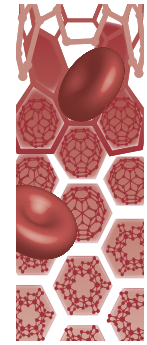
Review

Nanomedicine



Disulfide-bridged cleavable PEGylation in polymeric nanomedicine for controlled therapeutic delivery

Haiqing Dong[1], Min Tang[1], Yan Li[1], Yongyong Li*[,1], Dong Qian[2] & Donglu Shi**[,1,3]

[1]Shanghai East Hospital, The Institute for Biomedical Engineering & Nano Science (iNANO), Tongji University School of Medicine, Shanghai, China

[2]Department of Mechanical Engineering, University of Texas at Dallas, TX 75080, USA

[3]The Materials Science & Engineering Program, Department of Mechanical & Materials Engineering, College of Engineering & Applied Science, University of Cincinnati, Cincinnati, OH 45221, USA

*Author for correspondence: yongyong_li@tongji.edu.cn

**Author for correspondence: donglu.shi@uc.edu

PEGylation in polymeric nanomedicine has gained substantial predominance in biomedical applications due to its resistance to protein absorption, which is critically important for a therapeutic delivery system in blood circulation. The shielding layer of PEGylation, however, creates significant steric hindrance that negatively impacts cellular uptake and intracellular distribution at the target site. This unexpected effect compromises the biological efficacy of the encapsulated payload. To address this issue, one of the key strategies is to tether the disulfide bond to PEG for constructing a disulfide-bridged cleavable PEGylation. The reversible disulfide bond can be cleaved to enable selective PEG detachment. This article provides an overview on the strategy, method and progress of PEGylation nanosystem with the cleavable disulfide bond.

Keywords: cleavable PEGylation • disulfide bond • drug delivery • gene delivery • micelles • prodrug • vesicles

Background

Poly(ethylene glycol) (PEG) is undoubtedly the most important nonionic hydrophilic polymers for surface modification, bioconjugation, drug delivery and tissue engineering [1–4]. PEGylation describes a process of covalent attachment of PEG chains to another molecule, which can be a therapeutic agent [3] or nanosystem (such as nanoparticles, colloids, vesicles etc.) [2] in medical therapy. PEG inherently exhibits biocompatibility, nonimmunogenicity and ease of excretion from living organisms [1,5]. PEGylation has shown several advantages in biomedical applications. The primary benefit is to impart resistance of the nanosystem toward protein adsorption. PEGylation can provide a robust steric effect that keeps the inner core from proteins in circulation. PEGylation can also regulate pharmaceutical kinetics for anticancer drugs of poor bioavailability and improve biocompatibility of a synthetic or bioderived system. So far, many PEGylated products have been approved for clinical applications in market, such as Doxil/caelyx (PEGylated liposome of doxorubicin), PEGASYS and Pegintron (both are PEGylated interferon alpha), and several additional products are summarized in the reported work [2]. Some typical products such as NK105 and Genexol-PM for paclitaxel delivery; NK911 and SP1049C for doxorubicin (DOX), and EZN-2208 for SN38 are under clinical trials [2,3,6,7].

Resistance to protein adsorption provided by PEG is of critical importance for a nanoparticulate delivery system that requires a blood contact. When exposed to blood, the nanoparticles (NPs) without shielding coatings have been shown to be opsonized by proteins within minutes and recognized by the mononuclear phagocyte systems [8,9], followed by rapid clearance by liver and spleen [10,11]. Ogris *et al.* reported on the DNA/transferrin-polyethylenimine (800 kDa) complexes before and after covalent coupling of PEG. Upon incubation with plasma, the positively charged non-PEGylated DNA complexes form aggregates. *In vivo* gene delivery trails also showed a strong aggregation of erythrocytes while PEGylation of the complexes strongly

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reduces plasma protein binding and erythrocyte aggregation [12]. Thus, shielding of NPs from opsonization and blood clearance is essential, known as the stealth effect [13]. At present, PEGylation represents the gold standard for the stealth polymers. PEG is highly hydrophilic, which can help 'shield' hydrophobic NPs from opsonizing by blood proteins [12,14–16]. However, the shielding layer of PEG can create steric hindrance that may negatively impact cellular uptake and intracellular distribution at the target site [17,18]. Furthermore, the PEG layer may also pose a significant diffusion barrier to the release of encapsulated payload, and adversely affecting therapeutic efficacy [17,18].

Several chemical approaches have been developed to address the above critical issues so as to improve biological efficacy of therapeutics nanosystem with the shielding PEG layer (mostly short-chain PEG) [8,11]. In general, the main objective of these strategies is to remove the PEG shell upon arrival at the target site (i.e., cleavable PEGylation) [15]. To meet this specific demand, one of the key strategies is to incorporate a disulfide bond (S–S) between the PEG segment and substrates. The disulfide bond can be selectively cleaved in tumor milieu, especially in the intracellular region, by the significant concentration gradient of glutathione (gamma-glutamyl-cysteinyl-glycine; GSH) [19–21]. The intracellular GSH concentration is almost 3 orders of magnitude higher than that of cellular exterior [19,22]. Extracellular low GSH concentration renders a high stability of disulfide-based nanovehicles [23]. The disulfide bonds are cleaved by high intracellular GSH leading to rapid release of payloads for effective cancer cell killing (Figure 1). The concept, as shown in Figure 1, has been confirmed to be viable and effective in a variety of nanoformulations for different biomedical applications [24–27]. The previous works have shown effective localization of the cleavable PEGylated system in tumor area [28–30].

Recently, considerable efforts have been devoted to the development of the disulfide-bridged cleavable PEGylation for both anticancer drug and gene delivery. This review summarizes the strategy, method and current progress on the design and development of cleavable PEGylation nanosystems including micelles,

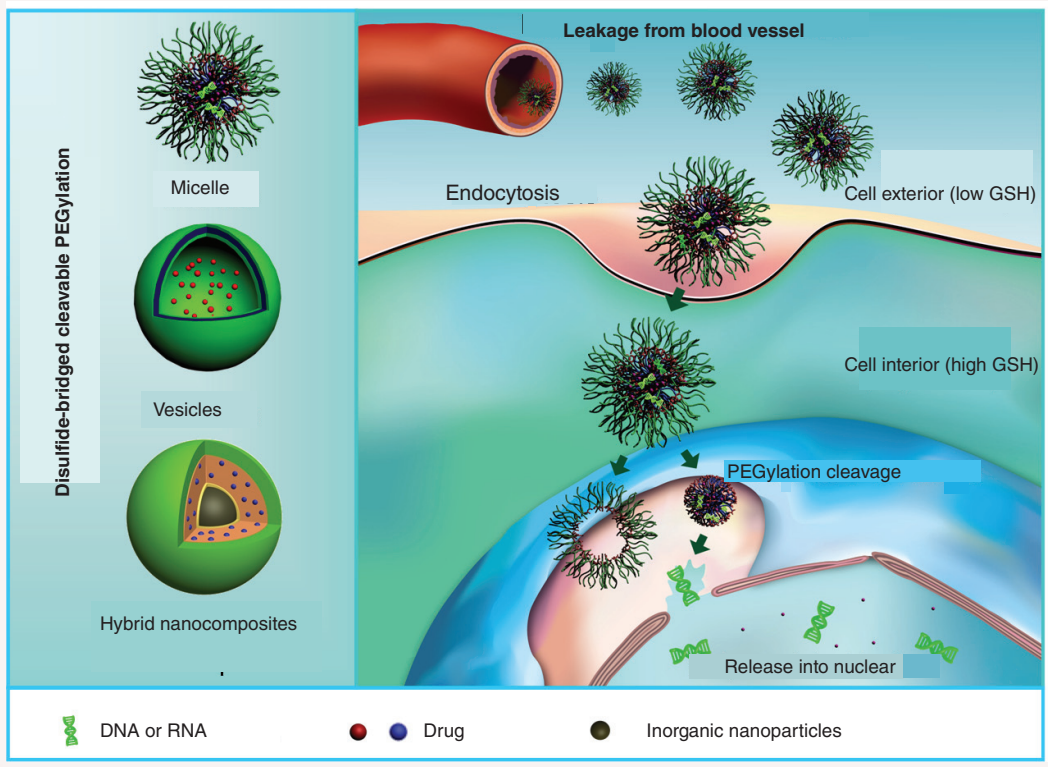

Figure 1. Nanoformulations engineered with disulfide-bridged cleavable PEGylation (left) and their function pathway from blood vessel leakage, and cell endocytosis to drug/gene release inside the cell. The release behavior can be regulated by selective intracellular PEGylation cleavage.
GSH: Gamma-glutamyl-cysteinyl-glycine.
For color images please see online at: www.futuremedicine.com/doi/full/10.2217/NNM.15.38

vesicles and prodrug nanosystem. The challenges and perspectives are also presented.

GSH-sensitive disulfide for cleavable PEGylation

One of the key issues in cancer therapy deals with efficient drug delivery at the pathological site. Drug delivery systems (DDs) based on polymeric micelles, vesicles, prodrugs and organic-inorganic nanoparticles with selective drug release behavior in response to a specific signal (from tumor milieu or external stimulus) have been developed and comprehensively summarized in other reviews [31–36]. For example: those physical signals can be temperature, pH, light, magnetic field, ultrasound and so forth [33,37]. Some of the stimulus sensitive DDSs have been clinically evaluated (ThermoDoxs®) or even already approved for clinical use (NanoTherm®) [31]. Despite enormous efforts devoted with a variety of strategies, most of works are not viable for potential *in vivo* applications. For instances: UV light [38] as a stimulus is not biocompatible and the high temperature [39] environment required is hardly achieved *in vivo*. The pH or temperature variations between the abnormal and normal tissue are generally not sufficiently high for selective drug release [32,34,40]. GSH, a tripeptide, is the most abundant low-molecular-weight thiol in organism such as animals, for regulating the cellular reductive microenvironment. GSH concentration in the intracellular space, such as cytosol, mitochondria and cell nucleus, reaches as high as 3–10 mM, almost three orders of magnitude higher than that in cellular exterior such as plasma (~2.8 μM) [19,22]. Furthermore, GSH in tumor tissues is at least fourfold higher than that in normal tissues [41,42]. The sharp differences in GSH levels between tumor and normal cells provide the possibility for the structure design of the carrier system based on the disulfide-bridged nanoparticles. The nanosystems, based on the GSH-sensitive disulfide bond, can enable intracellular drug/gene delivery and regulate the intracellular fates of the delivered therapeutic agents.

Polymeric nanosystem with disulfide-bridged cleavable PEGylation

Among various polymeric nanocarriers, the disulfide-bond-linked cleavable shells have recently attracted broad research interests [17,43,44]. These particulate formulations are generally composed of an inner core with encapsulated therapeutic agents and surrounded by a hydrophilic, cleavable PEG shell [30,44,45]. The detachable process occurs at a threshold GSH concentration in a targeted region, for instance, inside a cancer cell. Normally, these particulate formulations with disulfide bond-linked cleavable shells are chemically stable without obvious drug leakage due to structural integrity. The shedding of the shell takes place in redox environment via disulfide cleavage as a result of GSH variation [17]. The disassembly of the system would trigger fast drug release intracellularly. Different from the conventional stimuli-responsive formulations, the structural disassembly of the PEGylated nanovehicles is fast and complete. The entire PEG shell can be completely removed from the core, exposing therapeutic payload intracellularly. Therefore, cleavable PEGylation is capable of much more efficient therapeutic agent release in a controlled fashion.

Strategies of disulfide-bridged cleavable PEGylation

Pegylation has been employed on a variety of substrates, resulting in conjugates with combined functionalities of PEG and the other polymer [46–48]. The common pegylation is based on the PEG derivatives, such as terminal hydroxyl, primary amine, carboxyl acid and thiol groups, that can initiate a reaction with the matrix functional groups. For instance, poly[bis(ε-amino-L-lysine) Glut-PEG] was obtained by carbodiimide-assisted amidation reaction between *N*-hydroxy succinimide (NHS) on NHS-PEG-NHS and bis(ε-amino-L-lysine) [49]. Disulfide-bridged cleavable PEGylation can also be achieved in the similar fashion. The general strategy is to introduce S–S linkage into PEG. The cross-linker is typically employed with the S–S moiety. These include cystamine dihydrochloride [22,25,26,43,50–54], 2,2-dithiodiethanol (DTDE) [55,56], 3,3′-dithiodipropionic acid (DPA) [57–60], *N*,*N*′-cystaminebisacrylamide [61], 2,2′-dithiodipyridine [27,44,62], 2-(pyridyldithio)-propionic acid [63], cystamine bisacrylamide [64], *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) [65,66] and sulfosuccinimidyl 6-(3′-[2-pyridyldithio]-propionamido) hexanoate (sulfo-LC-SPDP) [67]. Alternative approaches involve conjugation of the thiol group (-SH) with PEG or other polymers, and subsequent oxidation of –SH with oxidants such as *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) [68] and pyridyl disulfide carbonate [69] in order to form the S–S bond. The disulfide agents employed as a cross-linker in a variety of cleavable PEGylated nanosystems are summarized in Table 1 with information of their key physiochemical features and the biological model.

Cleavable PEGylated nanosystems in drug delivery system

Polymeric micelles

PMs have been proven to be a promising and clinically relevant platform for drug delivery [70]. PMs are generally formed by supramolecular assemblies of amphi-

Table 1. Overview of representative PEGylated nanosystems engineered with disulfide bond for drug or gene delivery.

System	Materials	Disulfide agent employed	D_{hydro} (nm)	ζ (mV)	Payload	Model	Ref.
Polymeric micelles	6sPCL-SS-PEG	DPA	35	–	DOX	MCF-7 cells	[71]
	mPEG-SS-PzLL	DPA	302	–	DOX	MCF-7 cells	[72]
	mPEG-SS-Pleu	Cystamine dihydrochloride	160	–	DOX	HepG2 cells	[73]
	PEG-SS-PBLG	Cystamine dihydrochloride	137	–	DOX	SCC7 cells	[54]
	PEG-SS-PBLG	Cystamine dihydrochloride	107	–	SN-38	L929 cells	[56]
	PEG-SS-PLys-PLeu	Cystamine dihydrochloride	150	-3.52	CPT	HeLa cells	[22]
Vesicles	PEG-b-PLys(Z)-SS-PCL	SPDP	256	43.9 ± 1.61	DOX·HCl; CPT	SCC7 cells	[66]
	PzLL-SS-PEG-SS-PzLL	Cystamine dihydrochloride	380	–	DOX·HCl; GC·HCl	MDA-MB-231 cells	[26]
	PEG-SS-PCL	2,2′-dithiodipyridine	210.0	0.40 ± 0.2	Cytochrome C (CC) proteins; recombinant human granzyme B	MCF-7 cells, HepG2 cells	[44]
	PEG-SS-PDEA	Cystamine dihydrochloride	54.5–66.8	–	FITC-BSA; FITC-CC	MCF-7 cells	[74]
Prodrug system	$(MTX)_2PEG(MTX)_2$	Cystamine dihydrochloride	278	–	MTX	HepG2 cells	[25]
	CPT-SS-PEG-SS-CPT	Cystamine dihydrochloride	226	–	CPT	HepG2 cells	[45]
	P(PTX-DTPA-HEMA)-*co*-PPEGMEA	DPA	135	–	PTX	HEK-293 cells; HeLa cells	[75]
Organic–inorganic nanocomposite	NGO-SS-mPEG	Cysteamine hydrochloride	220	–	DOX·HCl	HeLa cells	[76]
	MSNs-SS-mPEG	mPEG-SS-Pyridine	100	-27.8	Fluorescein dye	MCF-7 cells	[24]
	S-P-βCD; S-T-βCD; S-MT-βCD	SPDP; SPT; SMPT	<100	–	Rhodamine B dye	Zebrafish (Danio rerio)	[77]
Gene vector	mPEG-SS-Lys_n-r-His_m	Cystamine dihydrochloride	200	Positive at the weight ratio >2	pDNA; VEGF-siRNA	Mice bearing HepG2 tumor	[50]
	mPEG-SS-PLH	Cystamine	180	-30 + 20	VEGF-siRNA	Mice bearing HeLa tumor	[30]
	RGD-PEG-SS-PEI	SPDP	339.5 (N/P = 4)	0.4 ± 0.03 (N/P = 4)	pDNA	Mice bearing U87 tumor	[65]
	4-arm PEG-SSPHIS	CBA	135–150	+(5 – 10)	pDNA	Mice bearing HepG2 tumor	[78]

ζ: Zeta potential; CBA: Cystamine bisacrylamide; CPT: Camptothecin; D_{hydro}: Hydrodynamic diameter; DOX: Doxorubicin; DPA: 3,3′-Dithiodipropionic acid; FITC-BSA: Fluorescein isothiocyanate labeled bovine serum albumin; FITC-CC: Fluorescein isothiocyanate labeled cytochrome C; GC: Gemcitabine; HCl: Hydrochloride; –: Not reported; N/P: Ratio of nitrogen to phosphorous; MTX: Methotrexate; PTX: Paclitaxel; SPDP: (*N*-succinimidyl 3-(2-pyridyldithio)-propionate.

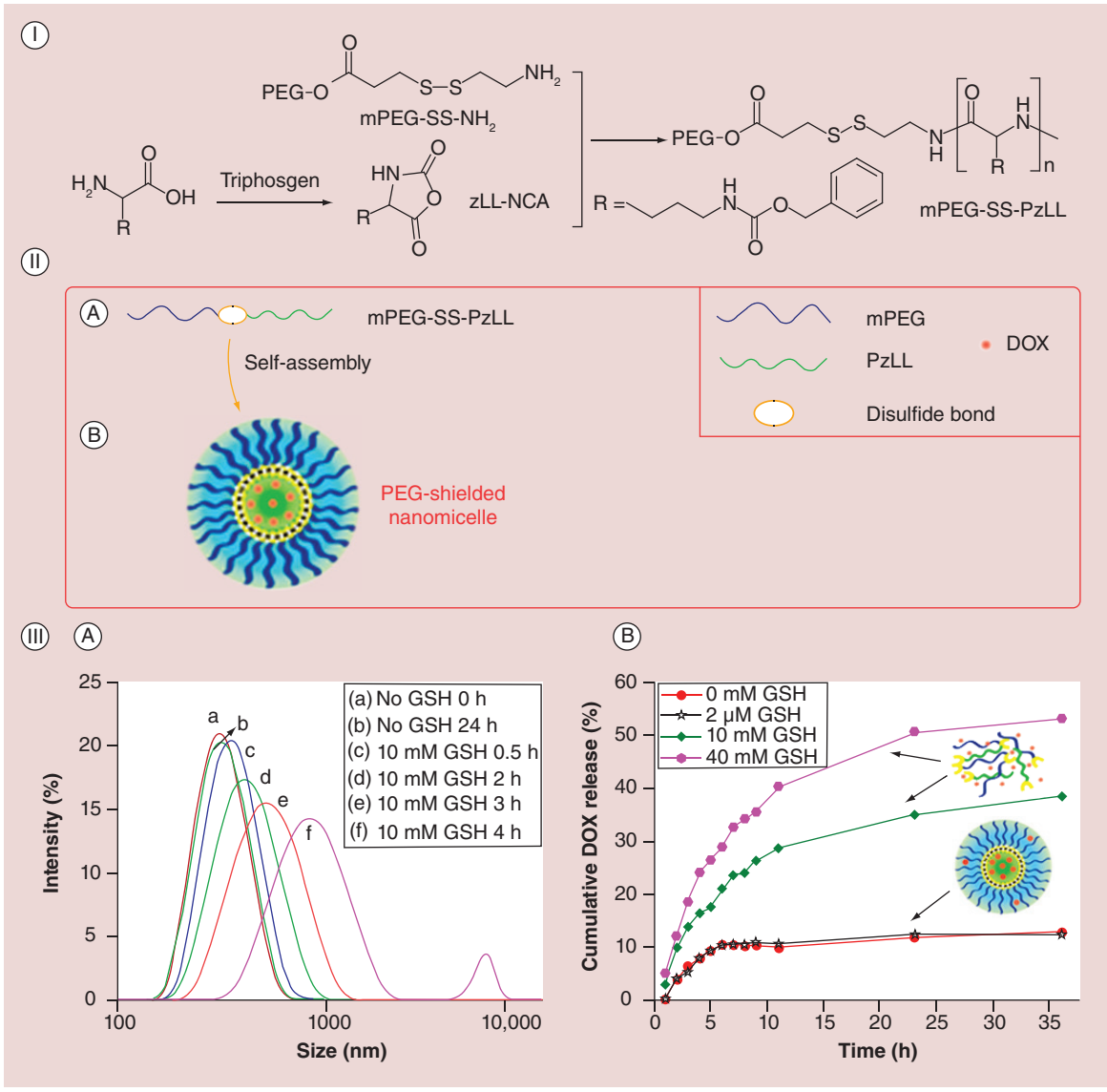

Figure 2. Polymeric micelles with cleavable poly(ethylene glycol) shell. (I) Synthesis of mPEG-SS-PzLL copolymer via disulfide bond linkage. **(II)(A)** Schematic illustrations of amphiphilic mPEG-SS-PzLL with disulfide linkage; **(B)** PEG-shielded nanomicelle; **(III)** Results of the GSH triggered micellar structure arrangement as well as drug release of redox-sensitive, DOX-loaded mPEG-SS-PzLL nanomicelles. **(A)** Time-dependent size change of mPEG-SS-PzLL15 micelle upon exposure to 10 mM GSH as determined by DLS; **(B)** GSH-mediated drug release from DOX-loaded mPEG-SS-PzLL nanomicelles in phosphate-buffered saline.
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philic polymers that possess unique core-shell structure. The inner core can be an efficient reservoir for drug encapsulation, which is protected by the hydrophilic shell, a necessary interface between the core and the external environment. PMs are highly multifunctional including enhanced drug solubility, extended circulation time *in vivo* and passive/active targeting. Compared with conventional liposome, they are more stable due to lower critical micelle concentration. The introduction of disulfide bond to PEGylated polymeric micelles enables more efficient drug release, making it an ideal bioresponsive delivery system.

Our group has made considerable efforts in the past few years in the development of redox-responsive micelles. In these unique micelles, PEG was used as the hydrophilic and polypeptides as hydrophobic segments [22,25,45,72,79,80]. A disulfide-bridged diblock copolymer: poly(ethylene glycol) methyl ether-*b*-poly(ε-benzyloxycarbonyl-L-lysine; mPEG-SS-PzLL) was synthesized via ring-opening polymerization of ε-benzyloxycarbonyl-L-lysine *N*-carboxyanhydride, initiated by mPEG-amino. The amphiphilic copolymer self-assembled in aqueous solution resulting in bioreducible redox-responsive micelles. At a given

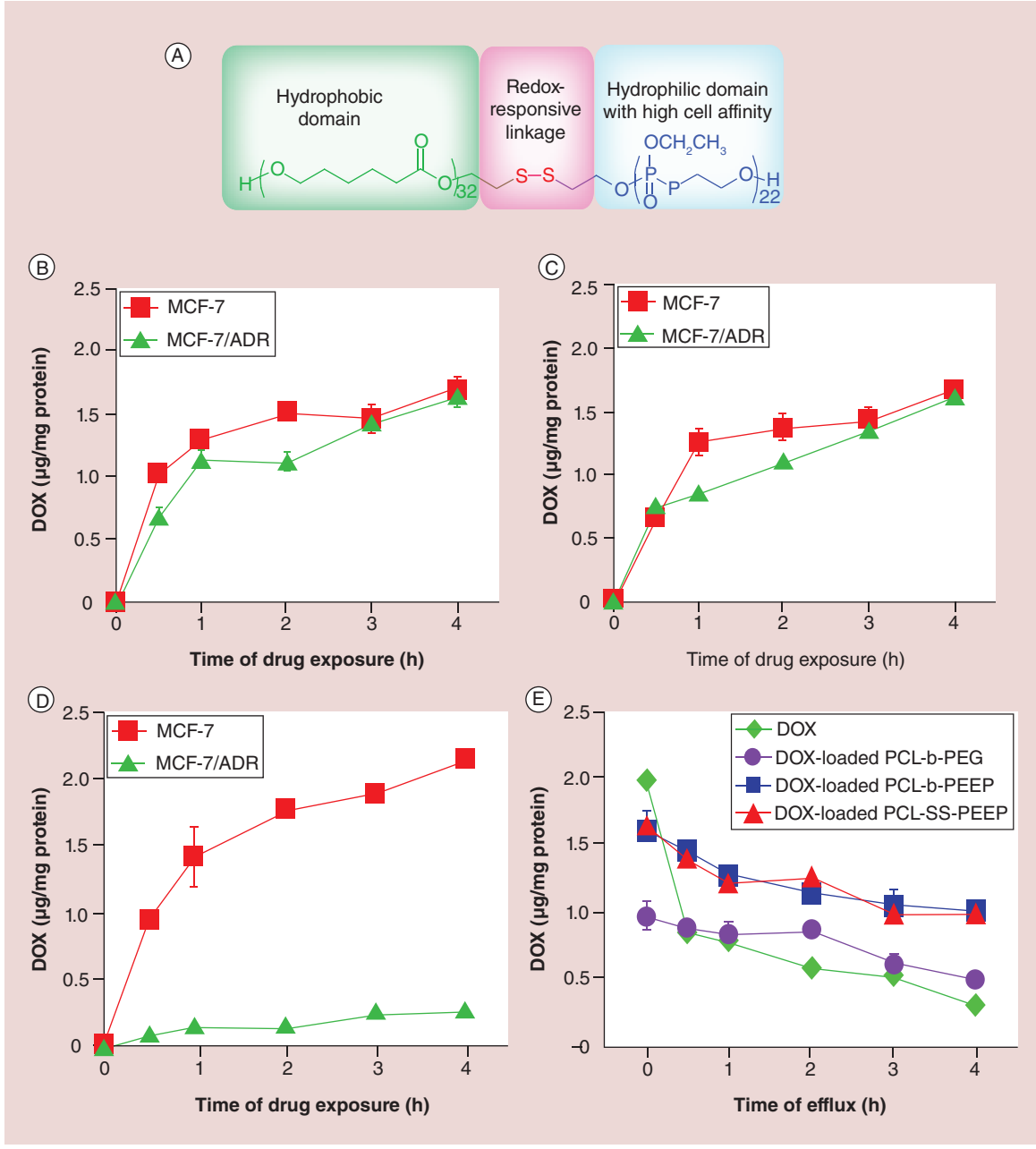

Figure 3. Polymeric micelles with cleavable poly(ethyl ethylene phosphate) shell. (A) Chemical structure of disulfide-bridged PCL-SS-PEEP block copolymer. DOX accumulation in wild-type MCF-7 and drug-resistant MCF-7/ADR breast cancer cells after incubation with **(B)** DOX-loaded PCL-b-PEEP, **(C)** DOX-loaded PCL-SS-PEEP nanoparticles and **(D)** free DOX and **(E)** retention of DOX in MCF-7/ADR cells after preincubation with free DOX (DOX), DOX-loaded PCL-b-PEG, DOX-loaded PCL-b-PEEP and DOX-loaded PCL-SS-PEEP nanoparticles for 4 h. The concentration of DOX in the free DOX preincubation is 40 μg mL–1, and 5 μg mL–1 for DOX-loaded nanoparticles. DOX: Doxorubicin; PEEP: Poly(ethyl ethylene phosphate).
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GSH concentration (2 μM; equivalent with GSH concentration in human blood), the micelles exhibited high stability without obvious size alteration and a nearly identical drug (DOX) release behavior in buffer solution without GSH. By contrast, much higher intracellular GSH concentration (10 mM) triggered PEG layer detachment with the exposure of the inner core and thus resulted in a faster release of fourfold, as compared with the control groups (Figure 2) [72]. The size increase in DLS monitoring in 10 mM GSH concentration is due to gradual aggregation of the hydrophobic inner cores as they are not thermodynamically stable. DOX release triggered by different extracellular GSH concentrations further

affected MCF-7 tumor cell viability. Cytotoxicity is more pronounced at higher GSH concentration.

Micelle stability is critically important for its *in vivo* performance since dilution in blood can result in structural disassembly and premature release of the encapsulated drug. To improve the stability of micelles, cross-linking is a commonly employed strategy. Tri-block copolymer poly(ethylene glycol)-*b*-poly(L-lysine)-*b*-poly (rac-leucine) (PEG-SS-PLys-PLeu) with the disulfide bond between PEG and the other segments was proposed and developed, in which the primary amine groups on PLys chains can be further linked by a disulfide-bond cross-linker. After encapsulation of anticancer drug camptothecin, the micelles were found to exhibit not only reduced drug loss in extracellular environments, but also drastically accelerated drug release at the cytoplasmic GSH level, leading to enhanced growth inhibition toward HeLa cells [22]. These results demonstrated an important role of the disulfide bonds in controlled intracellular drug delivery.

As an alternative to PEG, poly(ethyl ethylene phosphate) (PEEP) can also be used as the hydrophilic chains for the micelle shell (Figure 3). The micelles with the PEEP shell exhibit higher affinity to the cancer cells than its PEG counterparts [81]. Disulfide-bridged block copolymer of poly(ε-caprolactone) and poly(ethyl ethylene phosphate) (PCL-SS-PEEP) were found with high drug accumulation and retention in multidrug resisting cancer cells [81]. It was demonstrated that micelles with the PEEP shell increased the influx but decreased the efflux of DOX by the multidrug resistant MCF-7/ADR breast cancer cells, in comparison with the direct incubation of MCF-7/ADR cells with DOX.

Active targeting can also be achieved by taking advantages of highly accessible functional groups of many polymers. Galactose (Gal) has been conjugated onto PEG-PCL and self-assembled together with PEG-SS-PCL to afford ligand-directed redox-responsive shell sheddable biodegradable micelles as shown in Figure 4 [27]. *In vitro* 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of HeLa and HepG2 cells showed apparent targeting ability of the DOX-loaded PEG-SS-PCL-Gal micelles and significantly enhanced growth-inhibition efficacy toward asialoglycoprotein receptor-overexpressing HepG2 cells. Flow cytometry revealed much higher cellular DOX level in HepG2 cells when treated with DOX-loaded PEG-SS-PCL-Gal micelles, compared with redox-insensitive PEG-PCL-Gal and nontargeting PEG-SS-PCL controls. These results indicate the pronounced effects of combined shell-shedding and active targeting.

Vesicles

As one of the important self-assembled nanostructures, vesicles, characterized with a hollow morphology surrounded by a bilayered membrane, have attracted ever increasing attention for promising applications in drug and gene delivery [82–84], nanoreactors [85] and artificial cell membranes [86]. Similar to micelles, polymeric vesicles are also self-assembled from amphiphilic macromolecules or lipids but with a larger hydrophobic/hydrophilic segment ratio. The hydrophilic membrane extends in water and forms an outlayer. The interlayer between the two outlayers is composed of a hydrophobic segment. Such a distinct structure enables a payload of the hydrophobic drugs (e.g., taxol, doxorubicin) in the interlayer and hydrophilic drugs (e.g., amino acids, peptides and proteins) in the inner hollow core.

In 1996, Kirpotin introduced pioneering work [87] on reduction-responsive polymeric vesicles which were subjected to rapid shedding of PEG outlayer upon reduction condition, concomitant with burst release of fluorescent dyes. Since then, considerable attention has been paid to the PEG containing vesicles, paving a new path to the application of drug vehicles. Park *et al.* [66] engineered vesicles based on triblock copolymer PEG-*b*-Plys-SS-PCL with PCL as a hydrophobic part and PLys

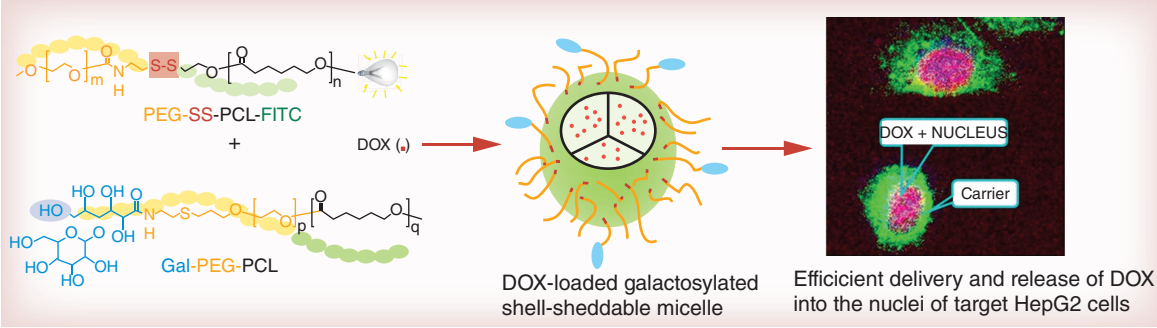

Figure 4. Ligand-directed, reduction-sensitive, shell-sheddable, biodegradable micelles based on PEG-SS-PCL and Gal-PEG-PCL copolymers actively delivering DOX into the nuclei of asialoglycoprotein receptor (ASGP-R)-overexpressing hepatocellular carcinoma cells.

DOX: Doxorubicin.

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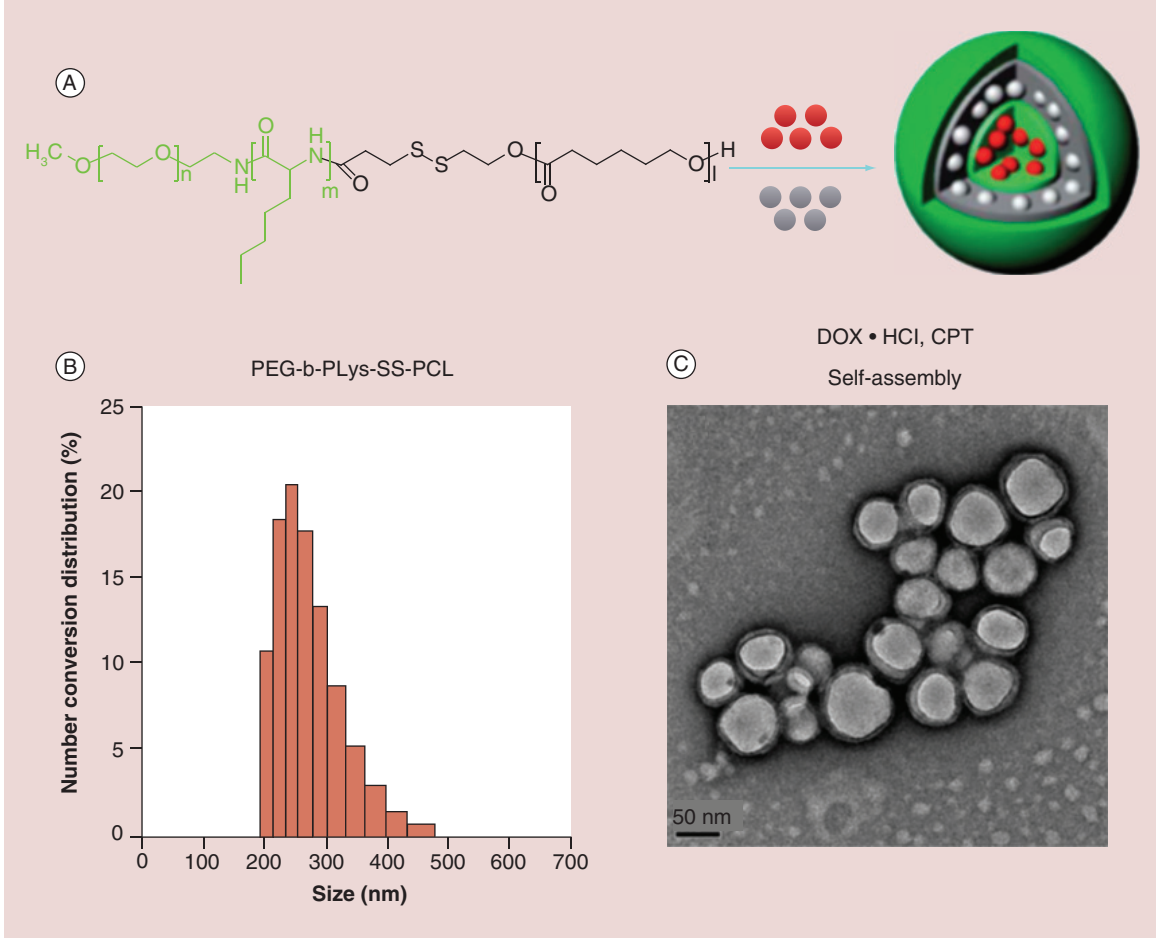

Figure 5. Cleavable vesicles. (A) Structure and schematic illustration of self-assembly of PEG-b-PLys-SS-PCL; **(B)** size distribution and **(C)** TEM images of PEG-b-PLys-SS-PCL.
CPT: Camptothecin; DOX: Doxorubicin; HCl: Hydrochloride.
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as a moiety for improved cell penetration (Figure 5). The vesicles can be used as a dual drug carrier for simultaneously loading of hydrophilic doxorubicin hydrochloride (DOX·HCl) and hydrophobic camptothecin (CPT). The vesicle size increases 1.85-times in the presence of 10 mM GSH while maintaining the same diameter in absence of GSH milieu or vesicles without S–S bond. A great advantage of these vesicles is their multiple drugs carrying ability for cocktail therapy.

By delicately tailoring the hydrophilic/hydrophobic segments, we constructed disulfide-bridged PzLL-SS-PEG-SS-PzLL vesicles with diameter around 380 nm *via* a so-called 'solvent switch' method [26]. These vesicles were developed for overcoming multidrug resistance (MDR) of cancer cells. The overexpression of protein (such as p-glycoprotein) and drug efflux pumps in the MDR cells have been the major obstacles to the success of chemotherapy [88]. The bioreducible vesicles described above were employed to load anticancer drug DOX·HCl or gemcitabine hydrochloride (GC·HCl). A significant acceleration of drug release was observed by GSH triggering (>threefold difference). In the control experiment, while the GC·HCl or vesicles was found insignificant in the GC·HCl-resistant MDA-MB-231 cells, in the concentration range of 0–250 mg/l, the cell viability was lower than 40% when exposing to 250 mg/L GC·HCl loaded vesicles [26]. The results demonstrated high effectiveness of the polymeric vesicles in overcoming MDR, and therefore a possibility to reverse the drug resistance by the drug-encapsulated vesicles (Figure 6).

Therapeutic proteins have emerged as potent medicines for their high specificity, superior anticancer efficacy and low side effects. However, their application has been limited by several challenges including rapid degradation and elimination following iv injection, and poor bioavailability. Zhong *et al.* [44] developed hepatoma-targeting reduction-sensitive vesicles based on complexation of PEG-SS-PCL and a protein binding copolymer for efficient intracellular delivery of proteins (Figure 7). The loading mechanism relies the active interactions of electrostatic and hydrogen bonding between proteins and poly(2-(diethylamino)ethyl methacrylate chains

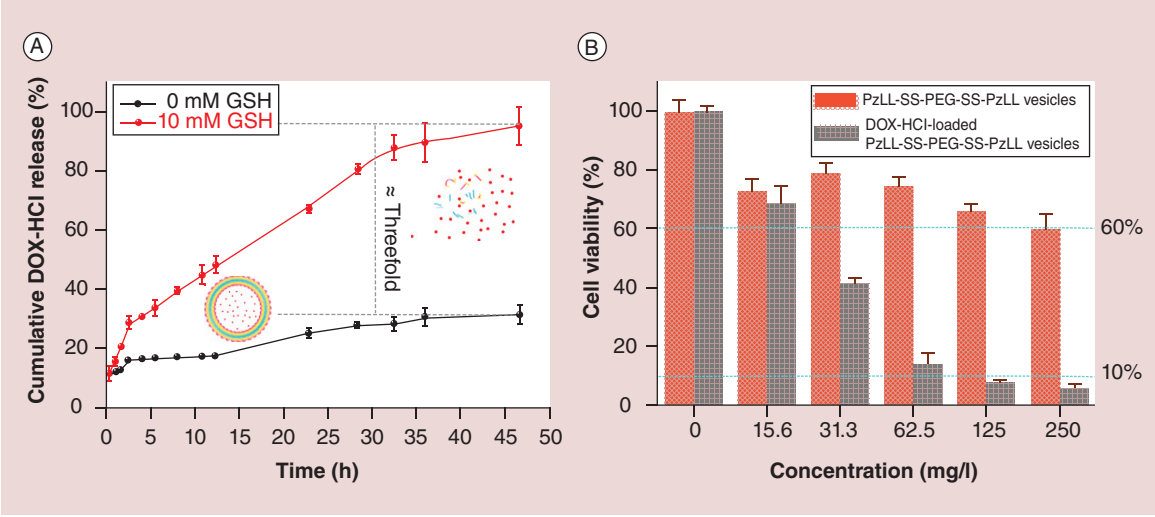

Figure 6. Redox sensitive vesicles. (A) *In vitro* DOX•HCl release from polymeric vesicles in presence and absence of GSH in PBS (pH 7.4). Data are presented as mean ± SD (n = 3). **(B)** Dose-dependent cytotoxicity of PzLL-SS-PEG-SS-PzLL polymeric vesicles 2 alone and DOX·HCl-loaded PzLL-SS-PEG-SS-PzLL polymeric vesicles 2 after 24 h co-incubation. Data are presented as mean ± standard deviation (n = 5).
DOX: Doxorubicin; GSH: Gamma-glutamyl-cysteinyl-glycine; HCl: Hydrochloride; PEG: Poly(ethylene glycol).
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in the protein binding copolymers [74]. The Gal moiety on the vesicle surface facilitates targeting capability to asialoglyco-protein receptor overexpressing hepatoma cells. The *in vitro* release study showed accelerated protein release under a reductive condition of 10 mM dithiothreitol. The cytochrome C loaded, Gal-decorated reduction sensitive vesicles exhibited apparent target-ability and pronounced antitumor activity to HepG2 cells [44]. These reduction-sensitive and biodegradable vesicles offer a robust platform for efficient intracellular protein delivery.

Prodrug systems

The concept 'prodrug' was first proposed by Albert in 1958 to signify pharmacologically inactive chemical derivatives that could be used to alter the physicochemical properties of drugs, in a temporary manner. The prodrug approach gained intensive attention as a technique for improving drug therapy in the early 1970s [89]. Numerous prodrugs have been designed and developed since then to overcome pharmaceutical and pharmacokinetic barriers in clinical drug applications, such as low oral drug absorption, lack of site specificity, chemically instability, toxicity and poor patient acceptance. Many US FDA-approved prodrugs such as protein–polymer drugs are currently being studied in clinical trials [90]. The prodrug approach is generally achieved by conjugation of active drug with different compounds to alter its chemphysical properties, for instance, to make it inert temporarily. When in organisms, the prodrugs may suffer from enzymolysis or chemical degradation (such as GSH).

By incorporation of disulfide-bridged PEGylation into hydrophobic anticancer drugs, our group engineered several reductive responsive prodrug-based micelles by hydrophilicity/hydrophobicity driven self-assembly [25,45]. The micellar prodrug has several major advantages: integration of drug into the carrier (as

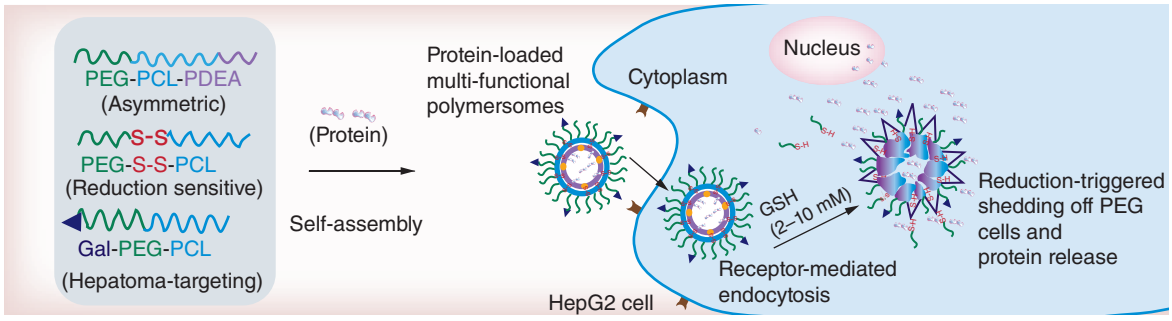

Figure 7. Illustration of the hepatoma-targeting reduction-sensitive biodegradable chimaeric polymersomes for active loading and intracellular release of proteins.
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hydrophobic segment of micelles) that minimizes the use of polymers; drug encapsulation into inner core can avoid premature exposure to body fluid, which is different from the conventional prodrug and selective drug release of the active drug in reductive milieu. The above design is first accomplished by conjugating CPT onto double ends of PEG via disulfide bond to afford CPT-SS-PEG-SS-CPT with CPT loading efficiency up to 20.3% [45]. Hydrophobic CPT enabled the formation of nanomicelles with a size of around 200 nm. Under tumor-relevant reductive conditions, reductive cleavage of the disulfide linker initiates micellar rearrangement associated with the rapid release of the therapeutic payload. It subsequently elicited more pronounced cytotoxicity toward HepG2 cancer cells based on *in vitro* evaluation. In a later work, an H-shaped pegylated methotrexate (MTX) conjugate was synthesized for intracellular drug delivery [25]. The conjugate exhibited a constant MTX loading efficiency up to 26 wt%. The cleavable S–S linkers exerted high therapeutic activity in the intracellular concentration of GSH (Figure 8).

Organic-inorganic nanocomposites

The organic-inorganic nanocomposites are known for their combined advantages of the structural stability and multifunctionality. In recent years, abundant inorganic matrices have been utilized for developing drug carriers, such as nanographene oxide [91], mesoporous silica [92], magnetic iron oxide (Fe_3O_4) [51] and gold nanoparticles [93].

Graphene oxide (GO) exhibits a myriad of unique chemical and physical properties that is being harnessed

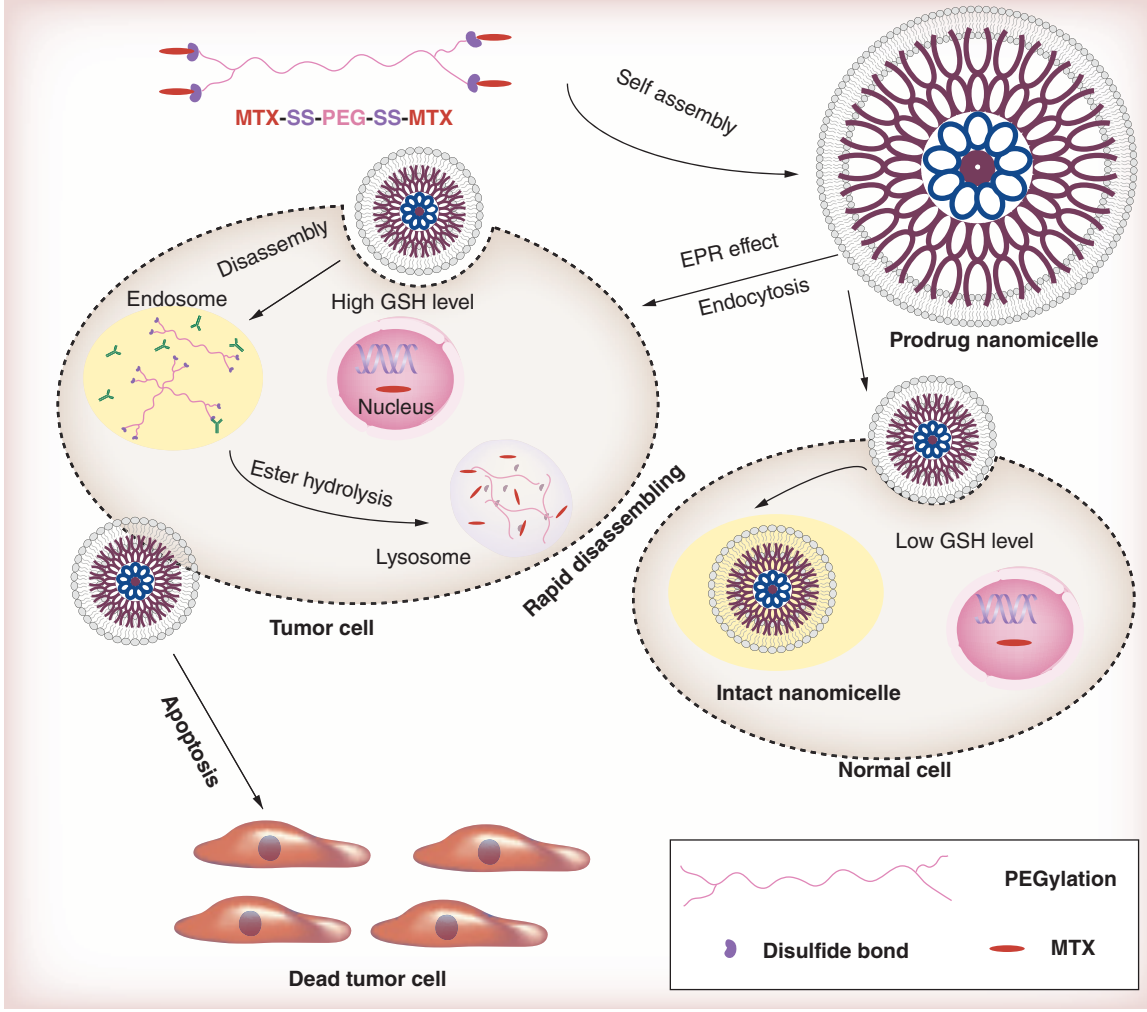

Figure 8. Predicted antitumor activity of redox-sensitive micelles based on H-shaped poly(ethylene glycol)-methotrexate conjugate. The prodrug nanoparticles are internalized from the plasma membrane first and then to endosomes, where they disassemble, triggered by higher concentration of GSH. They are subsequently subjected to lysosomes where MTXylation with ester bond is degraded.
GSH: Gamma-glutamyl-cysteinyl-glycine; MTX: Methotrexate.
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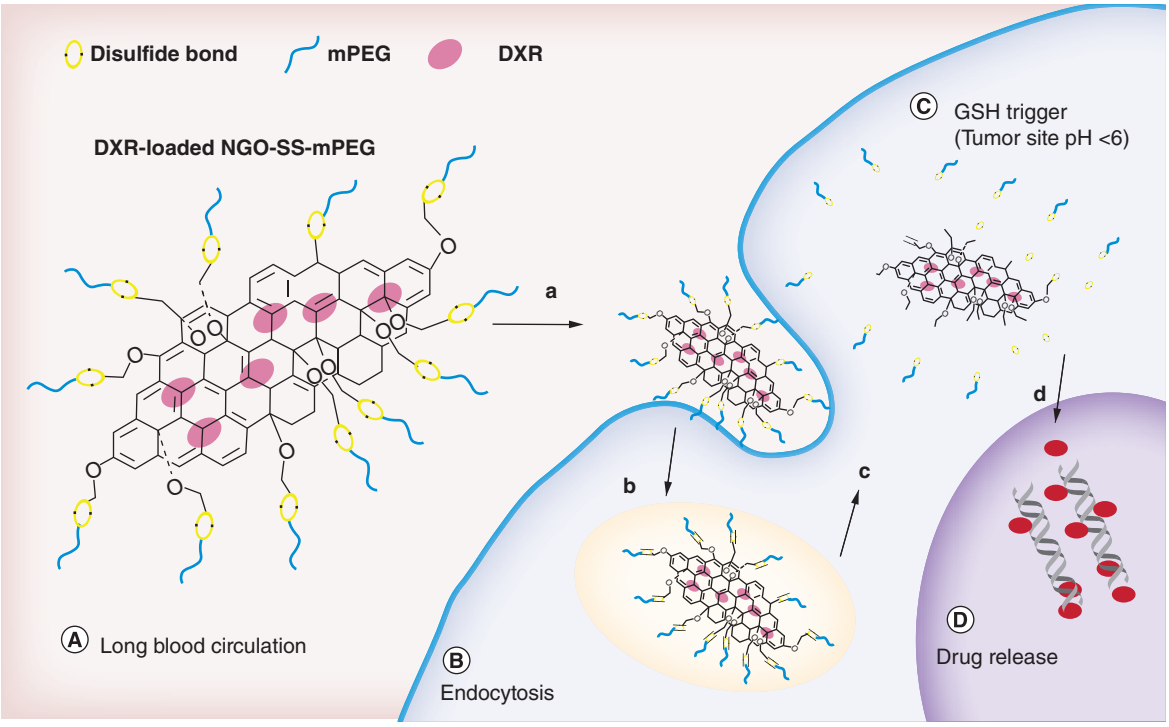

Figure 9. Antitumor activity of redox-sensitive, DXR-loaded NGO-SS-mPEG. (A) PEG-shielded NGO with disulfide linkage for prolonged blood circulation; **(B)** endocytosis of NGO-SS-mPEG in tumor cells via enhanced permeability and retention effect; **(C)** GSH trigger (GSH >fourfold than normal cells) resulting in PEG detachment, and **(D)** rapid drug release on tumor site.
DOX: Doxorubicin; GSH: Gamma-glutamyl-cysteinyl-glycine.
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for highly versatile applications including drug carrier, gene delivery, etc. [94]. GO surface has an abundance of oxygen-containing groups such as carboxyl, hydroxyl and the epoxy groups. The richness of these oxygen-containing groups of GO affords its highly hydrophilic property and respectable stability in aqueous solution. To improve its stability in biological environment, surface coating via chemical or physical method has been attempted for biomedicine applications. Our group [76] engineered PEGylated nanographene oxide (NGO-SS-PEG) with redox-responsive detachable PEGylation for surface functionalization and intracellular drug delivery. In this work, the unique structure design enabled fine dispersivity of the system in various salt-rich solutions-cell culture medium, PBS, etc., which is essential for primitive GO. Meanwhile, it loaded aromatic drugs efficiently via π-π stacking and hydrophobic interaction, and subsequently released the drug into cell cytoplasma at tumor-relevant GSH levels (Figure 9).

Mesoporous silica nanoparticles (MSNs) have been extensively investigated on drug delivery systems for their unique porous structure, tunable pore size, biocompatibility, ease of surface functionalization and overall versatility [77,95]. To achieve effectively controlled drug release, switchable gatekeepers on the MSN surface pore have been proposed. The controlled release can be regulated by the on–off of the pore via a gatekeeper. Nadrah *et al.* used β-cyclodextrin as the pore capping agent to coat the surface of MSN via S–S bond. Three S–S bonds with different levels of hindrance were synthesized to precisely regulate the drug release kinetics of the redox-responsive drug release systems. Results showed that the drug release efficiency was dependent on disulfide bond with different steric hinderances. It was found the larger the hindrances, the slower the drug release rate [77]. In a similar strategy, we developed a disulfide-bridged PEG gatekeeper to assess the control of the drug release. Compared with the group without GSH, in which less than 10% drug release within 5 h, release of the model drug loaded into MSNs showed more than 50% drug release at 10 mM GSH within the same time period, indicating the accelerated release due to opening of the pores, regulated by GSH [24].

Cleavable PEGylated nanovehicles in gene delivery

Cleavable PEGylation of gene vector has been shown to be most effective in extending *in vivo* circulation time of the genetic payload. It exhibits considerable resistance to undesired aggregation and unspecific interactions with serum proteins during *in vivo* circulation.

Selective release of gene payload was also achieved upon arrival at a specific milieu [96].

Polyethylenimine (PEI) represents a popular cationomer for gene delivery, but with major concern on toxicity limiting its broad applications [30,97]. Poly-L-lysine (PLL) as another regular cationic polypeptide for nonviral gene vector was chosen as a scaffold to incorporate disulfide-bridged PEGylation, in order to obtain PEG-SS-PLL via a facile ring opening polymerization [96]. Due to cleavable PEGylation, the transfection activity of PEG-SS-PLL is serum resistant after gene complexation, attributable to the PEG-shielding effect. However, the transfection activities of PLL50 in luciferase expression were significantly suppressed in the presence of 10% serum for both 293T and HeLa cell lines, respectively. The reason was presumably associated with poor stability of the PLL complexes in serum with positive surface charge. To further examine the effect of cleavable PEGylation, gene transfection activity of PEG-PLL without disulfide link was assessed. Expression for PEG-PLL was found to be three- to sixfold lower than that of PEG-SS-PLL against HeLa cells, attributable to the cleavable PEGylation.

We also carried out systematic studies on PEGylated PLL [98], and PEGylated PLL combined with hydrophilic poly(L-histidine) [30] or hydrophobic poly(L-histidine-Bzl) [50] as gene vectors. The PLL structure was optimized to enhance the biological efficacy of cleavable PEGylation. The PLL-based nonviral vector is generally not satisfactory as a result of its low amine group density and capability of endosomal escape. Most of the amine group has already been protonated at pH 7.4. To improve the capability of endosomal escape as well as efficient unpacking of genetic payload, a dual stimulus-responsive mPEG-SS-PLL_{15}-glutaraldehyde star (mPEG-SS-PLL15-star) cationer was developed and biologically evaluated [98].

In another work, mPEG-SS-PLL was partially replaced by the histidine groups on the PLL segments (mPEG-SS-PLH) for facilitating endosomal escape. The transfection efficacy of mPEG-SS-PLH was found to closely correlate with histidine substitution. The therapeutic efficacy of this tailored cationer was further evaluated using siRNA-VEGF as a therapeutic gene, and HeLa xenograft nude mice as the tumor model. A dose of 20 μg of siRNA-VEGF was intravenously and intratumorally administered in mice every 2 days. The tumor suppression effect is pronounced by both intravenous and intratumoral administration (Figure 10). Figure 10A & B shows the inhibitory effect exerted by *siRNA–VEGF* administration in terms of tumor weight and volume. Compared with the control group, tumor weight/volume is significantly lower. On the last day of experiment, the tumor weights for the groups intravenously injected with mPEG-SS-PLL52/*siRNA–VEGF* and mPEG-SS-PLH15/*siRNA–VEGF* have been, respectively, reduced to 50 and 26%. A representative *ex vivo* tumor from each group is shown in Figure 10C. These results are consistent with the *in vivo VEGF* expression levels as shown in Figure 10D, revealing the suppressed *VEGF* expression via both intravenous and intratumoral injection [30]. Meanwhile, hydrophobic histidine(Bzl), as an alternative group, was employed to substitute hydrophilic histidine for enhancing endosome escape. The hydrophobic benzyl group was simultaneously introduced to provide a 'phase separation' in a single gene/vector nanocomplex. Phase separation can stabilize nanocomplex due to the strong compact structure of gene/vector nanocomplex for high gene transfection [50].

PEI has been another widely used nonviral gene vector and presents advantages over other polycations for its strong DNA condensation ability and intrinsic endosomolytic activity. However, high molecular weight of PEI can induce serious cytotoxicity, and strong packing of DNA in PEI/DNA complex. It therefore becomes a critical hurdle to the release of DNA inside the cytoplasm. Cleavable PEGylation was introduced to PEI for improved biostability, prolonged *in vivo* circulation time and reduced toxicity [58]. Chitosan oligosaccharide-based disulfide-containing polyethylenimine derivative PEG-SS-COS-SS-PEI was found to effectively condense DNA into small particles with improved buffering capacity (~44%), compared with $PEI_{1.8k}$ (~20%). *In vitro* study showed much lower cytotoxicity of the PEGylated redox responsive copolymer, but high transfection efficiency as compared with the control branch of 25 KDa PEI [58].

Conclusion & future perspective

Cleavable PEGylation has been identified as an effective strategy to prolong circulation time and improve hydrophilicity. It has widely been utilized to develop the polymeric or hybrid drug delivery system. Cleavable PEGylation has been shown effective in overcoming drug resistance. The flexibility of disulfide bond formation allows for design of a variety of delivery systems including polymeric micelles, vesicles, prodrug, nanocomposites and nonviral vectors. Significant glutathione concentration differences between the tumor/normal cells and tissues play a key role in the triggering mechanism of cleavable PEGylation for controlled drug release.

Considerable efforts have been devoted to the design and development of the drug delivery systems that are functionalized with disulfide-bridged cleav-

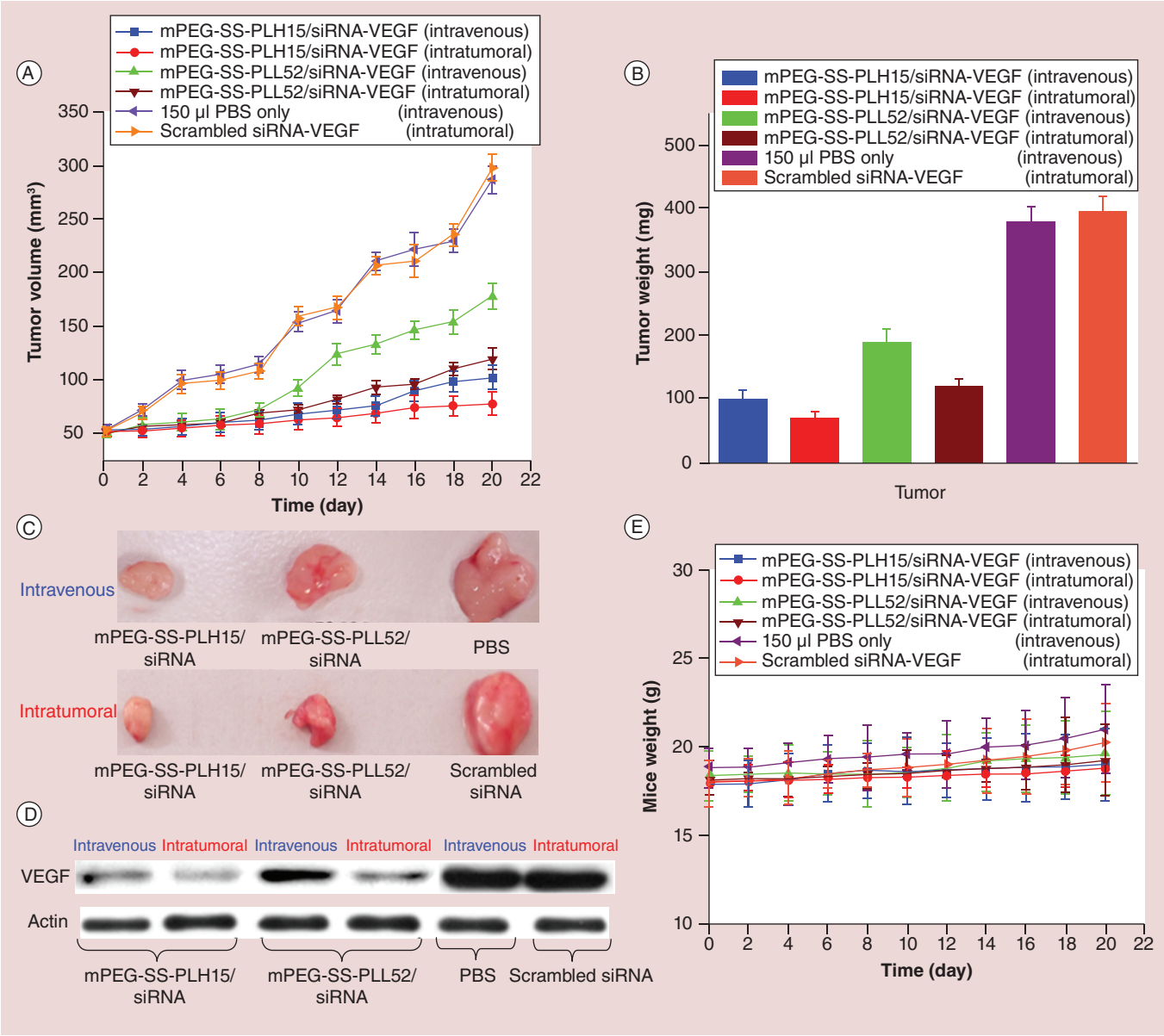

Figure 10. (A) Tumor volume and (B) tumor weight in HeLa xenograft nude mice after intravenous and intratumoral treatment with different vector/siRNA-VEGF complexes. (C) Photograph and **(D)** *VEGF* expression of tumors in HeLa xenograft nude mice after intravenous and intratumoral treatment with different vector/siRNA-VEGF complexes. **(E)** Mice weight of HeLa xenograft nude mice after intravenous and intratumoral treatment with different vector/siRNA-VEGF complexes. PBS and mPEG-SS-PLH15 loaded with scrambled sequence are served as negative controls.
PBS: Phospahte-buffered saline.
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able PEGylation. Versatile and effective nanosystems have been developed that demonstrate significantly enhanced efficacy. However, some critical issues still remain that need to be addressed by advanced design and structural optimization. For instance, some of the current designs appear quite complex that are not straightforward in the synthesis. The structural complexity also creates multiple factors that are difficult to control systematically *in vitro* and *in vivo*. Therefore the future study needs to focus on simplifying the carrier system for viable clinical applications. For example, searching for the S–S cross-linkers with new structures that can link PEG and polypeptide in one-pot process is a possible way to significantly simplify the synthesis procedure.

It is important to find ways to regulate the sensitivity of disulfide bond for different biological milieus. The reduction sensitivity should also be carefully optimized in accordance with the delivery systems and encapsulated therapeutic payloads. The physio-

chemical properties of polymeric chains constitute the delivery system and determine the diffusion of glutathione and subsequent sensitivity [77]. Nadrah *et al.* incorporated steric groups adjacent to the disulfide bond in order to regulate reduction sensitivity. Diselenium (Se–Se) represents another reduction-sensitive bond that can be functionalized in the cleavable PEGylation [99].

The exact cleavage mechanism remains unidentified for disulfide-bridged cleavable PEGylation. By conjugating a pair of quenched fluorescent dyes into both ends of disulfide bond, researchers have found cleavage taking place inside the cells [17]. However, disulfide-bridged cleavable PEGylation may not promote cellular uptake if cleavage only occurred inside the cell. More in-depth investigation is needed to identify the cleavage mechanism.

So far, only limited work on disulfide-bridged cleavable PEGylation has been carried out at the *in vivo* level. Future works will need to be devoted to animal studies in a preclinical setting. Investigations on the formation and regulation of a disulfide bond in a nanodelivery system will require interdisciplinary collaborations, particularly with medical researchers.

Financial & competing interests disclosure

This work was financially supported by 973 program (2013CB967500), research grants from the National Natural Science Foundation of China (NSFC 51473124 and 51173136),

Executive summary

GSH-sensitive disulfide for cleavable PEGylation

- The sharp differences in GSH levels between tumor and normal cells, as well as between the extracellular and intracellular provide the possibility for the structure design of the carrier system based on the disulfide-bridged nanoparticles.

Polymeric nanosystem with disulfide-bridged cleavable PEGylation

- Polymeric nanosystems with disulfide-bridged cleavable PEGylation are generally composed of an inner core with encapsulated therapeutic agents and surrounded by a hydrophilic, cleavable PEG shell. The detachable process occurs at a threshold GSH concentration in a targeted region, for instance, inside a cancer cell.
- These nanoformulations have been developed to address the critical issue of PEGylation limits (including steric hindrance and diffusion barrier), so as to improve biological efficacy of the therapeutic nanosystems with a cleavable PEG layer.

Strategies of disulfide-bridged cleavable PEGylation

- The general strategy to afford disulfide-bridged cleavable PEGylation is to introduce a S–S linkage to PEG. The cross-linker is typically employed with the S–S moiety, such as cystamine dihydrochloride, 2,2-dithiodiethanol (DTDE), etc.

Cleavable PEGylated nanosystems-polymeric micelles

- Micelles exhibit high stability without obvious size alteration and nearly identical drug (DOX) release behavior in buffer solution without GSH.
- Intracellular GSH concentration (10 mM) triggers PEG layer detachment with the exposure of the inner core and thus results in a faster release of fourfold, as compared with the control groups.
- After incorporation of cross-linking in the shell, the micelles exhibit not only reduced drug loss in extracellular environments, but also drastically accelerated drug release at the cytoplasmic GSH level, leading to enhanced growth inhibition toward HeLa cells.
- Active targeting can also be achieved by taking advantages of highly accessible functional groups of PEG.

Cleavable PEGylated nanosystems vesicles

- The vesicles can be used as a dual drug carrier for simultaneously loading of hydrophilic doxorubicin hydrochloride (DOX·HCl) and hydrophobic camptothecin.
- Disulfide-bridged PzLL-SS-PEG-SS-PzLL vesicles are developed for intracellular drug delivery and overcoming MDR of cancer cells.
- Hepatoma-targeting reduction-sensitive vesicles are developed to load cytochrome C, which exhibit apparent target-ability and pronounced antitumor activity to HepG2 cells.

Cleavable PEGylated nanosystems-prodrug

- The micellar prodrug has several major advantages: integration of drug into the carrier (as hydrophobic segment of micelles) that minimizes the use of polymers; drug encapsulation into inner core can avoid premature exposure to body fluid, which is different from the conventional prodrug and selective drug release of the active drug in reductive milieu.
- Camptothecin (CPT) is conjugated onto double ends of PEG via disulfide bond to afford CPT-SS-PEG-SS-PEG with CPT loading efficiency up to 20.3%.

Executive summary (cont.)

Cleavable PEGylated nanosystems-organic-inorganic nanocomposites

- Nanographene oxide with redox-responsive detachable PEGylation is developed for surface functionalization and intracellular drug delivery.
- Cleavable PEGylation is used as switchable gatekeepers on the MSN surface pore to control the drug release.

Cleavable PEGylated nanovehicles in gene delivery

- Cleavable PEGylation of gene vector exhibits considerable resistance to undesired aggregation and unspecific interactions with serum proteins during *in vivo* circulation. Selective release of gene payload is also achieved upon arrival at a specific milieu.
- Due to cleavable PEGylation design, the transfection activity of PEG-SS-PLL system is serum resistant after gene complexation, attributable to the PEG-shielding effect.
- Therapeutic siRNA-VEGF is loaded into the mPEG-SS-PLH system, which exhibits pronounced tumor suppression effect by both intravenous and intratumoral administration.

the Fundamental Research Funds for the Central Universities (2013KJ038) and 'Chen Guang' project founded by Shanghai Municipal Education Commission and Shanghai Education Development Foundation. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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