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Review

Covalently triggered self-assembly of peptide-based nanodrugs for cancer theranostics

Yamei Liu,[1] Ruirui Xing,[1,3,*] Junbai Li,[2] and Xuehai Yan[1,3,4,*]

SUMMARY

Covalently triggered peptide self-assembly is achieved through sequential integration of spontaneous covalent reaction and noncovalent interactions, thus both enhancing the physiological stability and extending unexpected functionality of the resulting peptide-based assemblies, different from popular supramolecular peptide self-assembly merely associated with noncovalent interactions. This review summarizes the recent progress on the development of covalently triggered peptide self-assembly for cancer theranostics. Especially, we propose the fundamental design principle of covalently triggered peptide self-assembly for constructing a variety of peptide-based assemblies including nanoparticles, nanofibers, hollow nanospheres, and other nanoarchitectures. Subsequently, the discussion is anchored in an overview of representative covalently assembled peptide-based nanodrugs for the cancer theranostics. Finally, the challenges and perspectives on the clinical potential of the covalently assembled peptide-based nanodrugs are highlighted. This review will provide new insights into construction of peptide-based nanodrugs through combination of covalent reaction and noncovalent self-assembly and prompt their clinical applications in cancer diagnosis and therapeutics.

INTRODUCTION

Biological systems perform advanced functions through the simultaneous interplay between covalent reaction and noncovalent self-assembly rather than through an inefficient step-by-step process.[1] The formation and breakage of covalent bonds and noncovalent self-assembly triggered by this process generally occur sequentially. However, separation of such a process *in vitro* is usually inevitable because the solvent environment required for organic synthesis is often incompatible with those required for noncovalent self-assembly.[2] Water is the medium in life system. Solving the compatibility of covalent reaction and noncovalent self-assembly in aqueous ambient remains highly desirable yet challenging.

Covalently triggered self-assembly is realized through sequential integration of covalent reaction and noncovalent self-assembly in water system. In this process, the self-assembly behavior is triggered by simultaneous chemical reaction in aqueous solution.[3–9] Compared with popular supramolecular self-assembly depending on weak noncovalent interactions,[10] covalently triggered self-assembly, hereinafter referred to as covalent self-assembly, introduces stronger covalent bonds into the assembly system that not only promotes the synergy between covalent bonds and noncovalent bonds but also offers an opportunity for self-adaptation due to the inherent timescale required for covalent self-assembly. These are unattainable for the two interactions that exist alone, thus affording robust and stable assemblies with highly uniform and controlled size.[5,7] In addition, covalent self-assembly avoids the separation of chemical reaction and self-assembly process, enabling to realize the one-pot preparation of the assemblies in aqueous system, which is conducive to large-scale preparation compared with the traditional complicated synthesis and separation processes. More importantly, covalent self-assembly combines covalent bond formation and noncovalent self-assembly to endow assemblies with unexpected functions that are often unavailable by the reactant on its own.[4,7,11,12] Finally, the covalent reaction not only creates the building blocks for self-assembly but also provides a facile way to modulate the self-assembly process for construction of nanoarchitectures with diverse structures and versatile functions by simply changing initial reaction conditions such as the molar ratio or concentration of reactants, the reaction time, as well as temperature and pH of solution.[7,11,13–15]

[1]State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China

[2]Beijing National Laboratory for Molecular Sciences, CAS Key Lab of Colloid, Interface and Chemical Thermodynamics, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, China

[3]School of Chemical Engineering, University of Chinese Academy of Sciences, Beijing 100049, China

[4]Center for Mesoscience, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China

*Correspondence: rrxing@ipe.ac.cn (R.X.), yanxh@ipe.ac.cn (X.Y.)

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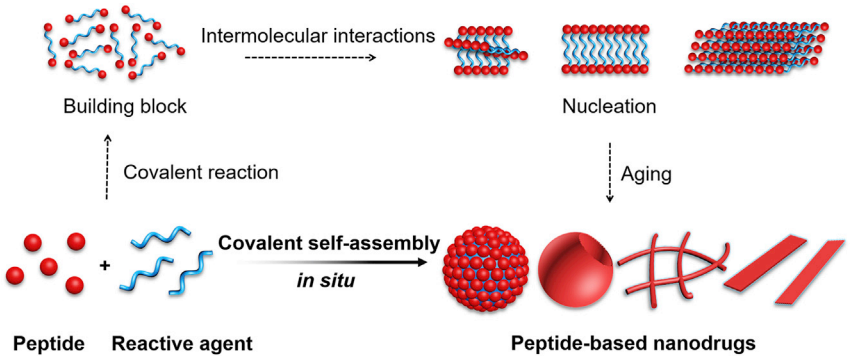

Figure 1. Illustration of the construction of peptide-based nanodrugs based on covalent self-assembly between peptide and reactive agent

Peptides are a kind of generally used building blocks for constructing nanodrugs due to their intrinsic advantages such as biocompatibility and biodegradability, programmability, and various bioactivities.[16,17] Noteworthily, peptides can provide many active reactive groups capable of spontaneous chemical reactions in aqueous solutions,[18,19] enabling to achieve the integration of covalent reaction and *in situ* self-assembly of the resulting products, and finally form nano-/micro-structures. Covalent peptide self-assembly highly enhances physiological stability of the resulting assemblies, avoiding the rapid clearance of molecular peptides by enzymatic degradation. Meanwhile, such a simple and mild self-synthesis process is beneficial to maintain the peptide activity. Since peptides are inherently nonpigmented molecules, the construction of photofunctional materials solely relying on peptides is challenging. The formation of covalent bond enables the construction of new chromophores based on nonpigmented molecular peptides.[4,7,11] Therefore, peptide covalent self-assembly might be a powerful tool for manipulating nonpigmented peptides into optical functional nanodrugs, which will apparently benefit the application of cancer theranostics, including either optical imaging or phototherapy. Comprehensively, covalently triggered peptide self-assembly strategy is expected to promote the preparation and antitumor therapy of novel peptide nanomedicines. Despite the significance of covalent self-assembly, the progress of peptide covalent self-assembly as a strategy for design and engineering of nanodrugs toward cancer theranostics has not yet been summarized until now.

In this review, we focus on recent advances in the construction of multifunctional nanodrugs based on covalently triggered peptide self-assembly strategy for cancer theranostics (Figure 1). We first classify different covalent reactions according to the types of peptide active groups, and propose a general design principle for covalently triggered peptide self-assembly. Subsequently, we summarize recent efforts in applying peptide covalent self-assembly to integrate multifunctionality and flexibility into a single platform for cancer theranostics, including drug delivery, optical imaging, multimode imaging-guided phototherapy, and immunotherapy. We discuss the significance of peptide covalent self-assembly in integrating robustness, responsiveness and degradability of the assemblies, endowing the peptide assemblies with fascinating optical properties, adjustable light energy dissipation pattern, the integrated diagnosis and treatment capabilities, and improved antitumor therapeutic effects. On the basis of the discussion of the precise design, functions, and applications of covalently assembled peptide-based nanodrugs, we finally aim to elucidate the critical role of covalent self-assembly of peptide-based nanodrugs in advancing tumor theranostics. Along with the challenges, the future application prospects of universal covalent self-assembly strategy in cancer combination therapy and other diseases therapy are also discussed.

COVALENTLY TRIGGERED PEPTIDE SELF-ASSEMBLY DESIGN

Peptides can provide a wealth of active reactive groups, affording some spontaneous chemical reactions in aqueous solutions. The ideal requirements for this kind of chemical reaction include high reaction rate, water or physiological solution as the reaction medium, and mild reaction conditions, thereby avoiding cumbersome purification processes, and lay the foundation for the subsequent *in situ* self-assembly of reaction products. In order to realize the subsequent self-assembly in aqueous solution, the precise peptide sequence design also needs to be extensively considered. In addition to the amino acids involved in the covalent reaction, such as lysine, cysteine, and polymerizable tyrosine, aggregation-prone amino acids

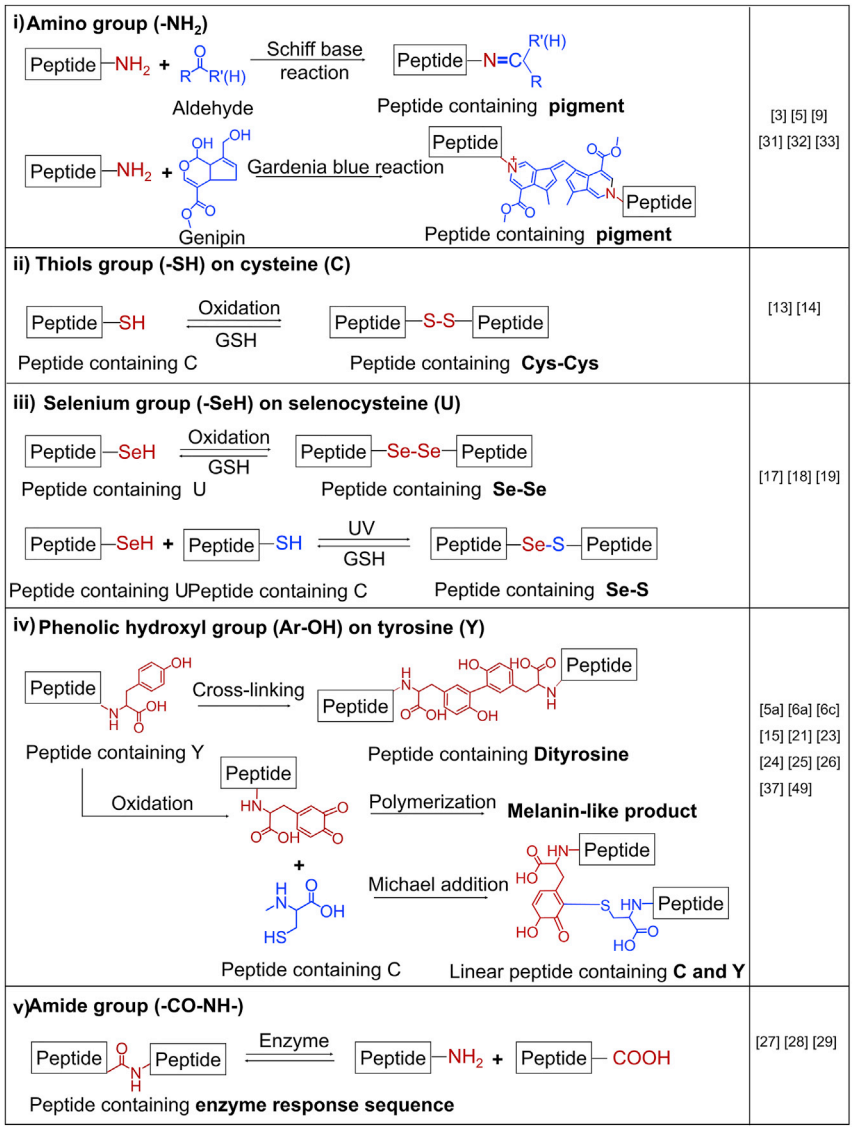

Figure 2. Covalently triggered peptide self-assembly design

i) Formation of Schiff base bonds and gardenia blue conjugated chemical bonds in primary amine (-NH_2)-related covalent reactions. ii) Formation of disulfide bond in thiols group (-SH)-related covalent reactions. iii) Formation of diselenide bond (Se-Se) and Se-S bond in selenium group (-Se)-related covalent reactions. iv) Formation of dityrosine and melanin polymers in phenolic hydroxyl (-Ar-OH)-related covalent reactions. v) Formation and cleavage of amide bond (-NH-CO-) in related covalent reactions.

such as phenylalanine and other amino acids with hydrophobic or aromatic side chains should be introduced, which can provide hydrophobic or π-π interaction to drive self-assembly. Additionally, hydrophilic or charged amino acids, such as aspartic acid and glutamic acid, are considered to be added to the peptide sequence for improving the solubility of peptide or providing electrostatic interaction. In short, the designed covalent reactions can trigger *in situ* self-assembly of the generated reaction products to form nano- and microscale peptide assemblies. It is especially emphasized that in the covalent self-assembly design of active peptides, the active sites should not be used as a reactive group to ensure the maintenance of its own activity after the covalent reaction. The following text summarizes the covalent reaction types of the active functional groups in peptides, the formed corresponding peptide-based nanostructures, and the new functions arising from them, which offer a basic guideline for the design of covalently triggered peptide self-assembly (Figure 2).

Amino group (-NH_2)

Amine group (-NH_2) is highly abundant in amino acids (side chain of lysine (K) and the N terminus), and its inherent nucleophilicity makes it a unique reaction site, which is widely used in the covalent modification of biomolecules such as protein and peptide.[19] The Schiff base reaction, refer to the reaction, in which aldehyde groups (or ketones) can condense with amine group to form imine group (−C=N−), has mild reaction condition and can be easily and efficiently carried out in aqueous solutions. In addition, Schiff base reaction only involves water as a byproduct, which is favorable for biomedical applications. Besides these excellent properties, the formation of Schiff base bond endows peptide assembly with autofluorescence and pH responsiveness, attributable to the n-π^* transition and dynamic covalent bond of the C=N bond, which is highly preferable for the self-tracking of peptide-based assemblies and specific drug release triggered by acidic pH in tumor, respectively.[20] For example, glutaraldehyde (GA), as a biomedical crosslinker with excellent water solubility, bifunctionality, and accessibility, induced dipeptide self-assembly into multifunctional assemblies with sizes from nano- to micro-scale through the formation of Schiff base bonds for tumor imaging and therapy.[3]

In the analysis of the Rubiaceae plant gardenia, it was found that nonpigmented iridoid molecules such as geniposide can react with protein to produce a blue product, which has been widely used as food coloring.[21] The essence of this reaction is that the pentanopyran group in the two iridoids undergoes various self-polymerizations, then spontaneously reacts with two primary amine molecules to achieve crosslinking of the primary amine-containing molecules, and finally forms highly π-conjugated blue chromophores with high stability, good biocompatibility and biodegradability, and near-infrared absorption capacity.[22] The integration of covalent reaction between peptides as well as iridoids and noncovalent interaction of reaction products could significantly improve the robustness of assemblies, which is highly advantageous for drug loading and delivery. Especially, this spontaneous color reaction-triggered self-assembly between primary amino group and iridoid molecule in aqueous solutions provides an opportunity for manipulating nonpigmented peptides and iridoid molecules into versatile pigment assemblies, which greatly expands the design concept of optical functional materials. At present, a variety of robust photofunctional nanodrugs with near-infrared absorption based on covalent self-assembly between primary amino-containing molecules and genipin, which is an iridoid molecule, have been constructed for optical imaging-guided tumor phototherapy.[4–7,9,14,23] It is worth noting that GA and genipin may exist in various self-polymerization forms in aqueous solution and may form other by-products after reacting with amino group.

Thiols group (-SH) on cysteine (C)

Thiols group (-SH) on cysteine (C) easily undergoes oxidation to form disulfide such as cystine (Cys-Cys), or more commonly, encounters disulfide exchange in water or physiological medium.[24] Disulfide bond can be responsive to UV light and reducing agents.[25] Such reaction remains stable in the extracellular environment while it undergoes a reductive bond-breaking process intracellularly due to its susceptible responsiveness to intracellular glutathione (GSH), which not only fulfills robust encapsulation of drugs in blood but also meets the need for on-demand drug release in tumor cells and subsequent material degradation.[6,24] Thiol also participates in several particularly efficient addition reactions. Among them, it is worth noting that the thiol group on cysteine can undergo a Michael addition reaction with the quinone-rich intermediates formed during the oxidation of tyrosine (Y).[26] This provides a tool for the preparation of peptide-based nanodrugs based on tyrosine-cysteine interaction.

Selenium group (-SeH) on selenocysteine (U)

Selenium and sulfur are in the same main group in the periodic table, and its chemical properties are similar to sulfur. While, because selenium has a larger atomic radius and lower electronegativity, the reactivity of selenium-containing compounds is often higher than that of sulfur with the same structure.[27] Selenocysteine (U) is the main form of selenium *in vivo*, which easily forms selenocystine (CysSeSeCys) with a diselenide bond (Se-Se). Studies have shown that diselenide bond (Se-Se) is likely to be a more sensitive dynamic covalent bond than disulfide bond, and can undergo exchange reactions under milder conditions.[28] It is worth mentioning that, as a weak bond, diselenide bond is not only responsive to visible light and temperature but can also be broken under the action of osmotic pressure,[29] which greatly broadens the stimulus response methods in drug release. Moreover, the Se-S bond can be obtained by an exchange reaction between a disulfide compound and a diselenide compound under UV light irradiation.[30] The formation of Se-S bond is expected to realize the cyclization between cysteine-containing peptides and selenocysteine-containing peptides to form new cyclic peptides, thereby triggering self-assembly *in situ* to form

pure cyclic peptide assemblies. This is conducive to the realization of organic solvent-free hydrophobic cysteine- and selenocysteine-based cyclic peptide self-assembly. These selenium-containing substances combined with active sulfur species are important members of cellular antioxidants and play an important role in the development and treatment of cancer. Therefore, the study of covalent self-assembly of peptides based on cysteine and selenocysteine is of great significance in cancer diagnosis and treatment. However, both thiols group (-SH) and selenium group (-SeH)-related reaction mentioned above are limited to specific amino acids including cysteine (C) and selenocysteine (U), showing certain limitations in the construction of peptide-based nanodrugs.

Phenolic hydroxyl group (Ar-OH) on tyrosine (Y)

Tyrosine is a versatile amino acid that plays an important role in regulating the structural conformation transitions of proteins and is related to the occurrence as well as treatment of pigment disorders and malignant melanoma tumors.[31] Tyrosine with redox-active phenol group as a side chain group has unique chemical reactivity and can be oxidized into various forms, such as 3,4-dihydroxyphenylalanine or melanin, or crosslink with other tyrosine residue, resulting in dityrosine bonds.[32] Dityrosine crosslinking is frequently found in many natural materials, which is used to enhance mechanical stability and functionality of materials.[33] The first step in the formation of dityrosine involves the removal of hydrogen atoms from the hydroxyl groups on the phenoxy ring of tyrosine to form free radicals. The tyrosyl radical then reacts with each other to form dityrosine bond with characteristic blue light emission. The tyrosyl radical may also couple to another tyrosine, leaving an unpaired electron, allowing for the generation of trityrosine and even polypeptides.[34] In addition to dityrosine formation, tyrosine can also polymerize into melanin-like substances that can convert light energy into heat.[15] There have been many different methods to induce or promote the reaction of dityrosine crosslinking or oxidative polymerization in aqueous solution, such as light crosslinking and enzyme catalysis.[32,35] Enzymatic reaction usually occurs under mild conditions such as neutral pH, aqueous solution, and physiological temperature. Overexpressed tyrosinase in tumors may trigger *in situ* self-assembly of tyrosine-containing peptides, enabling targeted imaging and photothermal therapy of tumors. Compared to enzyme catalysis, the method of photo-crosslinking is more flexible and versatile in material design, but harsh relative to the biological system.

The phenolic group on tyrosine serves as not only excellent chemical reactivity but also excellent assembly motif which contributes to hydrogen bonding and π-π interactions. Therefore, the unique characteristics of tyrosine-tyrosine crosslinking and noncovalent interaction provided by the tyrosine molecule itself can easily realize covalent self-assembly of tyrosine-containing peptides. Kim and co-workers construct peptide assemblies through a single-step covalent self-assembly approach with symmetrical tyrosine-rich short peptides (YYAYY) under UV irradiation.[36] The morphology of the resulting peptide assemblies can be adjusted by changing the reaction medium, forming hollow nanocapsules by dissolving YAAYY in the buffer or aqueous media, while obtaining thin films or lamellae by dissolving YAAYY in methanol.[13]

In addition to dityrosine formation, tyrosine also can polymerize into melanin-like supramolecular materials through catalysis of tyrosinase.[37] However, precise control of the tyrosine oxidation pathway remains challenging. Recently, we demonstrate tyrosine can be selectively oxidized into dityrosine or melanin by simply manipulating oxygen concentration in the reaction system.[15] Based on such selective oxidation reaction and the consequent self-assembly of the resulting dityrosine- or melanin-containing building units, assemblies with distinct morphologies and photophysical properties were readily constructed *in situ*. This study demonstrates covalent self-assembly is a versatile strategy for the design of tyrosine-containing peptide assemblies with functions of optional photoluminescence imaging and photothermal therapy.

Amide group (-CO-NH-)

Although amide bonds are widely present in peptide, the breakage and formation of amide bonds are difficult to occur spontaneously in aqueous solution because of their inherent stability. As an endogenous ubiquitous high-efficiency catalyst, enzyme can effectively catalyze the condensation and cleavage of amide bonds *in vivo*. Enzyme-catalyzed hydrolysis or polymerization of amide group enables a new possibility for researcher to move peptide covalent self-assembly from laboratory tube into tumor/intracellular microenvironment.[38] Therefore, enzymes, as one of the tumor-specific markers, provide an opportunity for the realization of tumor-specific accumulation and retention of peptide-based nanodrugs by covalent self-assembly strategy.

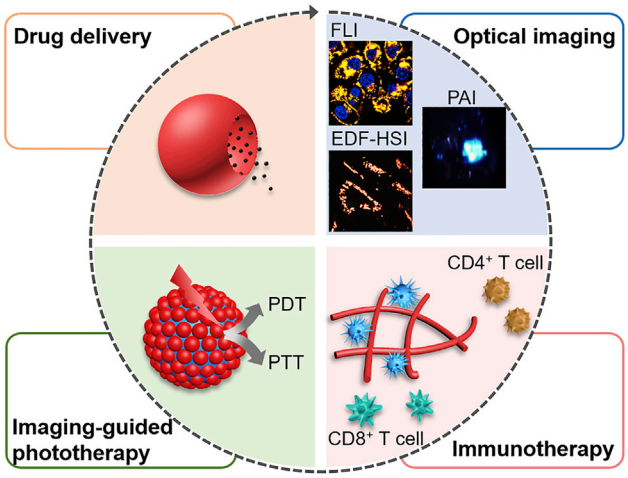

Figure 3. Schematic illustration of covalently self-assembled peptide-based nanoparticles for cancer theranostics

Applications for cancer theranostics include drug delivery, optical imaging, imaging-guided phototherapy, and immunotherapy. (FLI: Fluorescence imaging. Reproduced with permission from.[11] Copyright 2019 Royal Society of Chemistry. PAI: Photoacoustic imaging. Reproduced with permission from.[7] Copyright 2019 American Chemical Society. EDF-HSI: Enhanced dark-field hyperspectral imaging. Reproduced with permission from.[12] Copyright 2021 Elsevier. PDT: Photodynamic therapy, PTT: Photothermal therapy, $CD4^+$ T cell: CD4-positive T lymphocytes, $CD8^+$ T cell: CD8-positive T lymphocytes.)

A series of strategies with enzyme-triggered peptide self-assembly have been proposed. Especially, intracellularly instructed self-assembly of peptides have been considered as one of the most versatile strategies of developing novel biomaterials for disease treatment.[39] In general, peptide precursor includes three main modules: assembly module (photosensitizer or hydrophobic part), enzyme-responsive peptide sequence, and targeting peptide. They exist in the molecule form in blood circulation and can enter into tumor due to its small size. After reaching tumor microenvironment, under enzyme stimulation, the responsive peptide sequence is broken and the hydrophilic part is removed, thus breaking the hydrophilic-hydrophobic balance of the original molecule and inducing *in situ* self-assembly. In addition, self-condensation reaction between amino and carboxamide group triggered by enzyme such as transglutaminase (TGase) can form more hydrophobic polymers, thus also realizing the process of self-assembly *in vivo*.[40] This covalent self-assembly in tumor/intracellular microenvironment triggered by breakage/formation of amide bonds is favorable for some tumor treatment modes such as photothermal therapy (PTT) or immunotherapy because it can achieve self-assembly-induced retention effect of photosensitizer or antigen, which is conducive to enhanced photothermal conversion effect or immune response.

COVALENTLY ASSEMBLED PEPTIDE-BASED NANODRUGS FOR CANCER THERANOSTICS

With intrinsic advantages of this strategy, peptide covalent self-assembly is favorable for application of cancer theranostics. Hereafter, we summarize the progress of covalently assembled peptide-based nanodrugs in cancer theranostics, including drug delivery, optical imaging of tumor cells and tissues, multimode optical imaging-guided phototherapy, and immunotherapy (Figure 3), and elaborate the subtle design and functional enhancement mechanisms of peptide covalent self-assembly in each application.

Drug delivery

Enhanced cancer treatment efficiency and reduced system toxicity can be achieved through functional nanocarriers that can efficiently target and deliver drugs to tumor. The ideal nanocarriers usually process following properties, e.g., excellent biocompatibility and stability, controlled drug release in target region, and degradable over time. Spontaneous covalent reaction followed by *in situ* self-assembly in aqueous solution can be regarded as self-synthesis of nanomaterials, which can strengthen the colloidal robustness and stability in physiological system. In addition, as a delivery system, the on-demand unloading of drugs to the target region also needs to be considered. The design of dynamic covalent bonds is of great significance in solving the dilemma between stability and controlled release for drug delivery.[41] Spontaneous

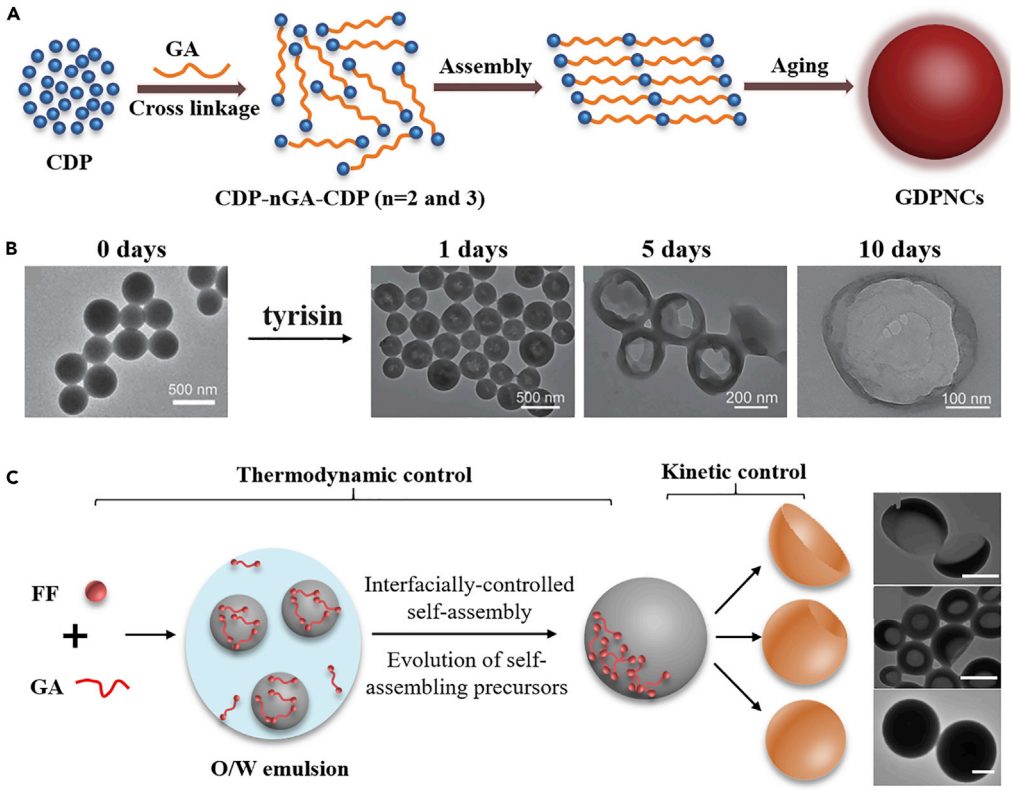

Figure 4. Covalently assembled peptide-based nanoparticles for drug delivery

(A) Schematic illustration of the covalent self-assembly of CDP nanocarriers (CDPNCs).

(B) TEM images of CDPNCs co-incubated with tyrisin in PBS for different times. Reproduced with permission from.[3] Copyright 2015 Wiley.

(C) Schematic illustration and TEM images of fabricating dipeptide-based concave at different time. Scale bar is 200 nm. Reproduced with permission from.[44] Copyright 2016 Royal Society of Chemistry.

synthesis under thermodynamic control endows the peptide assemblies with better stability and adaptability under physiological conditions, and at the same time has the characteristics of stimuli responsiveness and the capacity of degradation under the action of biological molecules or the changes of tumor microenvironment, which fulfills the requirements of robust encapsulation and on-demand drug delivery, thus avoiding the cumulative toxicity.

Schiff base bond is a dynamic covalent bond and possesses reversibility with the change of pH values, which is stable in physiological environment but stability decreases in acid pH.[20] This merit makes Schiff base reaction preferable in constructing drug delivery system, especially for on-demand release system.[23,42] Li group-mixed cationic phenylalanine dipeptide (CDP) and GA in an aqueous solution, aldehyde groups in the oligomers of GA, and amino groups in dipeptide can spontaneously react to form Schiff base bonds.[3] Then, the formation of Schiff base bonds in the aqueous solution and accompanying π-π interaction of aromatic rings together trigger bola-like unit CDP-nGA-CDP self-assembly *in situ* to form monodisperse nanoparticles (Figure 4A). The obtained CDP nanocarriers (CDPNCs) show high biocompatibility, high loading capacity for hydrophobic or hydrophilic guest molecules, and desirable tumor microenvironmental responsiveness. After being uptake by the tumor cells, CDPNCs-DOX enables the sustained responsive release of doxorubicin (DOX) triggered by tyrisin, and realizes enhanced killing efficiency against tumor cells and fast removal of the CDPNCs (Figure 4B).

Compared with sustained release, the burst release of drugs for emergency treatment is more needed and challenging for covalently assembled materials. Phenylalanine dipeptide-based porous nanocontainer has been presented through covalent self-assembly approach by using negatively charged phenylalanine

dipeptide (FF) and GA as building blocks.[43] The obtained FF-based porous nanocontainer is colloidal stable at pH 5.0, but can achieve "fast disassembly" within several seconds under a physiological pH (pH = 7.2) trigger due to enhanced hydrophilicity and destruction of intermolecular hydrogen bonding resulting from deprotonated carboxyl groups. The highlighted characteristics of such covalent-assembled nanoparticles are adapted for emergency medical treatments.

This time-dependent covalent bond formation and self-assembly process is conducive to the morphological manipulation of assemblies via synergistic kinetic and thermodynamic controls.[4] Our group prepared diverse concave assemblies with structures from crescent-like, bowl-like, to solid interior through controlled self-assembly at oil/water interface, associated with covalent Schiff base bond formation of dipeptide and GA.[44] This process is achieved by the synergistic effect of thermodynamic control (the formation of covalent FF-nGA-FF building blocks and interfacially controlled noncovalent self-assembly of FF-nGA-FF aggregated clusters) and kinetic control (the formation rate and interfacial nucleation rate of FF-nGA-FF) (Figure 4C). These concave nanostructures relative to solid spherical particles are more promising for biomedical application such as functionalized drug loading and responsive release.

Based on the reaction between peptide and iridoid, our group fabricated robust nanoparticles with both physiological stability and biodegradability by covalent self-assembly of genipin and amphiphilic peptide.[6] An amphiphilic peptide which contains two phenylalanine groups and one disulfide bond linkage has been designed. Phenylalanine, as a strong hydrophobic amino acid, has been shown to be a self-assembly motif that can regulate the structure and performance of assembly.[45] The disulfide bond, which is ubiquitous in proteins, remains stable in the extracellular environment but can undergo a bond scission process within the tumor cells due to its sensitive response to the overexpressed GSH in tumor cells.[46] Taking advantage of these molecules, it is possible to construct stable yet biodegradable drug delivery system with controllable disassembly capacity for delivery of photothermal agents. Overall, the covalent self-assembly that combines the stability conferred by covalent bond with the flexibility of noncovalent self-assembly processes obvious advantages in constructing adaptive multifunctional delivery nanoplatforms.

Optical imaging of tumor cells and tissues

Among natural amino acids, there are three aromatic amino acids including phenylalanine, tyrosine, and tryptophan to have photoluminescence property, but only excited by the UV light. In nature, biological system can construct pigment assemblies with broad absorbance from the UV to the near-infrared region by covalent self-assembly of endogenous nonpigment amino acids and other biomolecules. For example, as natural pigment molecules, porphyrins are self-synthesized through a series of chemical reactions of glycine with the assistance of succinyl coenzymes *in vivo*.[47] Inspired by this, photofunctional assemblies with different characteristics based on covalent self-assembly of nonpigment peptides have been constructed for optical imaging *in vitro* and *in vivo*.[7,11,48]

Tyrosine is one of the main sources of pigment production in organisms. Life only utilizes the metabolic reaction of tyrosine to create colorful fur, feathers, and eyes.[32] Tyrosine has a characteristic fluorescence emission at about 305 nm from phenolic groups. After self-crosslinking to form dityrosine bond, the absorption of dityrosine redshifts to ~330 nm and an intrinsic blue fluorescence at ~410 nm occurs.[49] Although tyrosine crosslinking can endow tyrosine-based peptide with characteristic of strong blue fluorescence, the development of tyrosine-based oligomeric peptide probes with long-wavelength emission is still challenging. Inspired by the chromophore of serine-tyrosine-glycine within fluorescent proteins, Zhang and co-worker designed tyrosine-based tripeptides which can form a robust dityrosine structure and then self-assembled into nanoparticles with the hydrodynamic diameter from 0.8 to 2.0 nm for the application of neuroglioma cells labeling.[48] Under the excitation of UV light, nanoparticles emit cyan fluorescence, which further redshifts the fluorescence of tyrosine-based peptide to the visible light region (~500 nm). This study also proves that both the chemical properties and the sequence of amino acids have a huge effect on the optical properties of tyrosine-based short peptides.

Fabrication of fluorescent assemblies through covalent self-assembly follows a way, in which its chromophore is generated through covalent reaction, so the light absorption characteristic of these assemblies is closely related to the reaction condition or reaction process. Therefore, changing the reaction condition (such as temperature and reaction time) is expected to control fluorescent property of the assemblies. Formation of a Schiff base bond endows materials with autofluorescence property. Our group prepared

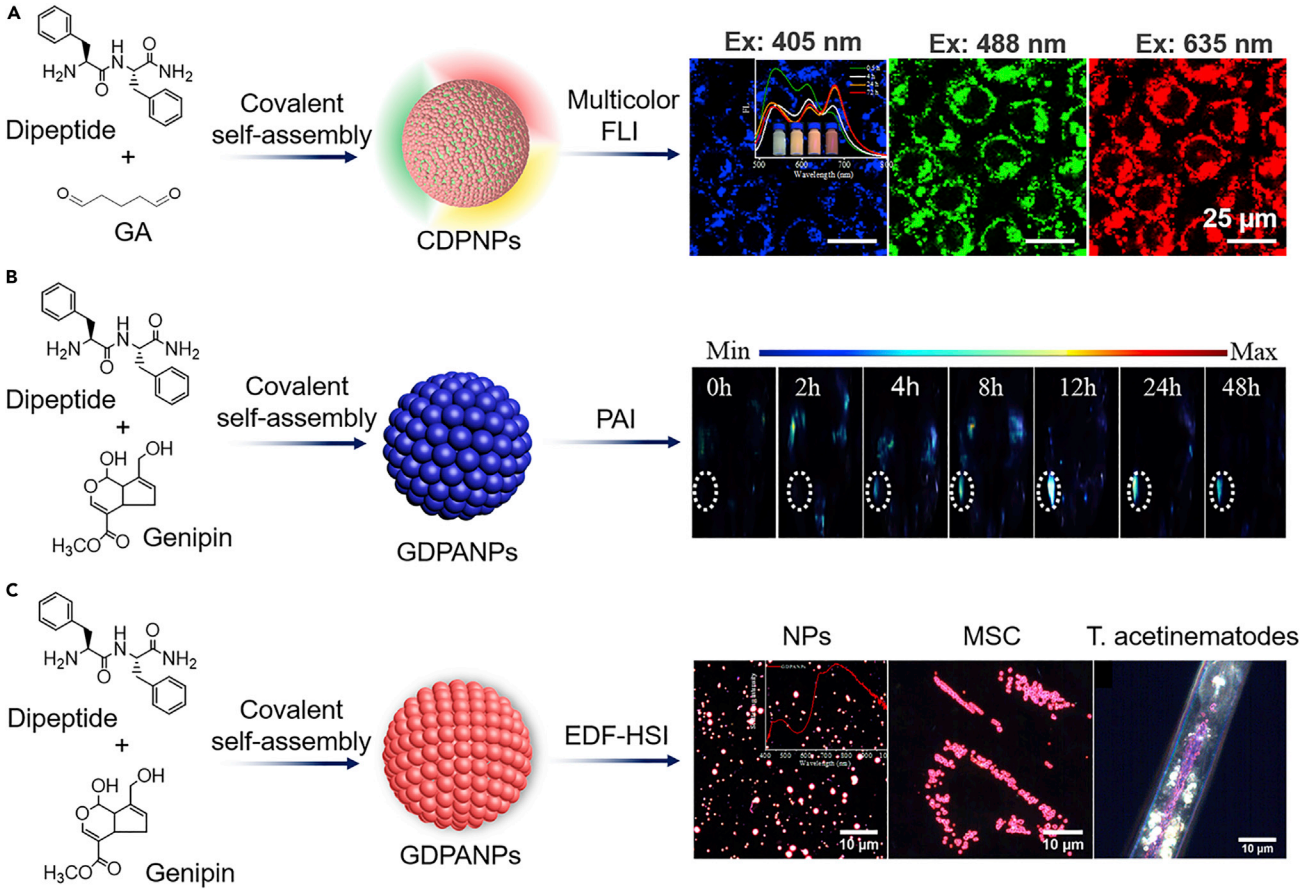

Figure 5. Covalently self-assembled dipeptide nanoparticles for optical imaging of tumor cells and tissues

(A) Multicolor fluorescence imaging (FLI), (B) photoacoustic imaging (PAI), and (C) enhanced dark-field hyperspectral imaging (EDF-HSI) of covalently self-assembled dipeptide nanoparticles. (A): Reproduced with permission from.[11] Copyright 2019 Royal Society of Chemistry. (B): Reproduced with permission from.[7] Copyright 2019 American Chemical Society. (C): Reproduced with permission from.[12] Copyright 2021 Elsevier.

dipeptide nanoparticles (CDPNPs) with autofluorescence properties due to the formation of Schiff base bonds and C=C double bonds.[11] Intriguingly, by changing the reaction time, the ratio of C=N bond and C=C bond of formed chromophores in the product can be flexibly modulated, enabling the adjustable fluorescence emission (from blue to green to red luminescence) of resulting nanoparticles. The three single channels and superimposed channels imaging by the laser confocal microscope can realize the co-localization of nanoparticles in cancer cells (Figure 5A). Due to repeated multichannel confirmation, interference signals are effectively avoided and the imaging accuracy is clearly enhanced.

Gardenia blue, as natural blue colorant, is formed by crosslinking of colorless iridoids and a primary amine in the presence of oxygen, serving as fingerprint reagent and food colorants.[22,50] The resulting blue pigments are known to absorb the light in the range from UV to visible wavelength and emit fluorescence in the 380–700 nm wavelength region.[51] The maximum fluorescence emission position shows a strong dependence on the excitation wavelength owing to a broad wavelength absorption caused by multi-component of this blue pigments.[52] The reaction of iridoids with primary amines to form blue chromophores plays an important role in the construction of peptide-based photofunctional nanomaterials.[5,14] Furthermore, the obtained pigment peptide assemblies with controllable fluorescence emission or photothermal effect can be selectively produced by manipulating the kinetics and thermodynamics of covalent self-assembly between peptides and iridoids, which can be selectively utilized for fluorescence imaging (FLI) or photoacoustic imaging (PAI).[4,7] Compared to FLI, PAI demonstrates deeper penetration depth, better spatial resolution, and lower background noise to monitor drug distribution over time *in vivo*

(Figure 5B). Especially, such multicomponent supramolecular assemblies originated from this reaction can cause the non-uniformity of refractive index, which will force the light to deviate from its linear trajectory to cause light scattering.[53] Imaging based on scattered light has many unique advantages such as stable signal and resistance to photobleaching. Enhanced dark-field hyperspectral imaging (EDF-HSI), integrated dark-field microscopy and hyperspectral imaging, is currently an advanced imaging acquisition method for nanomaterials, especially for non-fluorescent materials, because EDF-HSI is based on light scattering of optically anisotropic materials.[54] We recently demonstrated that the peptide nanoparticles (GDPANPs) formed by covalent self-assembly of dipeptide and genipin are a promising contrast agent for EDF-HSI.[12] The prepared GDPANPs showed strong signals in EDF-HSI both inside cells and nematodes due to their strong light scattering generated from non-uniformity of refractive index at the interface, thus realizing visualization and tracking of non-fluorescent peptide nanodrugs at the cellular and invertebrate level by EDF-HSI (Figure 5C).

In summary, this simple covalent self-assembly strategy offers a potent means to develop novel peptide-based optical imaging contrast agents. By integrating covalent reaction and self-assembly, the obtained peptide-based assemblies can be endowed with multiple photophysical and photochemical properties for multimode optical imaging such as FLI, PAI, and EDF-HSI, which greatly enriches the optical imaging methods of peptide-based nanomaterials.

Multimode optical imaging-guided cancer phototherapies

In addition to constructing multifunctional delivery systems and optical imaging agents, covalent self-assembly strategy based on peptide and iridoid also can be used to construct nanophotodrugs for multimode optical imaging-guided phototherapy.[23] Compared with traditional pigment molecules, the advantage of the covalent self-assembly system of iridoid and peptide is that its chromophores are self-synthesized *in situ* in the reaction process, and the optical properties of these chromophores are closely related to the reaction conditions. The reaction conditions play an important role in regulating the aggregation form of chromophores, which in turn regulates the optical properties of the assemblies. Under different assembly conditions, the aggregation form of chromophores differs. Depending on regulation of the aggregation form, it is hopeful to obtain the assemblies with near-infrared absorption, which is beneficial for improving the therapeutic depth.[55,56] In addition, the molecular aggregation can regulate the energy dissipation mode of the assemblies, and thus can be selectively used for FLI-guided photodynamic therapy (PDT) or PAI/photothermal imaging (PTI)-guided PTT (Figure 6A).[4,7] For example, by quenching fluorescence and reducing the production of reactive oxygen species, more light energy absorbed by chromophores can be converted into heat energy and thus the photothermal conversion efficiency can be significantly enhanced.[45]

Li et al. fabricated monodispersed dipeptide-based nanospheres with red autofluorescence through a covalent reaction-induced assembly by simply mixing a 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol solution of cationic diphenylalanine with an aqueous solution of genipin in the neutral pH, and then keeping the solution at room temperature for 7 days (Figure 6B).[4] The obtained nanospheres show the broad absorption from 500 to 700 nm with maximum absorption peak at 620 nm (Figure 6C). Unexpectedly, such covalently assembled nanospheres can act as an intrinsic photosensitizer to emit red fluorescence and convert O_2 to singlet oxygen ($^1O_2$) under 635 nm laser irradiation toward PDT (Figure 6D). By contrast, Yan et al. obtained robust photothermal nanodrugs by lowering the pH (pH = 4.7) and raising the temperature (70°C) in the reaction system of cationic diphenylalanine and genipin.[7] The resulting nanodrugs show intense absorption band in the near-infrared region (640–900 nm) (Figure 6E). Especially, the photothermal conversion efficiency of the nanodrugs can be as high as 50.7% through 660 nm laser irradiation (Figure 6F). *In vivo* experiments in mice confirmed that photothermal nanodrugs can effectively accumulate at tumor sites and consequently achieve optional tumor ablation via PAI-combined PTI-guided PTT (Figure 6G). Therefore, through fluorescence quenching and inhibiting the production of active oxygen, the light energy absorbed by the nanodrugs can be efficiently converted into heat toward PTT.[57,58] The difference in optical properties of the nanodrugs mainly attributes to the susceptibility of the covalent reaction and noncovalent self-assembly between dipeptide and genipin under the different conditions. It is thus clear that peptide covalent self-assembly is an efficient approach for constructing supramolecular nanophotodrugs toward optional cancer phototherapy.

Taking into account the versatility of peptide and the universality of chemical reaction, bioactive peptides can be rationally designed to participate in covalent self-assembly for additional therapeutic effects. Recently, Yan et al. reported the covalent self-assembly of genipin and tyroserleutide that is a therapeutic

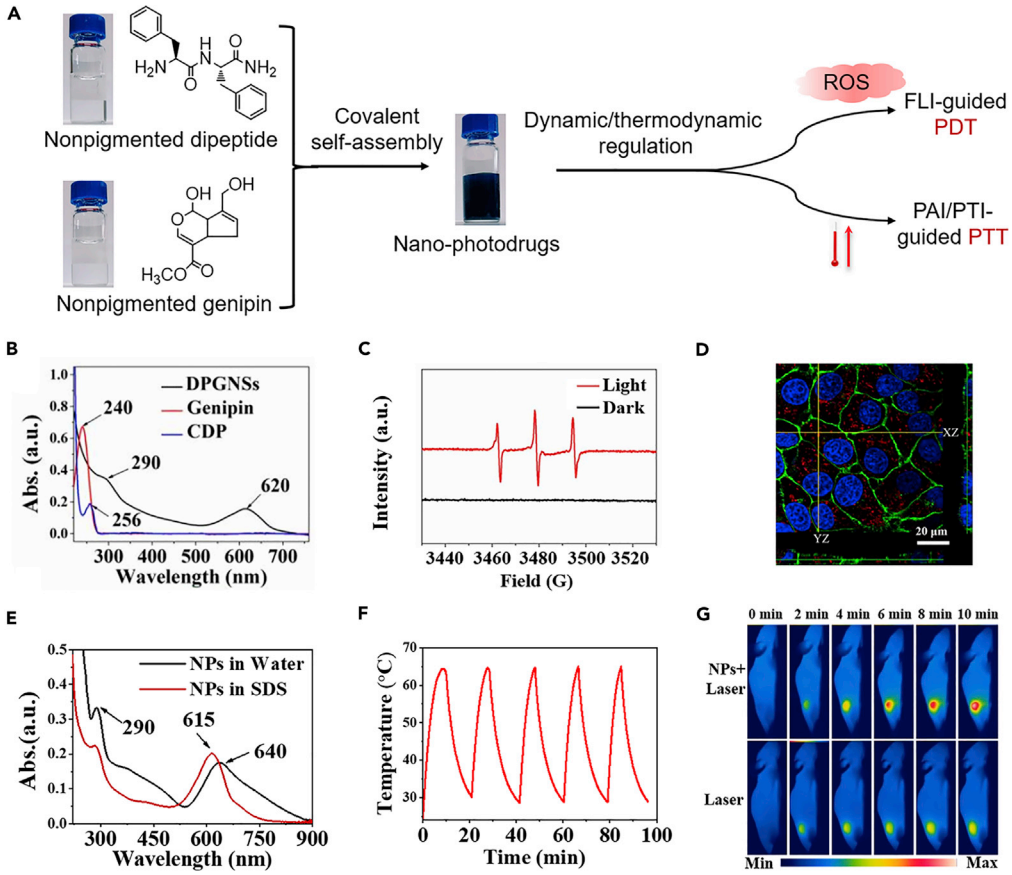

Figure 6. Covalently self-assembled dipeptide nanoparticles as nano-photodrugs for multimode optical imaging-guided cancer phototherapies

(A) Illustration of the preparation of nanophotodrugs based on covalent self-assembly between nonpigmented dipeptide and genipin for optical imaging-guided optional PDT or PTT.

(B) UV-Vis spectra of CDP, genipin, and dipeptide-genipin nanospheres (DPGNSs).

(C) Detection of $^1O_2$ for DPGNSs. (D) 3D-CLSM fluorescent image of the cancer cells endocytosing DPGNSs. Reproduced with permission from.[4] Copyright 2016 Wiley.

(E) UV-Vis spectra of photothermal NPs in water or 10% SDS.

(F) Photothermal activity of the photothermal NPs under five irradiation cycles by 660 nm laser (1.0 W cm^{-2}).

(G) Infrared thermal images of mice injected with or without photothermal NPs coated with BSA under continuous irradiation. Reproduced with permission from.[7] Copyright 2019 American Chemical Society.

tripeptide capable of chemically treating primary liver cancer.[9] The formation of peptide nanodrugs, triggered by the covalent reaction of therapeutic peptide and genipin, enhances peptide stability against the degradation by peptidases. Similarly, the obtained nanodrugs show intense absorbance in near-infrared region and high photothermal conversion efficiency. Covalent self-assembly of tyroserleutide and genipin leads to enhanced antitumor activity due to the formation of nanoassemblies and the introduction of supramolecular photothermal activity. In particular, the covalent self-assembly of therapeutic peptides achieves the synergistic anticancer therapy through the combination of chemotherapy (CT) and PTT.

Altogether, this simple and flexible covalent self-assembly approach offers an efficient way to direct synthesis of peptide-based nanophotodrugs with high stability, adjustable optical absorbance, and controllable light energy conversion for multimode optical imaging (FLI, PAI, and PTI)-guided PDT, PTT, and CT-combined phototherapy.

Cancer immunotherapy

With the development of tumor immunology and in-depth research on antitumor immune response, tumor immunotherapy has gradually become an important means against cancer.[59] The mechanism of antitumor

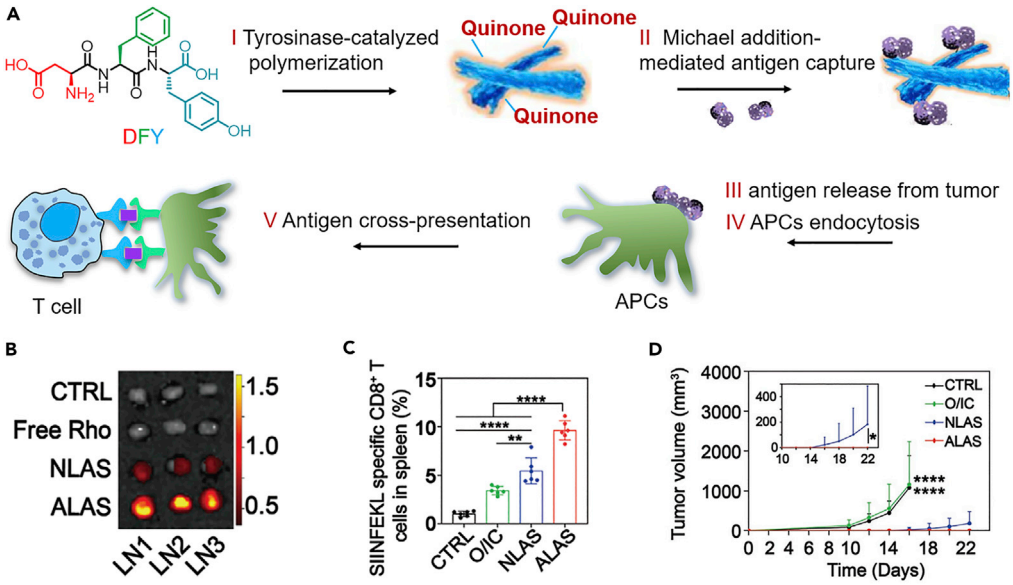

Figure 7. Peptide covalent self-assembly *in vivo* for cancer immunotherapy

(A) Schematic diagram for illustrating the mechanism of the Asp-Phe-Tyr tripeptide in anticipating intracellular oxidative polymerization and Michael addition with proteins for cancer immunotherapy. Reproduced with permission from.[62] Copyright 2021 American Chemical Society.

(B) IVIS fluorescence imaging of the isolated lymph nodes from the mice injected with different drugs.

(C) Flow cytometry measurement of SIINFEKL-specific $CD8^+$ T cells in spleen.

(D) The average tumor growth curves from the mice with different treatments. Reproduced with permission from.[66] Copyright 2021 Wiley.

immune response is that tumor-associated antigens are taken up and processed by antigen-presenting cells, and then present to T cells in lymph nodes in the form of antigen peptides and major histocompatibility complex, thus stimulating the activation of effector T cells to kill tumor cells.[60]

Systemic suppression of antigen presentation has been a substantial challenge to initiate robust antitumor immune response, thereby restricting the application of immunotherapy.[61] Zhang et al. constructed a tumor protein-engineering system based on the enzyme-catalyzed polymerization of Asp-Phe-Tyr tripeptide (DFY) for malignant melanoma immunotherapy.[62] Through intracellular tyrosinase catalysis, the phenolic hydroxyl group of soluble DFY is oxidized to form a quinone-rich intermediate that further undergoes a Michael addition reaction with the sulfhydryl group of cysteine in tumor-specific protein, causing a necrosis of tumor cells by covalent crosslinking of tumor-associated proteins. Therefore, during the formation of microfibers by oxidative polymerization, intracellular tumor-specific antigens are also captured and subsequently released from the disrupted cancer cells (Figure 7A). Overall, the DFY tripeptide can selectively accumulate in melanoma cells with overexpressed tyrosinase. The released antigen-loading microfibers with larger size are more likely to be engulfed by antigen-presenting cells and deliver antigens into immune cells for boosting immune effect.

Lymph nodes are the primary site for antigen presentation. A large number of antigen-presenting cells settle in the lymph nodes and these cells are adjacent to the initial T lymphocyte, which can achieve rapid presentation after ingesting antigen.[63–65] Therefore, the effective delivery and accumulation of tumor vaccines to lymph nodes is one of the important strategies to improve immune efficiency. In a recent study, Nie et al. developed an active-targeting strategy based on click chemistry between azide and dibenzocyclooctyne group to facilitate the accumulation of antigen peptide-based nanovaccines in the lymph node.[66] By mimicking the targeted delivery of vaccines based on the "ligand-receptor" interaction, the nanovaccines promote the accumulation of antigen peptides in lymph nodes (Figure 7B), thereby enhancing the efficiency of T lymphocyte activation and effectively inhibiting the proliferation of tumor (Figures 7C and 7D). Similarly, Wang et al. developed a click reaction-assisted peptide immune checkpoint blockade strategy, which realized deep penetration and subsequent construction of peptide assemblies *in situ*,

enhancing accumulation in solid tumors, prolonging programmed-death ligand 1 occupancy, and ultimately realizing high-performance immunotherapeutic effect.[67]

These studies again highlight the advantages of the covalent assembly strategy for improved cancer immunotherapy because it not only achieves the covalent reaction-induced efficient delivery of antigen peptide to lymph nodes but also achieves self-assembly *in-situ*-induced efficient presentation of antigen peptide to T lymphocyte, eventually amplifying antitumor immune response.

CONCLUSIONS

In summary, we have given a comprehensive overview of covalently triggered peptide self-assembly from the basic design principle to antitumor theranostic applications, including drug delivery, optical imaging, phototherapy, and immunotherapy. Compared with supramolecular self-assembly, the strategy of covalently triggered peptide self-assembly has demonstrated an effective and versatile tool for cancer theranostics with advantages: i) simple and environmentally friendly self-synthesis process in water system, ii) enhanced colloidal stability and controlled drug release, iii) endowing the non-pigment peptides with adjustable optical properties for applications in multimode imaging (FLI, PAI, and EDF-HSI) and multimode imaging (FLI, PAI, and PTI)-guided phototherapy, and iv) enhancing immunotherapy effect of peptide antigens through covalently triggered *in situ* self-assembly in the tumor. Also, peptide covalent self-assembly endows peptide nanomaterials and nanodrugs with self-synthesis capability, enabling the large-scale preparation of uniform and size-controlled particles ranging from nanometer to micrometer. These are highly appreciated in the clinical translation and application of nanoscale and microscale peptide assemblies. Therefore, covalent self-assembly can be considered as a potent strategy for design of peptide-based nanodrugs to advance cancer theranostics.

Although peptide covalent self-assembly strategy has shown advantages in cancer theranostics and made considerable progress, this field is still in its infancy. First, the types of covalent reactions and amino acids involved in the peptide covalent self-assembly are limited due to serious requirements such as mild reaction conditions, reactions in aqueous solutions, and high reaction rates, mainly focusing on the reactions of phenylalanine and tyrosine-related non-functional peptides. Highly reactive reactions of other amino acids need to be further explored for peptide covalent self-assembly so that diverse nanomaterials or nanodrugs can be constructed. Additionally, due to the ability of peptides to provide a variety of weak interactions including hydrophobic, π-π stacking, hydrogen bonding, and electrostatic, the precise peptide sequence design also needs to be extensively considered to realize the subsequent self-assembly of product *in situ*. Meanwhile, more functional peptide sequences, including targeted peptides, therapeutic peptides, antigenic peptides, and immunomodulatory peptides, are expected to be introduced to construct multifunctional assemblies for antitumor combination therapy. However, it is challenging to maintain and/or enhance the activity of functional peptides after covalent self-assembly, so it is necessary to avoid the active sites of bioactive peptides when designing reactions. Therefore, there is plenty of room for deep insights into the construction and functional exploration of covalently self-assembled peptide-based nanodrugs. Finally, in view of the universality of covalent reaction, the covalent self-assembly strategy is expected to be extended to other biologically active molecules (such as polysaccharides and proteins) or small molecule drugs which is expected to construct new functional nanodrugs and provide new ideas for the clinical treatment of other diseases.

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AUTHOR CONTRIBUTIONS

Conceptualization, X.Y. and J.L.; Writing – Original Draft, Y.L.; Writing – Review & Editing, all authors; Visualization, R.X. and Y.L.; Supervision, R.X. and X.Y.; Project Administration, R.X. and X.Y.; Funding Acquisition, Y.L., R.X., and X.Y.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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