

TME-Responsive Multistage Nanoplatfom for siRNA Delivery and Effective Cancer Therapy

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Abstract: Since the discovery of RNA interference (RNAi), RNAi technology has rapidly developed into an efficient tool for post-transcriptional gene silencing, which has been widely used for clinical or preclinical treatment of various diseases including cancer. Small interfering RNA (siRNA) is the effector molecule of RNAi technology. However, as polyanionic macromolecules, naked siRNAs have a short circulatory half-life (<15 min) and is rapidly cleared by renal filtration, which greatly hinders their clinical application. Furthermore, the anionic and macromolecular characteristics of naked siRNAs impede their readiness to cross the cell membrane and therefore delivery vehicles are required to facilitate the cellular uptake and cytosolic delivery of naked siRNAs. In the past decade, numerous nanoparticles (NPs) such as liposomes have been employed for in vivo siRNA delivery, which have achieved favorable therapeutic outcomes in clinical disease treatment. In particular, because tumor microenvironment (TME) or tumor cells show several distinguishing biological/endogenous factors (eg, pH, enzymes, redox, and hypoxia) compared to normal tissues or cells, much attention has recently paid to design and construct TME-responsive NPs for multistaged siRNA delivery, which can respond to biological stimuli to achieve efficient in vivo gene silencing and better anticancer effect. In this review, we summarize recent advances in TME-responsive siRNA delivery systems, especially multistage delivery NPs, and discuss their design principles, functions, effects, and prospects.

Keywords: nanoparticles, siRNA, TME-responsive, multistage delivery, cancer therapy

Introduction

Since the discovery of RNA interference (RNAi) by Fire et al,¹ RNAi technology has been rapidly developed into a powerful tool to clarify the body pathogenesis, and widely used for the clinical or preclinical treatment of various diseases such as malignant tumors, Hepatitis B, obesity, and multiple neuropathy. Small interfering RNA (siRNA) is an effector molecule of RNAi technology with a length of 21–23 nt.² Once the synthetic siRNA or double-strand RNA (dsRNAs) cleaved by enzyme Dicer enters the cytoplasm, it can bind to a protein complex called RNA-induced silencing complex (RISC). Subsequently, Argonaute 2 (Ago2), a multifunctional protein within the RISC, can unwind the siRNA double strand and the sense strand of the siRNA is cleaved. The antisense strand in the activated RISC selectively targets mRNA via complementary base pairing to achieve the enzymatic cleavage of target mRNA and gene silencing (Figure 1).³

As the activated RISC can repeatedly silence the target mRNA, the therapeutic effect usually last for several days in dividing cells and several weeks in non-dividing cells.⁴ In addition, siRNA inhibits mRNA translation but not transcription.

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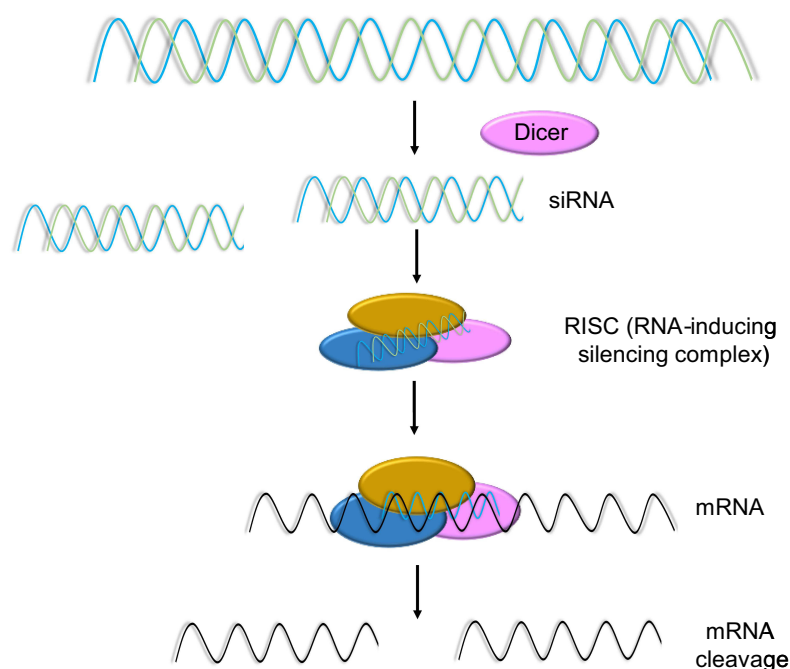


Figure 1 Overview of RNAi mechanism in the cell. dsRNA was processed by DICER to become short interfering RNA (siRNA), before being recruited into RISC. The complementary strand of siRNA will find complementary mRNA sequence. Once found, the ROSC complex would cleave the mRNA.

Therefore, it does not affect DNA functions and can avoid possible genetic mutations when using plasmid DNA as therapeutics.³ More importantly, siRNA can target and inhibit the translation of any disease-associated mRNAs, it therefore shows great potential for the treatment of various diseases, especially the genes that encode “undrug-gable” proteins.

In the past decade, tremendous investment in RNAi technology has been made by the governments worldwide. In parallel, most pharmaceutical companies have also paid much attention to develop siRNA drugs. In 2018, the US Food and Drug Administration (FDA) approved the first siRNA drug, Onpatro (Patisiran) developed by Alnylam for the treatment of Hereditary transthyretin amyloidosis (hATTR) (Trial 1/NCT01960348). Currently, another siRNA drug Inclisiran is undergoing a Phase II clinical trial for patients at high cardiovascular risk with elevated LDL cholesterol. In addition, there are around 30 siRNA drugs under clinical trial.⁵ However, most of these siRNA drugs focus on the treatment of liver diseases and the clinical translation of siRNA drugs for cancer therapy is still far from expected. The key challenge is to effectively in vivo transport siRNA to tumor cells. Naked siRNA has a short circulatory half-life (<15 min) and is easily degraded by nucleases and filtered by glomeruli.⁶ In addition, due to its polyanionic and macromolecular characteristics (Mw ~13

kDa), naked siRNA cannot deeply penetrate the tumor tissues and readily cross the cell membrane.^{7,8} Nanoparticles (NPs) such as liposomes and polymeric NPs exhibited unique advantages in tumor targeting, transmembrane delivery and tumor growth suppression.^{9,10} Introducing NPs to encapsulate siRNA can protect it from degradation and efficiently deliver siRNA to tumor cells, NPs-mediated delivery systems have been widely used for in vivo gene silencing and cancer therapy. In particular, because tumor tissues or tumor cells show different microenvironment (eg, acidic pH, overexpression of enzymes, hypoxia) compared to normal tissues or cells, much attention has recently paid to develop Tumor microenvironment (TME)-responsive NPs, which can respond to biological stimuli to achieve efficient in vivo gene silencing and better anticancer effect.^{11–13} In this review, we comprehensively summarize the recent development of TME-responsive siRNA delivery systems and discuss their application prospect.

NPs-Mediated siRNA Delivery

Viral vectors are the earliest carrier used for in vivo siRNA delivery. However, viral vectors show the drawbacks of potential immunogenicity, mutagenicity, and difficulty in scale-up.¹⁴ In the past decade, non-viral vectors, especially lipid and polymeric NPs, have been widely used as alternative carriers, due to their characteristics of good biocompatibility, low

immunogenicity and ease in scale-up.^{15,16} The representative example is the siRNA drug Onpatro, which is essentially a liposome nanoplatform with therapeutic siRNA encapsulated into its nanostructure. When using NPs to deliver siRNA for cancer therapy, they can passively target to the tumor sites via the enhanced permeability and retention (EPR) effect, which thus facilitates to improve the tumor siRNA accumulation in tumor tissues and enhance its gene silencing effect.¹⁷ However, increasing evidence have demonstrated that the traditional NPs cannot completely overcome the multiple physiological barriers in the tumor tissues during *in vivo* siRNA delivery (Figure 2).

Therefore, an ideal *in vivo* siRNA delivery nanoplatform should have the following features: i) good biocompatibility and low systematic immune response; ii) long blood circulation to protect siRNA from clearance and degradation; iii) high accumulation in the tumor sites and deep penetration into the tumor tissue; iv) higher uptake by tumor cells and efficient release of loaded siRNA in the cytoplasm to exert its biological function. To achieve this goal, the researchers have recently taken advantage of the distinguishing microenvironment in tumor tissue or tumor cell, and have utilized series of chemical strategies to design TME-responsive NPs to improve the *in vivo* siRNA delivery efficacy and enhance gene silencing effect.

TME-Responsive NPs-Mediated Multistage siRNA Delivery

Solid tumor is a systemic disease and its growth relies on the surrounding microenvironment.¹⁸ Besides the highly heterogeneous tumor cells, solid tumor also contains extracellular matrix, fibroblast, blood vessel/lymphatic, and immune cells, which together constitute the tumor microenvironment.¹⁹ The interaction between tumor microenvironment and tumor cells plays an important role in tumor genesis, progression and metastasis. Tumor cells act to the microenvironment by secreting growth factors and proteases, and the acclimated microenvironment cells feed back to the tumor cells.²⁰ This interaction makes the tumor tissues showing different physiological microenvironment from normal tissues, such as weakly acidic and hypoxic microenvironment. Based on this distinguishing microenvironment, designing TME-responsive NPs could address the issues encountered in the siRNA delivery by traditional NPs. These TME-responsive NPs are stable during blood circulation and in the normal tissues. After arrival to the tumor sites, siRNA is rapidly released in the tumor tissue or tumor cells, which could significantly improve the bioavailability of siRNA and enhance the anti-tumor effect. At present, the reported TME-responsive NPs mainly include pH-, hypoxia-, enzyme-, reactive oxygen species (ROS)-, and

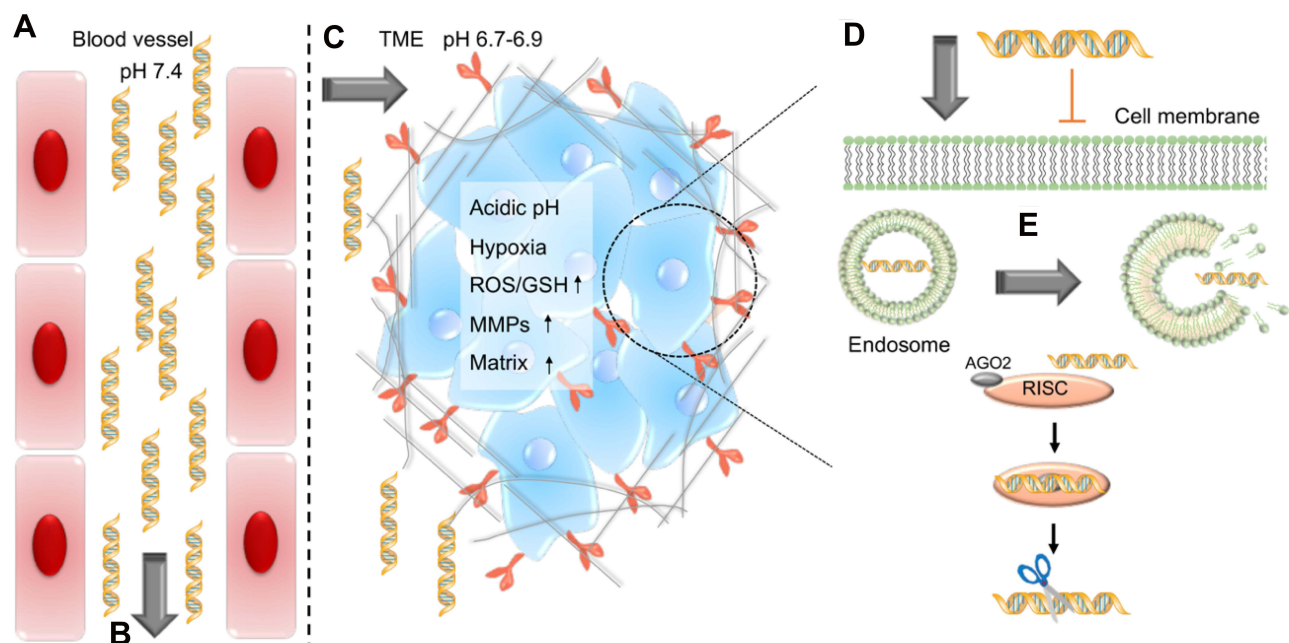


Figure 2 Major barriers in siRNA therapeutics. (A) siRNA is degraded within seconds in light of the abundance of RNases in circulation. (B) The short half-life of naked siRNA warrants a capable delivery carrier that could safely and effectively deliver siRNA to TME. (C) TME exhibited unique characteristics, including acidic pH, hypoxia, increased redox stress, elevated enzyme expression and abundant extra cellular matrix, which further impede the delivery of siRNA. (D) Even when siRNA is successfully delivered to the tumor site, being an anionic biomacromolecule, siRNA is unable to easily penetrate the negatively charged cell membrane. (E) After cellular uptake, siRNAs need to escape endosomes to exert its gene silencing mechanism in the cell cytoplasm.

reduction-responsive NPs (Table 1). The applications of these TME-responsive NPs for in vivo siRNA delivery are described in the following sections.

Targetable Attributes of the TME

The Acidic pH

As compared to the pH levels of normal tissue (~pH 7.2–7.5), pH levels within the TME are usually more acidic (~pH 6.5–6.9)³¹ because of the increased metabolic rate of cancer cells to support their high energy requirements for survival.^{31,32}

The Warburg effect describes the tumor's acidic environment as a consequence of glycolysis (the conversion of glucose into lactic acid).³³ By utilizing pH-responsive polymers,³⁴ acid-labile chemical bonds^{35,36} or ionizable chemical groups, nanocarriers can be designed to be pH-sensitive.

Upregulation of Enzymes

Another important target in the TME is the upregulation of enzymes from tumor cells and tumor stromal cells.^{37,38} Certain enzymes that are over-expressed by solid tumors

Table 1 siRNA-Based Drugs for Solid Tumor Therapy and Their Current Status in Clinical Trials

Drug	Method of Delivery	Disease	Clinical Trial No.	Phase	Status	Year Completed	Ref
ALN-VSP02 Alnylam Target: KSP and VEGF	Intravenous/LNP	Solid Tumors	NCT 00882180	Phase I	Completed	2011	[21,22]
			NCT01158079	Phase I	Completed	2012	
CALAA-01 Calandro Target: RRM2	Intravenous/ Cyclodextrin polymer-based nanoparticles	Solid Tumors, Cancer	NCT 00689065	Phase I	Terminated	2012	[23,24]
SIG12D Silenseed Target: KRAS	Implant/LODER polymer	Pancreatic Ductal Adenocarcinoma, Pancreatic Cancer	NCT 01188785	Phase I	Completed	2013	[25]
			NCT 01676259	Phase II	Recruiting	Est: 2020	
TKM-PLK1 Tekmira Target: PLK-I	Intravenous/LNP	Colorectal Cancer with Hepatic Metastases, Pancreas Cancer with Hepatic Metastases, Gastric Cancer with Hepatic Metastases, Breast Cancer with Hepatic Metastases, Ovarian Cancer with Hepatic Metastases	NCT 01437007	Phase I	Completed	2012	[26,27]
		Cancer, Neuroendocrine Tumors, NET, Adrenocortical Carcinoma, ACC	NCT 01262235	Phase I	Completed	2015	
DCR-MYC Dicerna Target: MYC	Intravenous/ LNP-formulated Dicer substrate siRNA (DsiRNA)	Solid Tumors, Multiple Myeloma, Non- Hodgkin's Lymphoma, Pancreatic Neuroendocrine Tumors, PNET, NHL	NCT 02110563	Phase I	Terminated	2016	[28,29]
siRNA- EphA2 MD Anderson Target: EphA2	Intravenous/LNP	Advanced Cancers	NCT 01591356	Phase I	Recruiting	Est: 2021	[30]

Abbreviations: KSP, Kinesin Spindle Protein; VEGF, Vascular Endothelial Growth Factor; RRM2, Ribonucleoside-diphosphate reductase subunit M2; PLK-I, Polo-like Kinase protein-1; KRAS, Kirsten Rat Sarcoma; EphA2, Ephrin type-A receptor 2.

and the TME include peptidases (eg, aminopeptidase), proteases (eg, matrix metalloproteinase and cathepsin B), and lipases (eg, phospholipase A2). In high concentrations, these enzymes serve a crucial role in the growth of tumor cells, angiogenesis, invasion, and metastasis, which serves as a key stimulus for a responsive nanoparticle system.

Hypoxia

The abnormal vasculature in solid tumors is unable to provide adequate oxygenation to the entire tumor tissue (especially to the inner regions with poor vascularization). In addition, the consumption of oxygen is further accelerated by the over-proliferation of tumor cells.³⁹ Hypoxia in the TME is mainly due to these two factors. Evidently, the hypoxic regions of the TME that are distant from blood vessels are unfavorable to large nanoparticles which possess low penetration ability into deep tissue. Hence, systems of hypoxia-sensitive nanoparticles have been developed, and can be classified into two major mechanisms. The first mechanism describes nitroimidazole-based micelle systems that are bio-reduced to aminoimidazole, creating a hydrophobic-to-hydrophilic transition, which then triggers micelle disassembly.⁴⁰ The second mechanism entails the use of azobenzene group-employed siRNA nanocarriers which are cleavable, leaving the polyethylene imine (PEI/siRNA) complex with a positively charged surface for the facilitation of cellular uptake.⁴¹

Elevated Redox Stress (ROS/GSH)

Due to the elevated levels of superoxide anion radicals, hydrogen peroxide and hydroxyl radicals in the TME, tumor cells experience an increased potential of oxidative stress.⁴² As a result, tumor cells will generally upregulate the reduction potential by the expression of redox species such as glutathione (GSH) and superoxide dismutase (SOD) to overcome the oxidative stress. Consequently, upregulated redox levels of the tumor create a high overall potential (oxidative/reductive) in the TME. This dysregulated potentials of oxidation and reduction in the TME position them as excellent candidates for the development of TME-targeted nanoparticles. Furthermore, the level of reactive oxygen species (ROS) in cancer cells is higher compared to normal cells due to aerobic metabolism as a result of oncogenic transformation⁴³ This is evidenced by recent studies that described the constant generation of high levels of intracellular ROS by cancer cells (ie, hydrogen peroxide, hydroxyl radicals, and superoxide) as compared to normal cells, due to oncogenic transformation.⁴⁴

Prospectively, the design and application of a novel siRNA delivery system which will respond to different pathophysiological properties in the TME, such as increased or unique levels of functional proteins, amino acids, and DNA fragments, will be available for tumor targeting.⁴⁵ As a whole, multi-responsive NPs can exploit the various targets present in the TME as compared to traditional NPs, as they are able to be activated at the target tumor site by the triggering of the unique characteristics present in the TME, as discussed above. The cargoes can then be released at a higher concentration, and on-demand, to the tumor site.^{33,36}

Development of Bioresponsive Multistage siRNA Targeting Strategy

Concept of Multistage-Responsive siRNA Nanocarrier

Conventional nanocarriers can be modified to be responsive and sensitive to the unique stimuli present in the TME. These carriers will receive the necessary stimulation which alters the physicochemical structures or features when it reaches the TME, consequently realizing their desired functions. In the following sections, multiple responsive modes of multistage responsive siRNA nanocarriers and their associated functions are discussed. Various TME stimuli, especially the upregulated enzymes and acidic pH, can activate approximately similar behaviors of these carriers in tumor tissue. In general, the responsive modes in the TME include targeting ligand exposure (re-emergence of targeting ligand), polyethylene glycol (PEG) detachment, particle-size shrinkage, and surface charge reversal.³⁶

As contrasted to drug delivery system (DDS), additional cargo preservation and endosomal release capabilities are required by siRNA delivery vehicles. If discharged extracellularly or in close proximity to tumors, small molecule drugs could still undergo passive diffusion through the cell membrane and be situated in the cell cytoplasm to carry out its functions. Due to charge repulsion, siRNAs, which are anionic biomacromolecules, cannot penetrate the cell membrane without aid. Hence, it is crucial to ensure the safe and efficient delivery of siRNAs across the cell membrane.⁴⁶ Upon safe transportation of siRNAs into the cell via nanocarriers, it must avoid endosomes to execute its potent mRNA degradation machinery. For these reasons, a “multi-stage delivery approach” is necessary for the development of an effective siRNA

delivery system, which guarantees the systemic delivery of siRNA.⁴⁷ Herein, we summarized the examples which fulfilled all three stages of the multistage siRNA delivery system (Table 2).

Multi-Stage Responsive Nanocarrier for siRNA Delivery

To achieve both stability in the circulation as well as rapid release of siRNAs in the TME, a number of nanocarriers were designed based on the abnormal TME stimuli such as a slightly acidic pH, elevated enzymes, hypoxia and increased ROS. Herein, we highlighted some designs of multi-stage responsive nanocarriers.

TME pH-Responsive Nanocarriers

Yang et al⁴⁸ designed a rapid pH-responsive sheddable nanoparticle (S-NP), which was prepared as follows. PEGylated tumor acidity-responsive anionic polymer PPC-DA was introduced to the surface of positively charged ssPEI800/siRNA

nanoparticles by electrostatic interaction. To obtain PPC-DA, the diblock polymer mPEG₄₅-b-PAEP₇₅ was first synthesized. Then, the mPEG₄₅-b-PAEP₇₅-Cya (PPC) was synthesized via the thiol-ene “click” reaction of mPEG₄₅-b-PAEP₇₅ with cysteamine. The PPC was further reacted with 2,3-dimethylmaleic anhydride (DMMA) to obtain the tumor acidity-responsive PPC-DA. DMMA-modified amines are usually stable under neutral pH, yet rapidly degrade in slightly acidic TME conditions. Upon exposure to slightly acidic pH, the amide bond of PPC-DA in S-NP was triggered, which led to degradation and the generation of positively charged amino groups, which further led to a strong electrostatic repulsion to positively charged ssPEI800/siRNA complex, resulting in the phenomenon of “sheddable nanoparticles” (Figure 3A).

Sun et al⁴⁹ created the “Acid-labile polymer” which is sensitive towards the pH in the TME. The Dlink_m Bridged Copolymer was constructed with PEG, Dlink_m and PLGA. Cyclohexanedimethanol (CDM) was first introduced to provide an additional reaction site to construct a bridged responsive copolymer. Also, it decelerated the linkage sensitivity to

Table 2 Multi-Stage Responsive siRNA Delivery Systems

STAGE 1 Physiological Changes Upon Stimuli in TME	STAGE 2 Efficient siRNA Delivery Through Cell Membrane	STAGE 3 Endosomal Escape Mechanism	STAGE 4 siRNA Release Mechanism	Reference
pH - sheddable PEG layer	Polycation/siRNA complex	Proton sponge effect	–	[48]
pH – acid labile polymer	CPP/siRNA complex	Proton sponge effect	–	[35,49]
	PEI/siRNA complex	Proton sponge effect	–	
pH – borate ester linker	PBA/siRNA complex	Proton sponge effect		[50]
pH – charge reversal polymer	TCPA peptide/siRNA complex	Proton sponge effect	–	[34]
pH – charge reversal polymer	bPEI/siRNA complex	Proton sponge effect	GSH	[51]
pH – charge reversal polymer	PEI/siRNA complex	Proton sponge effect	Redox sensitive linker (GSH)	[52]
pH – pH responsive polypeptide	Cell-penetrating peptide			[53]
pH – PPy nanoparticle	pH-triggered cleavable backbone	Size-tunable PPy/ pH-triggered drug release		
MMP enzyme – sheddable PEG	PASp(DET)/siRNA complex	PASp(DET) is an excellent endosome destabilizer	–	[54]
MMP enzyme – cleavable peptide linker	Polycation/siRNA complex	Proton sponge effect	–	[55]
MMP 7 cleavable peptide	DMAEMA/siRNA complex	Proton sponge effect		[56,57]
Hypoxia – PAPD polymer	PEI-DOPE/siRNA complex	Proton sponge effect	–	[41]
Redox responsive	Cationic lipid/siRNA complex	Proton sponge effect	–	[58]

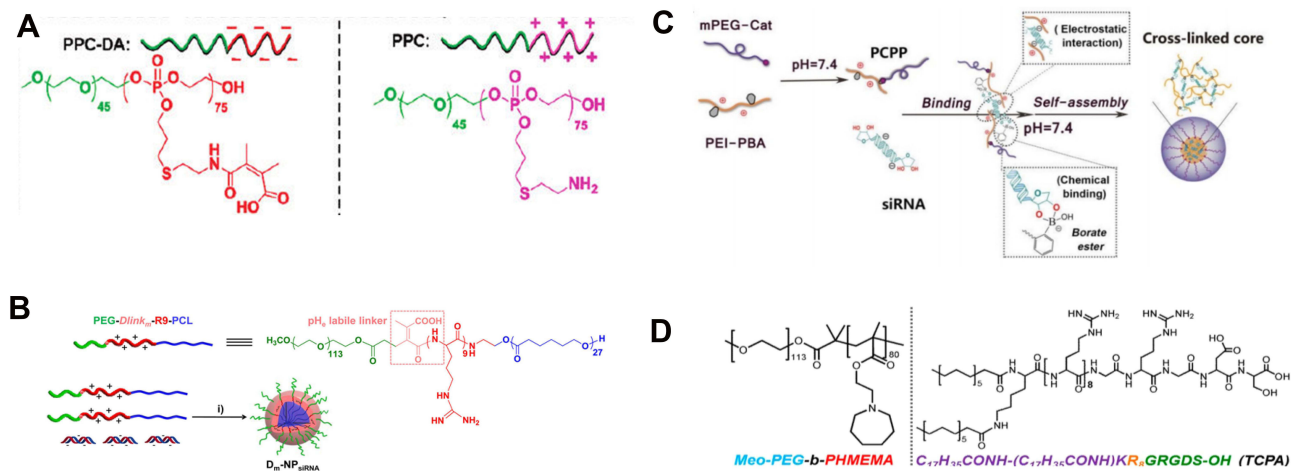


Figure 3 (A) The charge reversal properties of PPC-DA from negative charge in physiological pH to positive charge at TME pH; Reproduced from Yang XZ, Du JZ, Dou S, et al. Sheddable ternary nanoparticles for tumor acidity-targeted siRNA delivery. *ACS nano*. 2012(6);771–781. Copyright 2012 American Chemical Society.⁴⁸ **(B)** PEG-Dlink_m-R9-PCL-based siRNA delivery system with a TME pH labile linker which could be cleaved at pH levels lower than 6.8; Reproduced from Sun CY, Shen S, Xu CF, et al. Tumor acidity-sensitive polymeric vector for active targeted siRNA delivery. *J Am Chem Soc*. 2015;137:15,217–15,224. Copyright 2015 American Chemical Society.⁴⁹ **(C)** The combination of mPEG-Cat and PEI-PBA could be self-linked and displayed binding to siRNA at pH 7.4. This linkage could be broken at pH levels in the TME, releasing the siRNA complex; Reproduced with permission from Fan B, Kang L, Chen L, et al. Systemic siRNA delivery with a dual pH-responsive and tumor-targeted nanovector for inhibiting tumor growth and spontaneous metastasis in orthotopic murine model of breast carcinoma. *Theranostics*. 2017;7(2):357–376. IVYSPRING.⁵⁰ **(D)** Creation of TME pH-sensitive Meo-PEG-b-PHMEMA-based NPs, with T CPA/siRNA complex; Reproduced from Xu X, Saw PE, Tao W, et al. Tumor microenvironment-responsive multistaged nanoplatfrom for systemic RNAi and cancer therapy. *Nano letters*. 2017;17:4427–4435. Copyright 2017 American Chemical Society.³⁴

pH 7.4, thus satisfying the requirement of cellular uptake. After the well-established synthesis of chlorine substituted CDM, PEG was modified into PEG-DMA. Then, PEG-

DMA was coupled with an amine-functionalized poly(ϵ -caprolactone)-nonaarginine R9 (PCL-R9, Figure 4B). Under slightly acidic pH conditions, an increasing

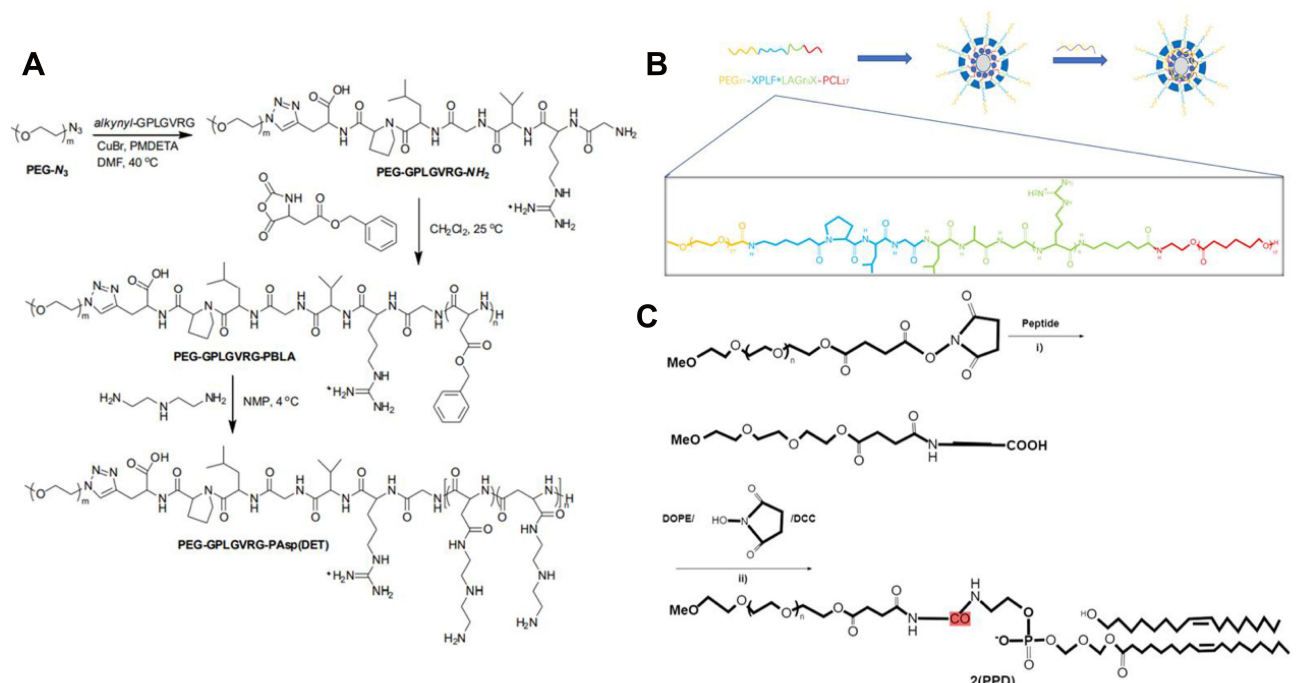


Figure 4 (A) Synthesis scheme of PEG-GPLGVRG-PAsp (DET; Reproduced from Li J, Ge Z, Liu S. PEG-sheddable polyplex micelles as smart gene carriers based on MMP-cleavable peptide-linked block copolymers. *Chem Commun*. 2013;49: 6974–6976, © 2013 The Royal Society of Chemistry.⁵⁴ **(B)** Synthesis of block copolymer of PEG and poly(ϵ -caprolactone) (PCL) with an MMP-2-sensitive peptide bridge, namely Pro-Leu-Gly-Leu-Ala-Gly-Arg-Arg-Arg-Arg-Arg-Arg-Arg-Arg (PLG*LAGr9). **(C)** Synthesis of PPD-MEND (constructed with PEG/matrix metalloproteinase (MMP)-substrate peptide₂₀/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) ternary conjugate (PPD)).

concentration of homopolymers was found with a prolonged elution volume (9.4 mL), which was consistent with the lower molecular weight of PEG and PCL-R9, indicating degradation of the polymer. After self-assembly, the R9 in the hydrophilic shell of Dm-NP and NP allowed them to bind with siRNA to form micelleplexes. Image analysis detected almost ~85% of the intact siRNA when the nanoparticles were treated by RNase A, suggesting the feasibility of Dm-NP in protecting the siRNA from enzymolysis and serum degradation (Figure 3B).

Wang et al⁵⁹ conjugated the cationic (PLGA)₂-PEI with 2,3-dimethylmaleic anhydride (DMMA) that reacted with the amines in PEI to generate amide bonds. The final nanoparticles were stable and exhibited negative surface charge at neutral or alkaline conditions, but could release the DMMA molecules in a weakly acidic niche due to the prompt hydrolysis of the amide bonds. The amino groups in PEI were exposed again, resulting in the charge conversion of the nanoparticles from negative to positive. Therefore, the nanoparticles could specifically deliver siRNAs into tumor cells with high efficiency and in turn, decreased the expression of Nogo-B receptor (NgBR) in cancer cells and tumor-associated endothelial cells.

Fan et al⁵⁰ developed a nanovector which consisted of α -methoxy ω -amino polyethylene glycol-catechol (mPEG-Cat) and PBA-grafted low molecular weight branched polyethylenimine (1800 Da) (PEI-PBA). Here, catechol (Cat) is an excellent cis diol-containing reagent to form complexes with boronic acids. The spontaneous formation of boronate linkages between PBA and Cat allows the facile attachment of mPEG-Cat onto PEI-PBA at neutral conditions. The PBA-functional polymer (PEG-CPB-PEI) could condense siRNAs into polyplex nanoparticles at pH 7.4 via a “synergetic assembly” approach of facile PBA-ribose chemical binding and an electrostatic interaction generated between the cationic PEI with the anionic phosphate group of siRNAs. After internalization into the endosomes/lysosomes, the borate ester bond of PBA-ribose in the cross-linked core would be completely disrupted as the pH value further declines to pH 4.5~5.5. Therefore, complete breakdown of the nanostructure would occur accompanied by the rapid and triggered release of siRNAs. Meanwhile, the PEI component promotes endosomal/lysosomal escape which allows siRNAs to migrate into the cytoplasm for gene silencing (Figure 3C).

We also developed a nanoparticle which was sharply responsive to the TME-responsive nanoparticle by

utilizing a unique charge-reversal polymer which could be triggered by the acidic TME pH to reverse the polymer charge from negative to positive. By introducing the hydrophobic poly(2-(hexamethyleneimino)ethyl methacrylate) (PHMEMA) which demonstrated a sharp response to pH levels in the TME, in combination with the PEG surface shell, which is a charge-mediated complex of siRNA and cell penetrating peptide, we developed an easy-to-control vector with high siRNA delivery efficiency triggered by pH change in a narrow range between pH 6.8 and 7.4 (Figure 3D).³⁴

Yang et al⁵³ provided a novel delivery tactic of building a new therapeutic agent delivery system by utilizing photo- and pH-responsive polypeptides (PPPs), and this delivery system could transport cargoes with greater selectivity and efficiency to tumor cells. In this system, the PPP consists of three elements: a cell-penetrating peptide sequence (CGRRMKWKK), a photo-decomposable group (4, 5-dimethoxy-2-nitrobenzyl group) and a pH-sensitive inhibitory peptide (EEEEERRR). After systemic administration, PPP-NPs accumulated at the tumor sites due to the enhanced permeability and retention (EPR) effect. The cell penetration ability of CPPs is effectively shielded by opposite electrical charges within the pH-sensitive inhibitory peptides in circulation. Upon Near Infra-red NIR light irradiation at the tumor location, the photo-cleavable group is cleaved. Simultaneously, the pH-sensitive inhibitory peptide eliminates the electrostatic attraction. After cleavage of the linker and elimination of the electrostatic attraction, the PPP could release its inhibitory peptides to expose the CPPs. Then, with the help of revitalized CPPs, the NPs could efficiently enter into the cancer cells.

Tubular polypyrrole (PPy) with tunable 60–200 nm diameters was successfully synthesized using a TiO₂ nanotube array template in the presence of FeCl₃ and pyrrole monomer. Aromatic imine bonds were introduced into the PPy nanoparticles via reaction with tetra-arm aldehyde (TAA) to prepare a novel pH-triggered cleavable backbone. One end of PEG was functionalized with FA as the targeting ligand to bind with α -FR on the surface of tumor cells, whereas the other end was chemically conjugated to the surface of PPy nanoparticles via a cleavable aromatic imine bond to control the release of the drug. Guided by the optimal size of PPy nanoparticles, the therapeutic efficacy of Doxorubicin-loaded PPy nanoparticles was demonstrated both in vitro and in vivo.⁶⁰

TME Enzyme-Responsive Nanocarriers

Matrix metalloproteinases (MMPs) are a group of proteases involved in tumor invasion and are significantly upregulated in the TME.⁶¹ With this characteristic, a TME-specific targeting strategy was developed. MMP-cleavable peptide was utilized as a linker to construct the backbone of nanocarriers. As a result, the nanocarrier demonstrated both stability in the bloodstream as well as rapid internalization and endosomal escape.

Li et al⁵⁴ constructed PEG-sheddable polyplex micelles as smart gene delivery vectors by utilizing a matrix metalloproteinase-cleavable peptide-linked block copolymer. This nanocarrier was demonstrated to exhibit higher cellular uptake, improved endosomal escape, and high-efficiency gene transfection in the presence of matrix metalloproteinase-2 (MMP-2). To achieve the goal, they utilized a MMP-2 cleavable peptide GPLGVRG to link the PEG and PAsp(DET) to synthesize the PEG-GPLGVRG-polymer backbone (Figure 4A). Upon arrival at the TME, the overexpressed MMP-2 will break the polymer backbone and remove the surface PEG layer to enhance the internalization ability of the nanocarrier. As PAsp(DET) is an excellent endosome destabilizer, the encapsulated siRNA would be able to escape from the endosome and therefore silence the target gene.

Wang et al⁶² reported site-specific decladding MMP-2-triggered sheddable polymeric micelles for siRNA delivery. They synthesized a block copolymer of PEG and poly(ϵ -caprolactone) (PCL) with an MMP-2-sensitive peptide bridge, namely Pro-Leu-Gly-Leu-Ala-Gly-Arg-Arg-Arg-Arg-Arg-Arg-Arg (PLG*LAGr9). The hydrophobic PCL segment forms the micellar core, while hydrophilic PEG constitutes the shell of the micelles. The peptide, PLG*LAG, has a specific cleavage site “*” for decladding the PEG layer from the micelleplex in tumors (Figure 4B). The cationic peptide polyarginine (R9) provides siRNA binding function and cellular membrane penetration. Micelleplexes based on such MMP-2-responsive nanoparticles are capable of enhancing siRNA delivery to tumor cells *in vitro* and *in vivo* and can efficiently inhibit breast cancer growth by delivering siRNA against Polo-like kinase 1.

Other MMP cleavable peptides were also utilized as linkers to create sheddable PEG layers, such as PPD-MEND (constructed with PEG/matrix metalloproteinase (MMP)-substrate peptide₂₀/1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) ternary conjugate (PPD)),⁶³ MMP-sensitive PAT element (poly[(DMAEMA)-

b-(DMAEMA-co-PAA-co-BMA)])⁵⁶ and its upgraded version (FA ligands were utilized to enhance the cellular uptake instead of the zeta potential shift, Figure 4C). These studies indicate that these MMP-responsive delivery platforms offer significant potential for environmentally targeted, tissue-specific siRNA delivery.

TME Hypoxia-Responsive Nanocarrier

Due to rapid growth of solid tumors, the dense extracellular matrix and the vascular dysfunction in the tumor region, the majority of solid tumors develop acute hypoxia (less than 1.4% oxygen) in the TME.³⁹ It has been widely accepted that hypoxia is related to tumorigenesis and therapy resistance.^{64,65} This scenario also provides a new specific delivery strategy by targeting the hypoxic TME. Several hypoxia-responsive prodrugs have been reported which were mainly based on nitroimidazoles, azo-groups, ferrocene and disulfide bonds.⁶⁶ Similar strategies have been used to design nanocarriers, for example the azo linker was introduced to construct a hypoxia-responsive sheddable PEG layer⁶⁷ or hypoxia degradable polymer.⁶⁸ 4-nitrobenzyl chloroformate (NBCF)-modified side chains were also utilized to construct a hypoxia-responsive charge shiftable polymer (the charge of the nanocarrier was neutral in circulation, and the charge became positive upon reaching the hypoxic TME).^{45,69} However, the majority of nanocarriers are designed for drug delivery and due to the instability and difficulty of encapsulating the siRNA, it is relatively difficult to prepare a hypoxia-responsive nanocarrier for siRNA delivery as compared with drug delivery.

The first reported hypoxia-responsive nanocarrier for siRNA delivery was the hypoxia-sensitive polymer PAPD. The PEI-DOPE conjugate was utilized to form siRNA complexation and promote formation of micellar nanoparticles, and to achieve the hypoxia-responsive ability, an azobenzene linker was introduced. When the nanocarrier arrived at the TME, the azobenzene linker would be degraded, and as a result, the positive charge of PEI would be presented, leading to the cellular uptake of the PEI-DOPE/siRNA complexes.⁴¹ This research sheds light on the possibility of utilizing hypoxia as a TME-specific target for siRNA delivery.

TME Redox-Responsive Nanocarrier

The redox environments are quite distinct between the cytoplasm and extracellular space, which provides an opportunity for the design of multistage responsive nanocarriers. For

example, the concentration of glutathione (GSH) in the cytoplasm is approximately $2\text{--}10 \times 10^{-3}$ M, while it decreases to $2\text{--}10 \times 10^{-6}$ M in the extracellular fluid.⁵⁸

The most commonly used redox-responsive functional group is the disulfide bonds which could be cleaved by GSH. Our group developed a disulfide bond-containing polymer (Poly (disulfide amide) (PDSA)). It was composed of a lipid-poly(ethylene glycol) shell and the PDSA/cationic lipid core which encapsulated the siRNA (Figure 5A). Once it reached the vicinity of tumors via the EPR effect and was internalized by tumor cells, the disulfide bonds in PDSA would be rapidly cleaved by the enriched GSH present in the cytoplasm, leading to the degradation of the nanoparticle and as a result, the release of the encapsulated cationic lipid/siRNA complex. Due to the proton sponge effect induced by the positively charged cationic lipid/siRNA complex, the encapsulated siRNA could finally escape from the endosome with subsequent silencing of the targeted gene.⁵⁸

Gao et al reported dual redox/pH responsive nanoparticles constructed with methoxy-poly (ethylene glycol)-polylactide-polyhistidine-ss-oligoethylenimine (mPEGb-PLA-Phis-ss-OEI)-based polyplex, which exhibited an enhanced endo/lysosomal escape ability (Figure 5B). The polyplex was designed to be a union between pH-sensitive PEG-b-PLA-

PHis and a redox cleavable disulfide bond linked by OEI, where this design provided two responsive sites to achieve efficient multistage responsiveness.⁷⁰

Several studies also reported a nanocarrier constructed by the conjugation of redox-responsive glucolipids (CSO-ss-SA) utilized for the co-delivery of siRNA and a small molecular drug. CSO-ss-SA demonstrated the ability to both condense the siRNA and form the nanocarriers, therefore offering the possibility for CSO-ss-SA to be utilized as a siRNA delivery vector.⁷¹

Conclusion and Future Outlook

This review has considered the barriers for siRNA delivery and the current solutions offered by nanocarriers. As illustrated in the beginning, siRNA suffers from first pass elimination, where more than 95% of siRNAs within the circulation would be eliminated by the liver, renal and RNases in the circulation during the few initial minutes. Even if the nanocarriers could arrive at the tumor region, the abundant negative charge of siRNAs would inhibit its cellular uptake. These barriers are the most important limitations plaguing the clinical application of RNAi technology.

To overcome these obstacles, a multistage nano-delivery system was invented. By utilizing specific stimuli

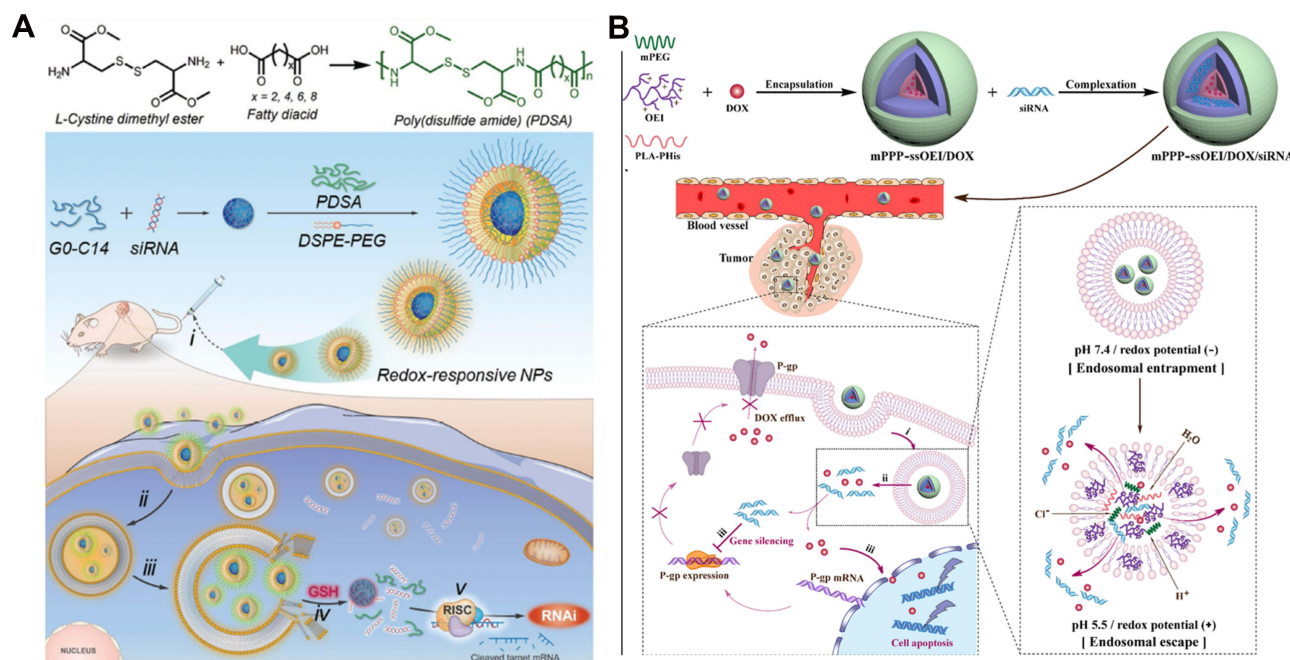


Figure 5 (A) The co-assembly of PDSA polymer with cationic lipid (G0-C14), siRNA, and DSPE-PEG to form stable NPs with an embedded hydrophobic PDSA core with DSPE-PEG on the surface of the NPs; Reproduced from Xu X, Wu J, Liu S, et al. Redox-responsive nanoparticle-mediated systemic RNAi for effective cancer therapy. *Small*. 2018;14:e1802565. Copyright 2018 John Wiley and Sons.⁵⁸ **(B)** Co-assembly of mPEG, OEI and PLA-PHis encapsulating Dox to formulate a mPPP-ssOEI/DOX/siRNA delivery system; Reproduced from Gao Y, Wang Q, Hu H, et al. pH/redox dual-responsive polyplex with effective endosomal escape for codelivery of siRNA and doxorubicin against drug-resistant cancer cells. *ACS Appl Mater Interfaces*. 2019;11:16,296–16,310. Copyright 2019 American Chemical Society.⁷⁰

responsive elements combined with a hydrophilic PEG surface layer, the multistage delivery system would be able to achieve both stabilization in circulation and enhanced cellular uptake in the TME. The most commonly targeted stimuli in the TME are the acidic pH, elevated enzymes, hypoxia and altered redox conditions. By introducing certain elements responsive to TME stimuli, the nanocarriers could change their physical or chemical characteristics (such as shape, charge, dis-PEGylation or dis-assembly) and rapidly release the encapsulated cationic/siRNA complex upon reaching the TME. For example, the amide bond is frequently used as the TME acidic pH responsive element, MMP cleavable peptide is usually used as the element responsive to the enzymes present in the TME, azo group is typically used as the hypoxia responsive element, while the disulfide bond is most commonly used as the redox responsive element.

During recent years, more and more studies are focusing on the development of multistage nanocarriers, especially regarding exclusive siRNA delivery or combination delivery. Due to the benefits offered by these multistage nanocarriers such as the ability of precise tumor targeting and high efficiency of siRNA delivery, increasing numbers of “untargetable” proteins which are crucial in tumorigenesis are now “targetable”. Therefore, it can be projected that the multistage siRNA delivery system would provide enormous potential for cancer therapy in the future.

Abbreviation

Ago2, Argonaute 2; CDM, Cyclohexanedimethanol; CSOss-SA, Redox-responsive glucolipids; DDS, Drug delivery system; DET, PEG-GPLGVRG-PAsp; DMMA, 2,3-Dimethylmaleic anhydride; DOPE, Phosphoethanolamine; dsRNA, Double-strand RNA; EPR, Enhanced permeability and retention; FDA, US Food and Drug Administration; GSH, Glutathione; hATTR, Hereditary transthyretin amyloidosis; MMPs, Matrix metalloproteinases; NBCF, Nitrobenzyl chloroformate; NgBR, Nogo-B receptor; NPs, Nanoparticles; PDSA, Polymer (Poly (disulfide amide)); PEG, Polyethylene glycol; PEI, Polyethylene imine; PHMEMA, Hydrophobic poly(2-(hexamethyleneimino) ethyl methacrylate); PPD, PEG-Peptide-DOPE conjugate; PPP-NPs, PPP-modified PLGA nanoparticles; RISC, RNA-induced silencing complex; RNAi, RNA interference; ROS, Reactive oxygen species; siRNA, Small interfering RNA; S-NP, Sheddable nanoparticle; SOD, Superoxide dismutase; TME, Tumor microenvironment.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81874226, 81803020, 82050410363, 82072930), the International Scientific and Technological Cooperation Program from Guangdong Science and Technology Department (2018A050506033), the Hundred Talents Program for Young Scholars of Sun Yat-sen University (1320318003) the grant from Guangzhou Science and Technology Bureau (201704020131), the Guangdong Province Outstanding Youth Award (2021B1515020066), The Three Million for Three Years Project of SYSMH (132090023), and grant from Guangdong Science and Technology Department (2017B030314026).

Disclosure

The authors declare no conflict of interest.

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