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PII: S0168-3659(20)30585-X

DOI: https://doi.org/10.1016/j.jconrel.2020.10.014

Reference: COREL 10579

To appear in: *Journal of Controlled Release*

Received date: 18 August 2020

Revised date: 6 October 2020

Accepted date: 7 October 2020

Please cite this article as: S. Karaosmanoglu, M. Zhou, B. Shi, et al., Carrier-free nanodrugs for safe and effective cancer treatment, *Journal of Controlled Release* (2020), https://doi.org/10.1016/j.jconrel.2020.10.014

Carrier-free nanodrugs for safe and effective cancer treatment

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Abstract

Clinical applications of many anti-cancer drugs are restricted due to their hydrophobic nature, requiring use of harmful organic solvents for administration, and poor selectivity and pharmacokinetics resulting in off-target toxicity and inefficient therapies. A wide variety of carrier-based nanoparticles have been developed to tackle these issues, but such strategies often fail to encapsulate drug efficiently and require significant amounts of inorganic and/or organic nanocarriers which may cause toxicity problems in the long term. Preparation of nano-formulations for the delivery of water insoluble drugs without using carriers is thus

desired, requiring elegantly designed strategies for products with high quality, stability and performance. These strategies include simple self-assembly or involving chemical modifications via coupling drugs together or conjugating them with various functional molecules such as lipids, carbohydrates and photosensitizers. During nanodrugs synthesis, insertion of redox-responsive linkers and tumor targeting ligands endows them with additional characteristics like on-target delivery, and conjugation with immunotherapeutic reagents enhances immune response alongside therapeutic efficacy. This review aims to summarize the methods of making carrier-free nanodrugs from hydrophobic drug molecules, evaluating their performance, and discussing the advantages, challenges, and future development of these strategies.

Keywords

Hydrophobic anti-cancer drugs; carrier-free nanodrugs; carrier-free nanoparticles; nanomedicine

Table of Contents

1 Introduction

Cancer costed an estimate of 9.6 million lives in 2018 alone and it is a major health concern worldwide [1]. In the last decades, there have been tremendous research efforts to develop efficient and powerful cancer therapies. Administration of anti-cancer drugs, i.e. chemotherapy, remains the most common strategy. Fig. 1 shows the chemical structures of some example drugs for anti-cancer therapy. These drugs are used as intravenous therapies for various forms of cancers, requiring them to be ideally dissolvable in water for administration. In general, water solubility of an organic molecule depends on the balance between its polar (hydrophilic) and non-polar (hydrophobic) functional groups. In all of the case in Fig. 1, this balance tilts towards non-polar groups due to the molecules' hydrocarbon heavy nature, which is essential to exert their mechanism of anticancer action but makes them poorly soluble in water [2].

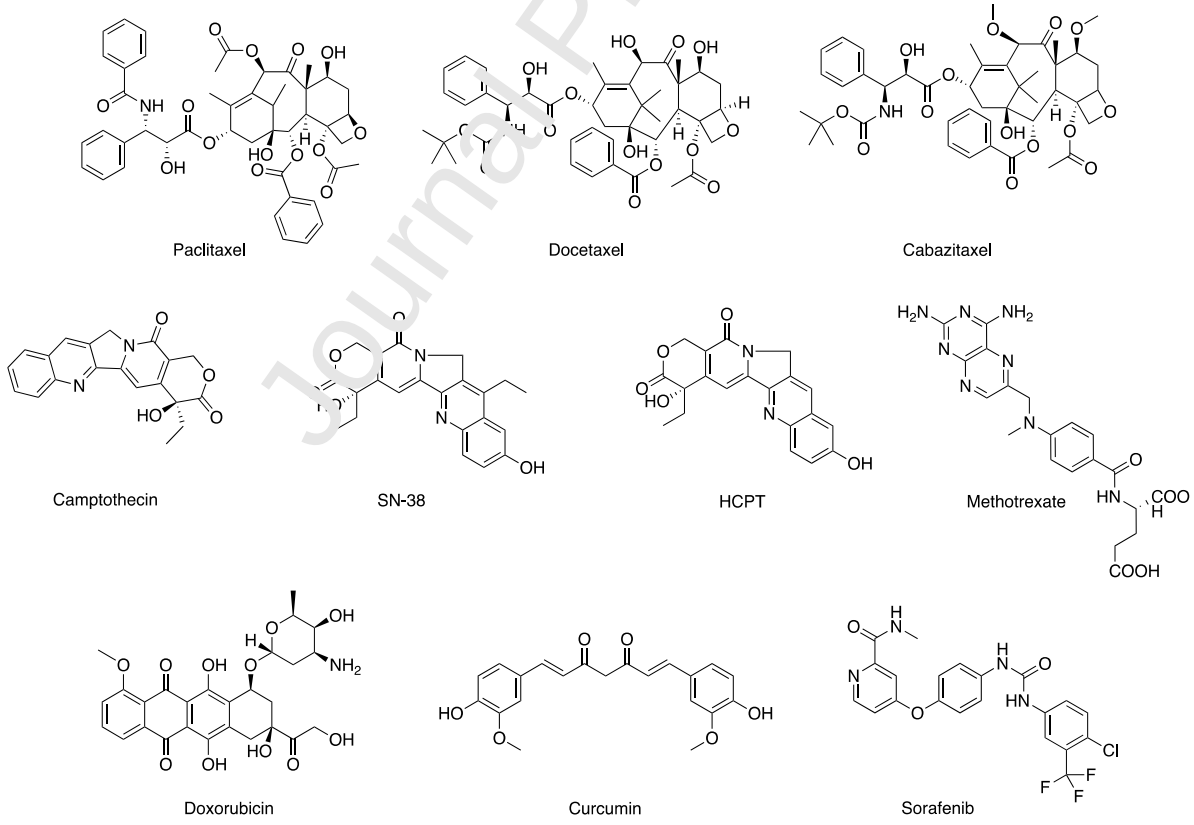

Fig. 1. Chemical structures of example hydrophobic anti-cancer drugs.

Taxanes are amongst the most promising hydrophobic anti-cancer drugs. They are anti-microtubule drugs which interfere with cellular mitosis through microtubule stabilization, resulting in apoptosis [3]. Among taxanes, paclitaxel (PTX), was first isolated from the bark of a Pacific yew tree, Taxus Brevifolia, which contains Endophytic fungi known to produce PTX [4]. It was approved by the Food and Drug Administration (FDA) in 1994 and is now widely used to treat breast, prostate, lung, head and neck carcinomas, malignant melanoma and Kaposi's sarcoma [4, 5]. PTX is a hydrophobic molecule and precipitates into needle-shaped crystals in water due to its low water solubility (0.3 μg/ml). This poses a challenge for its clinical application and necessitates the use of organic solvents such as ethanol, methanol, and dimethylsulfoxide (DMSO) [6]. Taxol, the first commercial formulation of PTX, contains 50% anhydrous ethanol and 50% Cremophor EL®. The latter was shown to cause side effects in the clinic including hypersensitivity, nephrotoxicity, and neurotoxicity, and it was recently reported that Cremophor EL® can even reduce the anti-cancer efficiency of PTX [7]. Docetaxel (DTX), isolated from Taxus baccata, has two-fold greater antimitotic efficacy than PTX and is used for the treatment of lung, breast and ovarian cancers. The water solubility of DTX is slightly higher compared to PTX (6-7 μg/ml) [8]. Clinical use of PTX and DTX, however, is hindered due to their affinity towards P-glycoprotein (P-gp), a multidrug resistance (MDR) protein [9]. Cabazitaxel (CTX), a semi-synthetic derivative, is another member of this family, approved by the FDA in 2010. CTX poorly binds to P-gp, preventing drug resistance, and demonstrated superior efficacy compared to PTX and DTX [10], but still suffers from the common hydrophobicity of taxanes (8 μg/ml) [11].

Camptothecin (CPT), extracted from Chinese ornamental tree, works by inhibiting topoisomerase-1 pathway which then prevents deoxyribonucleic acid (DNA) re-ligation, resulting in cell death [12]. CPT is mainly used for the treatments of lung, breast and brain cancers and has a planar pentacyclic ring structure which causes cylindric shaped aggregations in water. Biological environment causes the degradation of CPT in only thirty

minutes by hydrolysis of its lactone rings and CPT has very poor water solubility (2-3 μg/mL) [13, 14]. Therefore, derivatives of CPT have been developed in order to increase its solubility and stability in physiological conditions. 7-Ethyl-10-hydroxy-camptothecin (SN-38), a semi-synthetic derivative of CPT, is effective on a wide range of solid tumors by inhibiting topoisomerase-1 [15]. SN-38 has two hydroxyl groups which can bond with different molecules and produce more efficient formulations. 10-Hydroxycamptothecin (HCPT), another analogue of CPT is known to have an anti-tumor effect against gastric carcinoma, hepatoma, leukemia, and head and neck tumors [16]. Compared to CPT, it is demonstrated that HCPT is more potent and less toxic in animal studies and in human clinical evaluation. However, the clinical applications of both SN-38 and HCPT are limited, again, due to their low water solubility, 11-38 μg/ml and 7.2 μg/ml, respectively [17-19]. Doxorubicin (DOX), also a topoisomerase inhibitor which exerts its activity on topoisomerase II, causes DNA damage and prevents mitosis in tumor cells [20, 21]. It is widely used to treat breast cancer, urothelial cancer, hematopoietic malignancies and solid tumors.

Amongst other anti-cancer agents suffering from poor water solubility, pemetrexed (PEM) is an anti-metabolite agent exhibiting anti-cancer activity by inhibiting multiple folate-related enzymes, such as thymidylate synthase and dihydrofolate reductase. It was approved by the FDA in 2004 for the treatment of malignant pleural mesothelioma and in 2008 for non-small lung cancer [22]. Bortezomib (BTZ) contains a boronic acid moiety and is approved by the FDA to treat multiple myeloma by targeting proteasomes [23]. Sorafenib (SRF) is a novel small molecule multi-kinase inhibitor and used in the treatment of renal cell, liver and thyroid cancers. SRF has a water-repelling heavy ring structure [24]. Another chemotherapeutic drug, methotrexate (MTX), is a folic acid (FA) antagonist and exerts its effect by interfering with DNA, ribonucleic acid (RNA) and protein formation [25]. MTX is used in cervical, colorectal, lung and breast cancers as well as leukemia and osteosarcoma [26, 27]. BTZ, SRF and MTX are similarly having low water solubility with 53.2 μg/ml, 1.7 μg/ml, and 0.1 mg/ml, respectively [28-30].

Alongside these FDA approved anti-cancer agents, the naturally occurring compound curcumin (CUR) is not clinically approved, but is nevertheless widely investigated in cancer treatment due to its antioxidant properties. CUR, extracted from Curcuma longa, is an antioxidant, anti-inflammatory agent. It has been demonstrated that CUR can exert an anti-cancer effect by inhibiting transcriptional factor nuclear factor kappa B which has a key role in inflammation [31]. In particular cases, it was found that CUR has synergistic effects when applied in combination therapy with other anti-cancer agents overcoming MDR and inhibiting inflammation [32, 33]. However, it is extremely hydrophobic with 0.6 µg/ml water solubility and unstable, resulting in inadequate efficiency [34].

As seen from the above examples, poor water solubility is one of the main challenges for clinical applications of many anti-cancer agents. Beyond this, most anti-cancer drugs act as antiproliferative agents and have poor selectivity resulting in severe side effects [35]. These anti-cancer drugs can be taken up by frequently replicating cells, regardless of the cell type, which require high nutrient supply. Therefore, anti-cancer agents cause serious side effects on healthy tissues dividing rapidly such as mucous membranes of mouth, throat, stomach and intestines, resulting in gastrointestinal side effects. Hair follicles are also fast dividing cells, and correspondingly patients suffer from hair loss including facial and body hair [36]. They also harm bone marrow cells which are responsible for the production of white and red blood cells. This further reduces the ability of body to fight infections [37]. It is also demonstrated that exhibiting frequently proliferating cell profile in tumor masses can be misleading as this is due to the high number of cells in dividing state. In fact, many of the tumor cells, particularly solid tumor cells, multiply rather slowly [38, 39].

Anti-cancer drugs also often suffer from poor pharmacokinetic properties. First, anti-cancer agents face rapid blood clearance by the reticuloendothelial system (RES) which removes

foreign substances from the bloodstream. Once anti-cancer drugs are administered to the body, they exhibit extremely rapid renal clearance and therefore can barely reach cancer cells and have a rather short half-time [39, 40]. For example, the half-life of fluorouracil (5-FU) and PTX is only 16 mins and 5.8 h, respectively [3, 41, 42]. In addition to these, anti-cancer drugs are known to create kidney and liver damage. The former arises from excretion of drugs from the body and the latter results from the detoxification mechanism of liver [43]. All of these side effects limit the maximum tolerated dose and potentially result in early termination of treatment due to their life-threatening toxicities [43, 44]. Some of these side effects can cause long term damage, such as neutropenia, known to remain for years or even permanent in many cases [38]. Anti-cancer drugs also encounter the issue of MDR being developed by cancer cells, usually after an initial therapy via genetic or non-genetic mechanisms. Second treatment may still have some effect on a minority of patients, but there are few cases who respond to a third administration [38].

To solve these serious problems, nanotechnology has been extensively studied for delivery of anti-cancer drugs in order to maximize their therapeutic effects and minimize the toxicity and side effects of cancer treatments [45, 46]. Many novel nanotechnology-based approaches have been studied for biomedical applications such as liposomes, inorganic nanoparticles (NPs), or polymeric micelles [47]. These carrier-based methods bring systematic advantages [48, 49]. Firstly, the water solubility is significantly increased. Hydrophobic drugs loaded in amorphous nano-carriers demonstrated higher solubility due to the lack of a lattice energy barrier to dissolution. In addition to that, the surface area of these anti-cancer drugs is increased by the use of nanoscale formulations [50]. In this way, the characteristics hindering the application of hydrophobic drugs is altered by encapsulating them into nano-carriers [51]. Secondly, the systemic circulation of anti-cancer drugs can be enhanced. Doxil®, (DOX loaded polyethylene glycol-surface modified, PEGylated, liposomes) was one of the first nanomedicines approved by FDA and it significantly increased the elimination phase of DOX (45.9 h with Doxil®) in the bloodstream compared to

free DOX (10.4 h) [52]. Thirdly, these carrier-based drug delivery systems demonstrated an increase in tumor accumulation of NPs. An example of this, Abraxane®, first nano formulated PTX, bonded with albumin (130 nm), was approved by FDA in 2005, demonstrated 5-fold increased transport into cancer via endothelial cells resulting in higher tumor accumulation [51]. It was demonstrated that NPs between 10 nm and 200 nm are the most suitable candidates for accumulation at tumor sites [53].

Despite significant progress, carrier-based nanotechnology for anti-cancer drug delivery still often lacks the required efficiency in terms of pharmacokinetics and biodistribution [54], and the carrier component often comprises the bulk of the mass of these nanosystems. The loading efficiency of anti-cancer drugs remains low, and their long term toxicities remain largely unknown [55]. High amount of carriers are used to reach a required drug doses, resulting in low drug loading (usually ~10% or lower) and high carrier uptake [56-59]. For example, liposomes are cleared from the bloodstream in a relatively short time and accumulate in the liver [60]. On the other hand, some inorganic NPs have an inner core composed of heavy metals which limits clinical application due to their toxicity [51]. In a 2019 study, it was demonstrated that carbon nanotubes, an inorganic carrier, can promote metastasis in breast cancer [61].

To combat these new problems, one promising solution is to develop carrier-free nanodrugs. These nanodrugs are made from pure drug molecules without involving any organic or inorganic carriers. It is worth noting that organic molecules may still be used to work as surfactants to stabilize nanodrugs or to tune the physical/chemical properties of drug molecules so they can be self-assembled to nanodrugs, but the amount is generally much smaller in comparison with the cases of using as carriers. To synthesize carrier-free nanodrugs, coupling two drug molecules (either the same or different anti-cancer molecules) together or conjugating functional organic molecules, such as vitamins, fatty acids, photosensitizers, and proteins with drug molecules using covalent bonds and/or physical

interactions can be employed to enhance the biostability, targeting ability, and therapeutic efficacy of anticancer drugs. All of the components in this design play active roles and importantly the therapeutic drug loading is often over 80% [62]. Such strategies can be further improved by employing tumor microenvironment responsive linkages. This review highlights the recent progress in the vast and ever-growing area of carrier-free anti-cancer nanodrugs. We will highlight the various approaches to synthesize NPs directly from pure drug molecules, discuss their advantages over conventional nanomedicines with nanocarriers, as well as compare the effectiveness and limitations of different approaches. After the comprehensive review, we will provide our insights into the future development of carrier-free nanodrugs for potential clinical applications.

2 Synthetic strategies of carrier-free nanodrugs

2.1 Direct self-assembly of drug molecules

Preparation of nanodrugs via molecular self-assembly without any processing is one of the most convenient strategies for delivery of hydrophobic therapeutics [63-66]. Self-assembly occurs at molecular level with non-covalent interactions, such as hydrophobic interactions, π-π stackings and CH-π interactions, play essential roles. In this section, we will review the work of directly self-assembling chemotherapy drug molecules with different molecules to make nanodrugs to solve the problems such as drugs' low solubility in aqueous solution, low anti-cancer efficiency, and serious side-effects.

2.1.1 Self-assembly of pure chemotherapy drug molecules

To achieve this, one-step precipitation method is commonly employed [67]. As shown in Fig. 2a, generally, one or multiple types of clinical drug molecules are dissolved in a small

amount of organic solvent and then added dropwisely into an aqueous solution under vigorous stirring [68]. Due to the interactions between the hydrophobic small molecules, uniform nanoparticles are rapidly obtained [69]. Minimal amounts of surfactants can then be added to stabilize the obtained nanoparticles. The self-assembled nanodrugs tend to exhibit high drug loading capacities, improved water solubility, prolonged blood circulation half-life times, higher drug delivery efficiencies, and reduced side effects [70]. For example, we reported the synthesis of pure DOX NPs solely from DOX free drug molecules via the self-assembly method. PLGA-PEG was then added to functionalize the surface of the nanoparticles as a surfactant [71]. After PEGylation of NPs, DOX loading was determined to be 90.47% as shown in Fig. 2b. DOX has similar chemical properties to surfactants as it has abundant hydroxyl groups and hydrophobic anthracycline rings. Therefore, pure drug NPs can be obtained from DOX which can serve as both the pharmaceutically active ingredient and a surfactant [72]. The prepared NPs resulted in good biocompatibility and stability, and long blood circulation times (Fig. 2c). Compared to free DOX, the DOX NPs possess fast release in an acidic environment and high accumulation in tumors. As a result, our results showed that the tumor growth in the mice treated with free DOX and DOX NPs were 5.40 ± 0.30 -fold and 2.09 ± 0.25-fold, respectively. It was also explored that addition of HCPT (DOX-HCPT NPs) circumvented the MDR in DOX resistant breast cancer cell lines and showed higher cytotoxicity and tumor inhibition in vivo then DOX NPs only.

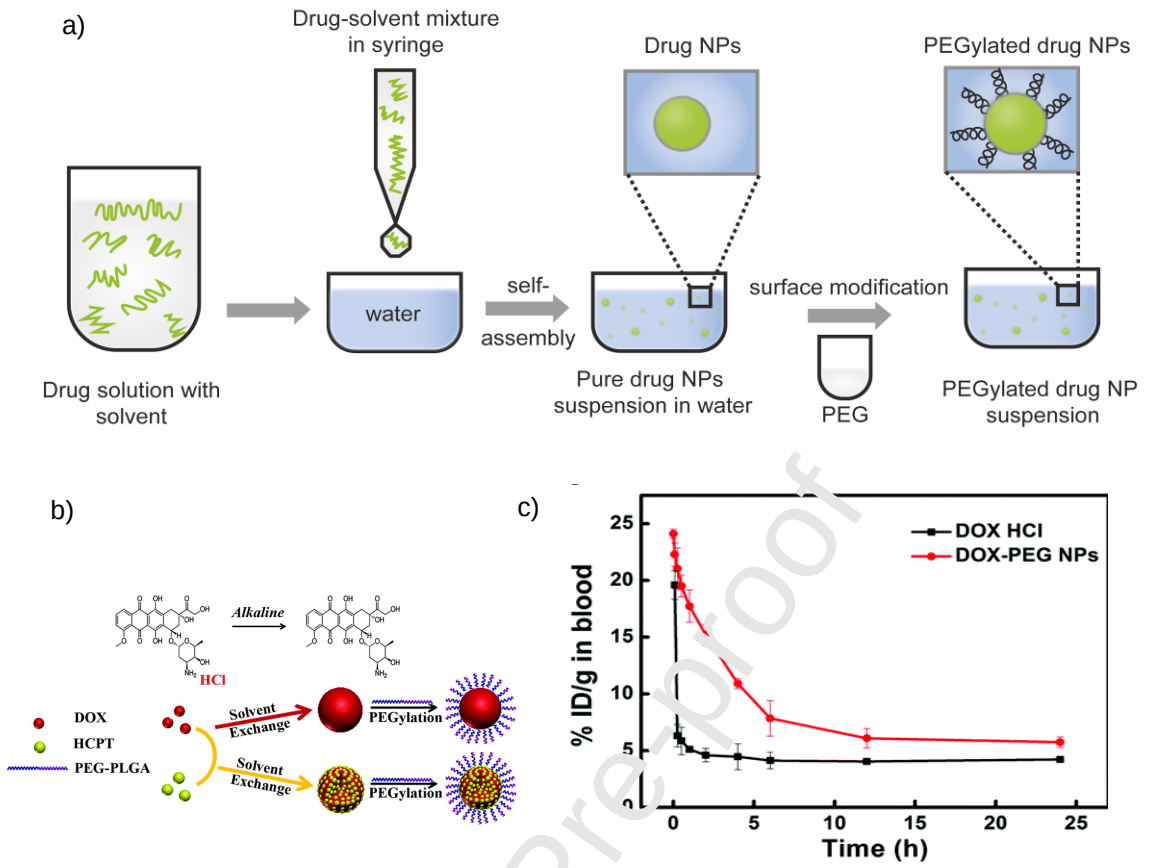

Fig. 2. (a) Schematic illustration of self-assembly of drug molecules and surface modification; (b) schematic illustration of the preparation and functionalization of DOX and DOX-HCPT NPs; (c) the blood circulation curve of DOX-PEG NPs and DOX·HCl determined by measuring the fluorescence of DOX in the blood at different time points post injection. Copyright 2015. Reproduced from ref [71] with permission from The Royal Society of Chemistry.

In a similar manner, we reported carrier-free multidrug nanocrystals (MDNCs) for combination chemotherapy [73]. Three widely used hydrophobic drugs, MTX, HCPT and PTX, were assembled into nanorods, and then conjugated with PEG to improve their bio-environmental stability. Our in vitro and in vivo studies showed that the MDNCs revealed almost 3-fold higher therapeutic efficacy than free drugs at the same dose and also efficiently suppressed MDR. Some other pure nanodrug formulations have also been

achieved with CUR, HCPT by using different solvents and anti-solvents [74]. This technique can also be improved with different therapeutic agents. We have also reported NP synthesis from DOX, triphenylphosphine and ionidamine which can inhibit energy metabolism by targeting the mitochondria [75]. The prepared NPs increased the half-life of DOX in bloodstream to 3 h. Some other representative examples of nanodrugs are summarized in Table 1.

Table 1. Examples of pure nanodrugs reported in the literature via anti-solvent method.

Drug	Solvent	Anti-solvent	Cancer type	Refs
DOX	DMSO	water	KB and 4T1 cells and 4T1 tumor	[71]
CUR	tetrahydrofuran	water	CT-26 cells and tumor	[74]
CPT, HCPT	DMSO	PBS	-	[76]
HCPT	ethanol	water	KB cells, 4T1 tumor	[77]

2.1.2 Self-assembly of chemotherapy with photodynamic or photothermal therapy drug molecules

Combination of photodynamic therapy (PDT) and photothermal therapy (PTT) with chemotherapy is an attractive area of research due to the advantage of increased therapeutic efficacy and alleviated drug resistance. PDT is a light-excited treatment method and shows promising results particularly with anti-cancer agents [78]. In PDT, a photosensitizer (PS), such as chlorin e6 (Ce6), di-iodinated borondipyrromethene ($BDP\text{-}I_2$), pyropheophorbide-a (PPa) and zinc phthalocyanine (ZnPc), is intravenously administered into the body. These molecules have a tendency to stay longer in tumor cells than healthy cells. After a certain time to ensure minimal damage to the healthy cells, a specific radiation

is introduced to the body to activate PS molecules which can convert dissolved oxygen into reactive oxygen species (ROS), resulting in cancer cell apoptosis and tumor eradication [79]. This method can improve the therapy by localizing the treatment to tumor sites and reducing the side effects to normal cells [80]. PTT is another light-excited treatment method, which converts the light energy into heat under specific near-infrared (NIR) laser leading to tumor ablation [81]. In spite of these advantages, phototherapeutic agents have some drawbacks such as instability stemming from their insolubility and poor selectivity which weakens the therapeutic effect [79]. To overcome the mentioned disadvantages of both anti-cancer chemotherapy drugs and phototherapeutic agents, a wide range of PDT and PTT agents were employed to form nanodrugs with anti-cancer agents via self-assembly method [82].

Yan et al. reported carrier-free chemo-PDT nanodrugs by using DOX and Ce6 [83]. The molecules self-assembled into NPs via electrostatic, π-π stacking and hydrophobic interactions**.** The size of Ce6-DOX NPs was determined as 70 nm with -20 mV zeta potential ensuring stability for 8 days. The encapsulation efficiencies of Ce6 and DOX were more than 95% and 99%, respectively. DOX Ce6 nanodrugs with high drug loadings demonstrated good cellular uptake and tumor depletion under laser irradiation due to the synergistic chemo-photodynamic therapy. In a 2020 study, a similar method was also applied with SN-38 and Ce6 which formed carrier-free NPs (154.87 ± 1.82 nm) due to π-π stackings and subordinate hydrogen bonds. SN-38-Ce-6 NPs demonstrated enhanced cellular uptake and anti-tumor efficacy with 85% inhibition rate with laser irradiation whereas this ratio was around 65% and <20% for without laser and only drug injected groups, respectively [84]. This study indicated the importance of combined PDT and chemotherapy. Yan et al. reported a chemo-photodynamic therapy with HCPT and Ce6, which were mixed to self-assembly into nanorods in water [85]. The prepared nanorods increased both HCPT and Ce6 cell uptake, resulting in enhanced tumor depletion via synergistic action.

In a 2018 study, another PS, BDP-I_2 was employed with PTX to self-assemble into nanorods with a size of nearly 200 nm. The NPs were stable for two weeks at room temperature [86]. The obtained nanorods exhibited higher cytotoxicity in vitro compared to Taxol when irradiated with laser.

We have reported a carrier-free nanodrug via a simple solvent exchange method by using CUR with a donor-acceptor pair which composed of 5,10,15,20-tetro(4-pyridyl)porphyrin (H2TPyP) and perylene, as photosensitizers, allowing PDT [87]. In our nanoplatform, CUR enabled the inhibition of cancer cell growth and the fluorescence state of CUR only became active after its release from the nanoplatform, enabling to self-monitor the drug release. The drug loading of CUR was high at 77.6%. NIR fluorescence emitted from the donor-acceptor pair also achieved real-time tumor imaging via Förster resonance energy transfer.

Anti-cancer nanodrugs with combinational PTT are also explored due to the aforementioned benefits. Indocyanine green (ICG) is the only FDA approved PTT agent, and is a promising candidate in self-assembly with anti-cancer drugs, due to its hydrophilic sulphate and hydrophobic indole skeletons [88]. For these reasons, a variety of anti-cancer nanodrugs conjugated with ICG and its derivatives (IR820, IR780 iodide) were reported. Shao et al. reported the self-assembly of PTX with ICG into uniform NPs, with 53.80 ± 3.79% drug loading content of PTX [89]. The PTX-ICG nanodrug increased the solubility of PTX and tumor accumulation.

2.1.3 Self-assembly of chemotherapy with immunotherapy drugs

Immunotherapy aims to stimulate immune cells to browse and eradicate malignant cells. It has revolutionized cancer treatment [90]. The first example of immunotherapy was reported more than a century ago by William B. Coley who administered streptococcal organisms into

a cancer patient to stimulate immune systems and shrink the tumor [91]. This discovery led to bigger developments and the 2018 Nobel Prize in Physiology or Medicine was awarded for the discovery of cancer therapy by the inhibition of negative immune regulation, i.e. cytotoxic T lymphocyte-associated antigen 4 (CAR T) and programmed death/ligand 1(PD-1/L1) to enable T cells. Tumor environment is usually immune suppressed and crucial responses such as T cell activation and antigen presentation are inhibited [88]. For these reasons, eight new immunomodulatory drugs based on the blockage of PD-1/L1 and CAR T cell therapy were approved by FDA from 2014 to 2018. However, their application still suffers from low patient response, dose limits, and low stability [92]. NPs can be used to increase the efficacy of immunotherapeutic drugs enabling targeted delivery by releasing their cargo at required sites such as tumor tissues and lymph nodes. With nanodrug formulations, pathophysiological barriers including compact extracellular matrix, renal clearance and endonucleases degradation can be tackled [93]. For these reasons, numerous immuno-nano drug delivery systems have been developed and showed promising results [94].

Indomethacin (IDM) is another drug which has immunomodulatory effects. IDM is a COX-2 inhibitor and nonsteroidal anti-inflammatory drug which decreases M2 polarization of macrophages by inhibiting the production of prostaglandin E2 (PGE2) which supports immune suppressant environment in tumor cells. Therefore, the use of IDM alongside chemotherapy may be beneficial. As shown in Fig. 3**,** a carrier-free PTX-IDM nanodrug was synthesized via one-pot assembly due to the known strong intermolecular interactions between two drugs including π−π stackings and hydrogen bonds [95]. Diverse morphologies including wire-like, net-like, honeycomb-like, sphere like, and capsule-like were prepared by changing the weight ratio and initial PTX concentration. The mean size of IDM/PTX nanodrugs was determined as 461.6 ± 57.72 nm and the zeta potential was -25.5 ± 0.702 mV. IDM/PTX nano-assemblies showed immunoregulatory ability in vivo by having the lowest expression of CD206, an anti-inflammation macrophage biomarker, and IL-10, which

has a proliferating effect on cancer cells, and the highest expression of CD86, an inflammatory macrophage biomarker.

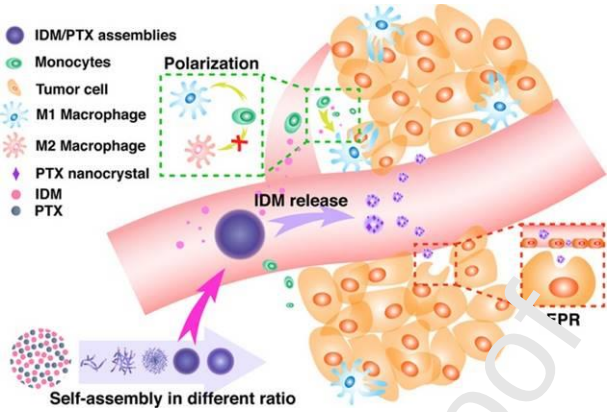

Fig. 3. Schematic illustration of fabrication of IDM/PTX nano-assemblies at different ratios and its mechanism of action. Copyright 2019. Reproduced from ref [95] with permission from the American Chemical Society.

Apart from these immunomodulatory agent, it is known that some anti-cancer drugs such as DOX and PTX can stimulate immune response by inducing immunogenic cell death [96]. To explore the immunotherapeutic effects of the combinational use of PTX and ICG, Li et al. demonstrated the self-assembly ability of ICG templating strategy by synthesizing stable NPs from a variety of molecules, including PTX, DTX, SRF, celecoxib, with ICG [97]. Drug solution was added to aqueous solution of ICG and this mixture self-assembled into NPs with a mean diameter of 112 ± 1.06 nm and 90.7% drug loading rate. Using low doses of PTX suppressed the immunosuppressive tumor microenvironment via downregulating T-regs whereas ICG increased immunogenic cell death under laser irradiation. In vitro and in vivo studies with triple negative breast cancer cells showed that synergistic therapy elongated the half-life by 3-fold and increased the intratumoral drug accumulation by 11.2-fold compared to free PTX. The therapy with laser irradiation also showed enhanced anti-

tumor immunity by exhibiting 3.1-fold higher dendritic cell maturation and significantly decreased T-reg infiltration due to the killing effect of PTX.

2.1.4 Self-assembly of chemotherapy drugs with other organic molecules

DOX and gossypol have been self-assembled with a very small amount of dopamine (PDA) with a weight ratio of 5:5:1 via π-π stacking and hydrogen bonding [98]. Gossypol is an anti-cancer drug exerting its effect by inducing tumor cell apoptosis. The prepared DOX-PDA-gossypol NPs exhibited the size of 59.6 ± 9.6 nm with a very high drug loading (91%). As shown in Fig. 4a, in vivo studies demonstrated that free DOX and free DOX-gossypol were quickly cleared from blood whereas DOX-PDA-gossypol NPS showed 458-fold and 228-fold higher elimination half-times than free DOX and free gossypol, respectively, due to the strong NPs structure. Furthermore, these NPs demonstrated >192 h blood circulation (Fig. 4b) and enhanced accumulation at tumor site when compared to free DOX and gossypol, which mostly accumulated in liver, kidney, lung and spleen.

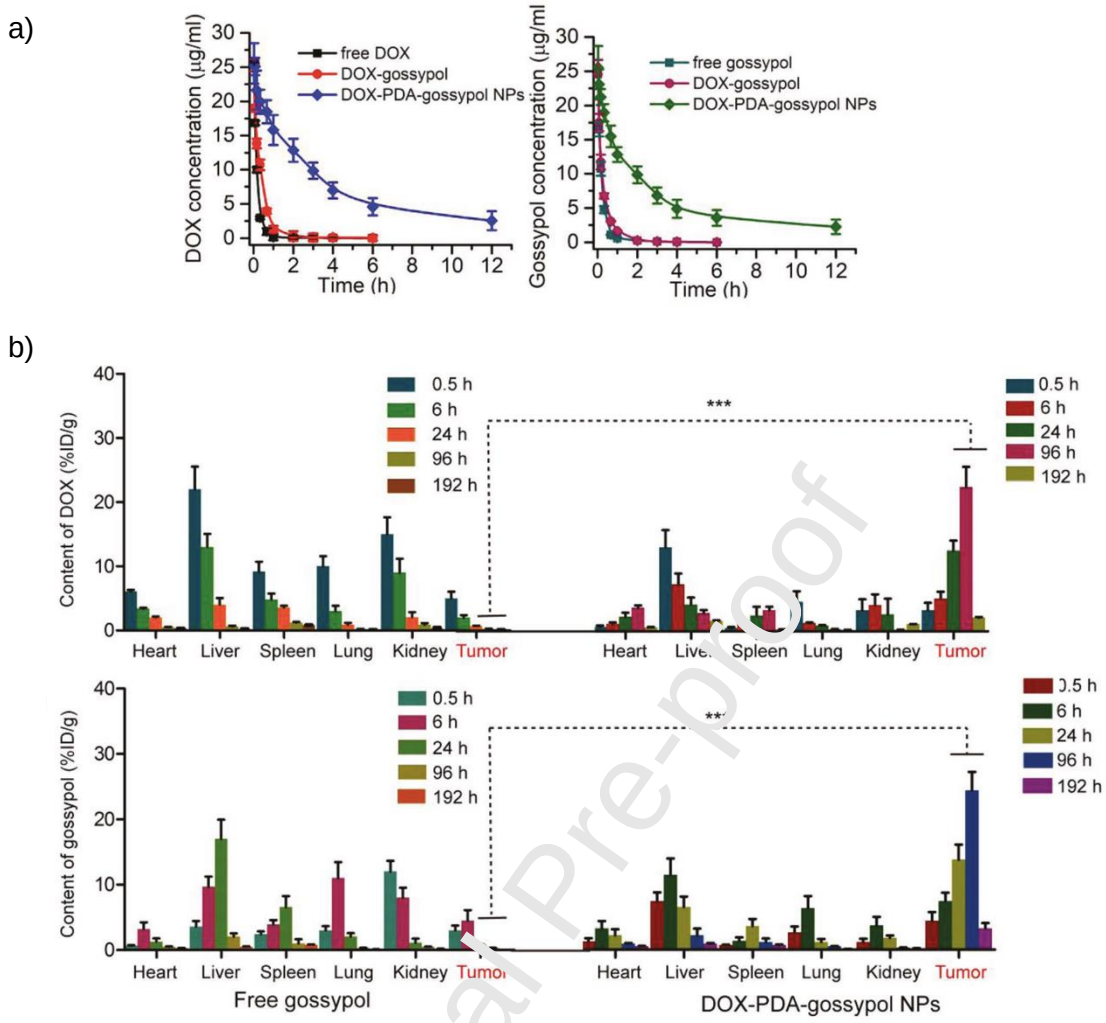

Fig. 4. (a) The pharmacokinetic properties of free DOX, free gossypol, free DOX and gossypol and DOX-PDA-gossypol NPs in mice after the injection at a drug dose of 2.5 mg/kg over 12 h. (b) Quantitative analysis of drug distribution (DOX-up, gossypol-bottom) over 192 h in major organs and tumors after intravenous injection. Copyright 2018. Reproduced from ref [98] with permission from John Wiley & Sons Inc.

Overall, the method described in this section is facile to use in making carrier-free NPs. In many cases, however, drug molecules cannot simply self-assemble into uniform and stable nanostructures using the above described self-assembly method due to limitations arising from their structures [95, 99]. Therefore, alternative approaches will be required, which are detailed in the next sections.

2.2 Self-assembly of clinical drug molecules with different conjugation

To solve the problems of many drug molecules not being able to self-assembly to uniform nanodrugs, covalent conjugation of multiple molecules of single anti-cancer drug or between different drugs or between drug and other organic molecules via different linkages has been widely investigated. These dimeric conjugations can convert hydrophobic anti-cancer drugs into less rigid molecules, disrupting crystallization and consequently enabling the formation of NPs via self-assembly. By regulating the connecting bridges, such as ester bonds, disulfide bonds and thioketal bonds, dimeric prodrugs can form stable nanostructures in biological environments, thereby improving drugs' water solubility, extending their blood circulation half-life time, and increasing the bioavailability [100, 101]. In this section, different conjugations with drug molecules will be reviewed to facilitate their self-assembly to high-quality nanodrugs.

2.2.1 Conjugation of homodimeric drug molecules with various linkers

PTX is known to aggregate in aqueous conditions due to the π-π stackings of aromatic rings. This results in the formation of needle-like crystals, instead of self-assembling into uniform NPs [102]. To solve this issue, two PTX molecules were covalently coupled to form a dimer. As shown in Fig. 5, two PTX molecules were covalently bonded via dicarboxylic acid linkers which contained either an aliphatic carbon chain or a disulfide bond (R: C4, C6, C8, C9, S-S) [103]. The synthesized dimers (PTX_2) were then used to form PTX_2 NPs in aqueous solution without using any carriers. PTX_2 NP formation increased the water solubility of PTX to a maximum of 1000 μg/ml. The NPs containing a disulfide bond were stable in physiological environment whereas they exhibited a rapid stimuli-responsive release in the tumor tissue due to the cleavage of glutathione (GSH)-sensitive bond. This resulted in enhanced anti-tumor effects and lowered systemic toxicity [104]. Another connecting bridge with a thioether

moiety was also reported for the preparation of PTX_2 NPs [105]. PTX loading in PTX-S-PTX NPs was as high as 94% and this nanodrug increased the solubility of PTX by 2000-fold. In 2018, Xie and co-workers reported thioketal linked dimeric PTX NPs [106]. The prepared NPs were coated with red blood cell (RBC) membranes for enhanced circulation and the mean diameter of NPs was determined to be 168 nm. In vitro and in vivo experiments showed that the prepared NPs displayed prolonged blood circulation, improved tumor accumulation, and enhanced therapeutic efficacy in cervical cancer cells. The RBC membrane coating also enabled to achieve 4.6-fold higher concentration of thioketal linked PTX dimer in tumor compared to the NPs without the coating at 23 h post-treatment.

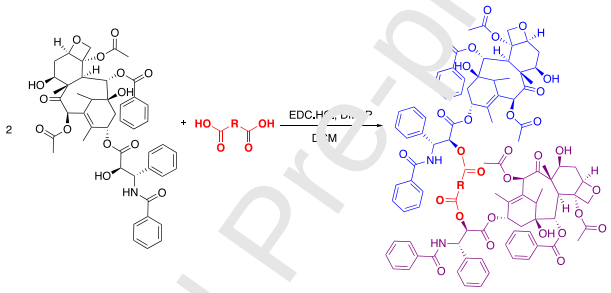

Fig. 5. Schematic illustration of PTX-R-PTX conjugates (R: C4, C6, C8, C9, S-S). Copyright 2017. Adapted from ref [103] with permission from Elsevier Science Ltd.

To further demonstrate the effect of self-assembly of dimeric drugs, Hou et al. reported the synthesis of a CUR dimer via a thioether bond [107]. This CUR-S-CUR prodrug was self-assembled into spherical NPs with 78% drug loading and PEGylated for prolonged circulation. The NP formation increased the water solubility of CUR thereby enhancing cellular uptake. In vitro studies showed that CUR-S-CUR NPs demonstrated comparable cytotoxicity to free CUR, with sustained release arising from thioether bond. Dimer preparation has also been extended to other anti-cancer drugs such as DOX. In a 2020 study, the synthesis of a DOX dimer was reported via an acid-triggered hydrolysable carbamate linker with 86% drug loading [108]. DOX dimer self-assembled into NPs which exhibited pH responsive drug release and enhanced anti-tumor efficacy in liver cancer cells.

Kasai et al. reported pure drug NPs below 100 nm by the preparation of SN-38 dimeric nanodrugs [109]. Two SN-38 molecules were coupled together via dicarbamate, diester and diether linkages. The SN-38 dimers transformed SN-38 molecule into a less planar structure which reduced the risk of crystallization and therefore successfully formed NPs via nanoprecipitation method.

2.2.2 Conjugation of heterodimeric drug molecules with various linkers

2.2.2.1 Conjugation of chemotherapy drugs

The above approach provides a way to make nanodrugs via preparing homodimeric drug molecules first followed by self-assembly. However, monotherapies with one type of drug molecule limit their application range due to the drug resistance of tumors [110]. To optimize the efficacy and safety of cancer treatment, a series of nanodrugs based on heterodimeric drug-drug conjugates have been developed [111]. Heterodimeric drugs can be amphiphilic with both hydrophilic and hydrophobic parts as well as formed by both hydrophobic drugs [112]. In addition to self-assembly into uniform nanostructures, the optimally designed heterodimeric drugs can also affect the interaction with cancer cells and release drug molecules in controlled ways at the tumor site, enabling efficient drug delivery to targeted tissues and cells without any external delivery vehicles [113]. Compared to single or sequential administration of anti-cancer drugs, different drugs in heterodimer drugs result in differences in pharmacokinetics and mechanisms of actions. This can then produce synergistic therapeutic effects and overcome MDR [72, 114].

Ni et al. reported the syntheses of alkyne terminated disulfide (-S-S-) introduced hydrophobic CPT and azide modified hydrophilic gemcitabine (GEM). GEM is an FDA-approved chemotherapy drug which works as a pyrimidine nucleoside antimetabolite, used

for the treatment of breast cancer, bladder cancer, pancreatic cancer and non-small cell lung cancer [115]. These two molecules were then reacted together in a Cu-catalyzed click reaction to form CPT-S-S-triazole-GEM for combination chemotherapy (Fig. 6a) [116]. This amphiphilic drug conjugate exhibited a high drug loading content (36.0% CPT, 27.2% GEM) and formed spherical nanoparticles (180 nm) in aqueous solution. In vitro studies showed that CPT-S-S-triazole-GEM NPs released two drugs simultaneously in liver cancer cells due to the cleavage of disulfide bond and exhibited higher toxicity compared to free CPT and free GEM within the 72-h test period. The prodrug NPs demonstrated moderate half-life time, high accumulation in tumor tissues and synergistic therapeutic efficacy in vivo.

Condensation reactions can also be used to form amphiphilic drug conjugates. Zhu et al. reported the preparation of MTX and GEM conjugate (MTX-GEM) via an amide bond as shown in Fig. 6b [117]. MTX-GEM self-assembled into NPs in aqueous solution via solvent exchange method with 100% drug loading. In vitro studies demonstrated that MTX-GEM NPs achieved enhanced anti-cancer effect and inhibited MDR in breast cancer cells compared to free drugs. In another study, MTX and CPT were conjugated via ester linkage for pH-/esterase-responsive cleavage [118]. MTX-CPT conjugate with amphiphilic and ionic properties self-assembled into MTX-CPT NPs in aqueous solution via hydrogen bonding and hydrophobic interactions. These NPs benefited from both the enhanced membrane permeability of MTX and the water solubility of CPT. The MTX-CPT NPs showed efficient tumor accumulation through both passive and active targeting (folate receptor-mediated). In vitro and in vivo studies showed that the prepared MTX-CPT NPs could specifically deliver drugs to achieve synergistic effect with distinct anti-cancer mechanisms.

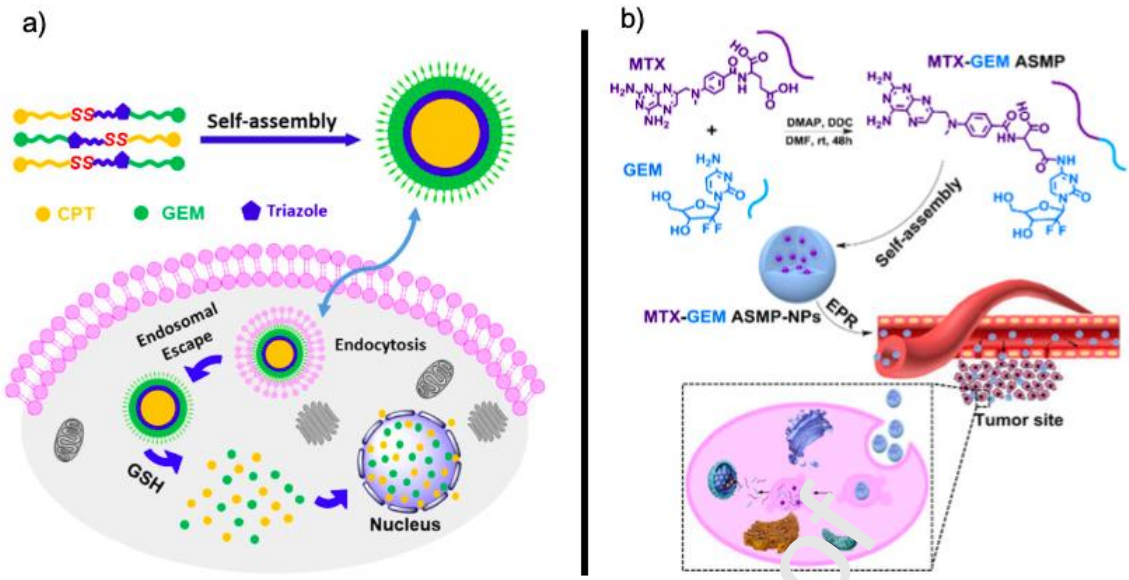

Fig. 6. a) The illustration of NP formation to form CPT-SS-triazole-GEM conjugate and its therapeutic mechanism in cells. Copyright 2019. Reproduced from ref [116] with permission from the American Chemical Society. b) Schematic of MTX-GEM synthesis and construction of self-assembled nanoparticles MTX-GEM NPs for cancer combination chemotherapy. Copyright 2016. Reproduced from ref [117] with permission from the American Chemical Society.

In addition, Dai et al. developed liposome-like nanocapsules based on amphiphilic camptothecin-fluorouridine (CPT-FUDR) molecules as shown in Fig. 7 [119]. The heterodimeric drug molecules were synthesized by two hydrophilic FUDR molecules and two hydrophobic CPT molecules via hydrolysable ester linkages. They self-assembled into uniform and stable prodrug nanoparticles in aqueous solution. In the presence of esterase and under acidic conditions of tumor tissues, the hydrolysis of ester bonds would trigger the release of FUDR and CPT, thereby inhibiting tumor growth.

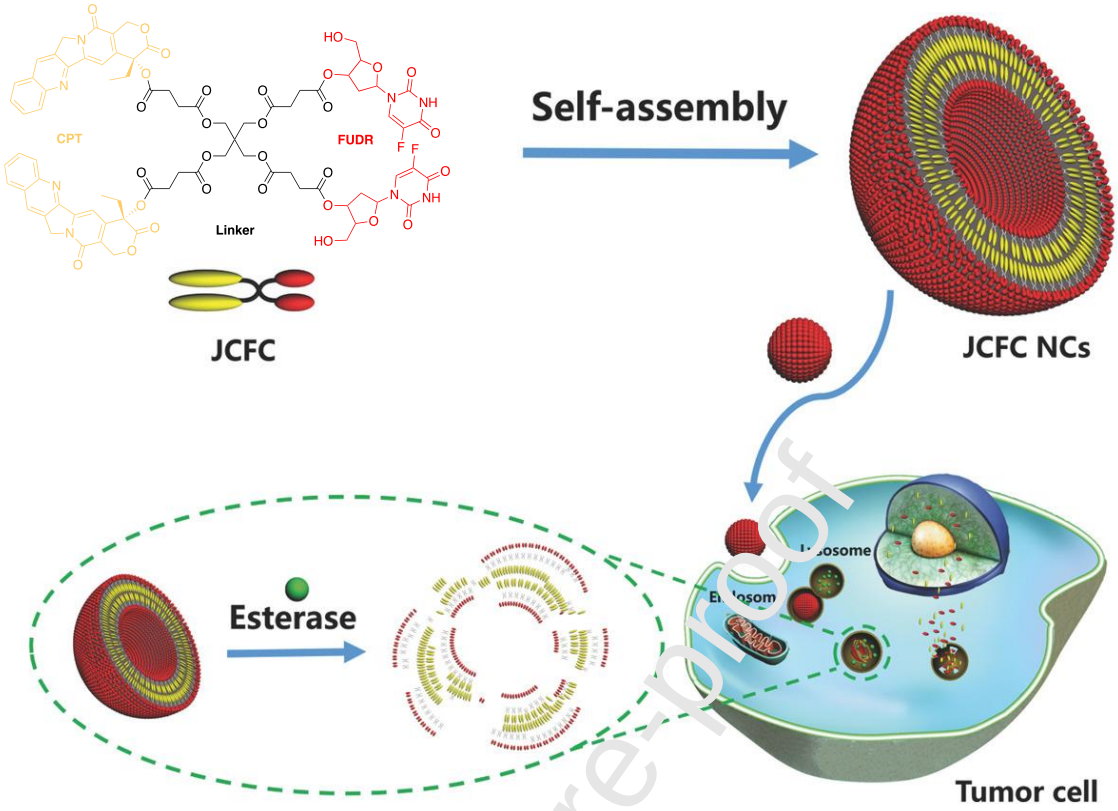

Fig. 7. Schematic illustration of the preparation of Janus camptothecin-floxuridine conjugate (JCFC) NPs. Copyright 2017. Reproduced from ref [119] with permission from John Wiley & Sons Inc.

In addition to hydrophobic-hydrophilic dimeric drug conjugates, heterodimeric nanodrugs from two poorly water-soluble anti-cancer drugs have also been explored. In a 2019 study, Wang et al. developed PTX-DOX heterodimeric nanodrugs [120]. PTX was conjugated with DOX by using a linking thioether bridge. The resulted conjugates formed uniform NPs via self-assembly. These NPs were further PEGylated for prolonged blood circulation time. The drug loading ratios of PTX and DOX were 46.5% and 30.0%, respectively. Tumor microenvironment triggered the cleavage of thioether bonds in the presence of breast cancer cells resulting in rapid release of PTX and DOX, thereby synergistic tumor inhibition. We have also reported the conjugation of PTX with pH responsive cis-aconitic anhydride (CA)-

modified DOX [121]. CA modification enabled GSH-responsive DOX release and the conjugate was modified with a layer of crosslinked surfactant based on hyaluronic acid (HA) which can bind to CD44 receptors which are highly expressed in certain tumor cells. Since DOX and PTX have different inhibition mechanisms and anti-tumor targets, the combination therapy enhanced therapeutic efficacy and inhibited MDR. In vitro and in vivo studies demonstrated that these CAD-PTX-HA NPs exhibited high stability with a half-time of 4h, excellent active targeting effect and controllable intracellular drug release. These NPs achieved significantly enhanced anti-cancer efficiency when compared with the individual administrations of DOX and PTX.

2.2.2.2 Conjugation of chemotherapy with photodynamic or photothermal therapy drugs

Sun et al. reported a non-carrier prodrug synthesized by bridging PTX with PPa via thioether bond [122]. Tumor-responsive nanodrug PPa-S-PTX was prepared by one-step nanoprecipitation method and PEGylated. The size of these chemo-photodynamic nanodrugs were around 90 nm with a zeta potential of about -30 mV. In the presence of laser irradiation, these NPs released more than 40% of PTX within 12 h and demonstrated effectiveness in generating ROS. To further increase the sensitivity of PTX, DOX was conjugated with PS pheophorbide a (Pa), via pH-sensitive hydrazone bond to form Pa-h-DOX (PhD) conjugates [123]. As shown in Fig. 8, the surface of PhD NPs was further modified with PEG via pH-sensitive Schiff base formation, resulting in NPs with an average size of 79 nm. After the pH-sensitive cleavage of the PEG surface, these NPs transformed into ultra-small strongly positively charged NPs (4 nm) which could achieve deep tumor penetration. PhD NPs remained stable in physiological conditions. Pa exerted both PDT and PTT effect and this combinational therapy demonstrated 100% cure rate as a result of its synergistic effect.

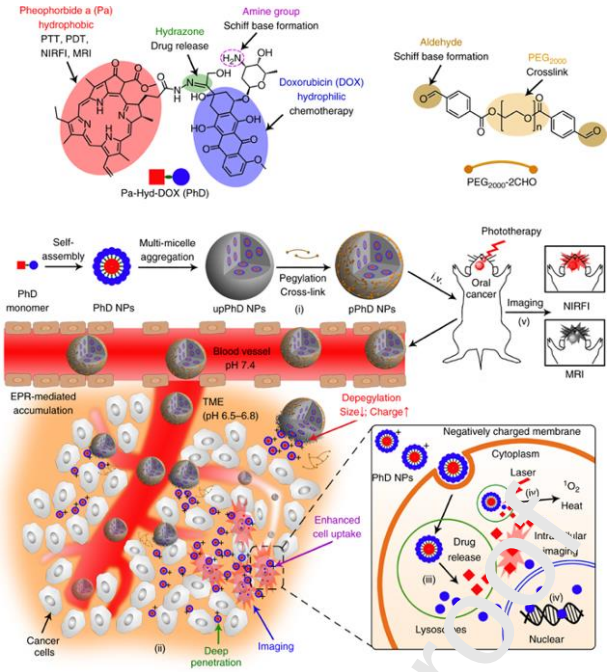

Fig. 8. Schematic illustration of properties of PDX-Pa conjugate nanoparticles. Copyright 2018. Reproduced from ref [123] with permission from Springer Nature.

Some lipid conjugated nanodrugs were also employed with PDT. Sun et al. reported carrier-free drug NP formation with PPa which formed uniform NPs with CTX-Se-OA and CTX-S-OA conjugates via π-π stackings arising from the phenyl groups on CTX [124]. It was hypothesized that pyrrole groups of PPa play a role in NP formation due to their intermolecular interactions with CTX. The NPs were further PEGylated with vitamin E polyethylene glycol succinate for enhanced systemic circulation. The release rates of both redox-responsive drugs were found to be equal in a 12 h period in the presence of 10 mM H_2O_2. However, under laser irradiation for combinational PDT, selenium linked NPs exhibited enhanced drug release in vitro compared to sulfur linked NPs and this further facilitated superior cytotoxicity of selenium linked NPs. Both NPs showed promising synergistic anti-cancer activities under laser irradiation in 4T1 breast cancer models.

Similar to aforementioned light activated OA conjugates, ZnPc was employed with CUR-S-OA conjugate [79]. ROS-responsive CUR-S-OA prodrug and ZnPc were self-assembled into spherical ZnPc@Cur-S-OA NPs (127.9 ± 1.5 nm) via hydrophobic interactions and π−π stackings between the two molecules. Nanoprecipitation method yielded NPs with 60.08% of CUR-S-OA content. Curcumin was particularly important here since it can inhibit HIF-1a whose high levels are correlated with PDT resistance. For this reason, CUR was employed as a synergistic therapeutic for PDT with ZnPc, by inhibiting HIF-1A and depleting GSH, as well as a chemotherapy agent. This combination therapy showed superior effects compared to free CUR and ZnPc and ZnPc@Cur-S-OA NPs without laser irradiation both in vitro and in vivo. Lipid conjugated carrier-free nanodrug examples will be discussed in later sections.

Among these promising spherical NP examples, Gao et al. explored the effect of shape transforming nanomedicines from spheres to nanofibers by combining PDT therapy with a dimeric nanodrug [125]. Nanofibers are known to have the best retention properties, but their linear shapes cause poor circulation due to being captured by many organs. To solve this problem, shape transformable, ROS- and light-responsive thioketal bond bridged PTX dimer (PTX_2-TK) nanoplatform was reported. PTX_2-TK co-assembled with two linear chimeric triblock molecules consisting of hydrophobic Ce6 or ROS-responsive bilirubin (BR), both conjugated with Phe-Phe-Val-Leu-Lys (FFVLK) peptide, and hydrophilic PEG chain forming Ce6-FFVLK-PEG and BR-FFVLK-PEG. FFVLK peptide favors the formation of β-sheet structure due to its tendency to form extensive intermolecular hydrogen bonds resulting in fiber formation. However, these conjugates form spherical NPs in aqueous solution due to their amphiphilic nature resulting in a strong affinity to form micelles. Upon ROS- and light-responsive cleavage, these spherical NPs are transformed into nanofibers as their amphiphilic nature weakens and the relatively weak hydrogen bonds start to dominate. PTX_2-TK@Ce6/BR-FFVLK-PEG demonstrated selective cytotoxicity in vitro and enhanced its effectiveness upon laser irradiation whereas the control group NPs, lacking both the thioketal linkage and the laser treatment, exhibited strong cytotoxicity from the very

beginning indicating poor selectivity. In vivo studies demonstrated that PTX_2-TK@Ce6/BR-FFVLK-PEG NPs demonstrated 2.21-fold and 4.26-fold higher tumor growth inhibition (61.8%) compared to free Ce6 and free PTX, respectively. This indicated the effect of the combinational therapy.

In a 2020 study, Li et al. reported a novel SRF conjugate, employed with hemoglobin (Hb) in combination with PDT [126]. In addition to be an anti-cancer agent, SRF can promote ferroptosis, a cell death mechanism dependent on the reaction of iron with excess ROS in tumor. Hb can bind to oxygen due to its iron content, and therefore serve as an oxygen supplement for ferroptosis and PDT. Ce6 was bridged with Hb via amido bond and mixed with SRF and tumor sensitive matrix metalloproteinase 2 (MMP2) peptide to self-assembly into SRF@Hb-Ce6 NPs (175 nm) via intermolecular forces. In vivo experiments demonstrated that SRF@Hb-Ce6 NPs exhibited tumor responsive release via MMP2 and high tumor inhibition with synergistic therapy. More examples of tumor sensitive/targeting peptides will be discussed in detail in later sections.

Luan et al. reported another PTX-based self-assembled chemo-photothermal nanodrug [127]. PTX was conjugated to IR820 via esterification, followed by self-assembly into pH-sensitive NPs. These NPs showed enhanced colloidal stability, tumor responsive drug release, and anti-cancer activity. It is also possible to achieve cocktail therapy with carrier-free nanodrugs using PTT agents together with dimeric drugs and human serum albumin (HSA), which the latter can increase the half-life of drugs in blood circulation. In a 2019 study, a thioether linked PTX dimer (PTX-S-PTX) was mixed with photothermal agent IR780 iodide and HSA to form uniform NPs via self-assembly method [128]. The resulting NPs were stable at 4°C for almost six weeks with a size of 129 nm. This approach enhanced the drug loading content of PTX from 6.6 wt% to 48.7 wt% compared to commercially available Abraxane® which is also known as nanoparticle albumin-bound PTX.

2.2.2.3 Conjugation of chemotherapy with immunotherapy drugs

A number of recent studies have focused on the synergistic use of immunotherapeutic agents with chemotherapeutics without any carriers via self-assembly strategy [129]. Indoleamine 2,3-dioxygenase (IDO) is an immunosuppressive enzyme in tumor microenvironment which works by breaking down an essential amino acid, tryptophan, and producing immune-suppressant kynurenine metabolites [93]. Although the exact mechanism has not been established yet, IDO overexpressing dendritic cells in tumor tissues favors the development of T-regulatory (T-reg) cells resulting in tumor growth [130]. PTX was incorporated with an IDO inhibitor, D-1-methyltryptophan (D-1MT), via an ester bond and the conjugate (MP) bonded with HSA [131]. PTX-D-1MT-HSA prodrug was then assembled into spherical MP NPs with a mean diameter of 116.6 ± 0.5 nm. These NPs improved the immune response and delayed tumor growth in melanoma, breast and lung cancer models when compared to free PTX and D-1MT. More importantly, PTX-D-1MT-HSA NPs increased $CD8^+$ T cells by 3.63-fold compared with PTX NPs with free D-1MT. The anti-tumor effect of the MP NPs was also compared with the FDA-approved Abraxane®. MP NPs demonstrated significantly higher inhibition of tumor growth compared to Abraxane® and the mixture of Abraxane® with D-1MT in mice bearing 4T1 breast cancer and LLC lung tumors. In addition, mice treated with MP NPs did not show any significant change in body weight after five injections which has 10 mg/kg of PTX and 2.56 mg/kg of D-1MT per dose.

Kang et al. reported a new chemoimmunotherapy nanodrug by co-assembling the immunotherapeutic agent imiquimod (R837), a small agonist of TLR7/8, with PTX which both are poorly soluble in water [132]. R837 induces immune response, via polarizing tumor-associated macrophages to M1 macrophages [133]. Stearyl alcohol (SA) was connected to PTX and R837, separately, via redox responsive linkers to form PTX-S-S-SA and R837-S-S-SA. This approach differs from other reported researches, which use redox-responsive linkers between two anti-cancer drugs. The prepared prodrugs were mixed with C18-PEG-

iRGD peptide which recognizes integrin receptors (e.g., αvβ3 and αvβ5) in cancer cells. This new formulation was able to self-assemble into NPs with the mean diameters of 140 ± 5 nm. The NPs showed increased cytotoxicity towards breast cancer cells as a result of the tumor-sensitive immunity. The release profiles of R837-acSS-SA and PTX-acSS-SA were studied by incubation with 0 mM and 10 mM DTT. As shown in Fig. 9A and Fig. 9B, both conjugates showed redox-responsive drug release due to the disulfide bonds, exhibiting a potential for a promising cancer therapy. Biodistribution studies (Fig. 9C) further supported this finding by illustrating a high tumor accumulation when compared to kidney, lung, spleen and heart. The nanodrug with iRGD peptide showed higher tumor accumulation than nanodrug free from the peptide indicating the targeting ability of the peptide.

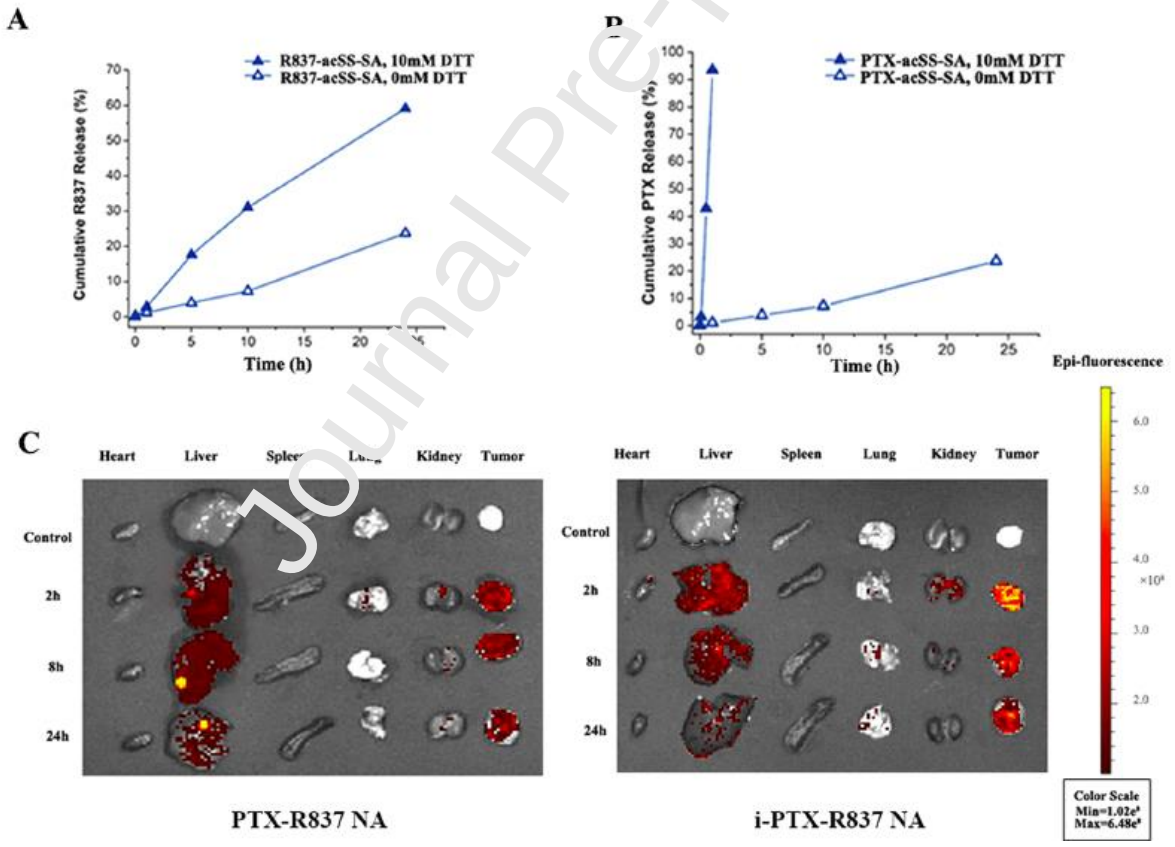

Fig. 9. Drug release studies of R837 (A) or PTX (B) from R837-acSS-SA or PTX-acSS-SA in buffer with or without DTT. (C) Biodistributions of PTX-R837 NA and i-PTX-R837 NA in main

organs and tumor site at different time points (2, 8, 24 h). Copyright 2019. Adapted from ref [132] with permission from the American Chemical Society.

2.2.3 Conjugation of drug molecules with various functional organic molecules

2.2.3.1 Conjugation with lipids

In recent years, anti-cancer drugs have been successfully translated into lipid-based formulations, such as liposomes for the encapsulation of drug molecules. Carrier strategies based on liposomes have been discussed in detail in the literature and are the subject of a previous review [134]. However, carrier based liposomal formulations still suffer from problems such as low drug loading, and pre-mature drug release. Efficient combination therapy by using two or more different drugs with liposomes as carriers remains challenging due to inefficient co-loading [135]. In this review, we focus on studies which did not use liposomes/lipid bilayers as carriers but those where lipid molecules are covalently conjugated with anti-cancer drugs via different linkages (-C-,-SS-,-S-, -2S, -Se-,-SeSe-) to modulate the physicochemical properties of drugs such as increasing their chemical stability and solubility, enabling the self-assembly due to enhanced amphiphilicity. Presence of redox responsive bonds also enables on-target drug delivery in tumor. Compared to liposomes, the lipids plays therapeutic roles such as enabling response release and targeted drug delivery, rather than functioning as carriers by forming lipid bilayers [1, 136]. It is possible to achieve high drug loadings due to the relatively low molecules weights of lipids. These conjugates improve the chemosensitivity of some drugs [137, 138] and are also known to penetrate deeper into tumors as lipids are used as an energy source for tumor cells [139].

A variety of lipids have been combined with anti-cancer drugs, such as fat-soluble hydrophobic vitamins, fatty acids, squalene and citronellol. For example, fatty acids contain

a free carboxylic acid moiety in their hydrocarbon chains [140]. They are commonly employed to form conjugates via their carboxylic acid group which reacts with a linker molecule or amine/hydroxyl groups on hydrophobic anti-cancer drugs [139]. These molecules with long flexible alkyl chains enable the formation of prodrugs improving the colloidal stability and cellular uptake of chemotherapeutic agents [141]. They can provide many advantages such as overcoming MDR in cancer cells and enhancing oral bioavailability of drugs by improving lymphatic drug transport [142, 143].

To explore the rationale behind the self-assembly of anti-cancer drugs with fatty acids, Zhang et al. studied the effect of lipophilicity and solubility of anti-cancer agents during self-assembly [144]. First, four different PTX conjugates were reported with different types of fatty acids, each having different extents of saturation. All these conjugates managed to self-assemble into NPs. Second, the hydrophobicity of the conjugates was determined, and it was revealed that all prepared conjugates had higher hydrophobicity compared to PTX alone. Third, they have calculated the hydrophilicity and lipophilicity values in a variety of PTX conjugates in their study and literature [5, 102, 145-148] which can self-assemble but was connected with different accessories and linkage. They suggested that when a hydrophobic molecule conjugates with PTX, hydrophobic interactions occur between hydrophobic parts of molecules creating the inner core whereas the hydroxyl group of PTX places on the outer region of self-assembled NPs (Fig. 10) which leads to a negative zeta potential.

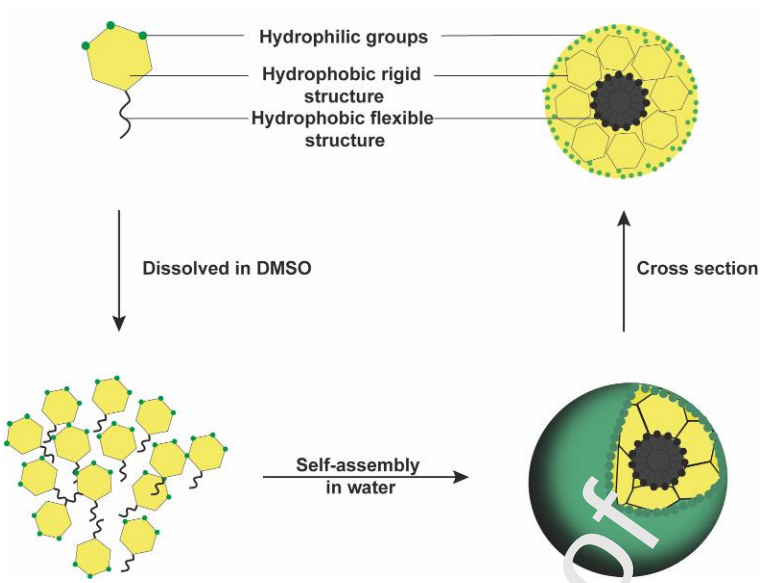

Fig. 10. Self-assembly mechanism of PTX-lipid conjugates. Copyright 2018. Adapted from ref [144] with permission from the American Chemical Society.

A number of other researchers also reported the conjugation of PTX with fatty acids due to the aforementioned benefits [149]. In a 2016 study, PTX was conjugated to linoleic acid (LOA) via an ester bond, forming ~105 nm NPs with 90% drug loading [150]. PEGylated PTX-LOA NPs remained stable for 9 months. Sun et al. reported conjugates with oleic acid (OA) [5]. Redox-responsive PTX-S-S-OA and ester linked PTX-OA conjugates enabled the preparation of spherical NPs in aqueous solutions which remained stable for three months. High drug loading of up to 66% was achieved due to the small molecular weight of OA compared to PTX. PTX-S-S-OA NPs released 27% of PTX in 2 h at pH 7.4 whereas, when exposed to 10 mM dithiothreitol, DTT, (a prevailing GSH stimulant), this triggered the cleavage of S-S bonds and 90% of the drug release. In comparison, there was no drug release from PTX-OA under the same conditions. The former demonstrated equivalent cytotoxicity with Taxol, where the latter showed no cytotoxicity due to very slow release in human epidermoid carcinoma, ovarian cancer and lung cancer cells. Correspondingly, PTX-S-S-OA had superior anti-cancer activity in vivo with complete tumor inhibition at day 10.

This result was attributed to the decreased off-target release of PTX with NPs of a desirable size (100 nm) for tumor accumulation and enhanced colloidal stability after PEGylation.

In a similar study, the same researchers compared the redox-responsive characteristics of thioether (-S-) and dithioether (-2S-) bonded PTX-OA conjugates (Fig. 11), capable of self-assembling into NPs. When PEGylated they remained stable for three months [146]. PTX-S-OA NPs demonstrated increased redox-responsiveness in vitro and anti-tumor efficacy in vivo when compared to PTX-2S-OA. They have proposed that the single thioether bond near PTX is the key in hydrolysis and -2S- bond consumes 2-fold more GSH/ROS compared to -S- and water molecules are unlikely to attack to -2S- due to longer hydrophobic chain arising from ethylidene between two thioether bonds.

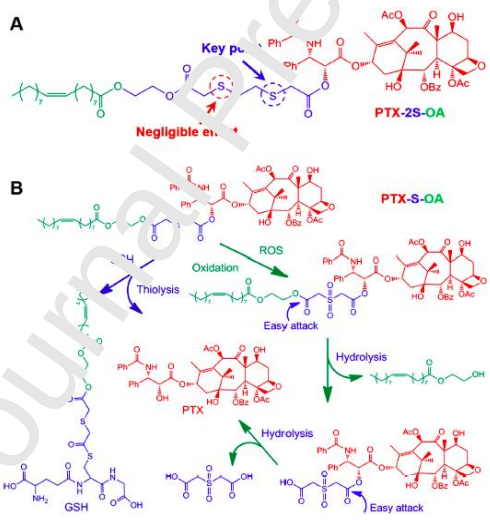

Fig. 11. Redox-responsive release mechanism of PTX-2S-OA (A) and PTX-S-OA (B) conjugates. Copyright 2016. Reproduced from ref [146] with permission from the American Chemical Society.

OA was also applied to DTX in 2017, where the conjugate DTX-S-S-OA was reported [151]. DTX-S-S-OA was able to self-assembly into NPs by nanoprecipitation method, demonstrated a prolonged half-time of 5.73 ± 6.0 h compared to free DTX (3.2 ± 1.1 h) and

enhanced anti-tumor effect on 4T1 breast cancer cells compared to free DTX [151]. In a 2019 study, Sun et al. reported another interesting example of this conjugation but this time via thioether linked DTX and OA. Oleate prodrugs could facilitate the lymphatic transport and enhance the oral bioavailability. In this study, a 6.2-fold increase in bioavailability was reported compared to free DTX solution. This study was particularly important since the oral formulation of DTX is not commercially available because of its poor oral adsorption.

Wang et al. reported the conjugation of LOA with DTX [21]. The redox sensitive DTX-S-LOA formed NPs in aqueous solutions due to its structural flexibility and intermolecular π-π interactions. The synthesized NPs were stable for three months exhibiting spherical morphology with a mean diameter of 100 nm. The reported zeta potential and drug loading were -21.5 mV and 53.4%, respectively. Gou et al. reported the conjugates of CPT with both OA and LOA. The conjugates were synthesized via various disulfide linkages including disulfanyl-ethyl carbonate (etcS-S), disulfanyl propionate (prS-S) and disulfanyl acetate (acS-S) in the absence of stabilizing agents [12]. It was demonstrated that only CPT-etcS-S-OA formed rod shaped NPs whereas CPT-acS-S-OA and CPT-prS-S-OA exhibited large aggregations in aqueous medium. Further to this finding, the hexyl carbonate (hecS-S) linked CPT-hecS-S-OA conjugate was synthesized to understand the importance of the carbonate ester linkage in NPs. The prepared conjugate again formed rod-shaped NPs with transparent appearance. Therefore, it was demonstrated that the carbonyl ester group facilitated the stabilization of NPs due to its high negative charge density. Researchers also investigated LOA by synthesizing CPT-etcSS-LOA which also formed nanorods. LOA containing nanorods showed 19.2-fold less toxicity in vitro against Lewis Lung carcinoma compared to free CPT due to the slow, sustained release of the active drug molecules. This hints a potential enhanced effectiveness in vivo anti-cancer therapy due to tumor responsive release. A similar linkage was also used for SN-38 where it was conjugated with OA via etcSS, forming nanorods in water, 100-200 nm in length and a diameter of tens of nm with 45% drug loading [15]. The prepared nanorods demonstrated one of the most redox-

sensitive nanodrugs in literature, with 100% release within 1 h in the presence of 10 mM DTT whereas only 1% release occurred at pH 7.4. This can be attributed to the high ROS sensitivity of the linking carbonate group. In vivo experiments revealed the superior effect of nanorods due to selective release whereas free CPT hydrolyses quickly into the carboxylate form in blood.

Hydrophobic vitamins including tocopherols (vitamin E, VE) and retinoic acids (RA) have also been extensively used in nanodrugs for cancer therapy due to their health benefits and chemical properties. Vitamin E succinate, a derivative of VE, not only enhances the therapeutic efficacy, but also increases the solubility of anti-cancer drugs by self-assembling into NPs [152]. In an example study, Wang et al. reported a conjugate of PTX and VE linked via a disulfide bridge and an ester bond [102]. Nanoprecipitation of the conjugate, PTX-S-S-VE, resulted in the formation of stable NPs with -29.2 mV zeta potential. Molecular simulation studies revealed that the oxygen atom close to -S-S- linkage has an impact on this negative charge density and the S-S-linker acted as a stabilizer (Fig. 12), enabling the folding of the molecule for self-assembly. NP formation increased the drug loading rate of PTX to 60 wt% whereas Taxol and Abraxane® have 1% and 10% drug loadings, respectively. The half-life of PTX in blood circulation was greatly enhanced, achieving 25.74 ± 7.66 h compared to the half-life of 1.47 ± 0.16 h for Taxol. In vitro and in vivo studies showed that PTX-S-S-VE NPs demonstrated redox responsive drug release in cancer cells while it remained stable in physiological conditions.

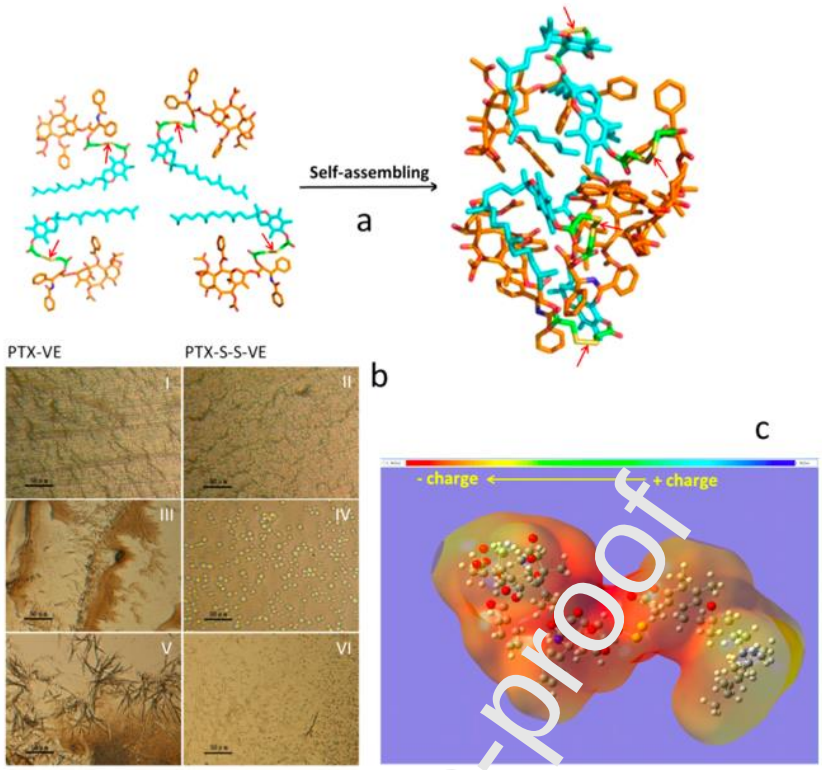

Fig. 12. (a) MD simulation of self-assembled PTX-S-S-VE conjugates. Blue, orange and yellow represent VE, PTX and S-S, respectively. (b) Crystallization of PTX-VE and PTX-S-S-VE NPs (c) the distribution of charges of self-assembled PTX-S-S-VE. Copyright 2014. Reproduced from ref [102] with permission from the American Chemical Society.

In a similar design, Xu and co-workers reported the conjugation of CUR with VE via -S-S- linkage, capable of self-assembling into NPs by the facile nanoprecipitation method [153]. Similar to this, DTX-S-S-VE and DTX-VE conjugates were also reported by He et al. to form spherical NPs from both conjugates [154]. The in vitro studies demonstrated that free DTX had the highest cytotoxicity followed by DTX-S-S-VE NPs and DTX-VE NPs, due to the cleavage mechanisms of conjugated nanodrugs. DTX-S-S-VE NPs improved the blood circulation time by 6.1-fold compared to free DTX. A similar approach was adopted by Duhem et al. to construct a VE derivative of DOX via an amide bond which self-assembled into 250 nm-sized NPs [20]. Among these nanodrug formulations with VE, a nano-formulation of PTX was also achieved with all-trans-RA, which is a non-toxic physiological metabolite of vitamin A, with 75% drug loading via nanoprecipitation method [155]. PTX-RA

NPs demonstrated lower cytotoxicity compared to Taxol in B16F10 and MCF-7 cells, while they exhibited similar toxicities at a concentration of 2 μM indicating high drug loading capacity.

Other commonly used lipid molecules are terpenoids such as citronellol (CIT) and squalene (SQ) [136]. CIT is a plant-based monoterpene alcohol with anti-inflammatory and anti-cancer activities [156]. Similar to fatty acids, it can provide increased sensitivity towards anti-cancer drugs [10]. Furthermore, it was demonstrated that it can increase immune response in cancer patients [156]. Sun et al. reported the synthesis of six different PTX-CIT conjugates, PTX-S-CIT, PTX-Se-CIT, PTX-C-CIT, PTX-S-S-CIT, PTX-Se-Se-CIT and PTX-C-C-CIT, forming uniform NPs (each around 90 nm) with 50% drug loading [157]. The bond angles around the linkages were calculated by dynamic simulations to investigate their effects in NP formation. -Se-Se- (89.9°/93.3°) exhibited the closest to 90^{o} in comparison to -S-S- (94.6°/97.9°); -Se- (95.4°); -S- (97.8°); -C-C- (111.6°/115.1°); -C- (112.6°). Further to this finding, dihedral angles of diatomic linkages were also calculated, and C-Se-Se-C was again much closer to 90° compared to C-C-C-C (177.4°). Selenium and sulfur bridged NPs showed much better colloidal properties and cytotoxicity over carbon bonded NPs. The oxidation responsive release rate of prodrug nano-assemblies in the presence of H_2O_2 was -Se- > -S- > -Se-Se- > -S-S- > -C-/-C-C-. The DTT-responsive drug release rate was however -S-S- > -Se-Se- > -S- > -Se-> C-/-C-C. Carbon bond linked prodrug NPs exhibited very slow and non-responsive drug release in H_2O_2 and DTT. Diselenide containing prodrug nano-assembly was proven to increase the intracellular ROS levels and showed rapid drug release in vitro and the strongest cytotoxicity over KB, A549 and 4T1 cells. PEGylated PTX-CIT prodrug NPs were further used in vivo to improve circulation times. PTX-CIT prodrug nano-assemblies exhibited lower cytotoxicity in vitro against human oral epidermoid carcinoma cells in the period of 48 h and 72 h compared to Taxol because of their responsive drug release and resulting superior blood circulation times. Amongst the prodrug

nano-assemblies, PTX-Se-Se-CIT NPs exhibited the longest circulation time and the highest tumor inhibition in vivo in KB and 4T1 tumor bearing mice.

Squalene (SQ), a natural lipid found in shark liver, is a precursor in cholesterol biosynthesis. SQ can be well tolerated and absorbed orally [158]. A squalenoylated nanodrug was prepared by covalently binding DOX and 1,1',2-tris-norsqualenoic acid [158]. The nano-assemblies were synthesized via nanoprecipitation method without PEG and displayed loop-train shaped morphology with a mean diameter of 130 nm and a zeta potential of + 35.5 mV. In summary, lipid conjugated anti-cancer drugs demonstrated great ability to assemble into NPs and showed enhanced blood circulations with increased efficacy. The studies are summarized in Table 2.

Table 2: Properties of recently prepared lipid anti-cancer conjugate nanodrugs with different chemical linkages.

Lipid	Anti-cancer drug	Linkage	Drug loading (wt %)	Size (nm)	Cancer cells	Refs
VE	PTX	-S-S-	60%	113 ± 5	KB-3-1 tumors	[102]
	CUR	-S-S-	27.3 ± 0.4 %	29.8 ± 0.9	HepG2 cells	[153]
	DTX	ester	75.4%	180.2 ± 3	A549 and PC-3 cells/ A549 tumor	[154]
		-S-S-	82.0%	173.8 ± 1.5		
	DOX	amide	34%	234	MCF-7 cells and CT26 tumor	[20]
RA	PTX	ester	75%	170.2	MCF-7 and B16F10 cells	[155]
LOA	PTX	Ester	90%	105	B16-F10 cells, MDA-MB-231, U87-MG cells	[150]
	DTX	-S-	53.4%	100	4-T1 cells	[21]
	CPT	-etcS-S-	-	-	Lewis lung carcinoma	[12]
OA	PTX	ester	66%		KB-3-1, H460, and OVCAR-8 cells/	[5]

		-S-S-	56%	100	KB-3-1 tumor	[146]
		-S-	57.4%			
		-2S-	54.9-66.5%			
	SN-38	-etcS-S-	45%	114.9	CT26 cells, murine 4T1 cells and B16-F10 cells	[15]
	CPT	-etcS-S-	-	220	Lewis lung carcinoma	[12]
		-hec-	-	240		
		Carbonate ester	-	231		
		ester	-			
	DTX	-S-S-	50.4%	75-95	4T1 cells and tumor	[151]
		ester	60.2%			
		-S-	60	30	-	[143]
CIT	PTX	-C-	50%	90	KB, A549 and 4T1 cells and tumors	[157]
		-C-C-				
		-S-				
		-S-S-				
		-Se-				
		-Se-Se-				
SQ	DOX	ester	57%	130	MiaPaCa-2 cells or M109 cells and tumors	[158]

2.2.3.2 Conjugation with carbohydrates

Carbohydrates, such as mannose (MAN), HA, dextran (DEX), are very appealing molecules in nanodrug delivery platforms due to their biodegradable, biocompatible, low immunogenic properties [159]. Different strategies have been reported to prepare conjugates of hydrophobic anti-cancer agents and hydrophilic saccharides to form stable nano-assemblies which results in enhanced efficiency [160]. Ionic saccharides can even form stimuli responsive systems due to their pH sensitivity [160]. Administration of nanodrugs containing

carbohydrates and anti-cancer agents provides tumor targeting properties due to the presence of various saccharide receptor-expressing tumor cells.

MAN, a C-2 epimer of glucose, is known to target tumor cells via over-expressed lectin receptors and was reported to increase the cytotoxicity of cisplatin and DOX [161]. In a 2020 study, MTX and MAN were coupled together via an esterification reaction to form MTX-MAN conjugates. Anti-solvent method resulted in the formation of uniform spherical NPs in aqueous solutions with a thin shell composed of large amounts of MAN on the surface [162]. The size and zeta potential of MTX-MAN NPs were around 100 nm and -28 mV, respectively. MTX-MAN NPs exerted long circulation times due to its MAN shell and released MTX under weakly acidic conditions. This was likely due to the hydrolysis of the ester linkage. MTX-MAN NPs demonstrated superior combinatory anti-tumor effect (around 60% tumor inhibition rate) compared to free MTX (around 35% tumor inhibition rate).

HA is another potentially tumor-targeting carbohydrate with known affinity to CD44 receptor, overexpressed in cancer cells. Yin et al. reported PTX-HA conjugates self-assembling into NPs (200 nm) in water [163]. In vivo studies demonstrated that PTX-HA NPs achieved 4-fold tumor inhibition on day 14 compared to free PTX. In a 2018 study, another HA-based nanodrug was reported. As shown in Fig. 13, PTX was covalently conjugated to HA and also to ICG-COOH, a derivative of ICG [164]. ICG-HA-PTX conjugates self-assembled into spherical nanomicelles with an average diameter of 130 nm. These NPs can release PTX via the cleavage of ester linkage in tumor microenvironment which has an over-expression of esterase by 2-3 orders of magnitude. A series of in vitro and in vivo studies revealed that the nanomicelles provided active tumor targeting via HA and esterase presence as well as minimal toxicity towards normal cells due to the targeting mechanisms. Nanomicelles showed elongated half-life in blood circulation and effectively inhibited 98.84% of the tumor with PTT which worked as a synergistic therapy with PTX.

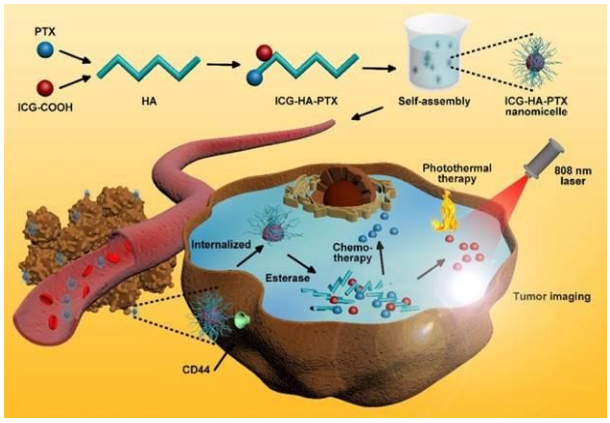

Fig. 13. Preparation of ICG-HA-PTX micelles via self-assembly method and its therapeutic effect**.** Copyright 2018. Reproduced from ref [164] with permission from the American Chemical Society.

Affinity of HA to CD44 was further demonstrated by Yao et al. [165] In this study, CUR was modified with HA (CUR-HA) and DOX was conjugated with low molecular weight heparin (LMWH) derivative via a pH-sensitive hydrazone bond to form L-DOX. LMWH is a natural glycosaminoglycan and can decrease tumor angiogenesis resulting in enhanced tumor efficacy. These two conjugates formed stable NPs in water. In vitro and in vivo studies with breast cancer cells revealed that the former achieved CD44 mediated deep tumor penetration and improved the delivery of CUR resulting in enhanced MDR reversion where the latter showed pH sensitive drug release of DOX. Hu et al. also reported a similar system where NPs were prepared by the self-assembly of HA-CUR and DOX in aqueous solution [166]. HA-CUR/DOX-NPs demonstrated good serum stability and reversed MDR in lung cancer cells. Targeting behavior of HA can be further improved by the incorporation of FA which is known to target folate receptors in tumor cells. In a 2019 study, DOX was self-assembled with natural HA-FA conjugates into HA/FA-DOX NPs (labelled as HA/FA-NP-DOX in Figure 20) [167]. HA/FA-NP-DOX demonstrated 5.6 h elimination half-life whereas this number was 0.4 h for free DOX (Fig. 14A). Furthermore, biodistribution studies revealed

that the drug accumulation into liver, lung, and kidney was lower compared to free DOX (Fig. 14B) and the DOX uptake with NPs into tumor was 4-fold higher (Fig. 14C) than the free drug group.

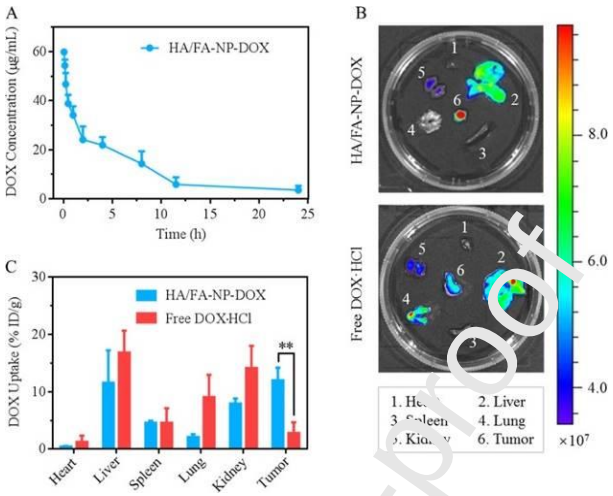

Fig. 14. In vivo pharmacokinetics and biodistribution of HA/FA-NP-DOX (n = 3). (A) Pharmacokinetics in BALB/c mice. (B) Fluorescence images and (C) quantification of DOX in major organs and tumors isolated from SKOV-3 tumor-bearing mice at 8 h after injection of HA/FA-NP-DOX or free DOX·HCl. Copyright 2019. Reproduced from ref [167] with permission from the American Chemical Society.

DEX is a carbohydrate and known to be useful for drug delivery systems due to its high-water solubility and long circulation properties similar to PEG. In a 2020 study, Yin et al. reported DEX-based self-assembled drug NPs. Amphiphilic dextran-deoxycholic acid (DEX-DOCA) conjugates self-assembled with PTX and silybin (SB), which can enhance the chemosensitivity for synergistic therapy, into NPs [168]. These NPs increased the circulation time from 1.04 h (free PTX) to 5 h. In vitro and in vivo studies revealed that the NPs enhanced tumor penetration by inducing tumor vascular normalization in lung cancer model. Li et al. reported the self-assembly of DOX with FA-DEX conjugate which formed tumor targeting NPs [169]. DOX@DEX-FA NPs reduced the side effects arising from DOX and enhanced cellular uptake.

2.2.3.3 Conjugation with peptides

In recent years, peptide conjugated drug delivery systems have attracted significant research interest due to their biocompatibility, biodegradability, producibility at large scales, and specificity as they can be employed as tumor targeting moieties by using particular sequences [170]. These sequences can target tumor-specific receptors which play important roles in tumor angiogenesis. Tumor-homing sequences were the subject of a review in 2010 [171]. So far, a variety of tumor targeting peptide ligands have been discovered, specific to particular cells and receptors such as integrin receptors [172].

Arginine-glycine-aspartic acid (RGD) is a tumor-homing tripeptide which can bind to integrin receptors, expressed in high quantities in tumor cells [173]. There have been many studies focusing on the incorporation of this targeting moiety onto anti-cancer drugs. Zhang et al. prepared tumor targeting CPT nanodrugs conjugated to peptide Arg-Gly-Asp-Ser (RGDS) via ester linkage. This cancer biomarker binds to αvβ3 integrin in cancer cells. Myristic acid was then coupled to the N-terminus of the tumor targeting moiety, as a hydrophobic group to facilitate pro-drug self-assembly. The conjugate was precipitated in cold ether, forming nanofibers which exhibited selective tumor inhibition due the presence of RGDS tetrapeptide targeting sequence. This study demonstrated that nanofibers are promising therapeutic agents as an alternative to traditional spherical NP approach.

Different RGD peptides were also used in the literature. Xu et al. reported the conjugation of PTX with iRGD peptide (CRGDKGPDC), forming NPs by dialysis [174]. In another study, Liu et al. reported a simple PTX conjugate by using tumor targeting peptide, cyclic Arg-Gly-Asp (cRGDyK), and Ce6, each bonded with HSA separately via amide bonds [175]. The conjugate generated NPs by adding equal amounts of HSA-RGD and HSA-Ce6 in phosphate buffered saline (PBS) into PTX solution in methanol. This co-assembly

mechanism occurred via the hydrophobic interactions between drug molecules and HSA. In vitro studies with human glioblastoma cells revealed that the prepared NPs provided superior cytotoxicity under 660 nm light irradiation at 2 mW/cm^2 for 30 mins compared to PDT or chemotherapy alone. This was attributed to the changed endosomal opening behavior from endosomal vesicles to the cytoplasm in the presence of photochemical reactions. In vivo studies demonstrated that the HSA-Ce6-PTX-RGD NPs was able to completely inhibit the tumor under light radiation and the mice survived more than 40 days with 100% survival rate, whereas all mice were dead within 30 days in the control group without tumor targeting peptide.

To further increase the targeting ability of RGD, Pan and co-workers reported the preparation of pH sensitive BTZ nanodrugs with a modified mussel derived tetrapeptide $(DOPA)_4$ as shown in Fig. 15 [176]. Boronic acid and ester groups can degrade in the presence of H_2O_2 [177] and BTZ with boronic acid group (on the drug molecule) was employed for pH sensitive drug delivery. The tetrapeptide was modified to have cancer-targeting sequence RGDS, fluorescein isothiocyanate (FITC) group, enabling the observation of cellular uptake, and a non-bioactive quintuple glycine G5 spacer. BTZ was conjugated via its boronic acid group to the catechol functionalities on $(DOPA)_4$. The conjugate was able to form NPs with a drug loading capacity of 37.4%. These characteristics enabled an enhanced cellular uptake and specific drug release in endo/lysosome. The blood circulation time of the NPs was around 24 h, whereas free BTZ molecule was cleared from blood almost immediately after administration.

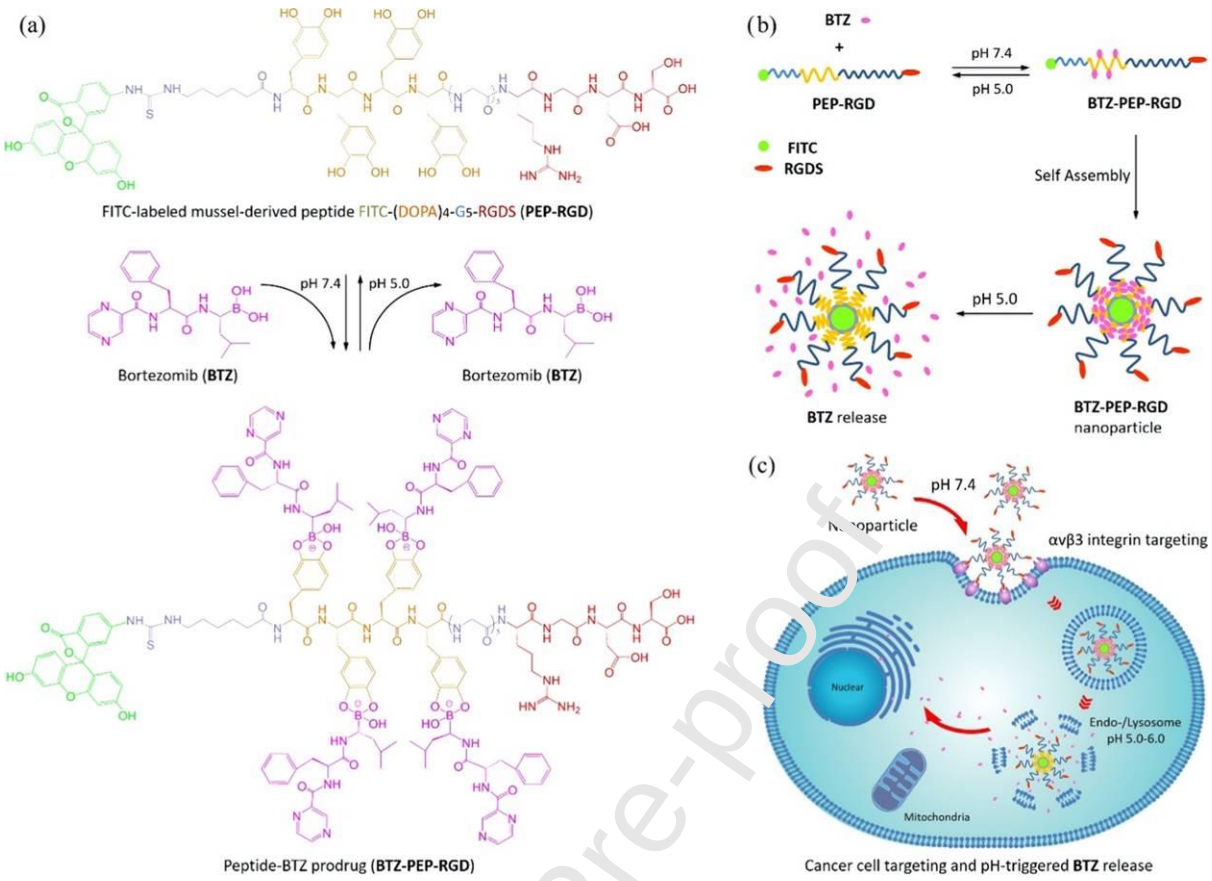

Fig. 15. (a) Conjugation of the mussel derived cancer-cell-targeting peptide with anti-tumor drug BTZ via pH cleavable linkages. (b, c) Schematic illustration of the peptide-prodrug-based nanoparticle with cancer cell targeting and pH-sensitive drug release property. Copyright 2019. Produced from ref [176] with permission from the American Chemical Society.

Cai et al. reported redox-responsive CPT conjugate bonded with hydrophilic PEG bridged iRGD, via disulfide linkage and loaded with IR780, a PS to achieve a glioma cell targeting therapy crossing the blood brain barrier (BBB) with the use of iRGD peptide [178]. The conjugate transformed into spherical micelles (140.68 ± 8.53 nm) via oil-in-water method due to the interactions between hydrophobic CPT and hydrophilic PEG moieties. In vitro studies showed that iRGD modified CPT micelles showed redox-response cleavage with 60% drug release in 3 days in the presence of 10mM GSH, whereas the drug release was

less than 5% in PBS. The control group without iRGD peptide remained limited in tumor periphery whereas the micelles accumulated in the center of tumor spheroids. In vivo studies demonstrated that PDT with IR780 provided higher anti-tumor effect and reduced toxicity. Pep-1 is another tumor targeting peptide particularly for brain cancer due to its ability to cross BBB [179]. Xu et al. reported Pep-1 modified PTX conjugates forming NPs [147]. The produced NPs demonstrated redox-sensitive drug release, exhibiting 74% drug release in the presence of 10 mM GSH, and almost no PTX release under 1 μM GSH. In vivo studies demonstrated that Pep-1 modified PTX NPs can target brain more efficiently and may be a promising nanodrug for glioma therapy.

Cathepsin B is a molecule overexpressed in breast, colorectal and prostate cancers [180]. Kim et al. reported cathepsin B-cleavable peptide Phe-Arg-Arg-Gly (FRRG) and its conjugation with DOX prodrug (FRRG-DOX) [181]. This conjugate produced stable NPs with an average size of 213 nm in water. This cathepsin B-cleavable system resulted in minimal toxicity to normal cells due their low expression of the molecule. To further enhance the cathepsin B responsiveness, another cleavable peptide sequence GFLG was used to bridge DOX and HSA, modified with K237 peptide, which is recognizable by vascular endothelial growth factor receptor 2 in tumor cells. A cyclooxygenase-2 (COX-2) inhibitor, celecoxib, was then introduced to the system to facilitate hydrophobic interactions between HSA-DOX and K237-HSA, further self-assembling into NPs with a mean diameter of 120 nm [182]. It was known that COX-2 inhibitors can increase the efficacy of some chemotherapeutic drugs including irinotecan and 5-fluorouracil (5-FU). Similar to those, celecoxib-DOX combination therapy showed enhanced anti-tumor activity by inhibiting the energy production and increasing GSH synthesis in cells more than DOX or celecoxib alone. In vitro and in vivo studies demonstrated that K237-HSA-DC exhibited longer systemic circulation and good anti-tumor efficacy with better tumor penetration. As shown in Fig. 16, pharmacokinetic studies demonstrated that K237-HSA-DC NPs remained at much higher drug doses in blood circulation over the period of 12 h, while free DOX quickly eliminated in the same period.

This finding also correlated with biodistribution studies which showed the accumulation of K237-HSA-DC NPs in tumor in 2 h after injection (retained 30% at 12 h), whereas the total free DOX was cleared from blood in the same period.

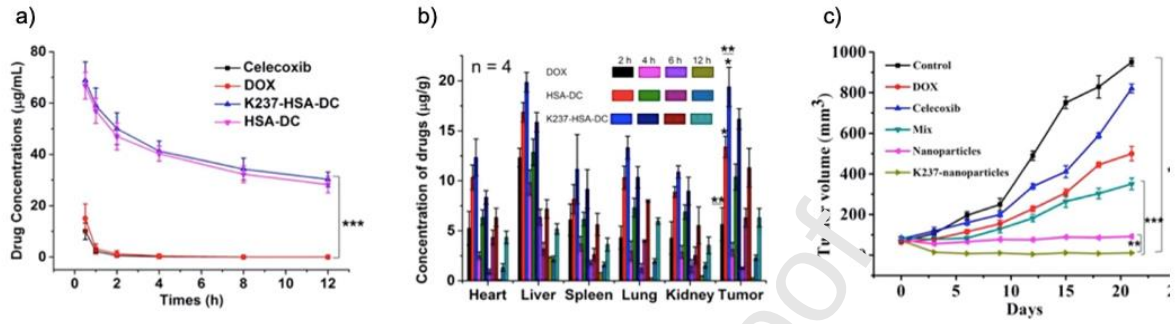

Fig. 16. (a) The pharmacokinetic profile of K237-HSA-DC NPs in vivo, (b) its drug distribution in major organs and tumor over 12h, (c) tumor volumes of different treatment groups. Copyright 2018. Reproduced from ref [182] with permission from the American Chemical Society.

In a 2020 study, CUR was co-assembled with tumor sensitive Ala-Thr-Lys-Thr-Ala (ATKTA) pentapeptides connected by cysteine [183]. The synthesis of nanodrug simply achieved by dissolving CUR and the cysteine bridge peptide (CBP) in DMSO, injecting into phosphate buffer at pH 5.5 and removing the sediment. The prepared nanodrug (220 nm) had spherical morphology. In vitro and in vivo studies on cervical cancer cells demonstrated that CUR-CBP nanodrug have almost 2-fold higher tumor inhibition rate and showed reduced side effects on healthy cells.

The effect of various linkers on tumor-homing peptide nanodrugs have also been investigated. In a 2018 study, different DOX conjugates with cRGDfC tumor targeting peptide were reported by using different linkages, including -S-S-, -S- and cathepsin B cleavable valine-citrulline dipeptide (VC). These conjugates were self-assembled into NPs by using the facile nanoprecipitation method [184]. The in vivo studies revealed that

thioether linked NPs and VC linked NPs exhibited superior anti-cancer activity around 1.5-fold and 2-fold compared to free DOX and disulfide linked NPs, respectively. It was hypothesized that the reduced activity of disulfide linked NPs was due to the generation DOX-SH but not DOX, as the prodrug degrades.

Recently, peptide nanodrugs have also been explored to increase the thermodynamic stability of drug NPs [185]. A conjugate of hexadecapeptide modified with a derivative of ICG, three cholic acids and a pH sensitive boronate ester moiety, was mixed with SN-38. The hexadecapeptide, KEKEKEKE, where K and E represent lysine and glutamic acid respectively, was employed as a shell and formed the hydrophilic segment. This particular peptide has negligible interactions with other proteins and a stronger ability of entangling with water molecules when compared to PEG. The mixture self-assembled into positively charged small NPs in water (~23.8 nm, +13.6 mV) via van der Waals interactions between SN-38 molecules and hydrophobic inner core of the conjugate. To circumvent the short blood circulation, these NPs were further coated with HA modified FA and pH sensitive dopamine, forming negatively charged large hICP NPs (127 nm, -19.5 mV). This enabled active cellular uptake by tumor cells. HA can further cleave into cancer cells which have over-expressed hyaluronidase. The tumor responsive size and charge transformable drug release occurred after the degradation of boronate ester and HA moieties in hICP NPs. In vivo studies demonstrated that hICP NPs have a half-life of 7.97 h whereas free SN38 cleared from blood circulation within 1.44 h. It was demonstrated that hICP NPs exhibited enhanced tumor penetration and additional PTT and PDT sensitive drug release via ICG. However, it was revealed that immune memory did not occur since the same tumor was grown after second challenging. This study is particularly important due to its specific coating system which possessed not only size and charge transformability for better tumor penetration, but also tumor sensitive cleavable molecules for cell targeting.

3 Advantages and challenges of carrier-free nanodrugs

3.1 Drug loading capacity

High drug loadings can be achieved in carrier-free nanodrugs. These carrier-free nanodrugs provide remarkably high drug loadings, usually between 50-90% [102, 186], and even up to 100% in particular studies [97, 150]. Dimeric and heterodimeric nanodrugs have demonstrated enhanced drug loading rates, such as 100% for MTX-GEM nanodrugs, 78% with CUR-S-CUR NPs, 86% with DOX dimer NPs. We have also reported 77.6% drug loading (CUR) with CUR-photosensitizer carrier-free nanodrug. Beyond this high CUR loading percentage, the nanodrug also contained photosensitizer as a PDT drug. All these values are significantly higher when compared to carrier-based nanoplatforms which usually have approximately 10% drug loading capacity or lower [72]. In an example study of carrier-based nanodrugs, researchers used block copolymers of either poly(lactic acid) (PLA) or poly(lactide-co-glycolide) and PEG to encapsulate DTX for the preparation of targeted polymeric nanoparticles [187]. The results showed that the drug loading capacity did not exceed 1% when nanoprecipitation method was used. This is usually due to the differences between the solubilities of carrier and drug molecule resulting in different precipitation times.

In addition to this, simplicity of the nanoprecipitation method makes large-scale production feasible. Preparation of high drug loading carrier-based methodologies require extensive synthetic work, often hindering their applications. In a rare high drug loading example, Zhao et al. reported a controlled nanoprecipitation method to form drug-core polymer-shell NPs by using multiple solvents together to increase the drug loadings of polymeric NPs [188]. It was shown that this method can reach up to 58.5% drug loading by using different range of drugs and polymers, however, it requires extensive experimentation and analysis of polymer and drug solubility data to find the “perfect” solvent mixture and drug concentration.

3.2 Improved pharmacokinetic profile and stability

Carrier-free nanodrugs increases the half-life of anti-cancer drugs, remaining stable in systemic circulation and overcoming rapid clearance [88]. Representative examples on how long anti-cancer drugs remain in circulation are shown in Table 3. A dramatic increase in half-time values are clearly observed with nanodrug formulations when compared to free drug solutions. Prolonged systemic circulation time can promote drug accumulation in tumors which in turn improves the efficacy of chemotherapy. This is in addition to a reduction of side-effects on healthy tissues and organs. Addition of covalent linkages between drug molecules and/or functional molecules also enhance their anti-tumor efficacy by on-target release of the active drug molecules.

Table 3. Representative examples of half-time comparisons between carrier-free nanodrugs and free anti-cancer drugs.

Carrier-free nanodrug	Nanodrug $t_{1/2}$	Free drug $t_{1/2}$	Refs
PTX-S-S-VE NPs	25.74 ± 7.66 h	1.47 ± 0.16 h (Taxol)	[102]
UA-PTX NPs	6-fold longer	No specific value (PTX)	[189]
hICP NPs	7.97 h	1.44 h (SN-38)	[185]
PTX-ICG NPs	3-fold longer	No specific value (PTX)	[97]
DOX NPs	3 h	<10 mins (DOX)	[71]
PhD NPs	31.8 min	4.4 min (DOX)	[123]
DOX-PDA-gossypol NPs	50.381 h	0.110 h (DOX)	[98]

Carrier-free nanodrugs also exhibit excellent stability at 2-8°C whereas some carrier-based drug NPs can cause drug leakage in long term storage due to being physically loaded and the presence of carriers [1, 190]. It was also pointed out in a number of in vivo studies that

premature drug release from carrier-based NPs is a problem of concern [191]. In a 2013 study, it was shown that PEG-PLA micelles and physically encapsulated hydrophobic drug molecules followed different distribution pathways within 10 mins of injection, resulting in premature release of the drug molecules [192]. Such burst releases cause biodistribution and toxicity profiles similar to free drug molecules leading to serious side effects. Polymeric micelles suffer the most from these premature drug releases mainly because of the adsorption of hydrophobic drug along the micelle core/shell interface and micelle dissociation [193]. This hints their ability to remain stable in circulation. Although stability issues of carrier-based nanodrugs have been improved through cross-linking and stimuli-responsive release resulting in on-demand release [194, 195], the potential accumulation of a large amount of carriers in patients is still a major concern in drug delivery.

3.3 Enhanced safety profile

Carrier-free nanodrugs minimize the use of carriers and excipients, therefore overcome the carrier induced toxicity which is a burden to our body. For instance, although PLA is approved by FDA as a biomaterial, it can produce a local inflammatory response when administrated as a NP carrier in vivo [196]. Another example, polyethyleneimine, often used in polymeric NPs displayed damage to mitochondrial membrane which further suppresses the electron transportation [197]. There is also a reported case where carrier based liposomal DOX nanoparticles resulted in unexpected additional side effects, such as hand-foot syndrome [198]. The release of these NPs into tumor sites was unsatisfactory, mainly due to the DOX crystallization in the core of liposomes. Considering long term clinical applications, the use of organic nanocarriers will increase the concern of the toxicity of accumulations of nanocarriers in patients. The interaction between unwanted accumulation of carriers and the biomolecules can form aggregation forming a protein corona, disturbing the mechanism of action of nanodrug formulations [199].

When inorganic materials are used as carriers, the side effects may be even more significant. Gold NPs [200], silver NPs [201] and iron oxide NPs [201] are found have toxicities in vitro and in vivo. The slow degradation of these inorganic nanocarriers also contributes to long-term cytotoxicity [202, 203]. In a 2019 study, carbon nanotubes were found to induce metastasis in breast cancer by mimicking the toxicity of asbestos which is a carcinogenic nano-fiber [61]. As shown in Fig. 17, the exposure to carbon nanotubes caused strong metastasis signals in three out of five mice while no signal was detected in control group at day 4. This number increased to all five mice in CNT-treated group at days 8 and 12, whereas there was a slightly detectable signal in two of five mice in the control group.

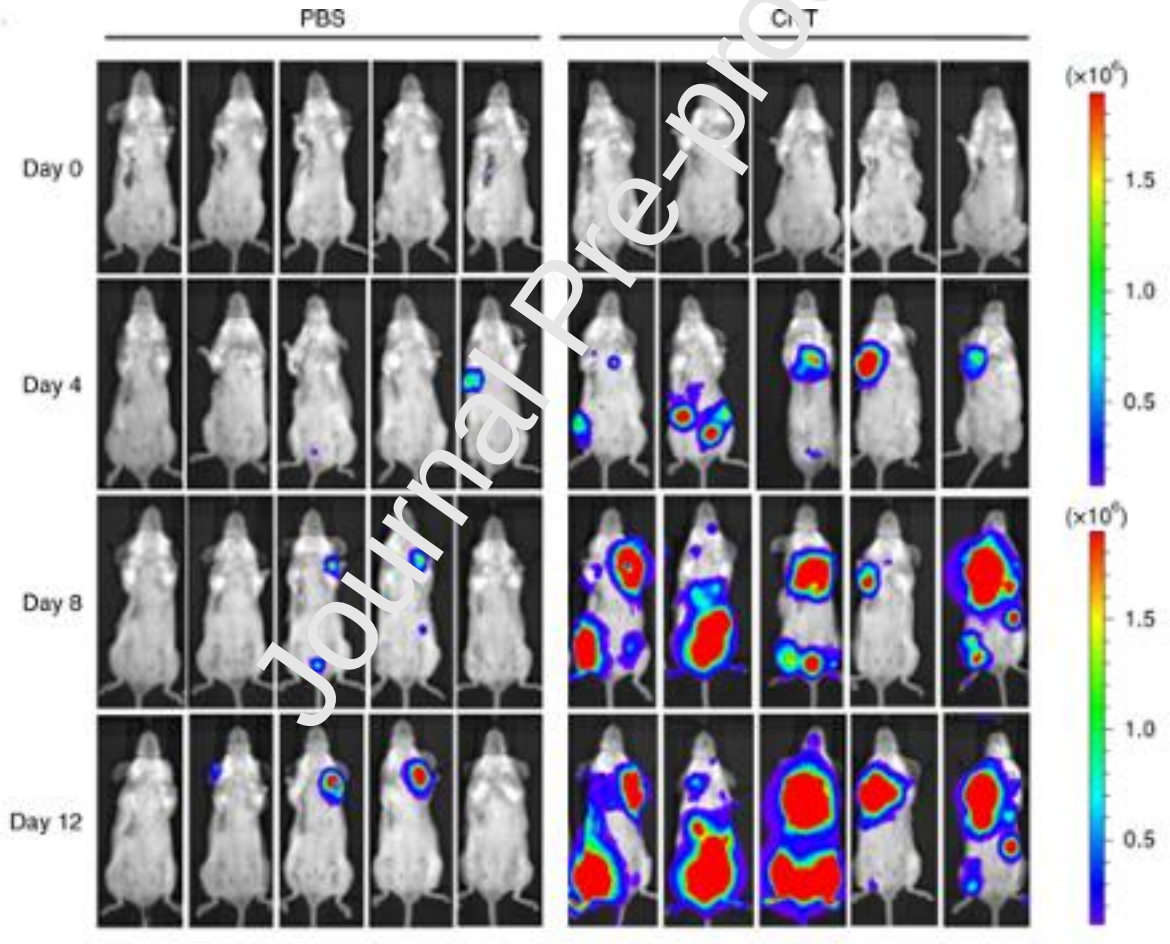

Fig. 17. Metastatic lung lesion formation by bioluminescence imaging at days 0, 4, 8 and 12 after 4T1-Luc cells tumor removal (n = 5 biologically independent mice for the PBS and CNT groups, respectively.) Copyright 2019. Adapted from ref [61] with permission from Springer Nature.

It is also important to consider that the drug loadings are generally very low with carrier-based systems, which means a large amount of nanocarriers with no therapeutic role would need to be administered into the body to achieve effective chemotherapy doses. A detailed analysis of the toxicity profiles of drug delivery nanocarriers was provided in a previous review [52].

On the other hand, carrier-free nanodrugs provides a relatively safe method to avoid carrier induced toxicity while only requiring simple synthetic procedures, such as the preparation of dimeric and heterodimeric drug conjugates. This increases the safety of nanodrugs and limits the use of non-FDA approved products [72]. For instance, aforementioned a heterodimeric nanodrug, DOX-PDA-gossypol NPs, showed significantly enhanced safety profile compared to free DOX. As shown in Fig. 18A, the survival rate of mice was 100% after the injection of DOX-PDA-gossypol NPs with an overall dose of 60 mg/kg (30 mg/kg DOX and 30 mg/kg gossypol) whereas only 30% of mice survived after the injection of mixture of free DOX and gossypol at the same doses. Furthermore, the body weights of mice (Fig. 18B) reduced in the groups treated with free drugs. An opposite trend was observed with the mice treated with DOX-PDA-gossypol NPs which exhibited an increase in weight. Histopathological analyzes (Fig. 18C) further demonstrated that the groups treated with free DOX and free gossypol exhibited several necrotic cardiomyocytes and myocardial fiber breakage alongside kidney damage whereas DOX-PDA-gossypol NPs showed no obvious damage in heart, liver and kidney.

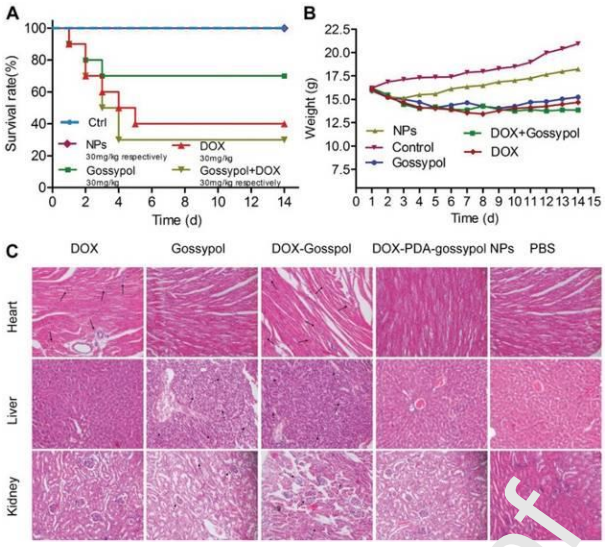

Fig. 18. (A) Survival rates of mice treated with different formulations, (B) the change in body weight over 2 weeks after treated with free DOX (30 mg/kg), free gossypol (30 mg/kg), free DOX-gossypol (30 mg/kg each) and DOX-PDA-gossypol NPs (30 mg/kg each, 60 mg/kg overall), (C) histological examination of hearts, livers and kidneys of mice treated with different groups. Copyright 2018. Reproduced from ref [98] with permission from John Wiley & Sons Inc.

3.4 High flexibility for responsive drug release and synergistic combinatorial therapy

The recent studies discussed in this review reveal that carrier-free nanodrugs have unique advantages due to their unprecedented synthetic versatility. Anti-cancer drug molecules containing hydroxyl, carboxyl, amine or halogen groups can be elegantly conjugated with different drugs or functional molecules including fatty acids, vitamins, photosensitizers, immunotherapeutic agents. Tumor-responsive carrier-free prodrug NPs can be constructed via using ROS-and GSH-responsive linkages, pH sensitive bonds, and coupling drug molecules with tumor-targeting peptides. These carrier-free NPs would remain stable until

reaching to tumor sites. Upon exposure to tumor microenvironment, tumor cleavable chemical bonds would break to release their drug loadings.

In addition, these NPs can be employed as carriers but with therapeutic role for different drug molecules, paving the way for combinational therapies, such as PDT and PTT, and further enhancing their anti-tumor efficacy with laser and/or NIR irradiation. Furthermore, carrier-free nanodrugs can be constructed with immunotherapeutic agents which inhibit the tumor recurrence and strengthen chemotherapy. For instance, as shown in Fig. 19, aforementioned PTX-IDM nano-assemblies [95] showed almost by 2-fold tumor inhibition compared to free PTX and mixture of IDM and PTX treated group (a, b). The treatment with PTX-IDM nano-assemblies increased the apoptosis of the tumor cell (TUNEL) resulting in effective inhibition on proliferation (c). It is also demonstrated that the amount of PTX in tumor site was much higher (d). This efficacy was then demonstrated with analysis of macrophage phenotype in tumor microenvironment (e) by analyzing CD68, CD206 (a biomarker of antiinflammation macrophage) and CD86 (a biomarker of proinflammation macrophage). It was shown that concentration of TNF-α, which has a cytotoxic effect on cancer cells, was higher in the groups treated with PTX-IDM mixture and PTX-IDM nano-assemblies. Attractively, the concentration of IL-10, which can promote angiogenesis and proliferation of cancer cells, was dramatically decreased only in PTX-IDM nano-assemblies treated group whereas all the control groups represents high concentrations (f).

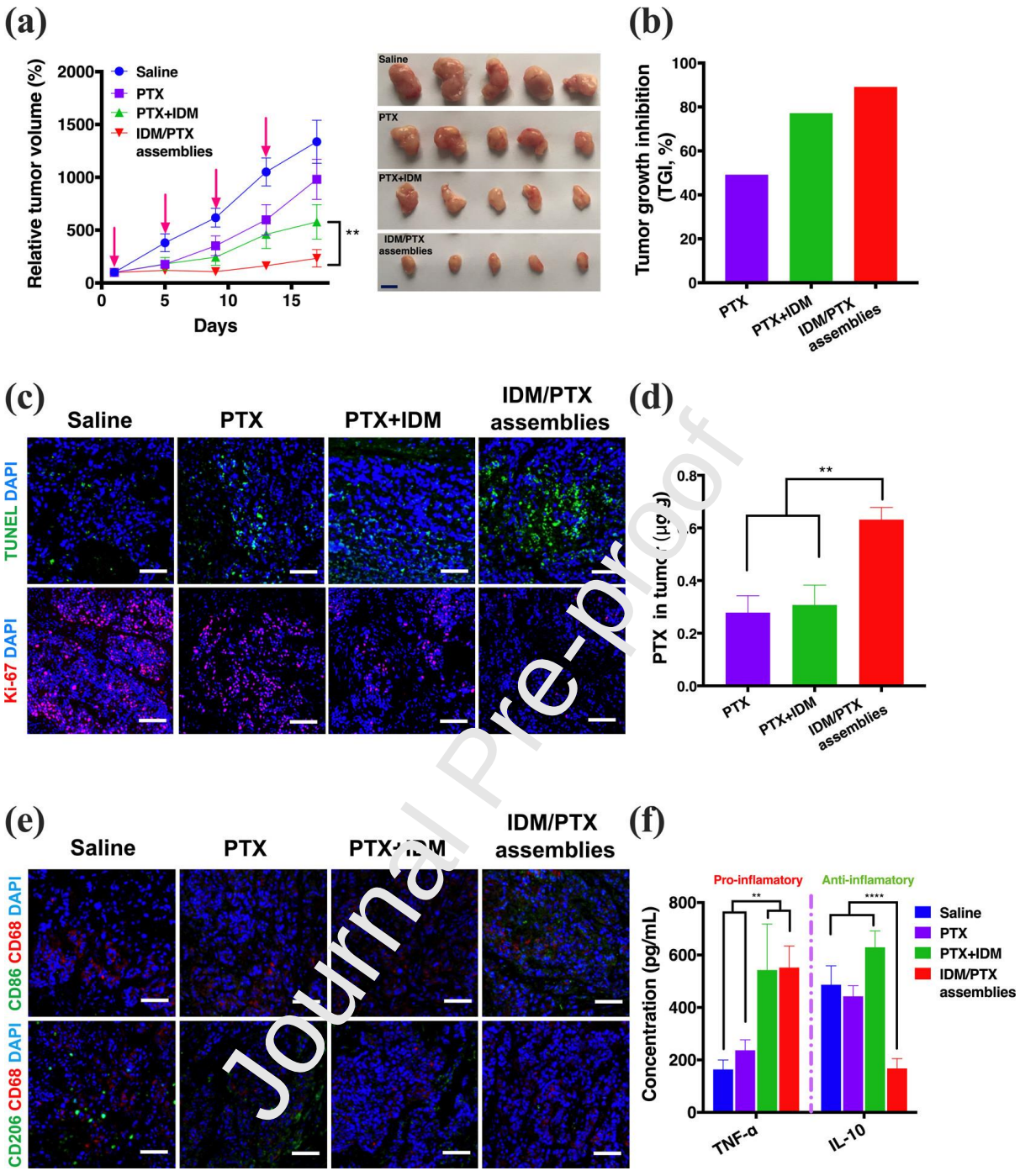

Fig. 19. The anti-tumor effect of PTX-IDM nano-assemblies. (a) tumor volumes (b) tumor growth inhibition (c) Representative immunofluorescence images of Ki-67-positive cells (red) and cells undergoing apoptosis (TUNEL stain; green); scale bars represent 50 μm. (d) the amount of PTX in tumor (e) macrophage staining results (f) the concentrations of pro and

anti-inflammatory cytokines. Copyright 2019. Reproduced from ref [95] with permission from the American Chemical Society.

3.5 Challenges

Despite to all these advantages, there are four key points that should be considered for the design of nanodrug platforms. One of the major challenges in these nanoplatforms is long-term nanotoxicity. At present, carrier-free nanodrug research is at its initial stages and we have not witnessed any clinical translation of these nanodrugs due to the lack of safety and toxicity studies [204]. Second, the properties of carrier-free nanodrugs should be consistent every time they are prepared, i.e. same or similar sizes, shapes, etc., to eliminate batch to batch variations [1]. Recently, microfluidic studies were employed to increase the reproducibility of these nanoparticles by precisely controlling the particle size and composition [205]. These microfluidic devices are also advantageous as some of them can mimic physiological conditions. Traditional preclinical studies are heavily relied on a high number of animal models due to the lack of poor recapitulation in cell culture dishes [206]. Third, the EPR effect requires a better understanding since tumors grow quickly and blood vessels are leaky in mice models resulting in efficient EPR effect. In reality, tumors in human body are different, usually biologically heterogenous consisting of a variety of biological barriers such as irregular vascular networks in the same tumor which we have evaluated in another review, causing inefficient therapies [207]. There are only a few clinically approved carrier-based nanoparticle formulations, such as Abraxane® and Doxil®.

Finally, most carrier-free nanodrugs reported in the literature were focused on therapeutic outcomes rather than the self-assembly mechanism of hydrophobic anti-cancer drugs. A variety of small molecules have been extensively studied as a platform to self-assembly numerous anti-cancer agents. Although there are many successful attempts, the principle for

selection of small molecular agents and drugs seems serendipitous [208]. It is important to rationally design nanoplatforms to achieve efficient cancer therapy; as it becomes clear that all these engineered approaches work in a small percentage of patients. We would like to highlight the importance of: i) π-π stacking interaction which could occur with the use of anti-cancer drugs containing heavy aromatic structures, ii) ionic interactions by using molecules with ionic groups and iii) hydrogen bonding between molecules which have oxygen and nitrogen atoms. These interactions should be considered to achieve stable nanoplatforms and also was a subject of another review [209].

4 Conclusions and future outlook

In this review, we have highlighted the recent progress in the synthesis and applications of carrier-free nanodrugs. The summarized work clearly demonstrates the advantages and versatility of carrier-free nanodrug systems for cancer therapy. Facile tuning of hydrophobic drug molecule properties by combining them with functional molecules, such as fatty acids, vitamins, carbohydrates, photosensitizers, immunotherapeutic agents and tumor-targeting peptides, enables the preparation of nanodrugs with improved functional performance. Different self-assembly behaviors can be observed based on the strength and nature of non-covalent interactions such as van der Waals, hydrogen bonds and π-π stackings, resulting in different nano-assembly sizes and morphologies. Incorporation of linkers onto the anti-cancer agents enables the preparation of stimuli responsive nanomedicines with applications in on-target drug release. Tumor-responsive linkers are dominated by ester, disulfide and thioether bonds but selenium and diselenide bonds have been recently emerging. With more synthetic approaches for the preparation of carrier-free nanodrugs than ever, we expect a range of new carrier-free formulations to be developed and employed in cancer therapy in future.

Studies to understand the precise mechanism behind the self-assembly of hydrophobic drugs will help to achieve more rational carrier-free nanodrug designs. It is predicted that a majority of these new formulation will target the tumor-microenvironment using various linkers, peptide sequences and/or other targeting molecules. Such systems have the potential of overcoming side effects on healthy cells and tissues and achieving deep tumor penetration thereby increasing survival rates. In addition, with more knowledge about immuno-suppressive and activating cells and pathways, the combinational use of immunotherapy with anti-cancer agents will promote the success of carrier-free nanodrugs. Eventually, these nanodrugs should be tuned to optimize their key properties such as size, shape, surface chemistry and long-term stability. Further studies should focus on the clinical safety of both pharmaceutically active ingredients and materials used for surface modifications, enabling their clinical practice in a timely manner. Finally, preparing stable nanodrugs at high drug concentrations is amongst the most important necessity for clinical applications alongside the need for them to be relatively easy to prepare and cost-effective. With the mentioned foundations in this review, this field is only expected to grow even more rapidly with exciting novel nanodrugs emerging with an overarching goal of developing a cancer therapy capable of overcoming MDR, achieving prolonged circulation times, enhanced anti-tumor efficacy, and deep tumor penetration, and possessing safe profiles to facilitate their clinical applications.

5 Acknowledgements

This work was supported Royal Society Edinburgh (RSE)-National Natural Science Foundation of China (NSFC) International Joint Project Grants, Cancer Research UK, National Natural Science Foundation of China (2167315, and 11811530640). Sena Karaosmanoglu would like to thank the Republic of Turkey Ministry of National Education Directorate General for the Higher and Overseas Education Studentship.

6 Declaration of competing interest

The authors declare no conflict of interest.

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Drug	Solvent	Anti-solvent	Cancer type	Refs
DOX	DMSO	water	KB and 4T1 cells and 4T1 tumor	[71]
CUR	tetrahydrofuran	water	CT-26 cells and tumor	[74]
CPT, HCPT	DMSO	PBS	-	[76]
HCPT	ethanol	water	KB cells, 4T1 tumor	[77]

Lipid	Anti-cancer drug	Linkage	Drug loading (wt %)	Size (nm)	Cancer cells	Refs
VE	PTX	-S-S-	60%	113 ± 5	KB-3-1 tumors	[102]
	CUR	-S-S-	27.3 ± 0.4 %	29.8 ± 0.9	HepG2 cells	[153]
	DTX	ester	75.4%	180.2 ± 3	A549 and PC-3 cells/ A549 tumor	[154]
		-S-S-	82.0%	173.8 ± 1.5		
	DOX	amide	34%	234	MCF-7 cells and CT26 tumor	[20]
RA	PTX	ester	75%	170.2	MCF-7 and B16F10 cells	[155]
LOA	PTX	Ester	90%	105	B16-F10 cells, MDA-MB-231, U87-MG cells	[150]
	DTX	-S-	53.4%	100	4T1 cells	[21]
	CPT	-etcS-S-	-	-	Lewis lung carcinoma	[12]
OA	PTX	ester	66%	100	KB-3-1, H460, and OVCAR-8 cells/ KB-3-1 tumor	[5]
		-S-S-	56%			
		-S-	57.4%			[146]
		-2S-	54.9-66.5%			
	SN-38	-etcS-S-	45%	114.9	CT26 cells, murine 4T1 cells and B16-F10 cells	[15]
	CPT	-etcS-S-	-	220	Lewis lung carcinoma	[12]
		-hec-	-	240		
		Carbonate ester	-	231		
		ester	-			
	DTX	-S-S-	50.4%	75-95	4T1 cells and tumor	[151]
		ester	60.2%			
		-S-	60	30	-	[143]
CIT	PTX	-C-	50%	90	KB, A549 and 4T1 cells and tumors	[157]
		-C-C-				
		-S-				
		-S-S-				
		-Se-				
		-Se-Se-				
SQ	DOX	ester	57%	130	MiaPaCa-2 cells or M109 cells and tumors	[158]

Carrier-free nanodrug	Nanodrug $t_{1/2}$	Free drug $t_{1/2}$	Refs
PTX-S-S-VE NPs	25.74 ± 7.66 h	1.47 ± 0.16 h (Taxol)	[102]
UA-PTX NPs	6-fold longer	No specific value (PTX)	[189]
hICP NPs	7.97 h	1.44 h (SN-38)	[185]
PTX-ICG NPs	3-fold longer	No specific value (PTX)	[97]
DOX NPs	3 h	<10 mins (DOX)	[71]
PhD NPs	31.8 min	4.4 min (DOX)	[123]
DOX-PDA-gossypol NPs	50.381 h	0.1[illegible] h (DOX)	[98]

Highlights

- Illustrated the necessity of making carrier-free nanodrugs for safe and effective cancer therapy
- Summarized methods of making carrier-free nanodrugs from hydrophobic drug molecules
- Evaluated the performance of carrier-free nanodrugs
- Discussed about the advantages, and challenges of carrier-free nanodrugs
- Envisaged the future development of carrier-free nanodrugs