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Peptide–Drug Conjugates as Effective Prodrug Strategies for Targeted Delivery

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Abstract

Peptide–drug conjugates (PDCs) represent an important class of therapeutic agents that combine one or more drug molecules with a short peptide through a biodegradable linker. This prodrug strategy uniquely and specifically exploits the biological activities and self-assembling potential of small molecule peptides to improve the treatment efficacy of medicinal compounds. We review here the recent progress in the design and synthesis of peptide–drug conjugates in the context of targeted drug delivery and cancer chemotherapy. We analyze carefully the key design features in choosing the peptide sequence and linker chemistry for the drug of interest, as well as the strategies to optimize the conjugate design. We highlight the recent progress in the design and synthesis of self-assembling peptide–drug amphiphiles to construct supramolecular nanomedicine and nanofiber hydrogels for both systemic and topical delivery of active pharmaceutical ingredients.

Graphical abstract

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Keywords

peptide–drug conjugates; conjugated chemistry; drug amphiphiles; drug delivery; cancer

1. Introduction

The primary goal of drug delivery in cancer therapy is to transport sufficient drugs to target disease site(s) while minimizing their exposure to healthy tissues [1–4]. To achieve this, two main strategies have been extensively exploited, both seeking to alter the pharmacokinetic properties of the drug in some way. The first involves the use of a delivery vehicle, such as well-defined nanoparticles, that encapsulate the drug and dictates the biodistribution through its own physicochemical properties [5–11]; the second is the covalent modification of the drug with some small moiety that temporarily masks or limits the drug's bioactivity and confers overall desirable pharmacokinetic properties (prodrug strategy) [12–19]. Compared to the delivery vehicle approach, the prodrug method features minimized premature drug liberation and fewer amounts of inert materials, both of which can lighten the metabolic burden of the patient and in turn enhance the therapeutic efficacy [20–26]. In light of these advantages and its somewhat simpler approach, it is perhaps not surprising that many prodrugs have been developed and approved by the Food and Drug Administration (FDA), in contrast to the relatively few examples for the delivery vehicle approach. In particular, antibody–drug conjugates (ADCs), such as Brentuximab vedotin (Adcetris®) and ado-trastuzumab emtansine (Kadcyla®), have drawn significant attention as a result of antibody's inherently selective binding ability [13, 27].

Peptide–drug conjugates (PDCs) are an emerging class of prodrugs, formed through the covalent attachment of a specific peptide sequence to a drug via a cleavable linker. The utilization of peptides allows for the incorporation of great degree of functionality into PDCs, as the amino acid sequence can be chosen to both control the physicochemical properties of the conjugate and to allow for active targeting of a particular receptor on the tumor cell surface. Since PDCs are prepared from amino acids and generally have short peptide lengths, they are biodegradable and should not elicit undesired immunogenic responses [28–30]. The diversity of amino acid combinations enables the facile preparation of many different PDCs, and the control over the sequence allows tuning of the overall conjugate hydrophobicity and ionization, both of which influence the bioavailability *in vitro*

and *in vivo*. Furthermore, the low molecular weight of PDCs allows purification to homogeneity via simple HPLC techniques. The ability to exert effective control of both molecular weight and purity of synthetic PDCs is critical for optimizing the pharmacokinetics of PDCs.

An emerging subset of PDCs seeks to combine the advantages of peptide-based prodrugs with those of a vehicular delivery approach, giving rise to self-assembling PDCs in which the individual conjugates possess the ability to form nanostructures with physicochemical properties unique to those of the components (Fig. 1) [14, 31]. In this novel design, the PDCs essentially form their own drug delivery vehicle (namely one-component nanomedicines), one that is capable of breaking down, either over time or due to some specific stimulus, and releasing the active drug. The drug retains its inherent bioactivity but also acts as a building block in the construction of nano-sized assemblies. Similarly, the peptide segment behaves like a traditional hydrophilic segment to stabilize the nanocarrier in water, but can also act as functional blocks for both assembly and targeting purposes. Manipulation at the molecular level also means the numbers and locations of reactive sites for drug conjugation can be precisely determined, resulting in control over the architecture of PDCs at the sequence level. In view of the elaborate design, PDCs retain all the advantages of conventional prodrug delivery systems, but also provide a number of additional benefits associated with drug delivery vehicles, such as increased tumor uptake through the enhanced permeation and retention (EPR) effect [32] and reduced renal clearance due to their nano-sized dimensions.

These unique properties of PDCs have motivated researchers to design and synthesize various types of PDCs. Although diverse in formulations, PDCs are typically composed of three essential parts: drug, linker and peptide (Fig. 1). It is easy to imagine that subtle changes made to one or all of these three segments would have a significant influence on the entire system's performance, and therefore careful selection of all the components is essential in determining the best drug delivery approaches [33, 34]. Up to now, a number of fields have benefited from the use of PDCs, including imaging and biosensing [35, 36] and biocatalysis [37], with numerous reviews published by experts in the area. Thus, the emphasis in this review will be on their applications in drug delivery. Since the internalized mechanism and bioavailability are highly dependent on the assembled state, water-soluble PDCs and self-assembling PDCs will be discussed separately. The ways in which the molecular design affects the conjugate's functions in drug delivery will be a particular highlight. It is our hope that the approaches presented here will serve as a guide for construction of novel peptide-based conjugates.

2. Water-soluble peptide–drug conjugates

A common trait among traditional small molecule anticancer drugs is their poor solubility in aqueous solutions. To circumvent this problem, excipients are often used to solubilize the insoluble drug but this strategy can introduce new problems such as the added solvent has toxicity of its own. For instance, the use of Kolliphor EL (formerly Cremophor EL) to “solubilize” paclitaxel (Taxol®) as an emulsion has been associated with allergic reactions and peripheral neuropathy, among other side effects [38]. Conjugating the anticancer drug to

a hydrophilic peptide can alleviate this solubility problem and provides the opportunity for the inclusion of greater functionality [31]. In this section, we will illustrate two commonly utilized peptide types that can enhance drug delivery—integrin-targeting and cell-penetrating peptides—and discuss the application of water-soluble PDCs for theranostic applications.

2.1 Integrin targeted peptide–drug conjugates

Given the goal of delivering chemotherapeutics to the disease site(s) with minimal collateral damages to healthy tissues, an effective approach is to consider the differences between normal tissues/cells and tumorous ones. Since the first point of interaction between any drug or delivery vehicle and a cell is at the exterior surface, any abnormally expressed receptors in cancerous tissues provide a focal point for targeted delivery. Thus, a great diversity of targeting groups can be integrated into the PDC design to achieve specific targeting to a particular cell type. Among such identified receptors, integrins are widely used since they are essential for the physiological development, maintenance, and repair of tissues, and feature prominently in the pathological processes of various diseases, particularly cancer [39, 40]. Accordingly, various kinds of integrin targeted peptide–drug conjugates have been designed in the past two decades [41], with the $\alpha_v\beta_3$ -targeting Arg-Gly-Asp (RGD) motif appearing almost ubiquitously in some form. For instance, coupling the free carboxylic groups of RGD4C to the 3'-amino position of doxorubicin (DOX) gave a conjugate that exhibits an enhanced *in vivo* tumor growth and metastasis inhibition efficacy over free DOX [42]. More importantly, the prodrug appeared to have less toxicity toward the liver and heart than free DOX in *ex vivo* histopathological assays. However, they did not clarify if the reported effect originated from the conjugates or the released DOX. In order to achieve the preferential release of drugs in cancer cells, Ryppa and coworkers introduced a metalloproteinase-2/9 (MMP-2/MMP-9) cleavable octapeptide between DOX and a divalent RGD peptidomimetic E-[c(RGDfK)₂] [43], observing significant drug release when compared with their amide-linked counterparts. However, the conjugate exhibited no or only moderate antitumor efficacy at relatively low doses, requiring a higher dose to exert any significant effect. The low efficacy may result from insufficient drug release since free DOX could not be recovered until further post-cleavage hydrolysis occurred [43]. Besides DOX, paclitaxel (PTX) [44, 45] and camptothecin (CPT) [46, 47] have also been conjugated to integrin targeting peptides, displaying a comparable *in vitro* anticancer effect to the free drug. Though these examples feature differing peptidic sequences, they illustrate the importance of the conjugation method in the efficacy of the resulting PDC. Both PTX and CPT are typically conjugated to peptides through ester bond formation, whereas DOX occurs through amide bond formation. Since amide bonds are significantly more stable to hydrolysis than esters, drugs conjugated via esters will degrade more readily in a physiological environment to release the free drug. Release of amide-conjugated drugs therefore requires additional complexity or an alternative strategy.

2.2 Guanidinium-rich peptide–drug conjugates

In nature, biological barriers are essential for all living systems, serving to isolate the intracellular structures from potentially harmful environments and pathogens. Although they are vital to survival, their inherent exclusivity can limit or preclude the uptake of a variety of

drugs. Accordingly, one important area in drug delivery research is finding solutions that enhance the internalization capability of drugs [48–54]. In early research on this topic, most studies aimed to find or develop drugs having a suitable hydrophilic–hydrophobic balance that would allow them to cross the nonpolar membrane of a cell. Typically, this would involve the adjustment of the drug's physical properties in order to optimize its log P value, where P is the partition coefficient and a measure of the drug's lipophilicity. In actuality, this is a time-consuming and low performance–price ratio task. Instead of concentrating efforts on the manipulation of the drug, Wender and his coworkers devoted great effort to finding an entirely new pathway for entry, by understanding these natural barriers and developing tools or strategies to breach them [52].

The identification of arginines, rather than lysines, and their number and spatial array as the essential features that allow the rapid cellular uptake of the Tat_{49–57} peptide (a basic domain from HIV-1 Tat protein) was a key discovery [55, 56]. By focusing on this, Wender and coworkers developed guanidinium-rich molecular transporters and demonstrated their use in a variety of applications [50, 53, 54, 56, 57]. For example, a cyclic peptide cyclosporin A (CsA) was chosen as the drug candidate because it is effective against several skin disorders, but causes off-target toxicity when delivered systemically [58]. By itself, CsA does not penetrate the skin and so to surmount this problem they conjugated a heptamer of arginine to CsA through a pH-sensitive linker to produce R₇–CsA. In contrast to unmodified CsA, topically applied R₇–CsA efficiently transported into cells in both mouse and human skin, reaching dermal T lymphocytes and inhibiting cutaneous inflammation. This represents a general strategy for enhancing delivery of poorly absorbed drugs across tissue barriers and provides a new topical approach to the treatment of inflammatory skin disorders [58]. In another example, Wender and coworkers expanded this method in order to help deliver the cell-impermeable octapeptide $\psi\epsilon$ RACK [59], a known ϵ protein kinase C isozyme agonist that can reduce ischemia-induced damage in cardiomyocytes. After conjugation with R₇, the resulting PDC readily entered cardiomyocytes and reduced ischemic damage when delivered into intact hearts either prior to or after the ischemic insult. Interestingly, this formula significantly outperforms $\psi\epsilon$ RACK conjugates of the transporters derived from HIV Tat and from Antennapedia [59]. Further research showed that after conjugation with guanidinium-rich peptides, some drugs could even breach less studied biological barriers, such as *Toxoplasma gondii* tachyzoites and *Chlamydomonas reinhardtii* [60]. One issue that arose, however, was that the increased release rate of cargos (CsA, $\psi\epsilon$ RACK) came at the expense of poor shelf stability. In an attempt to avoid this problem, bio-activatable moieties were investigated to allow triggered release through enzyme action (esterases, phosphatases, or proteases) or intracellular redox events. They attached the cargo to the transporter through a disulfide bond, which would cleave upon encountering intracellular glutathione. Luciferin was chosen as a drug surrogate to simulate drug uptake, release, and interaction with its intracellular target because it produces one photon of light per molecule, allowing the researchers to quantify the real time uptake and release in both cells and animals [57, 61].

When applying guanidinium-rich peptide-drug conjugates *in vivo*, it can be easily envisioned that adsorption by proteins in the blood stream would result in rapid clearance from the body. Recently, Zhang and coworkers reported their efforts to address this issue. Exploiting the ring opening reaction of 2,3-dimethylmaleic anhydride (DMA) by primary

amines, they reported an activatable cell-penetrating peptide-conjugated DOX (DOX-CR₈G₃PK₆-DMA) for tumor targeted drug delivery [62]. At physiological pH 7.4, the DMA shielding group blocks the cell-penetrating function of the polycationic CPP (R₈) through intramolecular electrostatic attractions. In sharp contrast, hydrolysis of DMA occurs at the tumor extracellular pH of 6.8, leading to recovery of the cell-penetrating function of R₈ and enhanced cellular uptake. *In vivo* results showed that this peptidic prodrug could significantly inhibit tumor growth. The same group also applied this strategy to an MMP-2-sensitive activatable cell-penetrating peptide (R₉GPLGLAGE₈) conjugate with protoporphyrin (PpIX) for tumor-targeting photodynamic therapy. Similar to the DMA example, electrostatic attractions between the cationic R₉ and anionic E₈ segments block the cell-penetrating function in normal tissues. Exposure to the overexpressed MMP-2 protease at the tumor site then results in proteolysis of the oligopeptide linker (GPLGLAG) between the CPP and the polyanionic peptide, releasing CPP-PpIX for photodynamic therapy (PDT). They found the released CPP-PpIX could effectively accumulate at the tumor site and significantly inhibit solid tumor growth [63].

An area that guanidinium-rich molecular transporters can greatly assist with is in overcoming the emergence of multi-drug resistance (MDR) in tumors. MDR is one of the main reasons for the failure of many cancer chemotherapeutic agents, particularly after recurrence following initial treatment [64]. One particular mechanism of resistance is the increased expression of membrane proteins that mediate unidirectional energy-dependent drug efflux [65], such as P-glycoprotein (Pgp), that can intercept and export the drug before it can reach its intracellular target. This type of resistance is observed for a wide range of drug types that operate through a variety of targets and pathways [66]. Moreover, resistance induced by one agent often results in cross-resistance to multiple agents [67], further limiting the treatment options for recurrent cancers. Guanidinium-rich PDCs can help circumvent these efflux proteins as their internalization does not involve passive diffusion across the membrane (the mechanism by which drugs enter cells), and can therefore avoid Pgp-mediated expulsion. While this provides a sound rationale for overcoming MDR, the overall effectiveness will still be determined by how efficiently the drug is released once it has been taken inside the cell. To demonstrate this strategy, Wender and coworkers conjugated a representative small molecule therapeutic agent (PTX) to an octaarginine peptide transporter through a bio-reducible linker, producing conjugates with significantly improved activity against malignant cells that were resistant to the therapeutic agent alone (Fig. 2) [50, 68]. They further demonstrated that this approach was effective in animal models of ovarian cancer, and explored the generality and mechanistic basis for this effect with coelenterazine, a Pgp substrate. Although coelenterazine itself does not enter cells because of Pgp efflux, its octaarginine conjugates does so readily. This general approach helps overcome the MDR elicited by small molecule cancer chemotherapeutics and could improve the prognosis for many cancer patients. It also fundamentally alters the approach to identifying novel therapeutic agents that are effective against resistant disease, given that their potentially susceptibility to efflux can be circumvented [68].

Recently, Cui and coworkers demonstrated an interesting finding that the attachment of an anticancer drug DOX to the *N*- or *C*-terminal of the Tat peptide can have a significant impact on their cellular uptake and cytotoxicity against both drug-sensitive and drug-resistant

cancer cells [69]. Specifically, they observed higher cellular uptake by both cell lines for *C*-terminal conjugates relative to the *N*-terminal analogue. In addition, the *C*-terminal conjugate partially overcame the multidrug resistance of cervical cancer cells, while the *N*-terminal conjugate showed no significant improvement in cytotoxicity when compared with free DOX. They also revealed that both *N*- and *C*-conjugates offered a mechanism to circumvent drug efflux associated with multidrug resistance. This illustrates how that the placement of the conjugated drug can influence the overall behavior of the PDC. Additional modifications to the molecular structure of PDCs can also lead to changes in behavior. Zhang et al., for example, reported that the covalent linkage of a hydrophobic unit near to the Tat sequence of a Tat–DOX conjugate can further improve the cellular uptake of the parental conjugate into cancer cells regardless of the membrane permeability of the unconjugated molecule [70]. In the case of Tat–DOX conjugates, conjugation of an extra hydrophobic piece, in this case palmitoylation, improved the conjugate's anticancer activity in both drug-sensitive and -resistant cervical cancer cell lines. They reasoned that the enhanced permeability was due to the lipid tail mimicking a hydrophobic domain on the Tat protein.

The utilization of guanidium-rich peptides offers an effective method that can translocate otherwise impermeable drugs across the cell membrane and allow circumvention of existing MDR mechanisms that would otherwise expel the cytotoxic agents. Clearly, such conjugates would be a valuable tool for the treatment of cancers, but their highly charged nature poses problems for general application and translation into the clinic. Strategies to address this are being developed, with Zhang's approach [62] being a good example to illustrate potential paths forward.

2.3 Theranostic peptide–drug conjugates

Although peptide–drug conjugates have proven successful in improving the efficacy of free drugs, the challenge of elucidating how and when the conjugated drugs are released after cell internalization remains [71–73]. In order to monitor this important process, fluorescent probes can be incorporated into peptide–drug conjugates [74]. However, conventional fluorescent molecules usually suffer from aggregation-induced quenching, a notorious effect that imposes limitations on their labelling efficiency or concentration to achieve the desired sensitivity. The recently emerged fluorogens with an aggregation-induced emission (AIE) feature offer a timely remedy to tackle this challenge. Incorporating AIE fluorogens (AIEgens) into “light-up” probes offers a number of advantages over conventional “always on” probes, including low background interference, a high signal-to-noise ratio (SNR) and superior photostability with activatable therapeutic effects [75–78], and in combination with peptide–drug conjugates can offer a powerful method of determining *in vitro* drug release characteristics.

Before constructing these theranostic conjugates, Zhang et al. tested the optical properties of tetraphenylethene (TPE) conjugated with different numbers of the hydrophilic amino acids (e.g. aspartic acid (D)) to reveal the importance of peptide design upon adjusting the fluorescence of AIEgen [79]. Their results showed that a minimum of five D residues was required to turn off the fluorescence of the TPE conjugates. On the basis of this critical

finding, a theranostic probe with a platinum (Pt)-based prodrug for tracking drug delivery/release in targeted cells and subsequent imaging of apoptosis induced by drug activation was designed (Fig. 3) [80]. Specifically, the two axial positions of a Pt(IV) prodrug were functionalized with the $\alpha_v\beta_3$ integrin targeting peptide, cRGD, and an apoptosis sensor composed of the AIEgen tetraphenylsilole (TPS) and a caspase-enzyme specific Asp-Glu-Val-Asp (DEVD) peptide. Upon selective uptake by integrin overexpressed cells, the probe was reduced by reducing agents such as ascorbic acid or GSH to yield separated TPS-DEVD and Pt(II) species. The released toxic Pt(II) species induced apoptosis in the cell, leading to an increased expression of caspases that activated the TPS-DEVD probe to yield a light-up response. In addition, the apoptosis-induced fluorescence intensity in the U87-MG human glioblastoma cancer cells showed a good correlation with the prodrug concentration and the cell viability. These results indicated that the theranostic drug delivery system with a built-in apoptosis sensor allows one to evaluate the effectiveness of the drug delivery and associated therapeutic response quickly, which is essential for guiding therapeutic decisions such as whether the treatment is successful.

While the AIE-based probe is useful for assessment of the therapeutic effect, it does not provide any information on the process of drug activation beyond the fact that the drug must have been released in order to induce the observed apoptosis. To capture the dynamic moment of Pt(IV) prodrug activation, a target specific probe was designed with tetraphenylethene pyridinium (PyTPE) conjugated directly with the Pt(IV) prodrug and cRGD [81]. In contrast to the previous example, five units of Ds were inserted between Pt(IV) and cRGD to provide the probe with a high water-solubility that precludes aggregation from occurring. Following selective internalization into integrin overexpressing cells, the reduction of the Pt(IV) prodrug released the highly emissive PyTPE residue, that provided a light-up response with high SNR and therefore a reliable method for quantitative analysis of the prodrug activation that yields the active Pt(II) drug. Significantly, greater cell death was consistently observed in MDA-MB-231 cells compared to MCF-7 cells due to the much higher level of integrin expression in the former. Very recently, Jin and Ji took advantage of TPE's light up property to design a multifunctional gemcitabine (GEM) prodrug, TPE-GEM-RGD, which can be used for targeted intracellular light-up imaging and selective release of GEM under an intracellular reductive environment [82]. They revealed the successful use of TPE-GEM-RGD for intracellular light-up imaging and glutathione (GSH)-responsive release of gemcitabine to suppress pancreatic cancer cell proliferation. Notably, in this system, GEM not only acts as drug, but also enhances the molecule's water solubility.

Currently, a single drug generally used in chemotherapy often fails to eradicate all cancer cells due to any existing or emergent drug resistance in the targeted cancer cells. Hence, non-cross resistant anticancer agents have been widely utilized for efficient cancer therapy. Based on these considerations and their previous studies of AIE, Tang and Liu's group exploited this strategy to construct a novel theranostic probe that contained both cisplatin and DOX and could be used for dual-drug tracking and monitoring of drug activation in target cells [83]. *In vitro* results demonstrated that the prodrug selectively killed cancer cells with overexpressed $\alpha_v\beta_3$ integrin receptors with no damage to normal cells. Moreover, the simultaneous DOX and cisplatin activation resulted in synergistic anticancer effects.

The second direct method to monitor drug activation is by attaching a quencher to eliminate any fluorescence emission from a fluorescent drug. Upon donor–acceptor (drug–quencher) separation, restoration of the drug’s fluorescence occurs to produce an observable signal. Recently, Lock et al. reported a novel type of enzyme-specific DOX drug beacon that could be used as drug-resistant theranostic molecular probes [84]. More specifically, covalent conjugation of the anticancer drug DOX to a black hole quencher (BHQ-2) and a cell-penetrating peptide (octa-arginine, R₈) through a lysine junction gave the drug beacon, R₈DB. In order to modulate the release of DOX while serving as a cathepsin B (CatB) sensor, the enzyme-cleavable tetrapeptide GlyPheLeuGly (–GFLG–) was inserted between DOX and BHQ-2. As expected, the fluorescence of DOX recovered upon addition of exogenous CatB, the implication being that the drug beacon design did not compromise the cleavage specificity of the GFLG substrate by CatB. It further demonstrated that specific activation of drug’s fluorescence by the CatB enzyme could serve as a clue for intracellular tracking and/or CatB sensing. Further cell experiments in a drug-resistant ovarian cancer cell line (NCI/ADR-Res cells) showed that the R₈DB drug beacon exhibited much stronger red fluorescence in cells than free DOX, indicating both effective cellular internalization and CatB activation. These results are consistent with the previous research on guanidinium-rich peptide–drug conjugates, in which conjugates functionalized by R₈ could overcome MDR[50]. Moreover, this theranostic beacon induced higher cytotoxicity to NCI/ADR-Res cells compared to free DOX at the same concentration. These exciting results indicate that incorporating a fluorescing drug into the beacon design opens up a new platform to explore the diagnostic functionality of therapeutic agents. This synergistic effect could potentially reduce the discrepancy between tumor diagnostic locations and actual drug-delivered sites, which is an important challenge in the current treatment paradigm.

Very recently, dual-Förster resonance energy transfer (FRET) was used by Zhang and coworkers to demonstrate an interesting prodrug (V-prodrug) [85], in which the fluorescence of both 5(6)-carboxyfluorescein (FAM) and DOX can be efficiently quenched by 4-dimethylaminoazobenzene-4-carboxylic acid (Dabcyl). Conjugation of DOX and FAM was accomplished *via* acid-labile (hydrazone) and caspase-3-cleavable (DEVD) linkers, respectively, and the incorporation of an RGD targeting moiety allowed selective binding to $\alpha_v\beta_3$ integrin overexpressed cancer cells. The acid-mediated DOX release of the V-prodrug correlated with an increase in the red fluorescence from DOX, allowing real-time evaluation of its release. Subsequent monitoring of the FAM fluorescence recovery (yellow-green) then provided information on the DOX-induced apoptosis, since the increased expression of caspase-3 triggered release of FAM *via* cleavage of the DEVD linker. Moreover, cell experiments showed no significant modification of the V-prodrug’s pharmacological profile when compared to free DOX, since the cleavage of the hydrazone bond in acidic environments releases unmodified DOX. Zhang’s platform illustrates the potential for theranostics, in particular the opportunities that the cascade imaging of real-time drug release and subsequent cell apoptosis offers for evaluating the cancer response to the drugs and the resultant therapeutic efficacy of the prodrug *in situ*. The therapeutic information acquired may reveal optimized therapeutic strategies to a certain disease, and allow the emergence of personalized medications in the near future. The *in vivo* applications of their V-prodrug, however, are currently limited by the low tissue penetration ability of the

fluorescence signal as well as the background noise of tissues in the visible region of the spectrum, although good *in vivo* applications using DOX and fluorophores with similar fluorescence properties can be found in the literature [86, 87]. The development of *in vivo* imaging instruments such as dorsal skin window chambers will expand these conjugates' *in vivo* applications for therapeutic decision-making and guidance of personalized cancer treatment.

3. Self-assembling peptide–drug conjugates

The design of peptide–drug conjugates was initially intended to take advantage of short hydrophilic peptides to improve the solubility and to provide additional functionality, i.e. the use of cell penetrating peptides to circumvent MDR as discussed in section 2.2 [68]. However, the combination of low molecular weight hydrophobic drugs and hydrophilic peptide segments can bestow overall amphiphilicity, introducing a tendency for self-aggregation in aqueous environments. This once undesired conjugation-induced assembly is now receiving rapidly growing interest in the drug delivery community, particularly with regard to gaining control over the assembly process. Herein, we will discuss the development of self-assembling peptide–drug conjugates that harness the potential of self-assembly for the creation of nano-vehicles that enable drug delivery *via* multiple modes of administration.

3.1 Supramolecular nanofiber hydrogels

Hydrogels are an important class of biomaterials for drug delivery due to their sustained drug release capabilities [1, 88–92]. Unlike the earliest phases of research in this field, which shed light on polymer-based hydrogels whose networks comprised covalently cross-linked natural or synthetic polymers [88, 93–95], most attention is now paid to supramolecular hydrogels [96, 97] formed by the aqueous assembly of low-molecular-weight gelators through noncovalent interactions [98–100]. Several unique merits are apparent, such as synthetic economy, biocompatibility, low toxicity, and inherent biodegradability. More importantly, changes in temperature, pH, or ionic strength can successfully trigger the formation of supramolecular hydrogels. These advantages make supramolecular hydrogels a promising alternative to polymeric hydrogels. Among the molecules that can act as the building blocks for supramolecular hydrogels, peptide-based hydrogelators are common candidates because of their biological relevance, their well-established synthetic chemistry (i.e., solid-phase synthesis), and the capacity to derive a diverse range of functionality from a relatively small array of amino acids.

Since subtle changes in stimuli can induce gel formation, it is advantageous to use inherent biological processes to create supramolecular hydrogels *in vivo* or *in situ* for certain biomedical applications [101, 102]. For instance, the Stupp group has developed a range of peptide amphiphile (PA) based hydrogels for biomedical applications [22, 25, 26], and has recently utilized this platform for drug delivery [103–106]. In one example, they conjugated the anti-inflammatory drug nabumetone to peptide amphiphiles through hydrazone bonds. They found this conjugate could form a gel that would release nabumetone in the low pH environment of the tumor [103]. More interestingly, they also expanded this strategy to

deliver dexamethasone. The molecule self-assembled in water into supramolecular nanofibers when mixed with a similar PA lacking the drug conjugate, and the addition of calcium salts to screen electrostatic repulsion between nanofibers promoted gel formation. These nanofiber gels demonstrated sustained release of soluble dexamethasone for over one month in physiologic media. The released dexamethasone maintained its anti-inflammatory activity *in vitro* and *in vivo* [104]. In another example, Matson et al. conjugated a solvatochromic dye 6-propionyl-2-dimethylaminonaphthalene (Prodan) at different positions of the peptide segment of a peptide amphiphile (E2) [105] (Fig. 4). The conjugated Prodan regardless of its conjugation site did not hamper the PA from forming supramolecular hydrogels. Their drug release studies revealed that all the Prodan-bearing nanofiber hydrogels have the near zero-order release kinetics but with different release half-lives depending on the conjugation position. This study demonstrates a conjugation strategy for controlled drug release from peptidic hydrogels. It is worth noting, however, that in these examples the drug does not play a critical role in the assembly process, merely acting as the cargo while the peptide amphiphile dictates the nanostructure formation.

By mimicking biomacromolecular self-assembly (e.g., formation of collagen fibrils [107]), the integration of enzymatic reactions with self-assembly of small molecules (enzyme-instructed self-assembly, or EISA) provides an effective means to form nanofiber networks and results in hydrogels under various conditions [91, 108–110]. Taking advantage of this strategy, Xu and coworkers have developed a series of hydrogels [111, 112]. In their first example, they aimed to prepare a taxol-bearing supramolecular hydrogel for chemotherapy using a phosphorylated naphthalene-Phe-Phe-Lys-Tyr precursor with conjugated taxol (Fig. 5) [113]. Upon treatment with alkaline phosphatase (ALP), cleavage of the phosphate group occurred and a translucent hydrogel spontaneously formed. They found that both the precursor taxol-NapFFKYp and the hydrogelator exhibited comparable anticancer abilities compared with free taxol, indicating that the drug's activity remains unaffected. Nevertheless, a notable aspect of this example is that the biostability is not robust enough for some long term used applications such as controlled drug release [92, 114], intracellular imaging [115], since peptides composed of L-amino acids are susceptible to degradation catalyzed by various endogenous proteases. In order to address this important issue, Xu and coworkers replaced L-amino acids with their D-enantiomeric counterparts and obtained D-peptide-based hydrogels [116]. Interestingly, the intrinsic resistance of D-peptide derivatives to proteolytic hydrolysis did not hinder the molecule from forming hydrogels when subjected to ALP. Further conjugation with taxol offered a biostable hydrogel that could sustainably release free taxol for up to 1 month, leading to significant suppression of tumor growth.

The two examples discussed above clearly illustrate that the preparation of supramolecular nanofibers and their hydrogels is feasible using the EISA method. Following this strategy, the groups of Yang [117] [118] and Liang [119] reported their respective contributions to this area. In 2014, Li and coworkers published an *in vivo* demonstration of EISA, finding that drug accumulation and retention in tumor sites through the *in situ* formation of supramolecular self-assemblies could be triggered by locally expressed phosphatases [120]. The self-assembled prodrug showed significant antitumor growth effects on a breast cancer

xenograft model based on 4T1 cells *in vivo*, but much lower toxicity towards the kidney, liver, spleen and other major organs than the free cisplatin drug in mice.

A great deal of evidence indicates that cancers are often associated with inflammatory conditions that require long-term management. Due to their excellent sustained drug release capabilities, hydrogels also make viable platforms for the delivery of anti-inflammatory agents. Thus Xu and coworkers utilized EISA to prepare anti-inflammatory agent-loaded supramolecular hydrogels [121] [122]. Similar to results found for anticancer drugs, using this method could maximally preserve the bioactivity of the anti-inflammatory agents, and even elevated the selectivity toward cyclooxygenase-2 (COX-2) over COX-1 by almost 20-fold at little expense of to the drug's activity. Similarly, Yang and coworkers used redox stimuli as a trigger to prepare supramolecular nanofiber hydrogels [118, 123–126]. In one example, disulfide bonds were integrated into the molecular design, serving as cleavable linkers to connect the molecular hydrogelators to the hydrophilic portions, affording the precursor Dexamethasone-FFFK(taxol/HCPT)E-SS-EE [127]. Here the two drugs have complementary purposes, taxol being an anti-cancer agent and dexamethasone an anti-inflammatory often used to reduce side effects associated with cancer chemotherapy. Upon addition of reductants such as glutathione (GSH) or dithiothreitol (DTT), the disulfide bond cleaves to induce hydrogel formation. They found that the stability of the drugs was greatly improved in hydrogels, with the active drug molecules being slowly released through ester bond hydrolysis.

A point to mention is that most of hydrogels discussed here are formed either with carriers (self-assembled molecules) or by drug derivatives, both of which require a long development time and high cost in order to evaluate the compatibility of carriers or compatibility and efficacy of drug derivatives. Actually, the only two FDA approved molecular hydrogel-based drug delivery systems are those formed by therapeutic agents themselves (Lanreotide and Degarelix) without any chemical modification [128, 129]. With this in mind, the Yang Lab rationally designed and synthesized a new molecule, namely taxol-succinic acid-oxidized glutathione (taxol-SA-GSSG) [118]. Upon incubation in PBS for 6 h, a hydrogel formed due to the hydrolysis process between taxol and SA. Animal testing illustrated that the administration of the hydrogel into solid tumors could hinder growth and prevent metastasis. The low blood concentration of taxol and high anti-cancer efficacy of their hydrogels suggested the great potential of the gels for chemotherapy. Although GSSG was used as the hydrophilic portion of the molecule in this example, they believed that other hydrophilic molecules such as PEG could also be used for the construction of precursors of taxol, which would further progress the possibilities for the clinical application of taxol hydrogels.

3.2 Supramolecular nanostructures

The effective treatment of both primary and secondary (metastatic) cancers requires the systemic delivery of drug(s) to ensure exposure of all cancer sites to the anticancer agent(s). Molecular hydrogels possess many advantages for local delivery and treatment of the primary tumor, but cannot efficiently affect any metastatic tumors that may have formed prior to treatment. A nanostructure-based strategy to this issue is the covalent modification of drugs in such a way as to form discrete nanostructures that can remain soluble without

further hierarchical assembly (aggregation of nanostructures). Early pioneering work by Couvreur and coworkers on lipid-conjugated nucleotides that could form nanoparticles [130], and later work by others that attached hydrophilic moieties to hydrophobic drugs [131–133], showed the promise that this approach possesses. This strategy utilizes the drugs as molecular building blocks for the construction of their own delivery vehicle, offering advantages over typical physical encapsulation and polymer-conjugation methods (e.g. higher drug loading, greater homogeneity). However, a number of issues were apparent, such as the need for organic solvents in the assembly process (kinetic trapping of nanostructures), and the lack of control that this gives over the assembly pathway and the resulting nanostructure, leading to a wide variation in properties such as particle size and shape. More recently, Cui and coworkers have reported a novel class of peptide–drug conjugates that can form nanostructures through a true self-assembly pathway. These PDCs, termed drug amphiphiles (DAs), are formed by covalently conjugating one or more anticancer drugs to a rationally designed/chosen peptide through a biodegradable linker [84, 114, 134–137]. The peptides in the resultant conjugates act as the functionally active and guiding unit, which modulate DAs to form a variety of supramolecular morphologies in aqueous solutions. Fig. 6 illustrates the three essential components for constructing a typical CPT-based DA: the anticancer drug CPT, a β -sheet-forming peptide sequence derived from the Tau protein, and a reducible disulfylbutyrate (buSS) linker. This strategy can be extended to other kinds of important drugs that possess functionalizable groups, such as hydroxyl, amine, thiol, or carboxylic acid. The ease of both designing and synthesizing these peptides also allows for facile incorporation of any biologically active sequences such as cell penetrating peptides, tissue-penetrating peptides, or tumor targeting peptides. The cleavable linker can be any chosen from a range of developed linkers, including, but not limited to: hydrolysable, enzymatically degradable, or reducible chemical bonds. More importantly, the small molecule nature of this platform makes it possible to obtain homogeneous conjugates with the aid of HPLC purification. Therefore, the resulting supramolecular nanostructures are essentially one-component nanomedicines with the drug loading precisely defined by the chemical structure of the conjugate.

Cui's initial report on this supramolecular strategy demonstrated the direct assembly of CPT DAs into discrete, stable, well-defined nanostructures with a high and quantitative drug loading [138]. Taking advantage of the two amine functionalities of the amino acid lysine, a branching point for the attachment of one, two or four CPT molecules could be created through the incorporation of cysteine, affording fixed drug loadings of 23%, 31% and 38%, respectively. With the variation of drug content, the morphologies of the nanostructures also changed from nanofibers to nanotubes. More importantly, nanosized assemblies formed by these DAs possess excellent stability, whose dissociation occurred only when the concentration dropped into the nano-molar range upon dilution. This could be a result of an increasing hydrophobicity in combination with possible π – π associative interactions among the CPT units. However, destruction of the nanostructure could be achieved through the addition of the cancer-relevant reducing agent GSH, which leads to release of free CPT. Cell studies confirmed that these DAs could inhibit proliferation of a number of cancer cell line phenotypes. Expansion of this concept led to the construction of paclitaxel (PTX) loaded DAs with a drug content up to 41% with activity comparable to free PTX [10]. Notably, their

results showed that conjugation of the bulky PTX to a short peptide does not prevent the peptide from assembling into one-dimensional nanostructures; though a later study noted that a similar conjugate possessing two PTX molecules could only form small, ill-defined structures due to steric hindrance preventing effective intermolecular hydrogen [139].

Drug release is an important factor in any drug delivery, and DAs are no exception to this. While the DAs described above exhibited good overall cytotoxicity, reduced activity in comparison with free CPT was apparent, with a likely explanation being retarded release of the active drug from the nanostructure. To gain more insight into this process, Cheetham et al. performed a more mechanistic study into the GSH-induced release of CPT [139]. They found that the compromised cytotoxicity of the DA with a disulfyl butyrate linker resulted from the nanostructure-promoted formation of CPT-disulfide dimer intermediates due to the effectively high local concentration of the β -sheet aggregates. The hydrophobic nature of these disulfide dimers protected them from aqueous hydrolysis through association with the remaining nanostructures or by self-aggregation, thereby preventing the rapid generation of the free drug. In contrast, a carbonate-based analog eliminates the possibility of dimerization through an intramolecular degradation pathway, thus restoring most of the drug's activity. Another factor that can influence drug release is that of steric hindrance, either with regard to the nanostructure acting as a barrier or within the monomeric units themselves. For example, Chen et al. found that the degree of drug conjugation per peptide could also regulate the drug release in addition to its apparent effect on drug loading of the resulting conjugates [134]. One, two, or four DOX molecules were covalently linked to the cell penetrating peptide Tat through a catB degradable tetrapeptide linker (–GFLG–), offering three different DAs (NTD, d-NTD and q-NTD). They found that the number of DOX within the conjugate largely impacts the release of DOX, with q-NTD showing the slowest release rate while NTD showing the fastest release rate. Interestingly, cellular uptake experiments revealed that q-NTD accumulated most effectively within cancer cells while NTD showed the lowest intracellular accumulation concentration.

As discussed earlier, the combination of two or more drugs in a rational manner could potentially generate synergistic effects, further improving drug efficacy with minimized side effects. More importantly, this cocktail treatment offers a way to overcome the MDR that cancer cells develop during the course of traditional treatment. Exploring the possibility of this approach with respect to the DA platform, Cheetham et al. reported the synthesis and assembly of a mikto-arm star dual DA containing both a bulky PTX and a planar camptothecin CPT [140]. With the help of HPLC, the conjugate with two different anti-cancer drugs appended to the *N*-terminal lysine of a β -sheet forming peptide (Sup35) could be separated from the other two kinds of conjugates, which led the resultant DA spontaneously associating into supramolecular filaments with a fixed 41% total drug loading under physiologically-relevant conditions (Fig. 7). The hetero dual DA was found to effectively release the two anti-cancer agents, exhibiting superior cytotoxicity against PTX-resistant cervical cancer cells. They believed that simultaneously delivering two drugs with completely different action mechanisms could help in reducing the acquisition of MDR and even the chances of tumor recurrence.

Co-assembly of two or more different amphiphilic molecules offers a robust way to regulate the size, shape and surface chemistry of the resulting nanostructures. Mixing of oppositely charged amphiphilic molecules can produce morphologies different from those formed by individual molecules. Lin et al. reported that multi-walled nanotubes with a fixed CPT content up to 36% were obtained through catanionic mixing of two different DAs [140]. They found that the molecular mixing ratio, the solvent composition, the overall drug concentrations, as well as the molecular design of the studied amphiphiles were all important experimental parameters contributing to the tubular morphology (Fig. 8). Although systemic delivery of CPT nanotubes through tail-vein injection did not show any improvement for tumor targeting, intra-tumoral injection of fluorescently labeled nanotubes revealed significantly enhanced retention of CPT nanotubes at the tumor site, offering the possibility for sustainable release of CPT over a long period of time that would be beneficial for local tumor treatment.

It is important to note that while the discussion Cui's drug amphiphiles here is in the context of developing a platform for the systemic delivery of drugs, the structure of the conjugates is such that they can be adapted for local delivery applications through the formation of hydrogels. Similar to peptide amphiphiles that can self-assemble into nanofilamentous structures [26], the drug amphiphiles could potentially be induced to form supramolecular hydrogels through screening of the peptide's charged groups, either through pH manipulation or the addition of screening ions. Such utility further expands the possible applications of the drug amphiphile platform, making them a versatile addition to the nanomedicine toolkit.

In recent years, increasing attention has been also paid to enzyme-sensitive drug delivery systems due to the typically high specificity towards substrates associated with this approach [141–144]. Early research focused on using enzymes in a similar fashion to other methods of stimulus applied in drug delivery systems [9], to induce morphology change or disassembly of nanocarriers, resulting in the release of a payload. Inspired by naturally occurring enzymatic reactions and observation that tumors often have unregulated enzyme expression, the application of enzymes to perform the reverse has garnered more interest, particularly since Xu's first report on enzyme-triggered intra-cellular assembly [115] (Fig. 9). It should be noted that although many papers have proven that nanofibers formed by enzyme-triggered assembly outside or inside of cells can regulate the fate of cells, these molecules usually do not contain drug segments [101, 145–148] and thus will not be included as examples here.

In the first section of this review, we illustrated that the attachment of drugs to molecular transporters, such as cell-penetrating peptides, can help facilitate cellular uptake through multiple mechanisms other than passive diffusion, which can be helpful in overcoming MDR. However, cells can adapt to resist the new agents after one cycle of treatment. Regarding their ability to load large amount of drugs, nanocarriers have shown unique advantages for overcoming MDR by local release of a high concentration of drugs inside cells after they translocate the cell membrane. However, the difficulties of their cell translocation and fabrication, poor reproducibility or physical stability, and long-term toxicity as well as the low bioavailability of the nanodrugs eclipse their advantages in anti-

MDR approaches [149]. Based on these considerations, Liang's group proposed a new strategy [150] for overcoming the MDR of drug resistant cells *in vitro* and *in vivo* by employing a biocompatible condensation reaction [151, 152]. Specifically, they designed and synthesized a taxol derivative Ac-Arg-Val-Arg-Arg-Cys(S^tBu)-Lys(taxol)-2-cyanobenzothiazole (CBT-Taxol), which could be subjected to furin-controlled condensation and self-assembly into taxol nanoparticles (Taxol-NPs). *In vitro* and *in vivo* studies indicated that, compared with taxol, CBT-Taxol showed a 4.5-fold or 1.5-fold increase in anti-MDR effects, respectively, on taxol-resistant HCT 116 cancer cells or tumors without being toxic to healthy cells or the mice. Their results demonstrate that structuring protease-susceptible agents and assembling them intracellularly into nanodrugs could become a promising strategy for overcoming MDR.

Although *in vivo* self-assembly enhanced biological functions have been convincingly validated, how the morphology of self-assembled nanostructures affecting their biological performances has rarely been reported [153]. The morphology of nanomaterials can markedly influence their pharmacokinetic (PK) and pharmacodynamic (PD) properties *in vivo*, for instance the fibrous nanostructures exhibit ten times longer blood circulation than their spherical counterparts [153]. Wang and coworkers developed a method to prove that *in situ* formation of morphology-defined self-assemblies in tumors might effectively improve their bioavailability and treatment outcomes [154]. Guided by this goal, they designed a responsive small molecule precursor P18-PLGVRGRGD that was composed of purpurin 18 (P18) as the functional molecule, Pro-Leu-Gly-Val-Arg-Gly (PLGVRG) as the enzyme-responsive peptide linker, and Arg-Gly-Asp (RGD) as the targeting ligand. Their results showed that the as-prepared molecule could readily diffuse, extravasate, and target cancer cells with overexpressed $\alpha_v\beta_3$ integrins (U87 cells). After the gelatinase-induced cleavage of the PLGVRG linker, nanofibers formed spontaneously and exhibited the assembly induced retention (AIR) effect, which resulted in an improved photoacoustic (PA) imaging signal and enhanced therapeutic efficacy. They believed that this AIR effect based on the assembly of supramolecular structures in living systems might pave the way to developing novel functional nanomaterials for cancer diagnostics and therapeutics.

4. Conclusions and future perspective

In this review, we have surveyed two main groups of peptide–drug conjugates that differ in their states—molecular or self-assembled. The examples selected in this review illustrate that peptide–drug conjugates have tremendous potential in drug delivery. With the aid of peptides, the targeted delivery of drugs to particular sites is possible (active targeting or passive targeting) and can even allow a more effective internalization into cells. Once the conjugates reach the lesion site, liberation of the drug through stimuli-specific cleavage of a linker in a controlled manner occurs. Self-assembling peptide–conjugates offer further advantages over their water-soluble molecular counterparts as their nanostructured nature allows for alternative modes of administration (local, topical and systemic) and provides the means to tune the physicochemical properties through manipulation of the surface chemistry.

Self-assembling PDCs represent a logical connection from small molecule prodrugs to nanomedicines, and are reminiscent of antibody–drug conjugates (ADCs). To a great extent,

the design of self-assembling PDCs provide a more flexible platform in the optimization of pharmacokinetics than that of ADCs. First, PDCs are easy to synthesize and can be scaled up and purified to homogeneity. This would allow for cost-effective mass production. Second, molecular assembly provides a toolbox to fine-tune not only the physicochemical properties of the resulting nanomedicines but also the release rate of the therapeutic agents from the assembled structures. Third, all the PDCs components including both the peptide moiety and the therapeutic agent can be potentially metabolized except for the intended use of non-natural amino acids to circumvent premature degradation or to take advantage of their unique biological roles. Given the clinical successes of ADCs, we have many reasons to envision a promising future of PDCs for clinical translation.

There might be two potential hurdles for successful translation of PDCs into clinic. First, the biocompatibility of peptide-based assemblies should be better elucidated. It is clear that peptides made of natural amino acids are completely biodegradable in its water soluble form, and unless specifically designed to achieve immunological or other biological responses, peptides are generically biocompatible and do not generate adverse responses. It is, however, less understood if the peptidic assemblies could preserve the biocompatibility and biodegradability of the unassembled molecules, particularly for those containing β -sheets, given their similarity to the amyloid fibrils. Although there are some studies that suggest the biocompatibility of peptidic materials, more efforts are necessary to further elucidate their degradability and generic safety. In addition, all the transient metabolites and their potential toxicities should be identified and manifested. Second, the efficacy of self-assembling PDCs need to be demonstrated and improved. Improved efficacy over the current standards of treatment is a prerequisite for FDA approval. Like many nanomedicine platforms that are currently under development, self-assembling PDCs need to demonstrate improved efficacy or increased response rates in treating devastating diseases such as cancer. In this regard, there are many challenges such as specific targeting to the disease sites, overcoming the multidrug resistance mechanisms, controlled release of the therapeutic agents. These issues can be addressed only with the development of transformative, adaptive, and multiple responsive PDC systems. Most of the reported examples for drug delivery are only responsive to a single stimulus, and have limited ability to overcome the multiple biological barriers en route to the tumor sites. Thus, it is reasonable to anticipate that in the near future we will witness the development of self-assembled systems responsive to multiple orthogonal stimuli that harness the abundant and unique stimulus features in tumor microenvironment. Further optimization of these conjugates, as well as the translation from lab research projects to clinical trials, will continue in the future, as the treatment of human patients and their well-being remains the ultimate goal of nanomedicine.

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Fig. 1.

Scheme of peptide–drug conjugates bearing one drug (A) or two different types of drugs (B). All PDCs contain three essential components: the therapeutic compound, the rationally designed/chosen peptide, and the linker that bridges the two. An emerging class of PDCs are designed to spontaneously associate in aqueous solutions into a variety of well-defined nanostructures, such as nanofibers (C).

Fig. 2. Wender's cell penetrating peptide conjugation strategy to circumvent multidrug resistance. Lipophilic drugs that are substrates for Pgp export become highly water-soluble upon conjugation to molecular transporters, and are able to rapidly enter cells without recognition by the Pgp pumps that would otherwise expel them [50].

Fig. 3. Schematic illustration of the targeted theranostic platinum(IV) prodrug with a built-in aggregation-induced emission (AIE) light-up apoptosis sensor for non-invasive in situ early evaluation of its therapeutic response [80].

Fig. 4. Conceptual illustration of controlled release of therapeutics using the peptide amphiphile (PA) platform developed by the Stupp Lab. (A) Chemical structure of the studied PA. (B) The different PA regions are highlighted in a space-filling model. (C) Schematic illustration of a self-assembled PA nanofiber. (D) Proposed structural models of PA packing arrangements. The alkyl tail is shown in gray, with β -sheet region in blue, charged residues in red, and lysine-hydrazone-Prodan group in green.

Fig. 5. Example of a supramolecular hydrogel-forming peptide–drug conjugate bearing an enzyme-cleavable solubilizing group, developed by the Xu Lab. In the absence of enzyme (alkaline phosphatase, ALP), the progelator solution exhibits no defined nanostructure (A and D). Upon addition of ALP, cleavage of the phosphate group triggers self-assembly (B and E), resulting in hydrogel formation (C and F).

Fig. 6.

Schematic illustration of a representative camptothecin drug amphiphile (DA). (A) The self-assembled nanostructures contain the same drug content as that of the individual DA. (B) Illustration of the three key components: the hydrophobic drug CPT, Tau β -sheet-forming peptide, and the buSS biodegradable linker (or using a non-degradable maleimide linker). RP-HPLC chromatogram (C) and MALDI-ToF mass spectrum (D) of the mCPT-buSS-Tau DA reveals the high purity and monodispersity of the conjugate.

Fig. 7.
(A) Synthesis of CPT-PTX-Sup35, and homo-dual DAs, dCPT-Sup35 and dPTX-Sup35, from the reaction of dCys-Sup35 with a 1:1 mixture of activated disulfide drugs, CPT-buSS-Pyr and PTX-buSS-Pyr. Representative TEM images of dCPT-Sup35 (B), CPT-PTX-Sup35 (C) and dPTX-Sup35 (D) in water.

Fig. 8.

(A) Chemical structures and schematic representations of qCPT-Sup35-K2 and qCPT-Sup35-E2. (B) Schematic representations and TEM micrograph of the supramolecular structures formed by qCPT-Sup35-K2, qCPT-Sup35-E2, and the CAM of the two DAs. The CAM of qCPT-Sup35 (mixing ratio 1:3) results in the almost exclusive formation of tubular structures in 1:1 MeCN/H₂O. Total concentration for the sample = 400 μ M.

Fig. 9.

(A) Principle of imaging enzyme-triggered supramolecular self-assembly inside cells. ER, endoplasmic reticulum; G, Golgi apparatus; L, lysosome; M, mitochondria; N, nucleus. (B) The scheme for the generation of the fluorescent hydrogelator via an enzyme-catalysed dephosphorylation from precursor. (C) Enzyme-triggered self-assembly inside live cells. Fluorescent confocal microscope images show the time course of fluorescence emission inside the HeLa cells incubated with 500 or 50 μM of precursor in PBS buffer, which shows

the different distribution of fluorophores inside living cells. Scale bar, 50 μm for time course panels and 10 μm for the enlarged panels.

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