Recent advances in siRNA delivery for cancer therapy using smart nanocarriers

Penghui Zhang[1,2], Keli An[1,2], Xiaoman Duan[1,2], Hao Xu[1,2], Fei Li[1,2] and Feng Xu[1,2]

[1] The Key Laboratory of Biomedical Information Engineering of Ministry of Education, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an, China
[2] Bioinspired Engineering and Biomechanics Center (BEBC), Xi'an Jiaotong University, Xi'an, China

Small interfering RNAs (siRNAs) can selectively target and downregulate disease-causing genes, holding great promise in treating human diseases, especially malignant cancers. However, how to efficiently deliver siRNAs into target cell cytosol is a problem that has hindered their clinical application. Here, we review the recent strategies for siRNA delivery on the basis of smart nanocarriers by using stimuli-responsive materials. We highlight the rationales of how to design smart nanocarriers responsive to physiological and external stimuli to improve the delivery efficiency, targeting precision and gene silencing efficacy. Finally, we provide an outlook on the fundamental limitation for clinical translation of siRNA-based nanomedicine that should be overcome by the combination of chemistry, biology, material and medical science.

Introduction

RNA-based therapeutics have shown great potential for treating miscellaneous diseases through antisense oligonucleotides (ASOs), aptamers, microRNA (miRNA) mimics, small-interfering RNA (siRNA), synthetic mRNA and CRISPR-Cas9 [1]. Especially in cancers, these drugs bring promise to patients bearing undruggable mutation genes by knocking down the gene expression, altering mRNA splicing, upregulating target genes and editing the genome [2]. Different from small-molecule inhibitors or antibodies, once delivered to the specific cell or tissue, the devised RNA-based therapeutics can selectively target the single gene, and off-target effects can be eliminated, bringing great potential in enhancing therapy efficacy with minimized side effects [3].

Since Fire *et al*. reported that double-stranded RNA exerted 100-fold improved gene silencing as opposed to single-stranded RNAs [4], RNA interference (RNAi) has been widely used to assess the individual roles of genes in different cellular processes and diseases, and to develop RNA-based therapeutics to correct disease-causing genes in hepatitis, virus infection, cancer, among others. So far, the most efficient RNAi effectors are miRNAs and siRNAs. The single-stranded miRNA with 19–24 nucleotides always elicits functions in an endogenous pathway. It is initially transcribed as primary miRNA in the nucleus, and is processed into precursors and mature forms through a series of biogenesis machinery including the enzymes Drosha and Dicer. The miRNA is recruited to the RNA-induced silencing complex (RISC) in the cytosol and regulates the protein-coding genes either by mRNA degradation or translational repression [5]. By contrast, siRNAs come from solid-chemistry synthesis and consist of a mRNA sequence (sense strand) and its complement (antisense strand), and are 21–23 nucleotides in length. They can bypass Dicer processing and directly load in the RISC to recognize the target mRNA sequence through perfect base-pair complementarity, leading to efficient cleavage and degradation and consequently gene knockdown. The siRNA therapeutics have experienced booming development since being first administered in 2003 [6], and have attracted tremendous research interest and capital investment. Recently, several siRNA therapeutics have entered clinical trials and demonstrated potent and durable gene silencing in the treatment of solid cancers following systemic administration [7].

The challenge in turning siRNAs into drugs is similar to other DNA or RNA candidates. These large and highly charged macromolecules

Corresponding author: Xu, F. (fengxu@mail.xjtu.edu.cn)

https://doi.org/10.1016/j.drudis.2018.01.042

suffer from the issues of rapid degradation by nucleases in body fluids, fast clearance by kidney and liver scavenger receptors, potential threats to trigger innate immune response and inefficient delivery to cross the cell membrane [2]. To address this, new RNA chemistries, such as substituting the natural phosphodiester linkages or modifying the 2′-position of the ribose, have been developed to improve the stability of siRNAs, reduce the unintended off-target effects and maximize on-target pharmacologic activity *in vivo*. However, the siRNA macromolecules still lack the capability to traverse the membrane and escape from the endosomes into the cytosol, where they initiate gene silencing. To aid cellular uptake, cationic lipid- or polymer-based transfection reagents are widely employed in *in vitro* experiments, but show poor performance *in vivo*. In fact, ~70% of clinical trials in gene therapy carried out so far utilize modified viruses to deliver genes, which stagnates for safety issues such as carcinogenesis and immunogenicity [8]. Recently, a broad diversity of nanomaterials with unique physical and chemical properties has been engineered to construct nanocarriers for delivering siRNA therapeutics, including polymers, dendrimers, lipids, protein-, gold-, silica- and iron-oxide-based nanoparticles [9–11]. The most striking characteristic of nanocarriers is that their physicochemical properties such as composition, size, shape and surface chemistry can be precisely modulated [12]. The nanocarriers can not only efficiently protect the siRNAs from nuclease degradation and renal clearance but also significantly improve the pharmacokinetics and biodistribution, thus enabling targeted delivery to specific cells and spatiotemporally controlled release on command [13,14].

Recently, the rapid development of stimuli-responsive materials has significantly promoted the application of nanotechnology in chemotherapy and medical translation to the clinic [15]. Although these promising chemical and material tools have been well studied for drug delivery, there is still lack of comprehensive reviews to summarize the recent application of novel stimuli-responsive materials for siRNA delivery. In this review, we highlight the recent design and approach to solve the delivery problems by using smart nanocarriers responsive to the physiological environment and external stimuli, such as how to improve delivery efficiency, targeting precision and gene silencing. Then we provide insight to guide the design of future siRNA-based nanomedicines for clinical translation.

Recent advances in smart nanocarriers for siRNA delivery

Efficient delivery and intracellular release are prerequisites for siRNA-mediated gene silencing. Different from small-molecule drugs, siRNA therapeutics exhibit several different characteristics: (i) they are endowed with high nuclease susceptibility and thus special packaging is needed to protect them from blood and tissue RNases; (ii) the negatively charged macromolecules cannot traverse the membrane unless it is with the aid of transfection reagents; (iii) the condensed siRNAs must be completely released in the cytosol so that they can load on RNAi machinery. To enhance the therapy efficacy in patients, a number of siRNA-based nanomedicines have been approved for clinical trials [7]. Although most cases involve nontargeted lipids or polymers, multiple results demonstrated these nanocarriers have favored the siRNA accumulation in human tumors, presenting great potential in RNAi-based cancer treatment.

To further improve the therapy efficacy, modifying the nanocarriers with specific ligands (i.e., antibodies, aptamers, peptides, folic acid, hyaluronic acid, etc.) is a popular and promising strategy to target the malignant tissues [16,17], packaging the charged macromolecules, shielding them from damage, delivering the right amount to right location and releasing the cargoes in a controlled manner. However, a recent review reveals that most nanocarrier systems tend to be trapped in the liver, spleen and kidney, and only 0.7% (median) of the administered dose is delivered to the solid tumor [18]. This could be beneficial for liver disease, but negatively impact nanomedicine translation for cancer treatment with respect to cost, toxicity and therapeutic efficacy (Fig. 1). It should be noted that the poor delivery efficiency *in vivo* is attributed to the fact that the nanocarriers with unchangeable surfaces or composition cannot adapt to the complex biological system. To overcome the biological barriers, many smart formulations have been developed, in which stimuli-responsive moieties translate the chemical or external physical signals into significant behavior changes, such as swelling, aggregation, degradation, surface rearrangement and charge reversal [19]. By virtue of the powerful chemistry and material tools, as shown in Table 1, smart nanocarriers responsive to physiological stimuli (e.g., acidic pH, overexpressed enzymes, redox gradient or elevated metabolites) and external triggers (e.g., NIR light, magnetic field or ultrasonic sound) hold great promise in improving delivery efficiency, enhancing therapy efficacy and reducing side effects [20–33].

Smart nanocarriers for siRNA delivery based on physiological stimuli

Significant variations in physiological parameters exist at the organ, tissue and cell levels, and are highly correlated with various pathological conditions, which can serve as attractive biomarkers for diagnosis and natural endogenous stimuli for controlled drug release in cancer, infection, diabetes, cardiovascular and degenerative diseases.

Smart nanocarriers for siRNA delivery based on pH

The pH gradient in a local tumor is different from that in the normal counterpart because increased fermentative metabolism and poor perfusion lead to the accumulation of lactic acid and formation of an acid microenvironment, which is hypothesized to promote tumor invasion and metastasis [34]. The siRNA-based nanocarriers will undergo drastic pH variations before exerting gene knockdown in RISC machinery. They must extravasate from the blood vessels (pH 7.4), diffuse into the tumor center (pH 7.2–6.0), enter the endosomes (pH 5.0–6.5) and lysosomes (pH 4.0–5.0), and finally escape into the cytoplasm (pH 7.4). Numerous smart pH-responsive formulations have been developed, in which the drops in pH can protonate the ionizable polymers (e.g., polyacids, cationic lipid and polymers, and poly-amino acids) to change their solubility or electrostatic interaction, or degrade the acid-cleavable bonds (e.g., hydrazine, ketal, ester) and nanoparticles for cargo release [35–37]. In particular, after being internalized in an acidic endosome, the polyamine-derived polymers with pK_a range of 5–7 can be rapidly protonated and cause endosomal swelling and rupture via the proton-sponge effect, making them promising scaffolds to facilitate endosomal escape without the aid of additional endosomolytic enhancers (e.g., chloroquine), which can cause severe side effects in normal tissues.

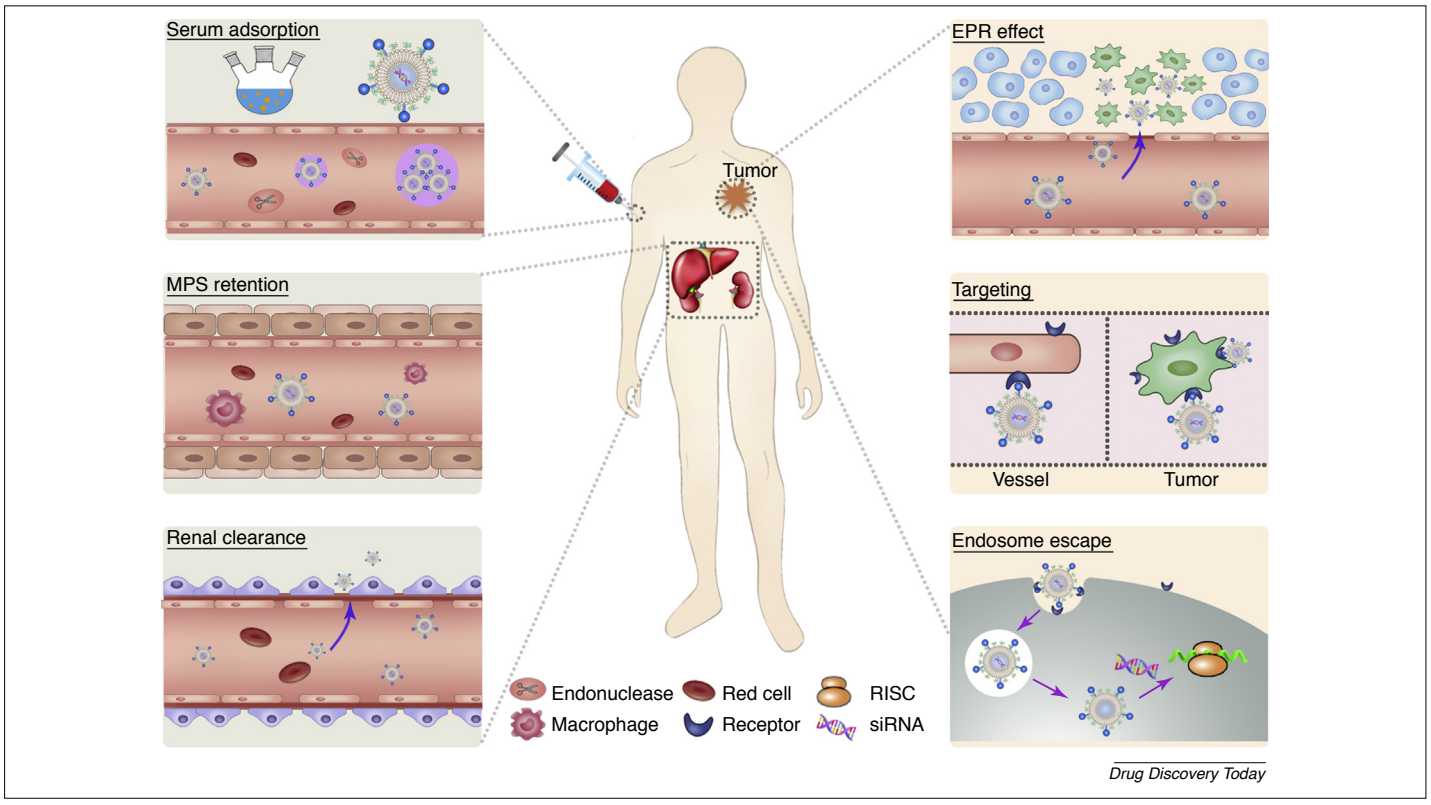

FIGURE 1

Biological barriers for siRNA delivery *in vivo* by using nanocarriers. The nanocarriers synthesized in the flask are equipped with PEG corona and targeting ligands. They are generally administered via intravenous injection, and thus the biological surroundings affect their *in vivo* circulation and biodistribution. Once in the bloodstream, distinct serum proteins adsorb onto the nanocarriers to form protein corona and bestow the nanocarriers new biological identity, which could lead to particle aggregation, MPS retention, liver and renal clearance. After accumulation in tumor sites via the EPR effect, the nanocarriers are internalized into the target tumor cells via active ligands or passive interaction and are always trapped inside the endosome. Most nanocarriers are designed to escape from the endosome into the cytosol by means of the proton-sponge effect, and subsequently release the siRNAs. Finally, the siRNAs are loaded in the RISC for gene silencing.

Lipid nanoparticles are the most popular platforms for siRNA delivery [38]. The positive charges on the surface of cationic lipid formulations (lipoplexes) can condense the genes and facilitate the cellular uptake and endosome escape. Lieberman's group developed a novel imaging approach to visualize the release process of siRNAs in lipid-based nanocarriers after endocytosis [39]. The results demonstrated that the free siRNAs could be rapidly released from the maturing endosomes during a narrow time window within 5–15 min after uptake. The efficient gene knockdown occurred within a few hours of release with the need for a minimum of 2000 copies in the cytosol, which established guidelines for designing and optimizing siRNA delivery strategies. To eliminate the unwanted toxicity, more smart motifs should be introduced into the cationic lipid to meet the demands for *in vivo* siRNA delivery. For example, tertiary amines with tunable pK_a values in the physiological range can be ionized at acidic buffers with sharp phase changes from hydrophobic to hydrophilic states [40]. By means of ultrasensitive tertiary amines, Nguyen's group crosslinked a poly(acrylic acid) shell onto a lipoplex core with diamine bonds. The dual pH-responsive behaviors of the tertiary ammonium head and acid-cleavable diamine linkers have facilitated the endosome escape and siRNA release [41]. Ultra pH-sensitive nanomicelles on the basis of polycations could enhance the early endosome escape and efficiently improve the transfection efficiency *in vitro* (Fig. 2a) [23]. However, it should be noted that the same pH distribution is shared inside normal and tumor cells, which means that it is hard to realize specific siRNA release by the acidic endosome.

The reticuloendothelial system (RES) in the living body can rapidly clear the cationic nanoparticles, further deteriorating the poor delivery. Equipping the nanocarriers with a PEG corona can minimize the nonspecific interaction and enhance the passive accumulation at tumor sites via the enhanced permeability and retention (EPR) effect [42], but in turn harm the targeted uptake by tumor cells. Recently, activatable strategies have attracted great interest in nanocarrier design, where the functional groups are protected by an inert shell in the circulating system and the surfaces are activated in the tumor microenvironment with the exposure of targeting ligands or reversal of surface charge. The minute pH distinction between blood circulation and extracellular tumor matrix is one of the most universal and practical tools, which can activate the nanocarriers for specific tumor penetration and targeting. As shown in Fig. 2b, Wang's group incorporated an extremely pH-sensitive moiety of 2,3-dimethylmaleamidic acid (DMMA) in a micelleplex delivery system [21]. The PEG corona collapsed in the acidic tumor microenvironment and exposed the cell-penetrating peptide, enhancing the cellular uptake and endosome escape, which exhibited superior gene silencing efficiency *in vivo*. More interestingly, a viral–nonviral chimeric nanoparticle

TABLE 1

Recent strategies for siRNA delivery using new chemistry and material

Formulations	siRNA target	Cell type	EPR effect	Targeting ligands	Endosome escape	Cargo release	Year
Passive targeting							
Gold nanoclusters	NGF	Pancreatic cancer	– (*in vivo*)	–	Proton sponge	–	2017 [31]
Dendrimers	MYC	Liver cancer	– (*in vivo*)	–	Proton sponge	Esterase	2016 [28]
Polycations	SCD1	Liver cancer	– (*in vivo*)	–	Proton sponge	Acidic pH	2016 [23]
RNA microstructure	GFP	HeLa	– (*in vitro*)	–	Proton sponge	Dicer	2016 [27]
Polymer	EGFP	HeLa	– (*in vitro*)	–	Proton sponge	Acidic pH	2016 [30]
NIR polymer	BRAF	Thyroid cancer	PEG (*in vivo*)	–	Proton sponge	Acidic pH	2016 [22]
Active targeting							
pRNA nanoparticles	MED1	Breast cancer	– (*in vivo*)	HER2 aptamer	Proton sponge	Dicer	2017 [24]
pH-responsive polymer	PHB1	Prostate cancer	PEG (*in vivo*)	ACUPA	Proton sponge	Acidic pH	2017 [26]
PolyMetformin	BCL-2	Lung cancer	PEG (*in vivo*)	Anisamide	Proton sponge	Acidic pH	2016 [101]
Polyurethane	GFP	Osteoblast	PEG (*in vivo*)	Periostin-peptide	–	–	2016 [25]
CaP	Ras	Glioblastoma cell	– (*in vivo*)	Apolipoprotein E3	Acidic degradation	Acidic pH	2017 [29]
LDH	Survivin	KB cell	– (*in vivo*)	Folic acid	Size effect	Acidic pH	2016 [33]
Activatable targeting							
Amphiphilic polymer	Survivin	Prostate cancer	PEG (*in vivo*)	iRGD/CRGDR (protease)	Proton sponge	Acidic pH	2016 [99]
Coplymer	Twist protein	Breast cancer	PEG (*in vivo*)	PEG/PEI (MMP-2/9)	Proton sponge	Acidic pH	2016 [20]
DNA nanotube	VEGF	Leukemia cell	– (*in vitro*)	Aptamer (lock/key)	Rigid structure	–	2016 [32]
Copolymer	CDK4	A549 cell	PEG (*in vivo*)	PEG/R9 (acidic pH)	Proton sponge	Acidic pH	2015 [21]

was prepared to simultaneously tackle two key BCR-ABL gene regulation pathways by coating the acid-degradable polymeric shell on the adeno-associated virus (AAV) core [37]. The sequential release of siRNA and virus by acid digestion in endosomes could avoid the generation of anti-AAV serum, and facilitate the simultaneous expression of BIM and silencing of MCL-1, synergistically suppressing the proliferation of BCR-ABL-positive K562 and FL 5.12/p190 cells *in vitro* and *in vivo*. Besides polymer, the degradable inorganic nanoparticles are also promising scaffolds for siRNA delivery. An acidic-degradable inorganic nanocarrier based on layered double hydroxide (LDH) demonstrated excellent biocompatibility, high delivery efficiency and gene silencing, presenting as a promising therapeutic system [33]. By virtue of the pH gradient in tumor tissues, pH-responsive nanocarriers have facilitated the specific cellular uptake, endosome escape and siRNA release. To promote clinical translation, new chemical and material tools should be introduced to make the design more simple and robust.

Smart nanocarriers for siRNA delivery based on enzymes

The initiation, progression, invasion and metastasis of a tumor are often associated with elevated expression of certain enzymes. The endogenous enzymes are classified into two categories: enzymes secreted into the ECM of tumors [e.g., matrix metalloproteinases (MMPs), furin, alkaline phosphatase, hyaluronidase] that are widely investigated as triggers to change the surface states of nanocarriers for massive retention or targeted recognition; and the overexpressed intracellular enzymes (e.g., esterase, cathepsin and kinase) that can cleave the specific chemical bonds or peptide substrates, so as to decompose the nanocarriers for cargo release [43].

MMPs, always overexpressed in tumor tissues with metastatic potential, are the most popular cues for designing enzyme-activating nanocarriers [44,45]. Two members of the MMP family: MMP-2 and MMP-9, can specifically recognize and degrade the

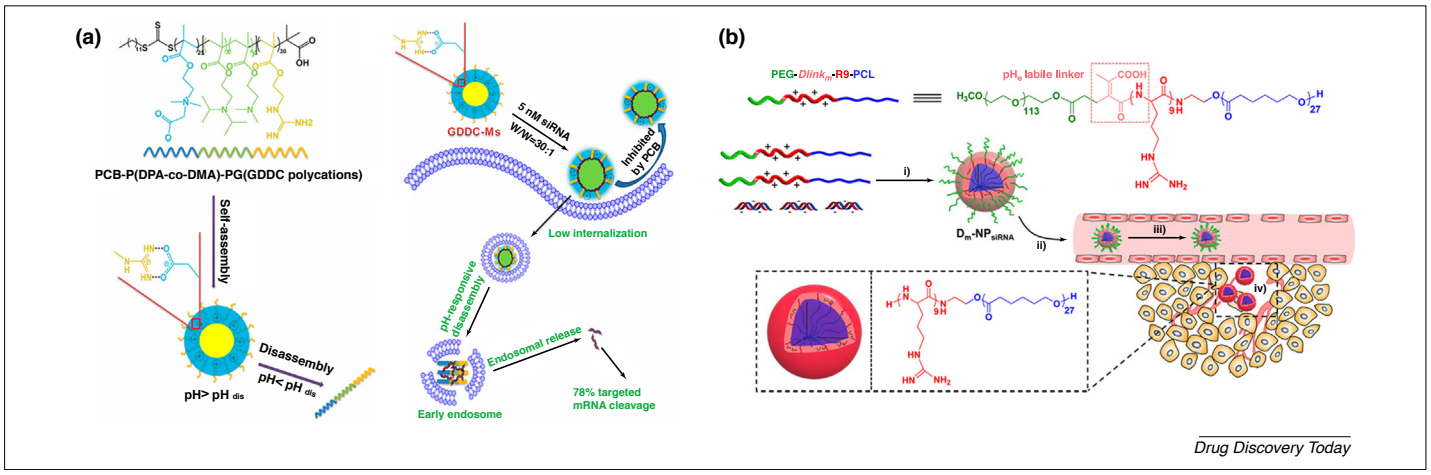

FIGURE 2

Recent advances in siRNA package and release with pH-responsive polymers. (a) pH-sensitive nanomicelles for high-efficiency siRNA delivery *in vitro* and *in vivo* [23]. (b) Tumor acidity-sensitive polymeric vector for activated siRNA delivery. (i) Self-assembly of PEG-*Dlink*$_m$-R9-PCL into nanoparticles to form D$_m$-NP$_{siRNA}$ for siRNA package. (ii) Systemic injection of D$_m$-NP$_{siRNA}$. (iii) Prolonged circulation of D$_m$-NP$_{siRNA}$ with PEG layer. (iv) Enhanced recognition of D$_m$-NP$_{siRNA}$ by tumor cells following degradation of the *Dlink*$_m$ labile linkage [21].

peptide sequence of Pro-Leu-Gly-Leu-Ala-Gly (PLG). In a typical design (Fig. 3a), the PLG peptide was inserted in the middle of the PEG-p-PDHA copolymer and the MMP/pH dual-sensitive nanocarriers were prepared for co-delivery of paclitaxel and siRNA in breast tumor cells. In a tumor microenvironment rich of MMP-2, the PEG layer was pulled off and the positively charged polyethylenimine (PEI) enhanced the penetration and cellular uptake, where simultaneous release of drugs and siRNAs synergistically inhibited the tumor growth and pulmonary metastasis [20].

Intracellular enzymes are also attractive triggers for tuning the release profiles of the nanocarriers. For instance, Meade *et al.* synthesized short interfering ribonucleic neutrals with phosphate backbones that were neutralized by phosphotriesters, allowing good serum stability and efficient delivery into cells [46]. Once inside the cell, the neutralized groups as well as the cell-penetrating peptides were efficiently cleaved by intracellular thioesterases, creating native siRNAs capable of gene silencing, with apparent EC_{50} values in the low nanomolar range. Shen's group reported an esterase-responsive gene carrier for potent cancer therapy with quaternary amines carrying *N*-propionic-4-acetoxybenzyl ester substituents [47]. The ester group underwent a fast esterase-catalyzed hydrolysis, subsequently triggering charge reversal from cationic to zwitterionic and cargo release. To better understand the influence of the molecular structures on delivery efficacy, Anderson and co-workers prepared 1400 esterase-degradable lipidoids and evaluated their transfection efficiency and structure–function activity [48]. Four structural and pK_a criteria were identified that could robustly predict the ability of nanocarriers to mediate >95% gene silencing *in vivo* without any prior biological testing. Different from other stimuli, the enzymes can specifically cleave their substrate to control targeting and triggering release. In the future, more tumor-associated enzymes should be screened for personalized clinical use.

Smart nanocarriers for siRNA delivery based on redox

Reactive oxygen species (ROS) are a group of highly reactive chemical species generated by all aerobic organisms in forms of hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$) and superoxide radical (O^{2-}), playing important parts in different cell signaling pathways. The normal cells must maintain the ROS homeostasis because overproduction of ROS causes oxidative stress, and subsequent cellular damage and organ function decline [49]. Redox balance between oxidizing and reducing species is achieved by various enzymes such as superoxide dismutase and glutathione peroxidase. Accumulating evidence suggests that the dysregulation of aggressive metabolism and ROS scavengers leads to detrimental accumulation of ROS in tumor cells, which will adapt to oxidative stress by upregulating glutathione (GSH) [50]. Ironically, the tumor cells utilize ROS to drive proliferation and invasion, but they are more susceptible to the reagents that damage the redox balance, whereas the normal cells are less sensitive owing to the low basal ROS and high antioxidant capacity [51].

The ROS level in tumor cells (~100 μM) reaches 10- to 100-fold levels higher than that in normal cells, which can be engaged for controlled drug release by cleaving thiolketal and aryl boronic acid bonds, or oxidizing thiolester and ferrocene units [52–54]. Murthy's group incorporated a ROS-sensitive thioketal bond into the polymer backbone to package the siRNA for oral delivery to intestinal tissue [55]. The thioketal linkages were stable to acid-, base- and protease-catalyzed degradation, but were broken down by elevated ROS at inflamed intestinal tissues, triggering the siRNA release. Similarly, Peng *et al.* reported a ROS-responsive fluorinated bola-amphiphilic dendrimer for delivering siRNA in cancer cells on command [56]. The ROS-sensitive thioacetal linkage in the dendrimer promoted the specific and efficient dissociation of the siRNA/vector by endogenous ROS in tumor cells for effective siRNA delivery and gene silencing.

To maintain the redox balance, tumor cells generate massive intrinsic antioxidants, such as GSH, to counteract ROS-induced oxidative stress. The intracellular concentration of GSH in tumor cells is 2–10 mM, which is fourfold higher compared with that in normal cells, but the extracellular GSH level drastically decreases to 2–20 μM in the tumor microenvironment. The distinct GSH

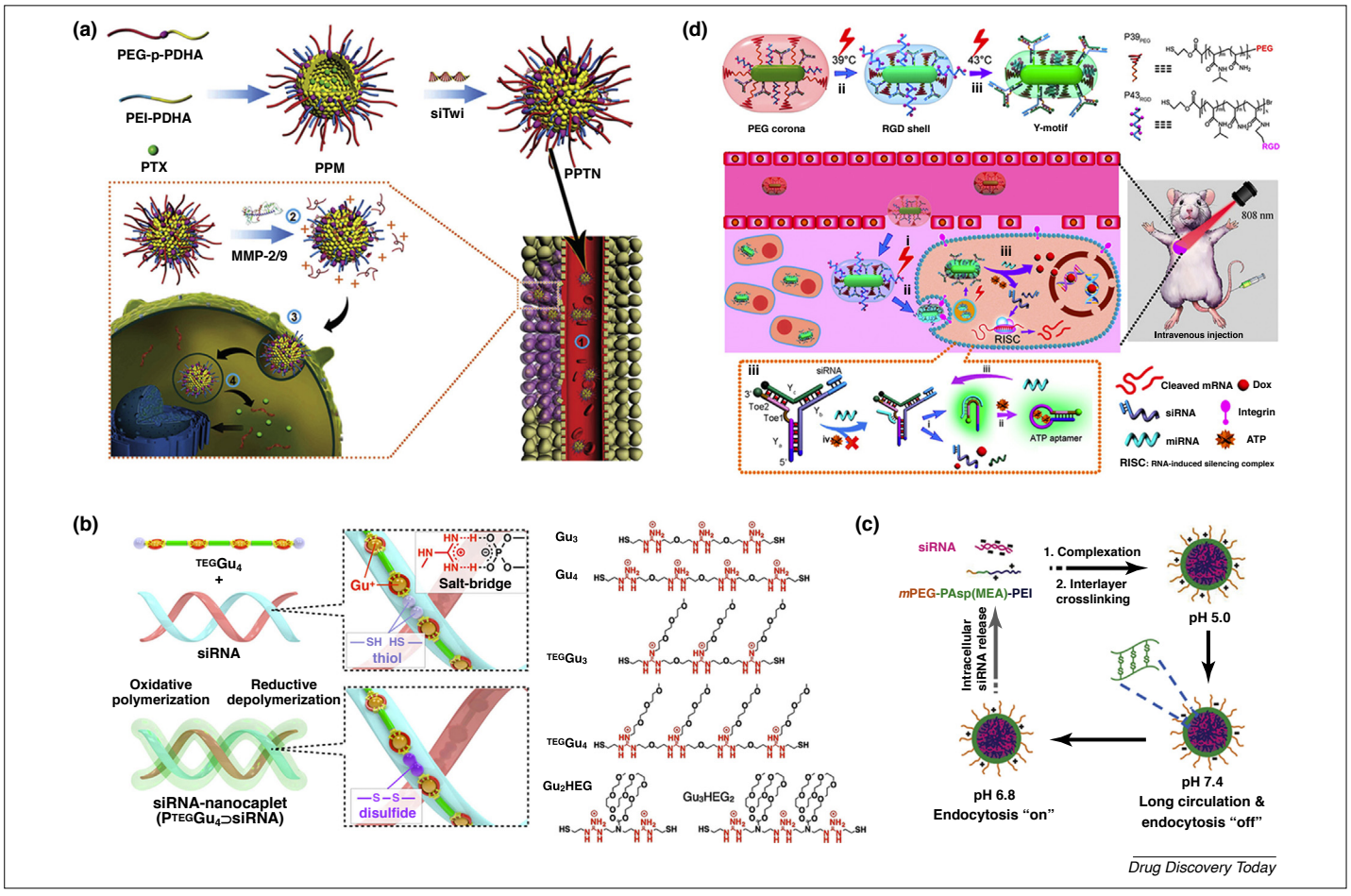

FIGURE 3

Novel smart nanocarriers for siRNA delivery with endogenous molecules as triggers. (a) The tumor-microenvironment-sensitive nanoparticle co-loading siTwi and PTX [20]. (b) Oxidative polymerization of $^{TEG}Gu_4$ with siRNA as the template to form siRNA-containing nanocaplet [59]. (c) Formation of charge-reversible polyplex for long circulation, tumor-specific cell uptake and easy intracellular siRNA release [58]. (d) Schematic design of the smart nanocarriers triggered by miRNA and fueled by ATP. Amplified dissociation of Y-motifs based on TMSD cascade reactions with miRNA as the trigger and ATP as the fuel: (i) Toe1-TMSD triggered by miRNA; (ii) ATP recognition by aptamer; (iii) Toe2-TMSD for miRNA regeneration [71].

gradient makes it convenient to design GSH-responsive nanocarriers for on-command drug release (Fig. 3b,c) [57–59]. The disulfide bond is the most promising motif that is stable in the extracellular space, but can be cleaved into thiol by reductive species or through thiol–disulfide exchange with thiol-bearing molecules [60]. To ensure deep tissue penetration through endothelial fenestrations (60–80 nm) and intercellular gaps (~10 nm), a series of nanocaplets with uniform size (~7 nm) were synthesized by oxidative polymerization of a water-soluble dithiol monomer carrying multiple guanidinium ion (Gu^+) pendants (Fig. 3b) [59]. The packaged siRNA could be liberated by GSH-mediated cleavage. Besides disulfide bonds, diselenide bonds also can be cleaved by GSH but undergo slower reduction kinetics because the sulfur is much more active as an electron acceptor than selenium. Gu's group designed a hierarchically ternary system to compact genes with diselenide and disulfide polymers [61]. These smart nanocarriers showed stepwise responses to the dual reduction gradients in the tumor sites and intracellular conditions, and exerted efficient genome release. In addition, GSH was also utilized to reduce the ferrocenium cation to ferrocene with a polarity shift from hydrophilic to hydrophobic, which could drive the disassembly of siRNA-loaded vesicles for rapid drug release and gene transfection [62]. Furthermore, the degradation of the redox-responsive nanocarriers consumes massive redox species and breaks the intracellular redox imbalance, which is neglected in most studies but would be promising to be combined with other strategies in tumor therapy [63].

Smart nanocarriers for siRNA delivery based on other metabolites

The aggressive proliferation and malignant metastasis of tumors also led to the dysregulation of other metabolites, such as oxygen, glucose, ATP and nucleic acids, which can be used to trigger on-command controlled drug release. Hypoxia is a general characteristic during tumor progression. Altered vasculature and consequent chaotic blood flow cause the gradients of oxygen tension and acute hypoxia (<1.4% oxygen) in fast-growing tumors, which poses a survival pressure to favor the upregulation of genes associated with tumor initiation, metastasis and resistance to chemotherapy and radiotherapy. Nitroaromatic, quinone and azobenzene derivatives undergo reduction-mediated cleavage in the hypoxia microenvironment, which is inhibited by molecular oxygen owing to radical depletion [64]. Torchilin's group

introduced a hypoxia-sensitive moiety of azobenzene in a PEG-ended cationic copolymer for hypoxia-induced siRNA uptake and gene silencing, representing a promising tumor-environment-responsive modality for tumor targeting and therapy [65].

As the molecular unit of currency for energy transfer in the living body, ATP is the essential biogenic molecule for cellular metabolism and signaling, which is highly concentrated within the intracellular cytosol (1–10 mM) but much lower in the extracellular niche (<5 μM). This great concentration gradient is a promising biological rationale for designing an ATP-triggered drug release system [66,67]. Phenylboronic acid (PBA) can form reversible covalent esters with 1,2-*cis*-diols incorporated on a ribose ring, which has been used for capturing RNA in affinity chromatography. Kataoka's group has conjugated the PBA pedants on a cationic polymer, which could efficiently condensate the siRNAs via electrostatic interaction and firmly lock them via PBA–diol recognition [68]. Once exposed in cytosol, the excess diols of ATP would competitively bind with the PBA motifs and release the siRNAs for gene silencing.

Another kind of attractive stimuli in tumors is the gene targets including DNA fragments, mRNA and especially miRNAs. miRNAs play important parts in the gene regulation network in all the cellular processes. Particularly during cancer initiation, progression and metastasis, tumors exhibit different miRNA expression patterns compared with normal tissues, providing promising biomarkers for cancer diagnosis and potential therapeutic targets. In our previous work, we have focused on the design of miRNA-responsive smart nanocarriers for controlled drug release and cancer detection [69–71]. To recruit low copies of endogenous miRNA, a Y-shaped DNA nanomachine was developed in Fig. 3d, by which the drugs and siRNAs could be efficiently released with oncogenic miR-21 as triggers and ATP as fuels, resulting in high tumor inhibition and minimum damage to normal organs.

Endogenous stimuli can selectively activate the targeting and trigger the drug release in the tumor sites. However, genetic and phenotypic heterogeneities are common features of tumors, leading to the dynamic changes of the tumor microenvironment [72]. The extracellular matrix, metabolites, chemokines, growth factors, as well as surrounding cells (e.g., fibroblasts, immunes cells, epithelial cells, etc.) can vary from patient to patient, and even dynamically change during tumor progression and therapy in the same person [73]. These uncertain factors make it difficult to predict the drug dosage and release kinetics, and more-efficient and smart formulations are needed to meet the demands of translation to the clinic.

Smart nanocarriers for siRNA delivery based on external stimuli

The external stimuli, such as light, temperature, magnetic fields, ultrasound and electric fields, can be applied with a high spatial and temporal resolution to mediate active targeting and controlled drug release, presenting great potential in enhancing therapeutic efficacy and minimizing off-target effects. Herein, we will introduce some external-stimuli-responsive strategies in drug delivery, which could also be beneficial to the translation to the clinic of siRNA therapeutics.

Smart nanocarriers for siRNA delivery based on light

Light with a broad spectral window from 300 nm to 900 nm is the most versatile tool as an external stimuli to modulate the targeting and release processes *in vitro* and *in vivo* owing to noninvasiveness and high controllability. The absorption of light with different wavelengths can induce physical or chemical property changes of the nanocarriers through photoisomerization, photocleavage, photosensitization or photothermal effect.

Ultraviolet (UV) and visible (Vis) light has sufficient energy to initiate photochemical reactions. For instance, the azobenzene derivatives conjugated on the mesoporous-silica-based nanocarriers underwent reversible photoisomerization between *trans* and *cis* on alternating irradiation by UV and Vis light, presenting photoregulated targeting activation and drug release [74]. UV and Vis light can also irreversibly cleave the photolabile groups such as *o*-nitrobenzyl and coumarin-4-yl-methyl groups to change the hydrophilic–hydrophobic balance and the stability of the polymers, release the drug molecules anchored on nanoparticles or remove the caging groups [75]. For instance, Garcia *et al.* designed light-controlled cell-adhesive interfaces by protecting the cyclic RGD peptide with a 3-(4,5-dimethoxy-2-nitrophenyl)-2-butyl ester (DMNPB) photolabile caging group [76]. On exposure to UV light (350–365 nm), the caging groups were cleaved to liberate the active cyclic RGD peptide, demonstrating a noninvasive, transdermal time-regulated technique to activate the cell-adhesive interfaces on implanted biomaterials so as to regulate *in vivo* cell adhesion, inflammation, fibrous encapsulation and vascularization. In early studies, different photoresponsive groups of DMNPE [1-(4,5-dimethoxy-2-nitrophenyl)ethyl], NPE [1-(2-nitrophenyl)ethyl], NPP [2-(2-nitrophenyl)propyl] and CD-DMNPE (cyclo-dodecyl DMNPE) were introduced on the terminal phosphate to sterically block the binding of siRNAs with RISC, whereas the UV irradiation could shed the caging groups and activate the gene silencing [77]. However, the UV and Vis light not only induced severe tissue injury but also showed poor penetration depth (<10 mm) in soft tissues owing to the strong adsorption by tissue chromophores (e.g., hemoglobin, myoglobin and melanin), resulting in poor light-triggered drug delivery *in vivo*.

Near-infrared (NIR) light with wavelengths of 650–900 nm can penetrate the tissue at the centimeter scale with lower scattering and minimal harm to tissues, making the NIR-responsive system promising for clinical application [78]. But the NIR photoenergy is too low to initiate the photochemical reactions. The upconversion nanoparticles (UCNPs), consisting of an inorganic crystalline host matrix and trivalent lanthanide ions embedded in the host lattice, are powerful tools to convert the absorbed NIR light to UV or Vis light through the unique ladder-like energy level structures of lanthanide ions [79,80]. Bian's group encapsulated DMNPE-caged siRNA in the mesoporous-silica-coated UCNPs for the remote controlling and real-time monitoring cell differentiation (Fig. 4a) [81]. The NIR light illumination cleaved the DMNPE groups and activated the gene silencing, which could be visualized by the aggregation-induced emission (AIE) of organic dyes. Kohane's group have designed polymeric micelles with a photosensitizer (PS) in the hydrophobic core to manipulate cell targeting *in vitro* and *in vivo* by using gentle laser irradiation (green light at 200 $mWcm^{-2}$ for 5 min) [82,83]. The energy of green light would be efficiently transferred to cleave a covalent bond by combination of triplet–triplet annihilation-based upconversion and Förster resonance energy transfer (FRET), with the releasing of RGD peptide for specific cell binding.

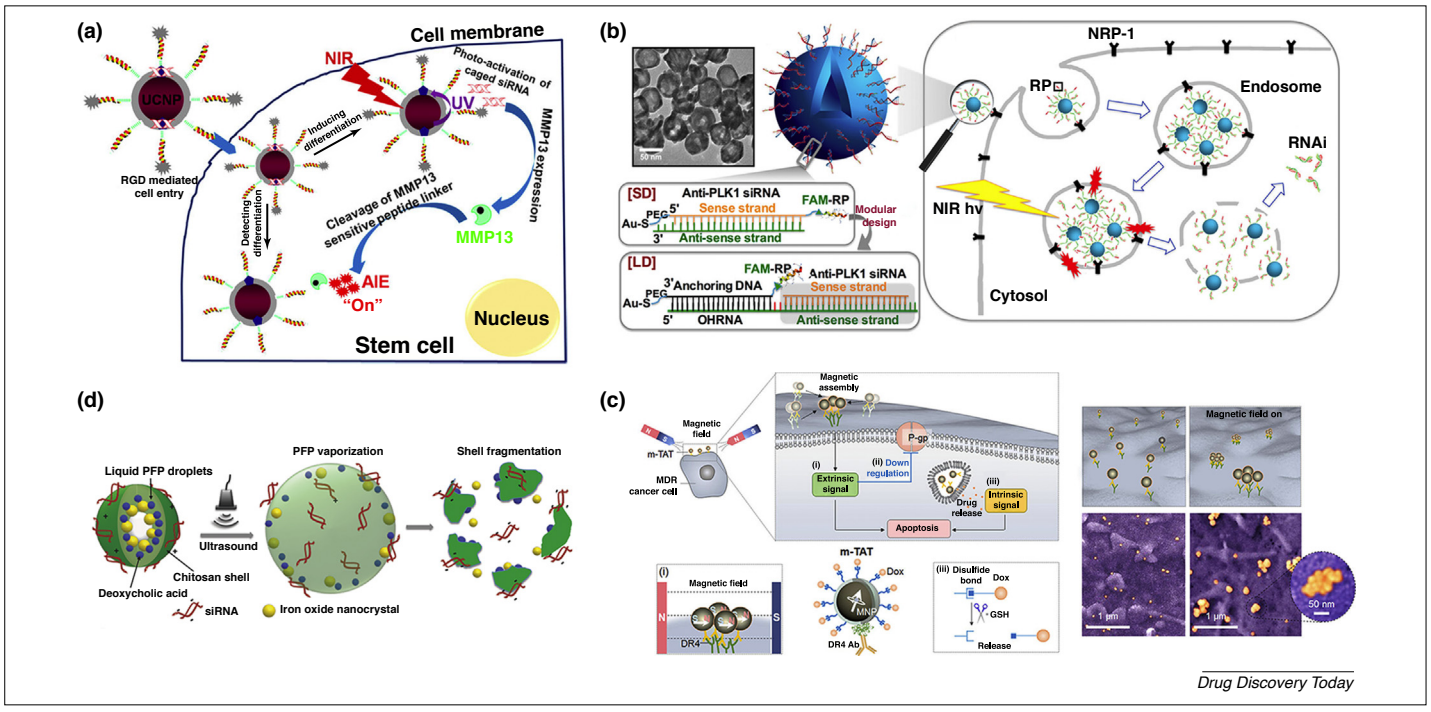

FIGURE 4

Novel smart nanocarriers for cancer therapy controlled by external stimuli. (a) Mechanism of controlling and detecting differentiation of hMSCs by UCNP-peptide-AIE-siRNA [81]. (b) Hollow gold nanoshell (HGN)-siRNA-targeting peptide (RP) architecture with original or modular design and schematic of nanoparticle uptake, laser-activated siRNA delivery pathway in PPC-1 cells [87]. (c) Magnetic tandem apoptosis trigger (m-TAT) and its tandem capability for magnetic clustering and Dox release [94]. (d) Schematic representation of chitosan-deoxycholic acid coated perfluoropentane nanodroplets (CNDs) [97].

The PS can adsorb the NIR light to produce ROS to kill cancer cells, which is called photodynamic therapy (PDT) [84]. The recent studies demonstrated that if the PS-containing nanoparticles are irradiated with NIR light for a short time, the PS energized surrounding molecular oxygen ($^3O_2$) to generate ROS that could induce lipid peroxidation, further leading to an increase of cell membrane permeability for photochemical internalization and endosome escape. Zhou's group constructed an all-in-one protoporphyrin-based polymer nanoplatform with a step-by-step multiple stimuli-responsive function for enhancing the combined chemotherapy and PDT [85]. Short-time irradiation facilitated the photochemical-aided uptake and endosome escape, whereas the long-time irradiation generated massive ROS species, which cleaved the ROS-responsive bonds and released the drugs, simultaneously inducing ROS- and drug-mediated cell death.

NIR light can also be adsorbed by photoabsorbing agents, such as different gold nanostructures, carbon nanomaterials and organic nanoparticles, to generate heat, leading to thermal ablation of cancer cells and subsequent cell death in terms of photothermal therapy (PTT) [84]. Here, we emphasize the applications of photothermal conversion to control targeting and drug release. Thermosensitive polymers (TSPs), usually poly(*N*-isopropyl acrylamide) (PNIPAM), undergo reversible phase transition from the hydrophilic to hydrophobic state at lower critical solution temperature (LCST). By incorporating different ratios of other monomers, the LCST of the PNIPAM-copolymer can be finely controlled between 32 °C and 50 °C, which provides a promising way to adjust the surface composition of the nanocarriers, thereby allowing selective cell entry in a controlled manner [86]. We have conjugated two TSPs (PEG-tailed $P39_{PEG}$ with LCST at 39 °C and RGD-conjugated $P43_{RGD}$ with LCST at 43 °C) onto gold nanorods and reversibly switched the surface states between PEG and RGD for specific cell uptake of nanocarriers carrying siRNA and doxorubicin (Dox), which showed preferred uptake and enhanced cell death in irradiated tumor tissues with minimal damage to normal organs [71]. Similarly, Reich and colleagues conjugated the siRNA on the hollow gold nanoshells by Au–thiol bonds to construct plasmonic nanocarriers, which could target the tumor cells using RPARPAR peptide at the end of the siRNAs (Fig. 4b) [87]. By femtosecond pulses of NIR light at 800 nm, the gold nanoshells converted the light to heat, which thermalized the thiol bonds and released the siRNAs. At the same time, vapor bubbles were rapidly formed and ruptured the endosomes for siRNA release with little damage to cell structure and function.

Smart nanocarriers for siRNA delivery based on magnetic field or ultrasound

Compared with light, magnetic fields have excellent tissue penetration and have been widely applied in whole-body MRI, which is one of the most popular medical imaging techniques. In 2007, Moore and co-workers first described the application of dual-purpose probes for *in vivo* siRNA delivery and simultaneously monitored its accumulation in tumors by high-resolution MRI and near-infrared *in vivo* optical imaging (NIRF) [88]. Zhang and co-workers assembled the siRNAs on an iron oxide core, which served as a MRI agent for treatment monitoring [89]. At the same time, the magnetic manipulation of the magnetic nanoparticles, usually the iron oxide nanoparticles (IONs), is useful for drug delivery, which can be externally guided to the desired cells or

tissues by means of a remote magnet [90]. Plank and co-workers developed a protocol to deliver nucleic acids into the cells by magnetic-force-assisted transfection using magnetic nanoparticles. The nanoparticles were deposited onto the surface of the cells with the aid of a magnetic gradient field, showing 1000-fold transfection efficiency compared with nonmagnetic gene vectors [91]. Recently, many novel strategies have been developed in cancer treatment by magnetic manipulation, which also showed potential application in siRNA-based therapy. Alternating magnetic fields (AMFs) cause the magnetic moment to rotate and return to equilibrium by dissipating thermal energy through Neel (dipole rotation) and Brownian (physical rotation) relaxations, which induce hyperthermia for tumor ablation or thermally controlled drug release [92]. Bhatia and co-workers encapsulated different protease substrates into thermosensitive liposomes to design a protease-activity nanosensor [93]. This sensor was remotely activated at disease sites via an AMF at 515 kHz and 15 kA/m, which could determine the tumor protease activity *in vivo* and identify the different profiles in substrate cleavage between two mouse models. Interestingly, Cheon and colleagues developed a magnetic tandem system to overcome multidrug-resistant cancer at a single-cell level (Fig. 4c) [94]. The magnetic tandem apoptosis trigger (m-TAT) consisted of magnetic nanoparticles conjugating death receptor 4 (DR4) antibody and Dox. Individual magnetic nanoparticles attached onto the tumor membrane by recognizing extracellular receptors such as DR4, which were induced to aggregate under an external magnetic field, activated the extrinsic death signal and induced receptor-mediated uptake. The internalized nanocarriers released the Dox owing to GSH cleavage and initiated the intrinsic death, presenting a new platform concept for cancer therapy.

Ultrasound is another noninvasive technique that can spatiotemporally control drug release at disease sites with deep penetration but slight harm to healthy tissues. It is convenient to regulate the penetration depth by tuning frequency, exposing time and cycles. Ultrasound can trigger the drug release through different mechanisms. The tissues and nanocarriers can absorb the energy of a small fraction of ultrasound and convert to heating, leading to thermally triggered release. Otherwise, the ultrasound can cause mechanical variations in terms of acoustic cavitation, where the nucleation, growth and collapse of gas bubbles destabilize the nanocarriers for drug release. Liu and colleagues prepared nanodroplets of perfluorocarbon as an oxygen shuttle to modulate the tumor hypoxic microenvironment by ultrasound-triggered oxygen delivery [95]. To minimize the side effects and prevent tumor recurrence in high-intensity focused ultrasound (HIFU), Shi's group constructed an organic–inorganic nanohybrid for co-delivery of perfluorooctyl bromide (PFOB) and anticancer drug camptothecin (CPT) [96]. The PFOB could enhance the HIFU ablation, as well as the HIFU-mediated drug release, which synergistically inhibited tumor growth. Lee *et al*. replaced the chemotherapeutic drugs with siRNA for magnetic-guided delivery and ultrasound-controlled release (Fig. 4d) [97]. The siRNAs were electrostatically bound onto the chitosan–deoxycholic-acid nanoparticles containing perfluoropentane and iron oxide, which promoted the cell uptake and apoptosis. So far, other external stimuli have also been studied for controlled drug release, including microwave, Cherenkov radiation, electrical field, among others. Although few of these techniques have been utilized in siRNA delivery, these pioneering designs accumulate a wealth of chemical, material and biological experiences, which will be beneficial to promoting the translation into the clinic of siRNA therapeutics in the future.

Concluding remarks and future outlook

With the development of chemistry, material science and biotechnology, thousands of novel stimuli-responsive nanocarriers have been designed during the past decade for *in vivo* drug delivery, along with a large capital investment and research interest. Especially in recent years, the nanocarrier-based delivery strategies have significantly promoted the application of siRNA in cancer therapy: (i) the siRNA therapeutics are efficiently protected after systematic administration and specifically delivered into the tumor cells for function; (ii) the biodistribution and pharmacokinetics of siRNAs are highly controlled by means of stimuli-responsive motifs in the nanocarriers; (iii) the combination of siRNA-based gene silencing and other treatment methods is widely studied to treat complex and malignant cancer. However, despite the booming scientific publications, few of these technologies have been successfully translated into clinical disease diagnosis and treatment. Several issues should be addressed in the future. First, existing smart nanocarriers have been designed to solve one or two problems during siRNA delivery (Table 1), and this is hard to adapt to the complex microenvironment in the living body. Passive targeting and active targeting strategies cannot meet the demands in complicated biological systems alone owing to massive nonspecific retention or poor cellular uptake issues. To further enhance treatment precision and efficacy, all-in-one activatable strategies responsive to multiple triggers (endogenous and external) are recommended during nanocarrier design: the synergistic stimuli-responsive systems in which either trigger A or trigger B initiates the tumor recognition and drug release to realize specific targeting and harness the tumor heterogeneity, and the sequential stimuli-responsive systems in which trigger A changes the behavior of the outer layer to activate the targeting and then the trigger B acts on the inner core for drug release [98]. For instance, a typical smart nanocarrier has been developed for *in vivo* siRNA delivery: the iRGD-encoded PEG shell prolongs the blood circulation and facilitates passive accumulation and active tumor targeting; the iRGD peptide is then cleaved by cell-surface proteases to expose the CRGDR sequence, which binds to neuropilin-1 for tumor penetration; after cellular uptake, the rapid protonation of the ternary amine segment induces the endosomal swelling for rapid escaping and efficient gene silencing [99]. By contrast, to precisely localize the lesion, assess the pharmacokinetics and monitor the treatment response, an imaging agent is a highly important component in nanocarrier design for clinical use. By incorporating quantum dots, gold nanoparticles, carbon nanotubes, UCNPs or magnetic nanoparticles, the multifunctional theranostic nanocarriers can not only possess different imaging modalities (e.g., optical imaging, positron emission tomography, MRI, photoacoustic imaging, etc.) but can also be endowed additional powerful tools for combination treatment (e.g., photodynamic or photothermal therapy) [100]. However, these exquisite designs are always considered as 'overdesigned' in the perspective of commercialization, which prefers simple structures with ease of scaling up and robust clinic safety and efficacy, thus requiring a trade-off between

function and design. Actually, only a few nontargeted nanomedicines (e.g., Abraxane® and Doxil®) have been approved for clinical treatment, and limited improvements in therapy and diagnosis are obtained. To face the dilemma between commercial drugs and fascinating proof-of-concepts, new ideas should be introduced. Recently, Huang's group utilized the antidiabetic drug metformin to synthesize a guanidination polymer for a siRNA package [101]. This interesting work inspires us to explore the new applications of FDA-approved materials (e.g., degradable PLGA polymer and fluorescent ICG dye) to fabricate multifunctional nanocarriers, which is much easier to solve the safety and translation issues.

Second, the majority of bioresponsive nanocarriers has been designed and tested *in vitro* or in animal models, and the large gap between laboratory and hospital hinders the clinical translation. Before translating the delicate nanocarriers in human trials, it should be noted that: (i) the levels of biological cues might be different between humans and the animal models, and also vary from patient to patient; (ii) the local biological stimuli are highly heterogeneous and dynamically changing at different stages of disease progression. These heterogeneities provide potential chances for personalized treatment, as well as great challenges for clinical translation. In the future, more-specific endogenous stimuli, such as enzymes and gene targets, should be screened to achieve specific targeting and drug release. Meanwhile, by combining with the external stimuli, which can be manually controlled, the precision of tumor targeting and controlled drug release will be greatly improved to adapt the complex clinical application.

Last but not least, the poor delivery efficiency of 0.7% (median) makes us rethink the current underlying principles of nanocarrier targeting. Most *in vivo* studies are built on the theory that the nanocarriers are passively accumulated at tumors via the EPR effect. But what if it is wrong? Current results indicate that the majority of the nanocarriers is sequestered by the MPS system and the EPR effect is not an efficient pathway for *in vivo* delivery in mice, let alone in humans. Meanwhile, it seems that the targeting ligands do not favor the desired active delivery because it is either buried by the protein corona or damaged by assorted enzymes in body fluids. If the nanocarriers cannot accumulate in solid tumors and reach their target tumor cells, all our work will be meaningless. Thus, in the future, it is urgent to explore new modification strategies to improve the EPR effect or find new mechanisms to favor accumulation in tumor tissues.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (21605118, 11372243, 11522219, 11532009), the China Postdoctoral Science Foundation (2015M580829, 2017T100735), the Open Funds of SKLACLS (1508), Postdoctoral Science Foundation of Shannxi Province (2016BSHTBSHTDZZ01) and the Fundamental Research Funds for the Central Universities (xjj2015081).

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