtain a supramolecular hydrogel. Therefore, there are many forms of stimuli or triggers for manipulating the weak interactions. The methods can be divided into physical methods (such as changing the temperature, applying ultrasound, or modulating the ionic strength) and the chemical methods (such as pH change, chemical or photochemical reactions, redox, and catalysis). Among them, the change of pH is the simplest method to gain hydrogel because a small amount of acid or base easily and rapidly can lead to a large pH shift via a diffusion-limited process. However, considering the biomedicine applications, the methods of great change of the environment (such as all the physical methods and the change of pH) may be useless. And the methods based on chemical reaction are promising. Chemical reactions can yield new products which have great different properties from reactants and turn the solution to gel. In polymeric hydrogels, click chemistry, [26] redox reactions, [27, 28] Michael reaction, [29] acid–base reaction [30], and ligation reaction [31] have already been developed. To supramolecular hydrogels, there are still much less attention. Considering that self-assembly is the molecular foundation of life, and soft and wet are another two obvious characteristics of most types of cells, it is not surprising that catalysis and enzymes are attracting increased attention and are achieving many unexpected successes in the generation and applications of supramolecular hydrogels. The typical experiment is reported by Xu group [30]. They synthesized the hydrogelator with good water solubility by the replacement of a carboxylate group with a hydroxyl group. By the hydrolysis of the carboxylic ester bond, it turned to be high hydrophobic and formed the gels, which is much stable over a wide pH(1–14) range. And this may be a possible application in designing a robust system of prodrug. Then, the group of Hamachi reported that the “retro-Diels-Alder” reaction can be used to trigger morphological transformation of supramolecular nanostructures, which triggered by heating [32]. Due to the complex environment in cells, the enzyme has greater advantages than other triggers on self-assembly.

4.2.3 The Development of Enzyme-Instructed Self-assembly

In 2015, the announcement of Precision Medicine Initiative (PMI) envisioned the new era of medicine to develop individualized care [33]. The establishment of PMI indicates that there are still many incurable or lack of effective treatment for a lot of diseases, especially cancer. The improvement in cancer treatment is calling for the urgent advances in new technology, such as nanotechnology. About three decades ago, the blossoming bionanotechnology has made remarkable progresses in

precision medicine [34–36]. When it starts, in this field, the major efforts are to fabricate multi-functional nanoparticles to realize diagnostic and therapeutic effect [37]. The vital concept is to carry out and maximize the targeting capability of nanoparticles while leaving the rest of body unaffected [38, 39]. Up to now, nanoparticle based on cancer theranostics has made tremendous progress which has been thoroughly reviewed [40–42]. Nevertheless, the nanoparticle-based strategy still remains in a dilemma. Before real application in market, there are a list of unavoidable issues to be solved, mainly including (1) the quality control of multi-step fabrication process; (2) the difficulty in clearance of nanoparticle; (3) the poor ability of penetration into the tumor because of the size of nanoparticle; (4) the high risky off target on account of frequent mutation of cancer cell surface proteins, and (5) the delivery efficiency of nanoparticle [43–45]. In contrast, the bottom-up strategy, which can generate nanomaterial directly in cancer cells rather than delivery the nanomaterial to cancer cells, has drawn more and more attentions in recent ten years [46]. Apart from the achievement of the in vivo formation of quantum dots for bio-applications, [47] one kind of nanomaterial which uses enzyme to activate self-assembly process of small molecules [48] has already been demonstrated the great potential applications ranging from cancer diagnosis to therapy or their combination (so-called theranostics). In this chapter, we summarize the recent progress on the EISA and classify the enzymes underlying enzymatic reactions in details. At the same time, if the readers have interests in various application of EISA, there are a few excellent reviews on the topic of supramolecular self-assembly for potential anticancer therapeutics for further reading, [49–51] which emphasize on supramolecular hydrogels as molecular biomaterial, the intersection of supramolecular chemistry, biomedicine science, and the biological functions of prion-like nanofibrils of small molecules, respectively. We just pay our attention to the biomedical application of EISA based on the difference of enzymes.

4.3 The Characteristic and Advantages of EISA on Cancer Theranostics

EISA is the multi-step process to form special functional structures, in which amphiphilic molecules undergo certain transformations usually triggered by enzymes before they stack with each other [48]. As a ruler, the formation of self-assembled objects can be kinetically controlled, which may be potential application on control drug release. In addition, there is still emerging example under mechanical control directed by catalytic action [52] or non-equilibrium transient state [53]. These structures of hydrogels display a variety of micromorphologies such as nanoparticles, nanofibers, ribbons, and so on [54–56]. And the different morphologies depend on the specific geometry of certain building blocks–molecular structures. Although it is different morphologies, all of them share some same features, such as the

intermolecular interactions which are noncovalent including hydrogen bonding, aromatic–aromatic interaction, electrostatic attraction, and other weak Van der Waals forces [57]. For example, the hydrogen bonding and aromatic-aromatic interaction are directional force, which eventually determine the diameter of nanoparticles, the infinite length along the prolate axis, well-defined width of nanofibers or tubes, repeated unit with fixed length in helical ribbons [58]. Among the various process of self-assembly, the unique feature of EISA is the involvement of enzymatic reactions [59]. In other words, all of the EISA is triggered by enzymes, and the difference lies in the types of enzyme. Enzyme-instructed self-assembly is quite common phenomenon but much critical process in living systems. As we known, the enzyme-catalyzed processes can regulate the formation of intracellular vesicles, the dynamics of cellular skeletons formed by F-actins and microtubules (MT). Further, the dynamic cellular transformations are largely dependent on enzymatic regulations, such as the disassembly and reformation of cell membrane, F-actin and MT during the mitosis, cell movement, and so on [60, 61]. Inspired by nature, the strategy using enzyme to instruct self-assembly to synthesize small molecules has already been developed a decade ago [62]. According to the reported documents, it is necessary to design a suitable hydrophilic substrate which is so-called precursor, just like as hydrogelator. By means of enzymatic reaction, such a precursor can be easily turned into corresponding amphiphilic form [63]. The generating amphiphilic molecules will self-assemble once its concentration reached the critical assembly/aggregation concentration (CAC) and yield various nanostructures eventually.

Considering the application in living system, EISA is irreplaceable process, specifically for mammals. For mammals, the basic physiological conditions remain almost constant. Therefore, this situation is improper to adopt usual sol-gel transition, which generally requires external physical or chemical stimuli including pH, temperature, and ionic strength. Comparing to them, EISA is not only the isothermal process but also performs in physiological condition, which means not necessary to change the environment. Based on these biocompatible features, the establishment of EISA makes it has great advantages to construct self-assemblies in biological milieu.

The main advantages of the application of EISA in cancer theranostics can be summarized as follows:

(1) The material can be easily synthesized by regular chemical methods. The precursor is mainly peptides and the derivative of peptides. And the different functional amino acid can be easily purchased by commercial channels. Then, the synthesis of peptides depends on the solid phase peptide synthesis (SPPS), which can be easily obtained at a large scale with high purity. Besides, the peptides can be simply modified because of the existence of the amino and carboxyl groups.
(2) The clearance can simply realize. Because the formation of the material is based on the noncovalent interactions, the self-assembly process can be reversible to disassemble to release the original small molecules into biological environment, which get cleared via reticuloendothelial system (RES).

(3) The adequate penetration into the tumor can be got because of the size of precursor. Compared to the shallow penetration of nanoparticle into tumor, which is limited by interstitial pressure, it is much easier for small molecules (precursor) to penetrate deep inside of tumor and even tumor cells by means of passive diffusion or active transportation. Besides, there are more choices to design the appropriate precursor to get better ability of penetration into tumor.
(4) The delivery efficiency is quite sufficient. The EISA depends on the over-expression of certain enzyme rather than cell surface receptor or vascular leakage, which indicate that it can increase the opportunity of entering cells. Because that overexpressed enzyme is the intrinsic component in tumor cells, ideally, the enzyme-instructed self-assembly will proceed only in tumor cells and get small molecules accumulated. When the concentration is over CAC, the intracellular concentration of small molecules would reach millimolar level and the self-assembly occurs, which can satisfy the need of preloaded or post-delivered therapeutic agents.
(5) Further, this strategy owns the potential to target "undruggable" targets or "untargetable" features of cancer cells and provides opportunities for simultaneously interacting with multiple targets [48]. EISA can greatly enrich the ways of release of drugs and enhance the solubility of some drugs.

Although there are as many as thousand kinds of enzymes in cells, the types of enzyme to trigger self-assembly is limited. Here in this chapter, we would like to elucidate the progress on the enzyme-instructed self-assembly in vivo for biomedicine application in the order of enzyme.

4.4 The Application of EISA Strategy in Cancer Theranostics

The EISA of small molecules has been proven to be a promising method in selective inhibition of cancer cells. However, just as everything has two sides, the EISA also have some problems and limitations to solve. One of the problems is that the inhibitory concentrations of those self-assembling molecules remain too high compared to traditional pharmaceuticals due to its high CAC [64]. And lack of understanding of the interaction between potential protein targets and the in situ assemblies or aggregates, as well as the limited techniques to identify and characterize the interactions between nanoscale assemblies of small molecules and proteins, remained another inevitable obstacle for further advances of EISA [65]. Therefore, it demands more researchers and scientists to work on it. The application of this concept in biomedicine was just getting started. Next, we introduce in detail the supramolecular self-assembly induced by different kinds of enzymes. For the convenience of readers, we summarize some important parameters of EISA in living cells and animals, such as concentration and incubation time in recent reported documents to more intuitively compare with each other. (Table 4.1)

Table 4.1 EISA in living cells and animals

Compound		1	2	3[a]	4[b]	5[c]	6	7	7	8	9	10	11[f]
Cell	Conc./μM	500	500	500	500	500	500	500	500	30	0.2	0.2	500
	Time/hour	12	12	0.5	0.5	0.5	0.5	24	3	24	48	48	7
Ref		73		71, 68, 67				68	74[e]	74	76		80

Cont'd

12	13	14[d]	15[d]	16[d]	17	18	19[a]	20	21	22	23	24
20	200	200	200	2	0.2	1 μCi	0.02 wt%	2500	2500	2	10	0.2 wt%
20	24	8	8	8	8	1/3	18	72	72	24	1	24
83	77	84			86	85	87	88		90	89	103

Cont'd

Compound		7	20	21	22	23	24
Animal	Dose	100 mg/Kg	36 mg/Kg	36 mg/Kg	5 nM	200 μM	1 wt%
	Time	48 h	6d	6d	24 h	24 h	14d
Ref		74 [g]	88		90	89	103

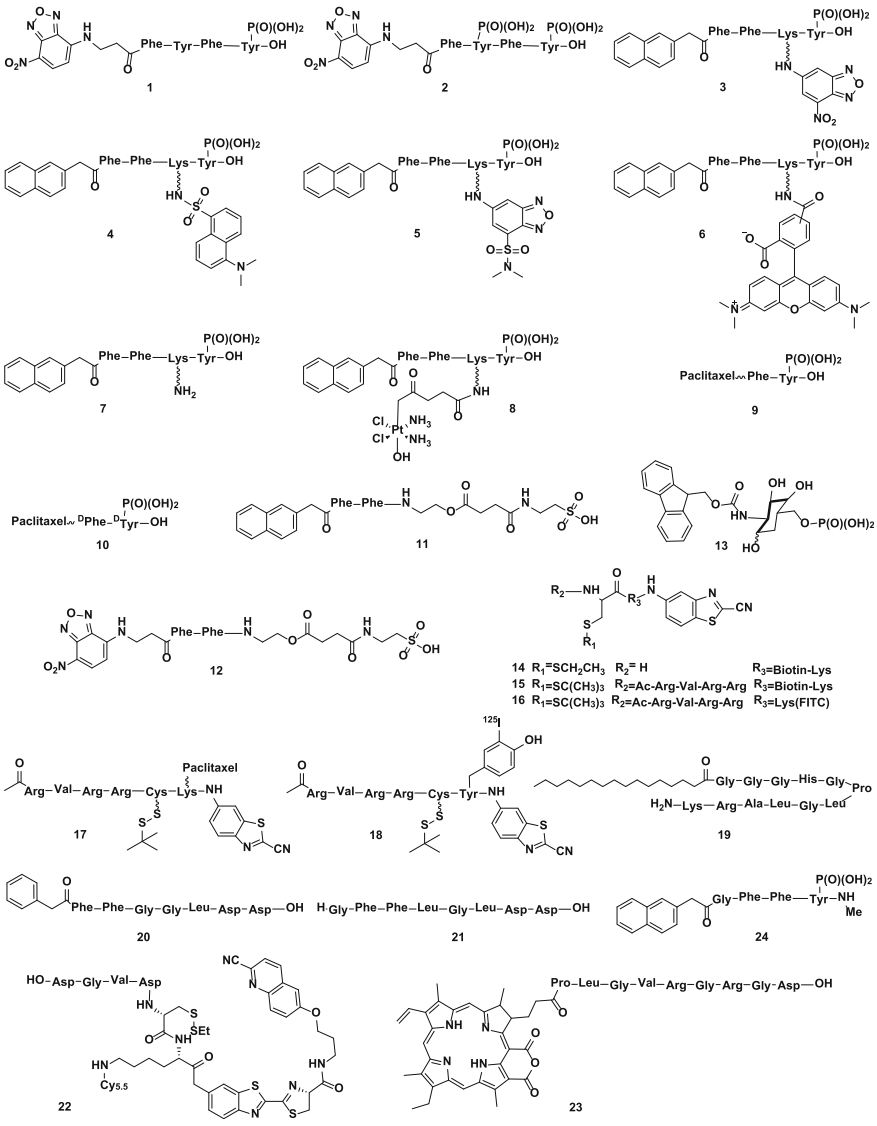
[a]inside cells
[b]membranes
[c]outside cells
[d]Glogi
[e]7 (500 *** g/mL) co-assembly with Indocyanine Green (10 *** g/mL)
[f]cell surface
[g]7 co-assembly with ICG(10 mg/Kg)

Besides, we rearrange the chemical structures of the precursors to directly distinguish the difference among them and easy to name them in this chapter (Scheme 4.1).

4.4.1 Hydrolysis of Esters

4.4.1.1 Phosphatase

As early as in 2004, a pioneer work finished by Yang and Xu reports the first example of enzymatic formation of supramolecular hydrogel [62]. It is a new way to turn the solution into gel using an enzyme (alkaline phosphatase) without further external stimuli. Though limited biological applications were envisioned at that time, the concept in this work has led the later-on tremendous development in this field. With proper design of the small molecules (hydrogelator precursors), it is possible to realize the hydrogelation in physiological conditions [59] and develop



Scheme 4.1 Chemical structures of each compound numbered

applications in various directions ranging from drug controlled release, controlling cell fate, tissue engineering, and so on.

Later the Xu group reports the use of phosphatase to confer a hydrogel of paclitaxel derivative. The paclitaxel derivative is made from paclitaxel and a peptidic self-assembly motif linked by a succinic acid. Once the paclitaxel derivative is dissolved in water, phosphatase could initiate the self-assembly process and yield

the formation of numerous nanofibers that result in a supramolecular hydrogel in macroscope (Fig. 4.1). Upon slow hydrolysis reaction, the hydrogel serves as a platform for controlled release of paclitaxel with adjustable release rates. Overall, this design provides a powerful method to create molecular hydrogels of clinically used therapeutics without compromising their bioactivities [66].

Then in 2012, the Xu group reported a method to image EISA of a small molecular (See Scheme 1 compound **3**) inside live cell [67] (Fig. 4.2). Aided with [31] P NMR and rheology, they demonstrated that enzyme-trigged conversion of the precursor (compound **3**) to a hydrogelator results in the formation of a hydrogel via self-assembly in vitro. Therefore, the same process is capable to perform inside living mammalian cells. The intracellular self-assembly is dependent on both the concentration of the precursor and the activity of protein tyrosine phosphatase 1B. Since the enzyme is localized on the endoplasmic reticulum (ER), it also dominates the location for the occurrence of intracellular self-assembly. The similar phenomenon is further confirmed via a co-assembly strategy to visualize the self-assembly of non-fluorescent small molecules (compound **7**) inside live cells in another work [68]. The cell fractionation experiment points out the cellular fraction containing ER triggers the fastest sol-gel transition, which implies the self-assembly occurs on ER with the highest possibility inside intact cells. Further immune co-staining shows the maximum distribution overlap between self-assemblies and ER tracker instead of lysosome or Golgi tracker. Although significant effort has been paid including correlated light and electron microscopy (CLEM), it remains difficult to identify the unambiguous fibrillar morphology of supramolecular assemblies in situ due to the intrinsic background noise from cellular components, which have similar chemical composition, the atoms of carbon, hydrogen, and oxygen. One possible way to solve this problem could be the involvement of small angle neutron scattering (SANS) which is the powerful technique investigating the internal structures of soft materials [69, 70]. To fulfill the specific aim, it requires further efforts on the correlation between SANS measurements and classical imaging data, the balance of water and heavy water to tune the scattering length density (SLD) mask the background from cell and so on, which demand too much and hard to realize.

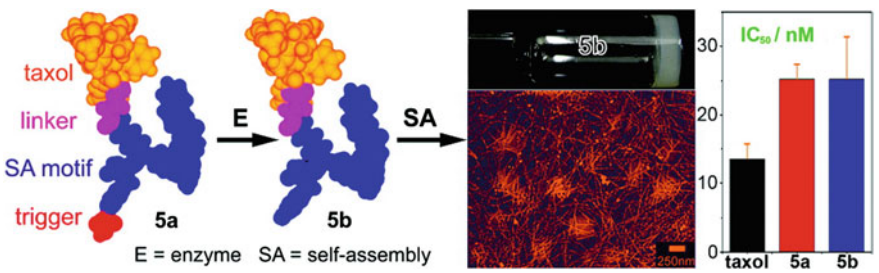


Fig. 4.1 Schematic representation of phosphatase-triggered self-assembly of a derivative of taxol and its microstructures and the histogram of the IC_{50} [66] Adapted with permission from Ref. [66]. Copyright 2009 American Chemical Society

Following the discovery of the intracellular supramolecular self-assembly, the Xu group tested a serial of different fluorophores labeled self-assembly motif against HeLa cells (Scheme 4.1, compound **3**, **4**, **5**, **6**) [71]. Interestingly, the slight structural difference among each fluorophore has great influence on the self-assembly propensity. Besides the NBD one which can self-assemble inside cells, DBD-derived molecules could form abundant nanofibers before in contact with cell/phosphatase; Dansyl-derived molecules show certain degree of toxicity and disrupt the integrity of cell membrane while Rhodamine-derived molecules fail to form nanofibers and remain homogeneous all the time. Overall, the variant self-assembly propensity induces the drastically different distributions of self-assemblies in the cellular environment (Fig. 4.2).

It is worth mentioning that the very recent result from the Xu group demonstrated a novel way to localize supramolecular assemblies to sub-cellular organelles. The attachment of a triphenylphosphonium to the self-assembly motif acquired targeting capability to intracellular mitochondrial, which eventually can selectively kill cancer cells via disrupting the cells' powerhouse [72]. This rational design shows the potential bioactivity of intracellular self-assembly via interacting with specific sub-cellular organelles.

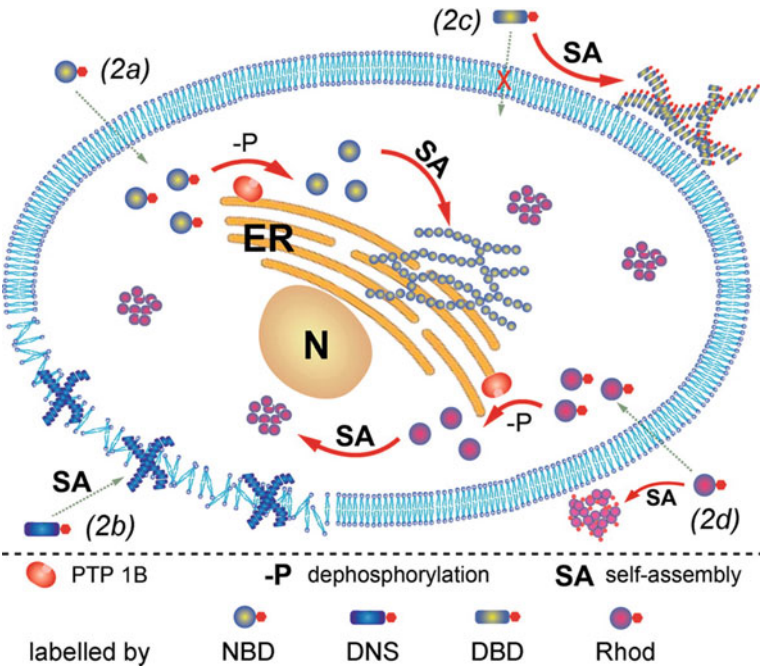


Fig. 4.2 Illustration of the distinct spatial distribution of the small molecules in a cellular environment because of their different propensities of self-assembly before or after dephosphorylation [71] Adapted with permission from Ref. [71]. Copyright 2017 American Chemical Society. Copyright 2013 American Chemical Society

Although the kinetics of the formation of molecular assemblies is one of important features of cells, it received little attention to develop anticancer therapeutics. In 2016, the Xu group designed a serial of tetrapeptide derivatives with same sequence but a different number of phosphorous esters within the molecule. A detailed comparison showed that the variation of phosphorous esters was able to regulate the rate of supramolecular self-assembly although both the enzymatic dephosphorylation and the final hydrogelator are exactly the same (Scheme 4.1, compound **1** and **2**) [73].

Besides the cell experiments, the Chen group further applied the phosphatase-instructed self-assembly to an animal study. They adopted ICG (indocyanine green) to nanofibers formed by compound **7** to achieve co-assemblies for cancer theranostics [74]. As the first example, they form tumor-specific ICG-doped nanofibers in vivo, which is capable to manipulate the spatiotemporal distribution of ICG in mice. As a result, the prolonged retention of therapeutic agent inside tumor eventually improves the cancer theranostics.

Since the D-peptides are presumably resistant to most of enzymes, a serial of valuable studies demonstrates that the formation of the nanofibers via enzymatic dephosphorylation is independent from chirality. That means D/L enantiomers undergo the quite similar enzymatic self-assembly process. Overall, D-peptide-based EISA could achieve both bio-stability and additional desired functions simultaneously. (See Scheme 4.1 compound **8** [75], **9**, **10** [76] and **13** [77]) Very recently, the Xu group reported that the D-peptidic nanofibrils can serve as multifaceted apoptotic inducers to target cancer cells in situ. With ALP as the catalyst, D-peptidic nanofibrils which formed in situ on cancer cells presented autocrine proapoptotic ligands to their cognate receptors in a juxtacrine manner, as well as directly clustered the death receptors, which eventually activate extrinsic death signaling for selectively killing cancer cells [78]. In another study, they co-cultured a group of cancer cells and stromal cells with a D-peptidic derivative. Based on differential EISA formation of fluorescent, non-diffusive nanofibrils, the significantly higher activity of ALP on cancer cells than stromal cells was confirmed. The inherent and dynamic ALP activity was determined by drug-sensitive or drug-resistant cancer cells and even with or without hormonal stimulation [79].

4.4.1.2 Carboxylesterase

It is well known that the cisplatin is one of the most successful therapeutic agents for the ovarian cancers. However, the emerging drug resistance still remains a major problem in its chemotherapy treatment. To overcome such a disadvantage, the combination of cisplatin with other therapies is in an urgent need. In 2015, the Xu group firstly demonstrated that enzyme-instructed intracellular self-assembly molecular is a new approach to boost the activity of CDDP against two drug-resistant ovarian cancer cell lines. They synthesized small peptide precursors (See Scheme 4.1 compound **11**) as the substrates of carboxylesterase (CES). CES efficiently cleaved the ester bond preinstalled on the precursors and initiated the

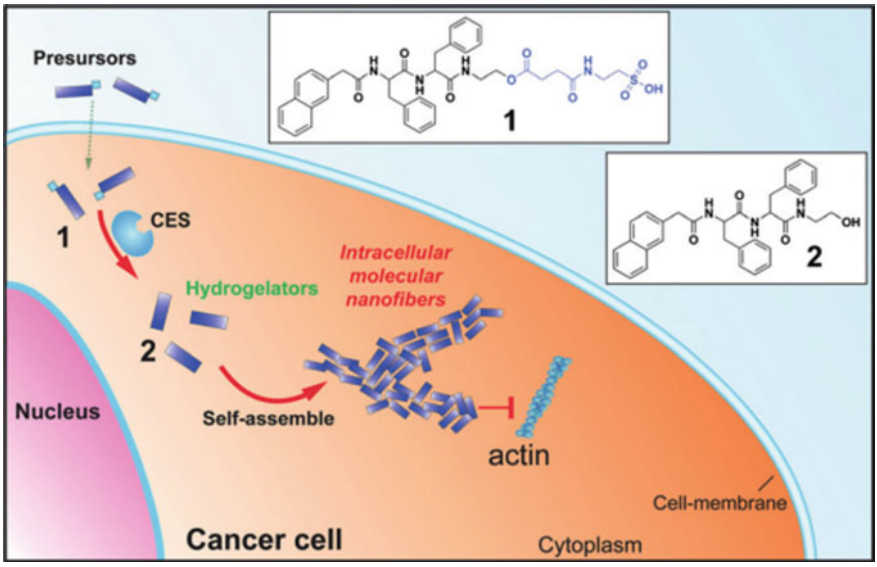


Fig. 4.3 Enzymatic transformation of the precursor (1) (compound **11**) as a substrate of carboxylesterase (CES) to the corresponding hydrogelator (2) for intracellular self-assembly. CES efficiently cleaved the ester bond preinstalled on the compound **11** and yielded the hydrolyzed peptidic part to form nanofibers in cancer cell [80] Adapted with permission from Ref. [80]. Copyright 2015 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

hydrolyzed peptidic part to form nanofibers in water via self-assembly. The precursors are innocuous to cells at the optimal concentrations, but they double or triple the activity of cisplatin against the drug-resistant ovarian cancer cells [80] (Fig. 4.3).

And recently, the Xu group designed peptidic precursors as the substrates of both CES and ALP to show the combination of enzyme-instructed assembly and disassembly. The precursors can turn into self-assembling molecules to form nanofibrils upon dephosphorylation by ALP, while the following CES catalyzed cleavage of the ester bond on the same molecules will result in disassembly of the nanofibrils [81]. Besides, they also illustrated a fundamentally new approach to amplify the enzymatic difference between cancer and normal cells for overcoming cancer drug resistance by establishing cytosolic EISA catalyzed by CES. The selectivity of EISA targeting cancer cells was validated in cancer and normal cell co-culture test [82].

4.4.1.3 Esterase

D-peptides have great applications in many areas of biology and biomedicine because of their bio-stability. However, it is ineffective for cellular uptake of D-peptides. To further explore the merits of D-peptides inside cells, it is essential to develop an effective strategy to enhance the cellular uptake of D-peptides. In 2015, the Xu group synthesized the conjugate of taurine and D-peptide which drastically

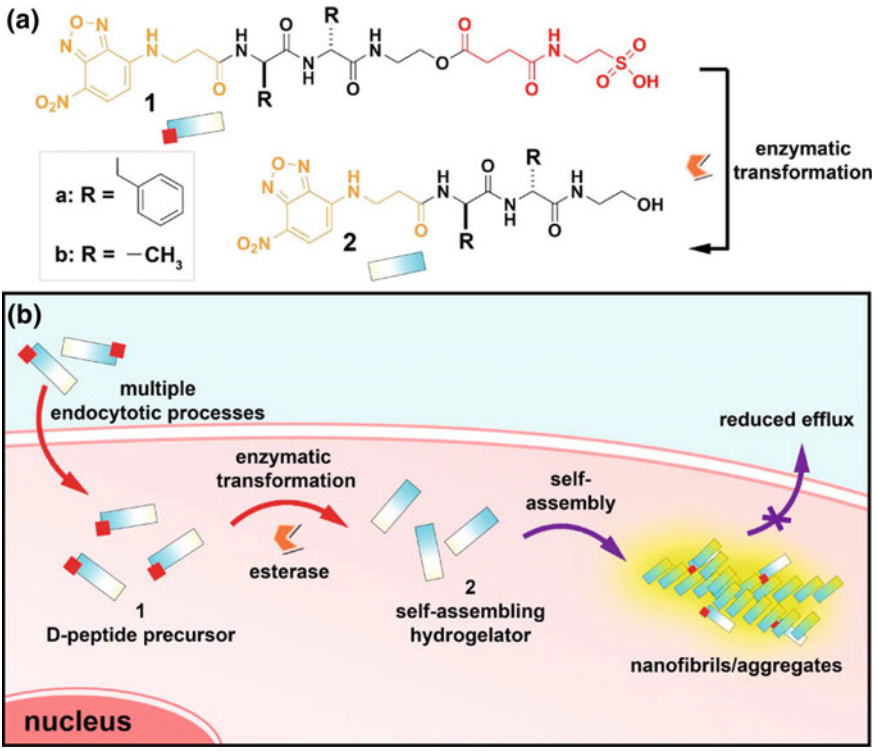


Fig. 4.4 Molecular structures of precursors (compound **12**) and the corresponding hydrogelators after enzymatic transformation. (B) Taurine conjugation boosts cellular uptake of D-peptide precursors and subsequent EISA to form nanofibers, accumulating inside cells [83] Adapted with permission from Ref. [83]. Copyright 2015 American Chemical Society

boosts the cellular uptake of small D-peptides in mammalian cells and allows intracellular esterase to trigger intracellular self-assembly of the D-peptide derivative, further enhancing their intracellular accumulation (Fig. 4.4). Using taurine for EISA is a new way to promote the uptake of bioactive molecules and generate higher-order molecular assemblies. This approach will be useful to facilitate the transport of functional D-peptides, and other bioactive molecules through the cell membrane into live cells for controlling the fate of cells [83].

4.4.2 Hydrolysis of Peptides

4.4.2.1 Furin

A controlled enzyme-triggered self-assembly in living cells was reported by Liang and the co-workers in 2010 [84]. Based on the chemical reaction between 2-cyanobenzothiazole (CBT) and D-cysteine, the authors designed a variety of

thiol- or amino-protected monomers via a disulfide bond and by a peptide sequence, respectively. The protecting group of the monomers can be removed by either pH, disulfide reduction or enzymatic cleavage in vitro and then the yielded product self-assembled into different structural patterns.

Then, the authors attempted the enzyme-triggered self-assembly of monomers in live cells. The direct imaging indicated that the self-assembled signals are closely located to the furin enzyme sites–the Golgi bodies. While in control experiments where furin inhibitor was added together with monomers, the fluorescence staining pattern previously observed was absent, confirming that furin was responsible for the localized condensation of monomers at the Golgi bodies.

Based on the biocompatible condensation and subsequent self-assembly, Liang group designed a radioactive probe to self-assemble into the radioactive nanoparticles (compound **18**) under the action of intracellular glutathione (GSH) and furin in living cells [85]. These as-prepared radioactive nanoparticles could concentrate the radioactive isotopes inside cells and hamper themselves to be eliminated from the cells due to the large size and hydrophobic property. The strategy provided a new method to design smart probes for molecular imaging. Following the successful strategy, Liang Group rationally designed a taxol derivative (CBT-Taxol, compound **17**) which could condensate and self-assembled into taxol nanodrugs (Taxol-NPs) under the control of furin [86]. In vitro and in vivo studies showed that the CBT-Taxol had a 4.5-fold or 1.5-fold increase in anti-multidrug resistance effects, indicating this strategy could be used for overcoming multidrug resistance (Fig. 4.5).

4.4.2.2 MMP

In 2015, Maruyama and co-workers reported the enzyme-triggered molecular self-assembly of a low-molecular-weight gelator for novel anticancer applications. Its precursor (**ER-C16**, compound **19**) exhibited remarkable cytotoxicity to several cancer cell lines and low cytotoxicity to normal cells. Cancer cells secrete excessive amounts of MMP-7(matrix metalloproteinase-7), which converted the precursor into a supramolecular gelator prior to its uptake by the cells. Once the supramolecular gelators entered inside the cells, they self-assembled to form nanofibers that greatly impaired cellular function and then caused the death of the cancer cells (Fig. 4.6). Due to the unique cytotoxic mechanism, cancer cells will be unlikely to acquire resistance to the present strategy [87] (Fig. 4.6).

Besides, in 2016, the Rein V. Ulijn group also has halted tumor growth by MMP-9 triggered self-assembly of doxorubicin nanofiber depots. They demonstrated that the fibrillar depots are formed where the MMP-9 is overexpressed. And this will enhance the efficacy of doxorubicin, resulting in inhibiting of the growth of tumor in mice [88].

Due to the intimate relationships between morphology of self-assembled nanostructures and their biological performances, Wang group rationally designed a responsive small-molecule precursor (compound **23**) that simultaneously

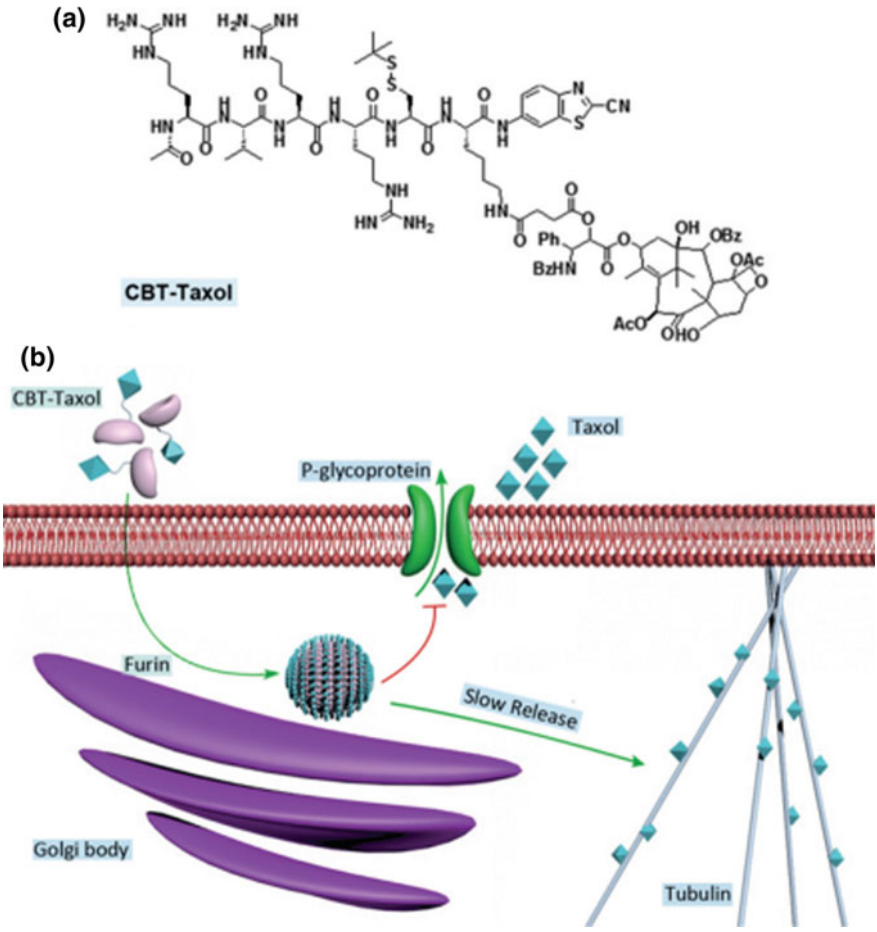


Fig. 4.5 **a** Chemical structures of CBT-Taxol. **b** Schematic illustration of intracellular furin-controlled self-assembly of Taxol-NPs for anti-MDR [86] Adapted with permission from Ref. [86]. Copyright 2009 American Chemical Society

self-assembled into nanofibers in tumor sites in 2015. The compound **23** consisted of P18, PLGVRG, and RGD. At first, the RGD can target to $\alpha_v\beta_3$ integrins, overexpressed on cancer cell membranes. Second, gelatinase, belonged to MMP-2, can cut the PLGVRD linker in tumor microenvironment. Therefore, the molecular become more hydrophobic, resulting in self-assembly of the building blocks. Eventually, the compound 23 simultaneously self-assembled into nanofibers in tumor site, and it exhibited prolonged retention time in tumor cell that directly led to an enhanced photoacoustic signal and therapeutic efficacy [89].

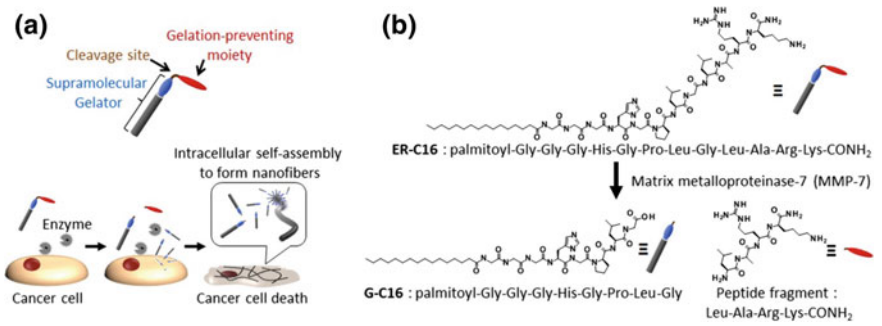


Fig. 4.6 **a** Cancer cell death induced by molecular self-assembly of an enzyme-responsive supramolecular gelator and **b** molecular structures of compound **19** [87] Adapted with permission from Ref. [87]. Copyright 2015 American Chemical Society

4.4.2.3 Caspase

Arguably, bio-orthogonal click chemistry (such as Staudinger ligation, azide-alkyne, and Pictet-Spengler ligation) has already been widely applied in living cells. In these examples, after activation, small molecules can enter cells and be self-assembly. However, there are few successful examples in living animals because of the more complex environment than cultured cells. Therefore, in 2014, Rao group report a new method to direct the synthetic small molecules into nanoaggregates in living mice. They designed a fluorescent small molecule (C-SNAF, Compound **22**) and initiated its self-assembly process via caspase

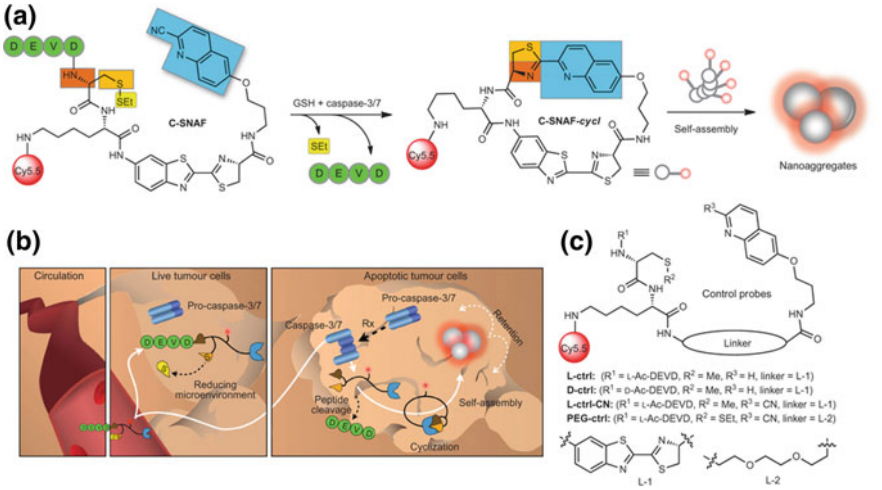


Fig. 4.7 Illustration of the mechanism of in vivo imaging by C-SNAF of caspase-3/7 activity in human tumor xenograft mouse models [90] Adapted with permission from Ref. [90]. Copyright 2014 Nature Publishing Group

activation (Fig. 4.7). The compound 22 comprised of two parts. One part was D-cysteine and 2-cyano-6-hydroxyquinoline moieties linked to an amino luciferin scaffold. The rest consisted of an L-DEVD (Asp-Gly-Val-Asp) capping sequence and a disulfide bond. The self-assembly process required a two-step activation, which was caspase-mediated cleavage and intracellular thiol-mediated reduction. The strategy combined the advantages offered by small molecules with those of nanomaterials and should find widespread use for non-invasive imaging of enzyme activity in vivo [90].

4.5 Vaccine Adjuvants

Vaccines play a very important role in our daily life and have been widely applied in medical fields by preventing the body against or treat the diseases through balancing the immune response to be immunity or silence [91]. With the development of vaccination, it probably serves as a medical intervention. The example is the first therapeutic cancer vaccine, which was licensed in 2010 [92]. Due to the complexity of human immune system and our lack of understanding of it, vaccine design still is challenging. Therefore, DNA vaccines may be an ideal method, which can generate long-term humoral and cellular immune responses [93, 94]. As far, there have developed many delivery systems to improve the efficiency of delivery DNA into mammalian cells, such as liposomes, [95] nanoparticles [96], and polymers [97, 98]. Despite of the advantages of these systems, there are still some disadvantages to overcome, including high toxicity, [99] low amount of antigen loading [100] and reduce the bioactivity of DNA in modification [101, 102]. Therefore, EISA may be an ideal system to deliver DNA and the self-assemblies base on EISA to produce vaccines have been studied and designed.

HIV infection is incurable so far, and developing efficient vaccines is urgent. Yang and co-workers designed a nanovector in 2014, which can condense DNA to result in strong immune responses against HIV [103]. This nanovector composed of peptide-based nanofibrous hydrogel can strongly induce both tumoral and cellular immune response of HIV Env DNA to a balanced level, which was rarely reported in previous studies (Fig. 4.8). The nanovector shows good biocompatibility both in vitro and in vivo, and the well-defined nanofibrous structure is significantly important for the enhanced immune responses. These nanofibrous hydrogels are potentially applicable to the development of efficacious HIV DNA vaccines.

And subsequently, Yang's group reported that in suit-formed peptidic nanofibers facilitate the induction of multiple crucial immunities against HIV DNA vaccine, including multi-functional T cell response, broad IgG subclasses response, and V1/V2-specific IgG response [104]. And then they reported the first co-assembly of peptide and protein (ovalbumin, OVA) upon alkaline phosphatase (ALP) catalysis [105]. The results show an obvious increase of the IgG production when compared to the clinically used alum adjuvant and thus have big potential for application in immunotherapy against different diseases as protein vaccines.

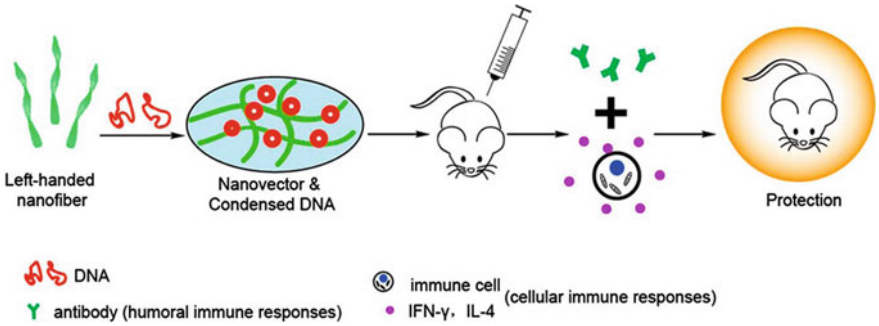


Fig. 4.8 Process of peptide-based nanofibrous hydrogel for enhancing immune responses of HIV DNA vaccines [103] Adapted with permission from Ref. [103]. Copyright 2014 American Chemical Society

The studies of using the peptide-based vaccines as physical carriers have showed great potential applications. And another approach was also used for designing such peptide-based vaccines by the previous conjunction of assembling peptide and model antigens or proteins. Collier and co-workers deigned peptide-based assembling supramolecular containing strong epitopes and demonstrated that the nanofiber induced strong antibody responses in mice [106–108]. MUCI proteins have a lot of tandem repeats, bearing tumor-associated carbohydrate antigens, which is the key problem to develop vaccines for epithelial tumors. To overcome the low immunogenicity of the short MCUI peptide, Li and co-workers designed the self-adjuvanting vaccine with self-assembly domains, which combined some vaccine candidates with a self-assembly peptide sequence. These vaccines can induce antibodies that recognized human breast tumor cells without additional adjuvant. It is reported that these vaccines can act through a T cell independent pathway and may be associated with the activity of cytotoxic T cells [109].

4.6 Outlook

Despite the rapid outcomes about enzyme-activated theranostics under the EISA strategy, it remains, as well as the whole precision nanomedicine field, lack of real applications for improving the overall benefits for patients. Thus, there is still plenty of room to be perfected. As of EISA, we would raise at least three aspects here.

(1) Elucidate the micromorphology of EISA in biological milieu. Various microscopic techniques, such as AFM, SEM, TEM, and so on are working on very well to study the micromorphology of EISA in vitro. However, because of the complexity of the cell itself, it remains impossible to directly capture a vivid snapshot of EISA with nanometer resolution in biological milieu. The attempts on heavy atom labeling (such as Iodine or certain metal element) may be an

option to allow the EISA visible under environmental electron microscope. The advancement in super-resolution fluorescent microscope may be another potential technique to conquer this task since multi-color fluorescent probe labeling is feasible and practical.

(2) Involve enzymes other than hydrolase, or even metabolites to instruct self-assembly. Although there have been plenty of strategies to generate EISA, however, all the enzymes involved in EISA monotonically belong to hydrolase —one of six type of enzymes based on catalyzed chemical reactions in the top-level enzyme classification. In comparison with hydrolase, some other enzymes may be of even greater importance in cancer therapy. For example, kinase is one of the most attractive targets in anticancer practice. This concept has drawn numerous money for the discovery of adequate kinase inhibitor which may eventually suppress the growth of cancer. However, more recent studies are likely to disclose kinase as "untargetable." Thus, if we can develop a system which uses kinase to instruct the self-assembly in vivo, it will be of great value helping the progress in kinase-oriented cancer therapy.
In addition to novel ways to instruct self-assembly, the precise localization remains of great interest which is critical to eliminate false-positive/negative results. Current EISA usually response to single stimuli such as abnormal enzyme expression and its activity. The involvement of two enzymes to instruct EISA process makes this strategy more delicate and potentially targetable to a broader range of cancers [49]. Since there are plenty of known hallmarks of cancers, a well-designed molecule which can respond to those stimulus and perform multi-step tandem transformation before self-assemble will definitely enhance the targeting capability and precision of EISA.

(3) Exploit the interaction between EISA and sub-cellular organelles. Most of the enzymes are associated with certain sub-cellular organelles, e.g., the alkaline phosphatase on the cell membrane, protein tyrosine phosphatase 1B on the endoplasmic reticulum, furin in the Golgi. As the formation of EISA is initiated by enzymatic reactions, the location of correlated enzyme dominates the distribution of EISA. We have realized this interesting phenomenon, but there is little discussion on the interaction between EISA and "host" sub-cellular organelles. The exploration on this point would be useful in controlling the cell fate by regulating the activity of its sub-cellular organelles.

(4) Incorporation with bio-orthogonal chemistry. EISA provides an excellent strategy for the construction of nanomaterial inside biological environment with targeting capability. But we notice that the required CAC is somehow deviated from the dosage of traditional drug molecules. If we covalently load drug molecule on self-assembly motif (that is 1:1 molar ratio), there must be an imbalance between severe overdose issue and initiation of self-assembly. To solve this inevitable dilemma, we think the incorporation of bio-orthogonal reaction would be a realistic solution. By linking a bio-orthogonal reaction handle (azide, alkyne, tetrazine, and so on) to a self-assembly motif, the in situ formed EISA material composed of those as-prepared functionalize molecule

will spontaneously deliver and localize bio-orthogonal reaction handles. A following therapeutic agent modified with the counter handle will efficiently target EISA in the desired location. We envision this pathway owns several advantages including the on/off target ratio, the adjustable dosing, and even the application of toxin which is too toxic by itself.

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