

## Stimuli-Responsive Polymeric Nanocarriers for Efficient Gene Delivery

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**Abstract** Gene therapy provides an alternative and effective method for treatment of genetic diseases and cancers that are refractory to conventional therapeutics. The success of gene therapy is largely dependent on the development of safe and effective gene delivery vectors for transporting genetic material from the blood stream to the cytoplasm or nucleus. Current gene vectors can be divided into viral and non-viral vectors. Although non-viral gene delivery carriers can offer some advantages, such as safety and facile fabrication, they do not possess the same high gene transfection efficiency as viral vectors due to a lack of functionality to overcome extra- and intracellular gene delivery obstacles. On the basis of these disadvantages, researchers are developing “smart” non-viral gene-delivery carriers in order to overcome the physiological barriers and realize efficient gene transfection. These “smart” stimuli-responsive carriers can undergo physical or chemical reactions in response to internal tumor-specific environments, such as pH conditions, redox potentials, enzymatic activations and thermal gradients, as well as external stimulations, such as ultrasound, light, magnetic fields, and electrical fields. Furthermore, “smart” carriers can also be triggered by dual or multiple combinations of different stimuli. In this review, we highlight the recent stimuli-sensitive polymeric

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nanocarriers for gene delivery, and we discuss the potential of combining multiple stimuli-responsive strategies for future gene therapy applications.

**Keywords** Stimuli-responsive · Gene therapy · Polymer · Non-viral vector · Nanocarriers

### Abbreviations

AAM	Acrylamide
AAV	Adeno-associated viruses
ADV	Acoustic droplet vaporization
AON	Antisense oligodeoxy nucleotides
ATP	Adenosine triphosphate
azoTAB	Azobenzene-trimethylammonium bromide
BAH	Bis-aryl hydrazone
C <sub>11</sub> F <sub>17</sub> -PAsp(DET)	C <sub>11</sub> F <sub>17</sub> -poly[ <i>N</i> -[ <i>N'</i> -(2-aminoethyl)]aspartamide]
CMD	Carboxymethyl dextran
CPDT	Cyclopentadithiophene
CTX	Chlorotoxin
DSPEI	Degradability of disulfide cross-linked short PEIs
dtACPP	Activatable cell-penetrating peptide
GALA	Glutamic acid-alanine-leucine-alanine
GFP	Green fluorescent protein
GILT	Gamma-interferon-inducible lysosomal thiol reductase
GNR	Gold nanorod
GSH	Gutathione
HCC	Hepatocellular carcinoma
HHV	Herpes simplex viruses
KALA	Lysine-alanine-leucine-alanine
KAPE	Ketal containing poly ( $\beta$ -amino esters)
LCST	Low critical solution temperature
MB	Microbubble
MF	Magnetic field
MMA	Methyl methacrylate
MMPs	Matrix metalloproteinases
MN core	Magnetic nanopartical core
MPAP	Membrane translocation peptides
mPEG- <i>b</i> -PLLys	Poly(ethylene glycol)- <i>b</i> -poly(L-lysine)
NBU	<i>o</i> -Nitrobenzyl urethane
NIR	Near infrared
NS	Nanoshell
PAA	Poly (acrylic acid)
PAO	Poly(alkylene oxides)
PATK	Poly(amino thioketal)
PBA	Phenylboronic acid
PBAA	Poly (butyl acrylicacid)

PBAE	Poly( $\beta$ -amino ester)
PDEA	Poly(2-(diethylamino)ethyl methacrylate)
PDMAEMA	Poly(2-dimethylaminoethyl methacrylate)
PEAA	Poly(ethyl acrylic acid)
PEEP- <i>b</i> -PDMAEMA	Poly(ethylethylene phosphate)-block-poly[2-(dimethylamino)ethyl methacrylate]
PEG	Polyethylene glycol
PEI	Polyethylenimine
PEO-PPO-PEO	Poly-(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)
PFP	Perfluoropentane
PGA- <i>g</i> -mPEG	Poly(glutamic acid)- <i>g</i> -MeO-poly(ethylene glycol)
PICM	PEG- <i>b</i> -P (PrMA- <i>co</i> -MAA)
PLH	Poly (L-histidine)
PLL	Poly-L-lysine
PLys	Poly (L-lysine)
PMA	Poly (meth acrylic acid)
PNIPAM	Poly ( <i>N</i> -isopropylacrylamide)
PPAA	Poly (propyl acrylic acid)
PPDDP	MPEG <sub>113</sub> - <i>b</i> -CP <sub>5k</sub> - <i>b</i> -PDMAEMA <sub>42</sub> - <i>b</i> -P(DMAEMA <sub>22</sub> - <i>co</i> -BMA <sub>40</sub> - <i>co</i> -PAA <sub>24</sub> )
PPMCs	Pluronic/polyethylenimine to embedded magnetite nanocrystals
PPPs	Photo- and pH-responsive polypeptides
PTX	Paclitaxel
RES	Reticuloe endothelin system
ROS	Reactive oxygen species
SDBS	Sodium dodecylbenzenesulfonate
SDS	Sodium dodecylsulfate
siGFP	siRNA molecules targeting GFP
SPIO	Superparamagnetic oxide nanoparticle
SSPEI	Disulfide-containing polyethylenimine
UCAs	Ultrasound contrast agents
UCST	Upper critical solution temperature
UNCs	Upconversion nanoparticles
uPA	Urokinase plasminogen activator
US	Ultrasound contrast agents
VP	Vinylpyrrolidinone

## 1 Introduction

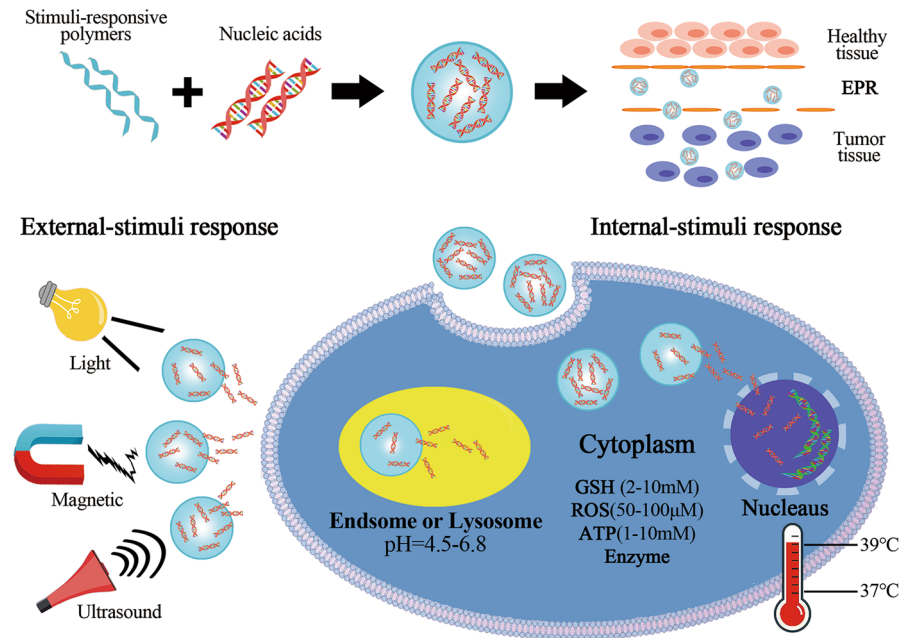
Gene therapy is a type of biological therapy that involves the transfer of genetic materials into target cells to repair or replace a disease-causing gene to treat human diseases [1]. Researchers are currently focused on designing effective gene carriers

that can protect and condense nucleic acids for gene therapy because naked nucleic acids are rapidly degraded by serum nucleases and intracellular substances after injection into the body [2]. In addition, negatively charged macromolecules such as DNA are difficult to bring close to the cell membrane, which carries the same charge [3]. Therefore, it is necessary to take advantage of gene vectors to deliver therapeutic genes into target cells. Gene therapy has the potential to revise abnormal genes that lead to disease at the source and can be selectively used to treat a variety of diseases that are serious threats to human health, including genetic disorders, such as hemophilia, cystic fibrosis, and hereditary hypercholesterolemia; malignant tumors; and cardiovascular disease. Gene therapy has shown certain therapeutic effects [4]. By 2015, a total of 2210 clinical trials for gene therapy had been approved, and currently, 78.1% of the 2210 protocols are at phase I or I/II [5]. Viral vectors, such as adeno-associated virus (AAV), retrovirus, lentivirus, adenovirus, and herpes simplex virus (HHV) vectors, are being used successfully in gene therapy at the clinical trial level. Viral vectors have high gene transfection efficiency for gene delivery but some disadvantages still exist, such as severe safety problems, the small amount of targeted gene being carried, the complex preparation processes, and the high cost. Compared with viral vectors, non-viral vectors have the advantages of low toxicity, larger gene carrying capacity, flexible design, and simple mass production. Because of these advantages, the first human phase I clinical trial using a non-viral vector, which delivers systemic administration of siRNA to patients, was started in 2010. This targeted nanoparticle-delivery system delivers siRNA designed to reduce the expression of RRM2 (M2 subunit of ribonucleotide reductase) to patients with solid cancers [6].

Non-viral gene vectors, including cationic lipids, polymers, dendrimers, porous materials, and peptides, have been extensively explored in the past few decades, and clinical attempts to treat inherited and acquired human diseases using non-viral vectors have also increased recently [7, 8]. Cationic liposomes as the most detailed studied materials for gene delivery have been commercialized as *in vitro* gene transfection reagents for over two decades. However, it still showed some disadvantages such as poor *in vivo* stability, low entrapment efficiency, and poor targeting capability. Porous nanoparticles as a new type of multifunctional materials were widely used for drug and gene delivery because of its stable structure, good biocompatibility, and high drug loading. However, for the porous silica nanoparticles with negative charge or weak charge, cationic modification must be carried out to improve their electrochemical performance in order to load negatively charged nucleic acids. The existing methods of the modification mainly include amine-based coupling, cationic polymer modification, and so on [9], which makes the preparation process of cationic porous material more complex. Compared to these vectors, polymers are particularly attractive due to their diversity in terms of polymer structures, compositions, and functional groups. When using polymeric nanocarriers to deliver genes safely to cell organelles (e.g., in the cytoplasm or the cell nucleus), the nanocarrier has to undergo a series of intracellular and extracellular obstacles. These gene vectors transport genes to cells, and during this process, it is necessary to cross the cell membrane by endocytosis, escape from endosomes, and deliver genes into the cell nucleus [10–12]. Genes ultimately being

sent to the cell nucleus is a key goal when trying to make a difference in gene transfection. These steps are very similar to the steps involved in viral or bacterial infection of cells. Therefore, it is necessary to understand the specific nature of the intracellular and extracellular barriers to provide a theoretical basis for further design of efficient gene vectors. One of the most promising and possibly indispensable strategies to overcome cell barriers is to use the extra- and intracellular environments as biological triggers. Extracellular barriers primarily include stability and circulation time in the blood circulation and targeting of specific cells. Intracellular barriers include efficient intracellular endocytosis, lysosomal escape, nuclear localization, and controlled release [13, 14]. The most widespread theory used to achieve efficient endosomal escape is the proton sponge effect. Cells process various endogenous biological signals that are used as the stimulus source for constructing stimuli-responsive polymers in the field of non-viral gene delivery. In addition, these obstacles are taken into consideration when designing polymers for gene delivery, and using extracellular and intracellular stimuli including pH, redox potential, temperature, and specific biomolecules can facilitate gene transfection [15–17]. Artificial stimuli, such as light, ultrasound, magnetic and electrical fields, have also been used to enhance the release of nucleic acids from vectors [18]. In contrast to internal stimuli that affect nanocarriers on the basis of environmental variations between pathological and normal tissues, external stimuli usually offer spatial and temporal controls for DNA release. For example, the ultrasound-responsive gene delivery system is able to cause a cavitation and sonoporation effect that enhances cellular uptake after exposure of the tumor site to ultrasound. Moreover, the light-responsive gene delivery system could control gene release through a light-cleavable linker as long as it was irradiated with a specific wavelength of light. The magnetic-responsive gene delivery system could also realize active targeting to the tumor site by encapsulating an iron particle. Furthermore, dual-responsive polymers are considered as a promising gene delivery system both *in vitro* and *in vivo* [19]. Because of the different mechanisms of drug delivery and gene therapy, the responsive release of the payload was not the only achievement that the stimuli-responsive carriers have to possess, the stimuli-triggered cellular uptake, the endosomal escape and cytosolic release, and the transportation of the nucleic acid to the cell nucleus have to be considered when stimuli-responsive gene carriers are designed. For example, the pH labile linkage was usually used as the drug-polymer linker in the pH-triggered drug delivery carrier, which can promote the drug release once it enters the cytoplasm; however, it might not work for the direct linkage between gene and polymer carriers, since the pH-triggered nucleic acid release might cause loss of function in the early stage of the endocytosis, resulting in low gene transfection. We will discuss this in detail in the following sections.

In this review, we focus on recent advances in the design of functional polymers that are responsive to internal stimuli and external triggers for efficient gene delivery with low cytotoxicity (Scheme 1). In addition to the traditional internal or external stimulation approaches, the use of systems responsive to multiple stimuli based on different triggers comes to the forefront in the polymer design for gene



**Scheme 1** The illustration of the strategies for internal- and external-stimuli responsive gene delivery therapy. In addition, the structure–function relationships of stimuli-responsive polymer carriers in gene delivery are reviewed as well.

## 2 Internal Stimuli-Responsive Systems

### 2.1 pH-Sensitive Polymers

It is well-known that the extracellular pH of tumor tissues (6.5–7.2) is slightly lower than that of normal tissues. Cancer cells also have acidic environments in lysosomes (4.5–5), endosomes (5.5–6), and cytosol [20]. This phenomenon occurs because of the Warburg effect, in which tumor cells proliferate rapidly, resulting in an inadequate supply of oxygen, and cancerous cells then generate most of their energy through high rates of glycolysis rather than oxidative phosphorylation, leading to an accumulation of lactic acid. Given the naturally acidic conditions, pH-responsive polymeric carriers have the ability to accelerate gene release within tumor cells and have been widely used for gene therapy [14].

pH-responsive gene vectors have attracted major attention, and great impetus has been directed towards using the subtle yet significant change in pH values within cellular compartments. pH-sensitive gene vectors can promote the release of genes from intracellular endosomal membranes. Researchers have reported a variety of gene delivery strategies to use the differential pH environment [21–23]. One approach is the use of polymers that have ionizable functional groups (cationic and

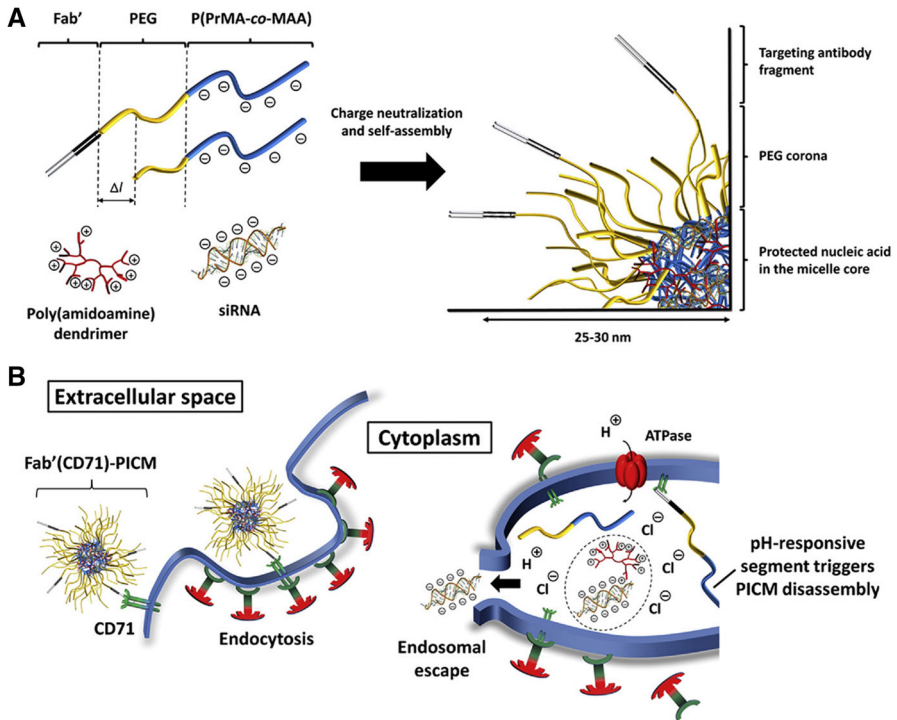
anionic polymers) that either accept or donate protons, leading to different physical characteristics in response to pH changes [24, 25]. Insertion of acid-labile linkages within the polymer is yet another approach for constructing pH-responsive nanoparticles. These linkages are stable at physiological pH but undergo cleavage at acidic pH, thus ensuring a triggered release in an acidic environment. Acid-labile linkages commonly used include imine, hydrazone, acetal, orthoester, and ketal acid bonds [26]. Each strategy is discussed in detail in the following subsections.

### 2.1.1 Polymers with Ionizable Functional Groups

Polymers with ionizable chemical groups, such as carboxylic acids and amines, are used to respond to pH changes. Anionic polymers, including poly(acrylic acid) (PAA), poly(methacrylic acid) (PMA), poly(ethyl acrylic acid) (PEAA), and cationic polymers, such as poly( $\beta$ -amino ester), poly(lysine), and poly(histidine), are sensitive to pH. Peptide vectors have been developed as pH-dependent non-viral vectors to mimic viral fusogenic peptides, which have the ability to penetrate the endosomal membrane due to pH changes. For example, GALA peptide (glutamic acid-alanine-leucine-alanine) and KALA peptide (lysine-alanine-leucine-alanine) have been reported to interact with endosomal membranes and release a vector into the cytoplasm [27, 28].

Anionic polymers, such as poly(acrylic acid) (PAA), poly(methacrylic acid) (PMA), poly(ethyl acrylic acid) (PEAA), poly(propyl acrylic acid) (PPAA), and poly(butyl acrylic acid) (PBAA), which possess carboxylic acid groups, were developed for enhanced intracellular gene delivery [29]. It is a new design strategy that combines a hydrophobic monomer and a more hydrophilic, ionized comonomer as pH sensitive gene vectors. Anionic polymers undergo a hydrophobic/hydrophilic transition induced by pH changes. When at high pH, these anionic polymers undergo deprotonation and become more hydrophilic due to carboxylic acid groups. Alternatively, when at low pH, the anionic polymers become more hydrophobic after protonation. Therefore, the phase transition occurs in the  $pK_a$  range of the carboxylic acid groups. For example, for the copolymers methyl methacrylate (MMA) and methyl acrylic acid (MAA), MMA is the hydrophobic site, and MAA is the hydrophilic site. MAA becomes hydrophilic in high pH due to deprotonation of the carboxylic acid groups; when the carboxylic acid group is protonated, it becomes more hydrophobic. Therefore, the phase transition occurs in the  $pK_a$  value range of the carboxylic group, which is in the pH range of 4.5–5.5. PAA has been used in combination with hydrotropic polymers to make pH-sensitive micelles for gene delivery. Felber [30] synthesized pH-sensitive core-shell type complex micelles, PICMs, by copolymerizing PEG-*b*-P (PrMA-*co*-MAA) with PAMAM. pH-sensitive PICMs showed good stability under serum conditions and effective protection of the siRNA cargo against enzymatic degradation (Fig. 1). The acidic pH in the endosome protonated the carboxylate groups of MAA, leading to disassembly of PEG-*b*-P (PrMA-*co*-MAA) from the PICM and left the unshielded PAMAM nucleic acid core, which promoted endosomal escape.

The hemolytic activity and pH sensitivity of these compounds can be controlled by adjusting the length of the hydrophobic alkyl chains; increasing the chain by one



**Fig. 1** Schematic illustrations of a PICM formation, **b** proposed mechanism of PICM entry through receptor-mediated endocytosis. Reproduced with permission from [30]

methylene group decreased the hemolytic activity of the polymers [29]. Because poly(propyl acrylic acid) (PPAA) with carboxylic acid groups can gradually be protonated, after the PPAA and hydrophobic monomer are copolymerized, the copolymers are able to transition from hydrophilic to hydrophobic within a specific pH range. PPAA successfully enhanced gene transfection efficiency through pH-dependent membrane-destabilizing activity. These results demonstrate that PPAA enhances cytoplasmic delivery when internalized through receptor-mediated endocytosis. To provide a more flexible conjugation route for therapeutic molecules, a new functionalized monomer was synthesized, a multifunctional copolymer based on backbone poly(propylacrylic acid) (PPAA) grafted with poly(alkylene oxides) (PAOs) with a varying extent of grafting and PAO chemistry [31]. The nanoparticle complexes with PPAA-g-PAO copolymers enhanced antisense gene silencing effects in A2780 cancer cells.

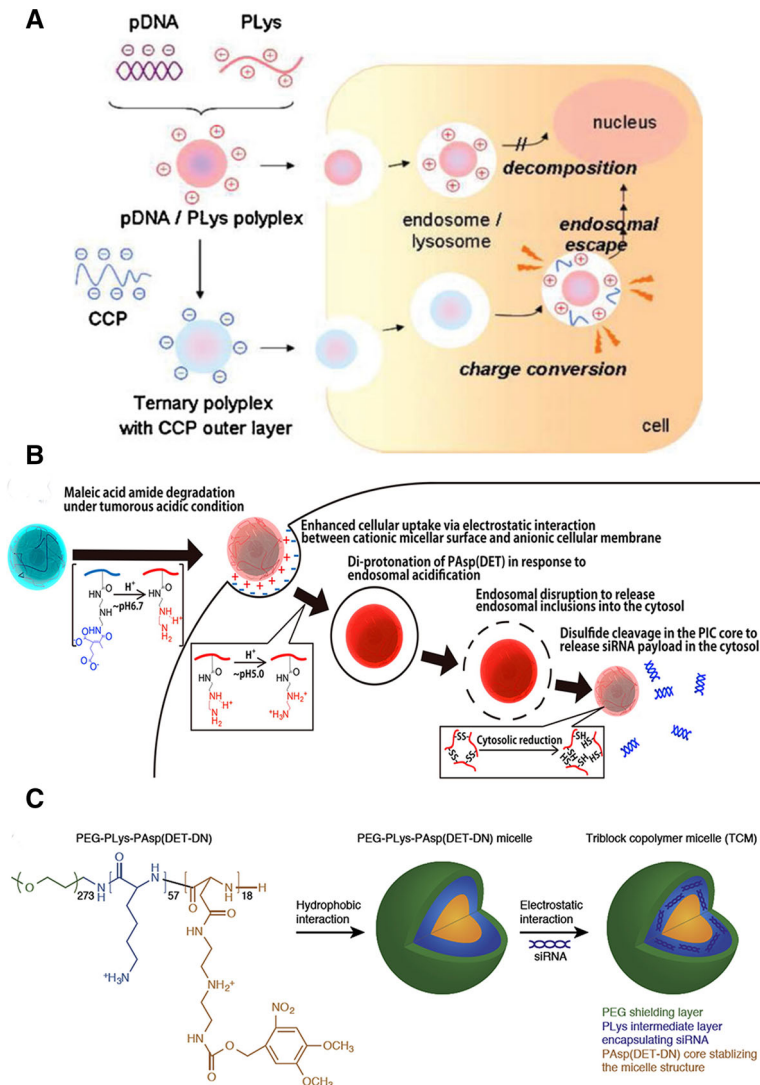
Cationic polymers maintain some free amine groups after complexation, which results in a wide protonation range. The remaining amine groups continue to become protonated in the endosome/lysosome, and they buffer the acidic pH and make the complex swell via osmotic pressure. This effect is known as the “proton sponge effect” [32]. Cationic polymers with amine groups, which induce the proton sponge effect, can facilitate endosomal membrane rupture, enhance the release of



DNA from the endosome to the cytoplasm, and improve the gene transfection efficiency, thus they can be used for gene delivery. Many studies have been conducted with polycations used for polyplex formation with DNA, siRNA, and oligonucleotides. Poly (L-lysine) (PLys) is a cationic polymer that has been used to form pDNA/PLys polyplexes to improve gene transfection efficiency. Based on the advantages of PLys, the Kataoka group [33–35] generated a new way to integrate a PAsp(DET) moiety that interacted with the cationic PLys polyplex to form a ternary polyplex. As shown in Fig. 2a, PAsp(DET-Aco) is considered a pH-sensitive, charge-conversion-controlled gene delivery system. The charge-conversional property of PAsp(DET-Aco) was essential to improve the transfection ability of the PLys polyplex. At a physiological pH, PAsp(DET-Aco) is expected to stabilize the ternary polyplexes due to reduced interaction with negatively charged serum proteins [34]. PAsp(DET) was installed on the cationic PLys polyplex. Compared with non-modified micelles, the PAsp(DET-CDM/DBCO)-installed micelles significantly improved the gene-silencing efficiency, especially after incubation at pH 6.7. PAsp(DET-CDM) undergoes acid hydrolysis of the CDM moieties to electrostatically bind to the negatively charged surface of cancer cells once the lowered-pH environment of a tumor (pH ~6.7) is reached [35]. The pH-sensitive function of PAsp(DET-CDM/DBCO)-installed micelles is shown in Fig. 2b. PEG-PLys-PAsp(DET-DN) [33] was synthesized in order to construct three-layered polymeric siRNA-loaded micelle intermediates, as shown in Fig. 2c. The formulation of PEG-PLys-PAsp(DET-DN) can be further developed toward stimuli-responsive siRNA delivery based on the DN moiety.

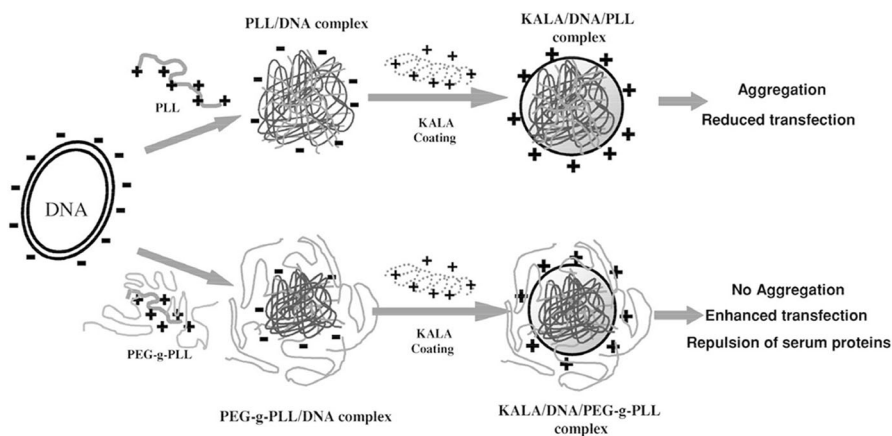
Cationic polymers, such as PLys and PLH [poly(L-histidine)], usually have membrane fusion capability. When beyond normal physiological pH conditions, PLys can induce membrane destabilization. PLH polymers are another group of pH-sensitive cationic polymers, and they contain an imidazole group. The pKa of the imidazole group of histidine is approximately 6.0, and PLH polymers have the ability to acquire a cationic charge below the pKa value and induce membrane fusion and destabilization of micelles. The fusogenic activity of PLH polymers allows nucleic acids to reach the cytosol and enhances delivery efficiency. Kawakami et al. [36] have already reported the synthesis of carboxymethyl poly (L-histidine) (CM-PLH) as a new pH-sensitive polypeptide to improve gene delivery efficiency. The synergistic effect of the pH-sensitive imidazole groups of PLH at endosomal pH and the anionic carboxymethyl groups at physiological pH in the CM-PLH copolymer facilitated efficient gene transfection. The researchers found that incorporating chloroquine into the PLys or PLH-g-PLL improved the efficiency of gene transfection [24]. PLys was inserted between PEG and PLys to form a triblock copolymer system, PEG-CH<sub>12</sub>K<sub>18</sub>, which formed DNA polyplexes and mediated significant gene transfer in vitro and in vivo while maintaining a favorable toxicity profile [37].

The membrane fusion peptide GALA, containing 30 amino acid residues, is also a pH-sensitive amphiphilic peptide. At low pH, GALA interacted with lipid bilayers. At acidic pH, it showed significant membrane fusion, resulting in high gene transfer efficiency [38]. The Ernst Wagner group [39] has described the use of the GALA peptide combined with a PLys polymer to enhance DNA delivery



**Fig. 2** **a** Schematic illustration of pH responsive of pDNA/PLys/PAsp(DET-Aco) ternary polyplexes. Reproduced with permission from Sanjoh et al. [34]. **b** Two-step acidic pH-responsive functions of PAsp(DET-CDM/DBCO)-installed micelles for tumor-targeted siRNA delivery. Reproduced with permission from Tangsangasakri et al. [35]. **c** Chemical structures of block copolymers developed in this study and schematic illustrations of hydrophobic triblock copolymer micelle. Reproduced with permission from Kim et al. [33]

efficiency (Fig. 3). KALA is a cationic membrane fusion peptide, which underwent a pH-dependent amphipathic  $\alpha$ -helix to random coil conformational change when the environmental pH decreased from 7.5 to 5.0. In a KALA/PLys/DNA system, KALA increased the transfection level of luciferase activity, and further substitution



**Fig. 3** Schematic diagram of the formulation of the DNA/PLL and DNA/PEG-g-PLL complexes with KALA coating. Reproduced with permission from Lee et al. [27]

of native PLys with poly(ethylene glycol) (PEG)-modified PLys kept the delivery system at a high transfection level, but clearly decreased the toxicity [27].

### 2.1.2 Acid-Labile Linkages in Polymers

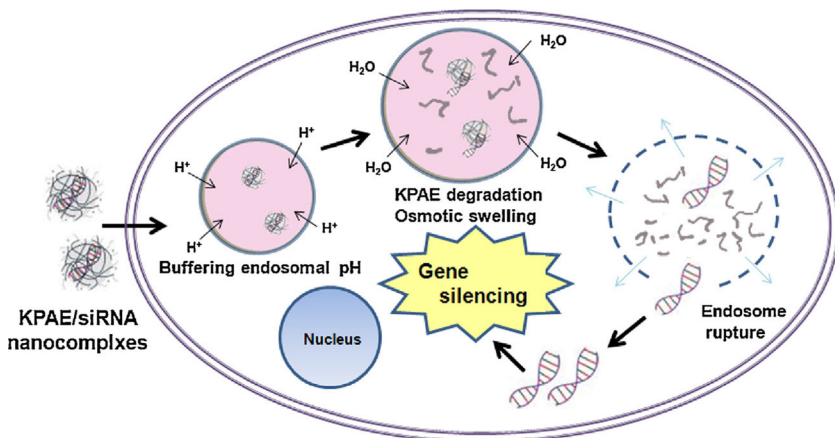
Acid-labile linkers respond to slight pH changes in acidic environments, such as in lysosomes and endosomes. Polymers that contain an acid-labile bond become more biocompatible and biodegradable and have a higher delivery efficiency, higher serum-resistant gene transport efficiency, and lower cytotoxicity than other polymers. Although PEG can neutralize the surface charge of a complex, prolong the circulation time of the complex and reduce the toxicity of extended complexes, there are still two major issues: one is reduced cellular uptake of the complex, and the second is incomplete release of the gene to target cells [19, 22]. The first problem can be solved by linking a targeted ligand to the polymer to enhance cellular uptake. The second problem can be solved by constructing an acid labile bond between the neutral shield and the core of the cationic polymer. Stealth PEG layers can be broken up by an unstable connection bond, such as imine, hydrazone, acetal, orthoester, and ketal acid bonds in response to acidic conditions in the endosomes. These connection bonds are hydrolyzed and trigger the shelter drop, which significantly affects the efficiency of gene transfection.

Hu Yang [40] reported a new conjugation link, bis-aryl hydrazone (BAH), for PEGylation of PAMAM dendrimers to facilitate gene transfer. The incorporation of bis-aryl hydrazone linkages supplied sufficient resistance to pH changes and likely was responsible for facilitating the endosomal escape of polyplexes, and these linkages significantly enhanced the buffering capacity even with a high degree of PEGylation. G4.0-BAH-PEG has a buffering capacity over a pH range of 5.5–6.0, which is sufficient to permit polyplexes to escape from the endosome.

The Wagner group [22] synthesized a novel acetal-based PEGylated polymer, which contained a p-pyridazine group benzaldehyde acetal connection linked to thiol-maleimide groups. PEG-acetal-MAL was conjugated to polyethylenimine (PEI) to form a PEG-acetal-PEI polymer with reversible shielding properties. PEG-acetal-PEI polyplexes tended to aggregate after incubation in medium at pH 5.0 for half an hour, which was due to rapid acetal bond hydrolysis in the acidic conditions and removal of the PEG shelter. PEG-acetal-PEI showed an approximately tenfold higher luciferase transfection efficiency in RENCA-EGFR cells and K562 cells compared to other stable PEG-shielding polymers.

Ketal-containing poly ( $\beta$ -amino esters) (KPAEs) are acid-degradable polymers that possess ketal linkages, as well as ester bonds, along with secondary amines in the backbone (Fig. 4) [41]. Hydrolysis of the ketal linkage occurs approximately 1000 times faster at the phagosomal pH 4.5 compared to the pH 7.4 environment of the cytosol. KPAE, which contains a number of secondary amines, induced rapid and efficient endosomal escape via the combined effects of the proton sponge effect and the rapid acid-mediated cleavage of the ketal linkages in its backbone. KPAE with ketal linkages had a higher siRNA transfection efficacy than other non-ketal-containing, PAEs.

In this section, we discussed recent advances in the development of pH-sensitive polymers, which have a close relationship with the genetic material. The design of the pH-responsive polymers including the following strategies, polymers contain ionizable function groups could not only stimuli-response to the external pH to tune their surface charge and hydrophobicities to promote cell internalization, but also could respond to the internal pH to help escaping the endosomal compartment by proton sponge effect. Polymeric nanocarriers with acid-labile linkages groups between stealth PEG and cationic segment would enhance the cellular uptake and intracellular transportation of nucleic acid. However, as an emerging gene delivery tool, pH-sensitive polymers still need further research and clinical studies to

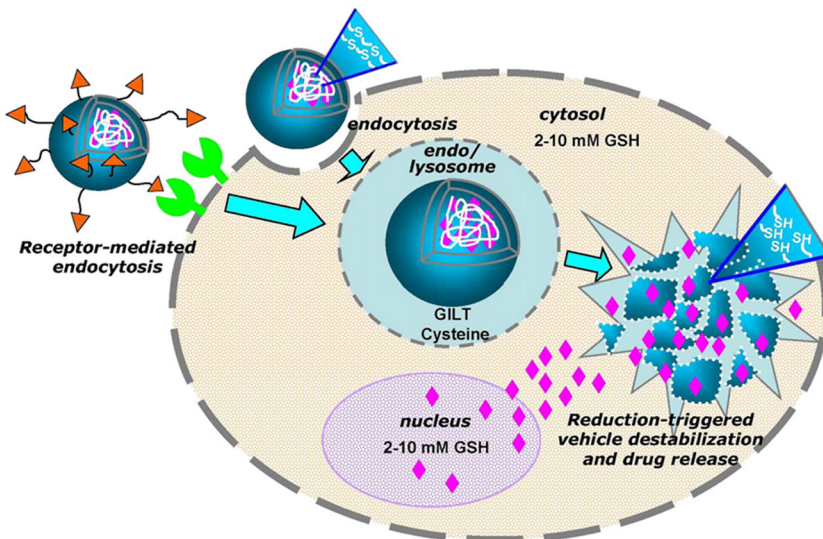


**Fig. 4** Schematic diagram of endosomal escape via proton sponge effect based on the buffering capacity of KPAE. Reproduced with permission from Guk et al. [41]

develop the potential of *in vivo* pH responsibility, the small pH variation from one tumor type to another should be taken into account in reasonable polymer design. Future research in this area will focus on the design of vectors that are easy to synthesize, detailed studies of the structure–activity relationship of vectors to discover better-performing gene vectors and clarification of the relationship between pH sensitivity and transfection efficiency.

## 2.2 Redox-Responsive Polymers

Glutathione (GSH), which is a tripeptide containing a thiol, is the main reducing substance in cells. Glutathione compose of a glutamate-cysteine-glycine peptide, and primarily maintains the stability of thiol-containing enzymes, prevents oxidation of hemoglobin and other auxiliary metal factors, scavenges free radicals and protects the integrity of the cell membrane [16]. According to the literature, the concentration of GSH in the cell is 2–10 mM, and in the normal blood environment, it is 2–10  $\mu\text{M}$ ; the concentration difference between these locations is as high as 1000-fold. In particular, due to the proliferation and metabolism of tumor cells, there is a large amount of GSH in tumor cells, approximately 10–7 times more than in normal cells [42]. Based on the difference in the redox potential between tumor cells and normal cells (Fig. 5), redox-responsive polymers were designed as efficient drug and gene delivery agents for cancer treatment. Redox-responsive nano-delivery systems release less drug, which is protected by the delivery system, into circulating blood and, instead, release the drug into the cytosol. Thus, there is a rationale for designing redox-sensitive systems for tumor targeting, as well as for



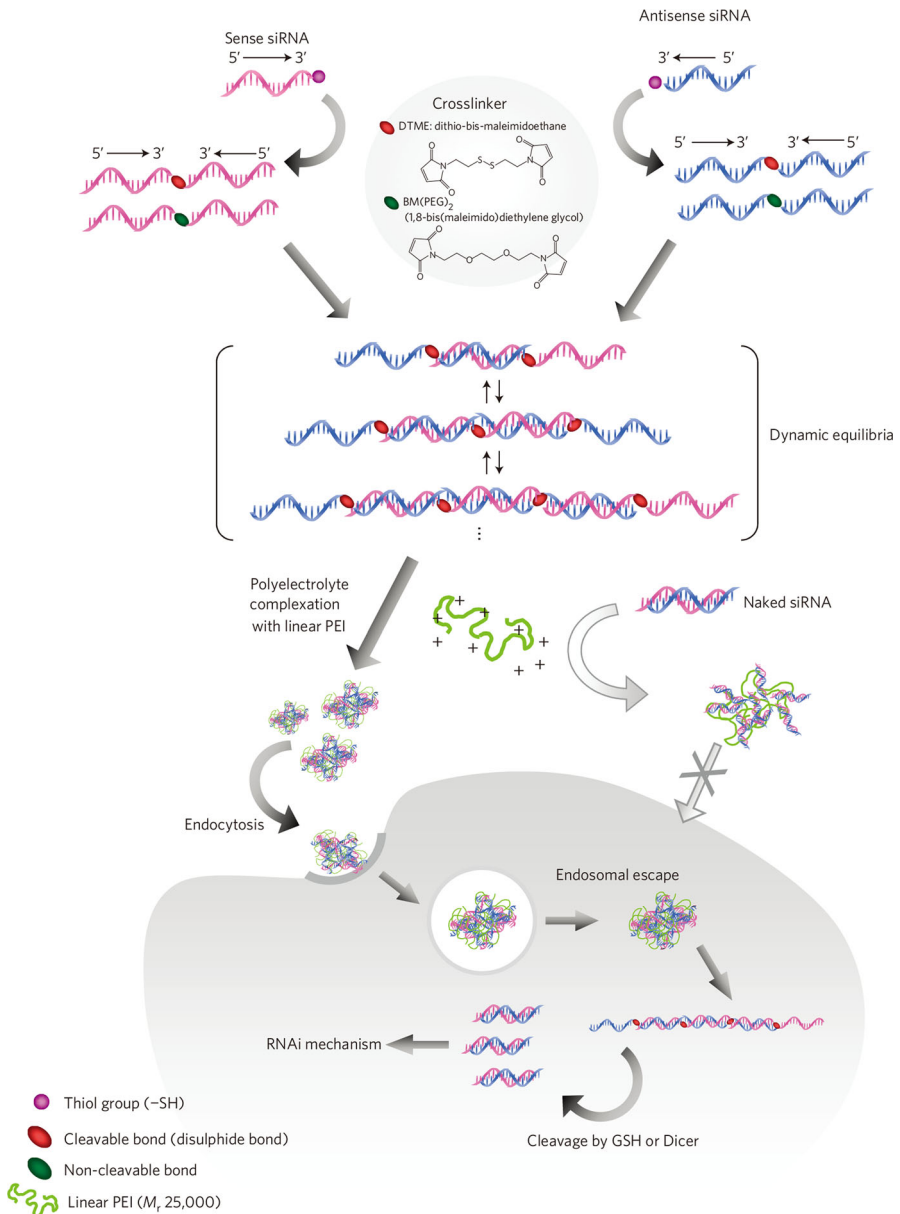
**Fig. 5** Schematic illustration of intracellular trafficking pathway of GSH-responsive nano-vehicles including steps of cellular internalization, endosomal escape, reduction-triggered vehicle degradation, and drug release. Reproduced with permission from Cheng et al. [42]

intracellular delivery of payloads. Redox-responsive polymers for drug delivery systems respond to redox potential using the reversible transformation between a disulfide linkage and a thiol [43]. Disulfide bonds can maintain the stability of the carrier in the blood circulation and the extracellular environment; however, under the effect of high intracellular concentrations of reducing substances, the thiol-disulfide exchange reaction leads to conformational deconstruction or structural change of the carrier, thus releasing the carried drugs into the tumor cells. Moreover, the characteristics of the disulfide linkage in the polymer are aimed at increasing gene transfection efficiency by releasing the gene from the complexes in cancer cells with high levels of GSH.

Different classes of cationic polymers containing disulfide linkages have been designed to prepare redox-triggered complexes containing siRNA. According to literature reports, a new class of biologically active siRNA structures that are chemically self-cross-linked through cleavable disulfide linkages have been developed. For instance, siRNA-grafted poly(aspartic acid) [PAsp(-SS-siRNA)] [44], PEG-siRNA conjugate [45], and poly-siRNA [46] are built on the disulfide bond. Tae Gwan Park [46] generated a novel multimerized siRNA (multi-siRNA) with a cleavable disulfide bond or non-cleavable linkages (Fig. 6). Because of the disulfide linkage used in the siRNA structure, the multi-siRNA/LPEI complexes were more stable than naked siRNA/LPEI complexes in the presence of serum and heparin. Multi-siRNA complexes also showed enhanced cellular uptake in PC3 and A549 cells, compared with naked siRNA complexes. The cleavable VEGF multi-siRNA/LPEI complexes showed sequence-specific gene-silencing activity on the mRNA level and nonspecific immune responses *in vitro* and *in vivo*.

Kataoka [44] has reported a method to link siRNA with poly(aspartic acid) via a disulfide bond. The PAsp(-SS-siRNA) structure induced smooth release of siRNA through the cleavage of the disulfide bond under reductive conditions. The gene silencing efficiency of PAsp(-SS-siLuc)/PAsp (DET) PICs was much higher than that of monomeric siLuc/PAsp(DET) PICs.

Various cationic polymers containing disulfide linkages have been designed to prepare redox-responsive complexes with nucleic acids. Cationic polymers, such as bio-reducible linear or branched poly(amido amine) (PAA), PEI, PLys, and poly(2-dimethylaminoethyl methacrylate) (PDMAEMA), have been investigated as gene carriers. Incorporation of bio-reducible disulfide linkages into polymer chains can make the gene delivery system respond to the intracellular reductive environment and then enhance the intracellular release of genes. Poly(ethylene glycol)-block-poly(L-lysine) [PEG-*b*-(PLys-IM)] with a disulfide linkage was prepared by the Kataoka group [47]. This thiolated copolymer was used to prepare disulfide cross-linked micelles incorporating siRNA. Importantly, these disulfide cross-linked micelles showed remarkable stability in the extracellular matrix and significantly improved cellular uptake, and they underwent disassembly upon the reduction of the disulfide cross-linkers to allow the release of entrapped siRNA and achieved a 100-fold higher transfection efficiency than micelles without disulfide cross-linkers. Reducible polyplexes of linear poly(amido amines) (PAA) have the ability to carry all major classes of nucleic acids: plasmid DNA (pDNA), messenger RNA (mRNA), antisense oligodeoxy nucleotides (AON) and siRNA. Redox-sensitive



**Fig. 6** Schematic illustration for the preparation of multi-siRNAs with cleavable and non-cleavable linkages and their intracellular behaviours with low charge-density cationic carriers. Reproduced with permission from Mok et al. [46]

polyplexes showed improved gene transfection activity compared to non-reducible polyplexes of PAA in response to intracellular GSH levels [48].

Current studies have shown that redox-responsive polymers can uniquely resolve the stability dilemma of genetic structures. The structure of the reduction-responsive

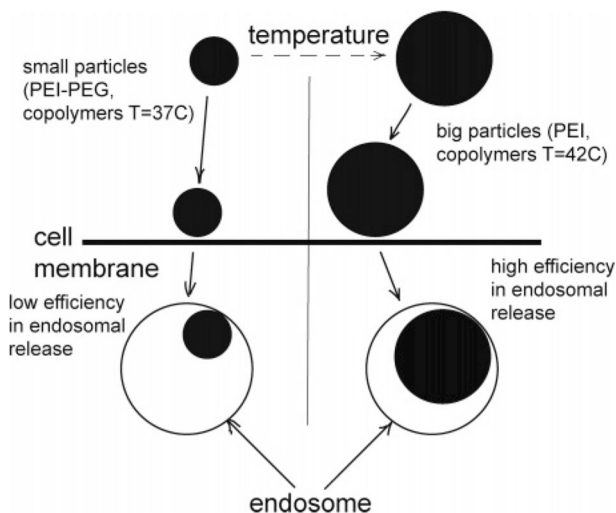
delivery system contains redox-sensitive chemical bonds, which can achieve effective delivery and specific release of genes while maintaining their stability in systemic circulation, improve gene efficacy, and reduce the related toxicity and side effects. Such reducible cationic polymers usually contain one or more disulfide bonds, either located in the polymer backbone or the connection of the polymer and the nucleic acid, which could quickly disassemble the complexes and unpacking nucleic acids under reductive intracellular conditions. It should be noticed that the redox-responsive polymers might work better for siRNA or miRNA delivery due to its small molecular weight and less electrical-binding requirement. The other strategy is using disulfide bond to cross-link either the shell or the core of the cationic micelles to increase their stability in the circulation, and response to the intraocular GSH to disassemble reductively the micelle structure. However, the use of redox sensitive carriers requires high concentrations of reduction, which may not be present in some diseased tissues. Moreover, when entering the cell via endocytosis, the reduction of disulfide bond is slower in mildly weak acid conditions. Although research on reduction-responsive delivery systems has led to great progress, there are still many problems to be solved: (1) most of the research is still at the cellular level, and the number of complete in vivo experiments is not sufficient. Particular attention needs to be paid to testing the in vivo stability of disulfide bonds; (2) given the complexity of the in vivo environment, the exact location and extent of disulfide bond degradation in tumor cells is not clear, and there is a lack of direct evidence; (3) reduction properties of the disulfide bonds themselves may be affected by steric hindrance of the support.

### 2.3 Thermally Responsive Polymers

In numerous stimulus strategies, temperature change is not only easy to control, but is also convenient and easy to apply in vivo and in vitro. Therefore, temperature-sensitive polymers have been widely studied. Malignant tumors are known to have a slightly higher temperature ( $\sim 40\text{--}42\text{ }^{\circ}\text{C}$ ) compared with normal tissues ( $37\text{ }^{\circ}\text{C}$ ) due to the Warburg effect in tumors [49]. Several thermo-responsive polymers that respond to hyperthermic conditions have been used in cancer-targeted gene delivery to enhance gene transfection efficiency. The critical solution temperature is the temperature at which the separation of polymer phase is achieved. Temperature changes around the UCST (upper critical solution temperature) or LCST (lower critical solution temperature) will lead to a phase transition of the thermo-sensitive polymers. Thermo-sensitive polymers were reported previously using poly(*N*-isopropylacrylamide) (PNIPAM) and its derivatives [50]. PNIPAM is a nonionic polymer that is known to undergo hydrophilic to hydrophobic phase transition when the temperature is increased to approximately  $32\text{ }^{\circ}\text{C}$ . As the temperature rises above the LCST, the thermo-sensitive polymer can bind DNA more densely. In such a situation, the complexes can better protect DNA from degradation in endosomes. When the temperature falls below the LCST, the water solubility of PNIPAM will increase, resulting in complexes that are less compact and facilitating DNA release from the polymer/DNA complex [50–52].



Ernst Wagner [53, 54] synthesized a set of block copolymers containing branched PEI and thermo-sensitive PNIPAM-based polymers. The PEI block is able to condense DNA, and NIPAM, with hydrophilic co-monomers such as acrylamide (AAm) or vinylpyrrolidone (VP), is known to increase the transition temperature. The complexation of DNA with these copolymers led to small, charge-neutral particles, which disassembled when the temperature increased from 37 to 42 °C. This aggregation was found to take up the enhanced transfection efficiency of these formulations under hyperthermic conditions. These data demonstrated that the copolymers AAm25 and PVP10 at temperatures above the LCST showed thermally induced high gene transfer and were suitable for activation by hyperthermia at a temperature of 42 °C. No significant cytotoxicity was detected even after eight hyperthermia cycles between 42 and 37 °C. When the higher temperature was reached, the PNIPAM-based polymer became hydrophobic and was not able to stabilize the particles and formed big particles. Big particles were more able to escape from the endosomal compartment compared to the small ones due to the proton sponge effect induced by PEI. Gene expression was nearly two orders of magnitude higher in cells incubated with copolymer/DNA treated by hyperthermia in comparison to cells transfected at physiological temperature [53]. PVP10-BPEI polyplexes (Fig. 7) have been used as thermo-responsive polymers for *in vivo* gene delivery [54]. BPEI and LPEI polyplexes showed significant liver toxicity. However, after sheltering with PEG or PVP10, PEG-BPEI, and PVP10-BPEI polyplexes showed no obvious liver toxicity. In this study, the tumor was heated to 42 °C for 5 min before polyplex injection to investigate the effect of tumor hyperthermia on the transfection efficiency of PVP10-BPEI polyplexes *in vivo*. The treatment gene transfection efficiency in tumors was, on average, tenfold higher in hyperthermia-treated tumors compared with untreated tumors.



**Fig. 7** Schematic representation of the mechanism of cell transfection by thermosensitive polyplexes. Reproduced with permission from Zintchenko et al. [54]

Temperature-sensitive polymers are highly sensitive to temperature changes due to their physical and chemical properties, and the phase transition temperature is easy to control. Therefore, temperature-sensitive polymers have become the research focus of gene delivery. The heat-sensitive carriers usually contain heat-sensitive hydrophilic segments and corresponding hydrophobic segments. The current study of heat-sensitive lipid also uses this property to modify thermosensitive polymers on the surface of lipids. Considering the enhanced drug cytotoxicity, temperature-sensitive carriers, because of their unique properties, reduce the dose and toxicity of several aspects of the system. Therefore, tumor chemotherapy, especially in the field of thermo-chemotherapy, has important prospects. Many types of thermo-sensitive polymers, including liposomes, polymer vesicles and polymer micelles, have been studied and optimized over the years. Their stability has further improved, while the phase transition temperature has also achieved a wide range, and free adjustment can be applied to the pathology of high fever, local artificial thermotherapy, and other methods of temperature-sensitive targeted release. One thing should be mentioned is that the thermo-responsive nanocarrier Termodox reached the clinical stage, which are in clinical trials for breast cancer treatment [55].

## 2.4 Biological Stimuli

In addition to the cells of tumor tissues that grow out of control compared to normal tissue, there are many other unique features in the tumor microenvironment, such as high metabolism that leads to partial acidity of the tumor, local hypoxia caused by imbalance between oxygen consumption and oxygen supply, and rapid growth and metastasis of tumors that leads to excessive secretion of enzymes [56]. Biomolecules, such as glucose, ATP, ROS, and enzymes, in specific tumor tissue differ from depending on the nature of the tissue. Several biomolecular responsive nanostructures, including enzyme-, ATP-, and ROS-responsive nanocarriers, have drawn attention lately as promising gene delivery systems.

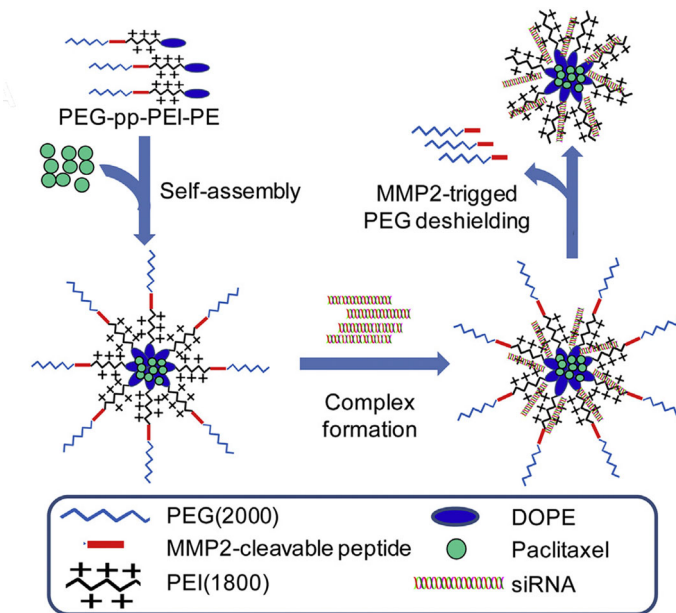
### 2.4.1 Enzyme-Responsive Polymers

Different from normal tissue, the mechanism of enzyme activity in tumor tissues is out of control, leading to excessive enzyme secretion in tumor cells. Enzyme-responsive polymers mainly rely on the cleavage of esters or short peptide sequences by esterases or proteases. The enzymes involved are primarily extracellular enzymes, such as urokinase plasminogen activator (uPA), matrix metalloproteinases (MMPs), hyaluronidase,  $\beta$ -glucuronidase, and intracellular cathepsins. Since some enzymes are overexpressed in tumor tissues, enzyme-responsive polymers have recently been developed for gene delivery [14, 57, 58].

The peptides for special enzyme substrate severed as a scaffold for nanoparticles or to bridge drug molecules in nanoparticles can achieve the release of a drug, for example, esterase severs ester bonds and proteases cleave short modified peptides [59]. Matrix MMPs are highly expressed in tumor cells and have become one of the hot spots in anti-tumor therapy research [60]. MMP2 and MMP9, in particular, are

known to be involved in cancer invasion, progression, and metastasis. Up-regulation of MMP2 is considered a diagnostic and prognostic biomarker in many cancers, and provides a strategy for tumor-targeted drug and gene delivery via an enzyme-triggered mechanism. Lin Zhu [61] developed an MMP2-sensitive self-assembling copolymer, polyethylene glycol-peptide-polyethylenimine-12 with dioleoyl-snglycero-3-phosphoethanolamine (PEG-pp-PEI-PE), for co-delivery of siRNA, and hydrophobic drugs for tumor therapy (Fig. 8). A synthetic octapeptide (GPLGIAGQ) was used as a stimulus-sensitive linker in micellar nanocarriers for MMP2-triggered tumor targeting. Compared to non-sensitive complexes, MMP2-sensitive micelles co-delivered siRNA and PTX to almost all cells as a result of enhanced cell internalization after MMP2-activated exposure of the previously hidden PEI. As reported in the study, PEG-pp-PEI-PE/anti-GFP complexes led to more significant GFP downregulation compared to non-sensitive complexes.

Kadi-Liis Veiman [62] reported a potent gene delivery platform in which condensed CPP and pDNA were shielded by PEG in an MMP-reversible manner for in vivo gene therapy. Complexes proceeded with a 50% PEGylation rate, using PF144/PF14 complexes activated after removal of PEG by MMP2 cleavage for in vivo tumor therapy. There are several reports of successful carriers that depend on enzymatic responses in the tumor-specific microenvironment. Activatable cell-penetrating peptide (designated as dtACPP) is dual-triggered by lowered pH and MMP2 for tumor-targeting gene delivery [59]. Because of the response of MMP2, dtACPP could be activated to expose CPP to enhance cellular uptake. By enhancing it with PEG, dtACPP/DNA could be protected and efficiently deliver DNA to the



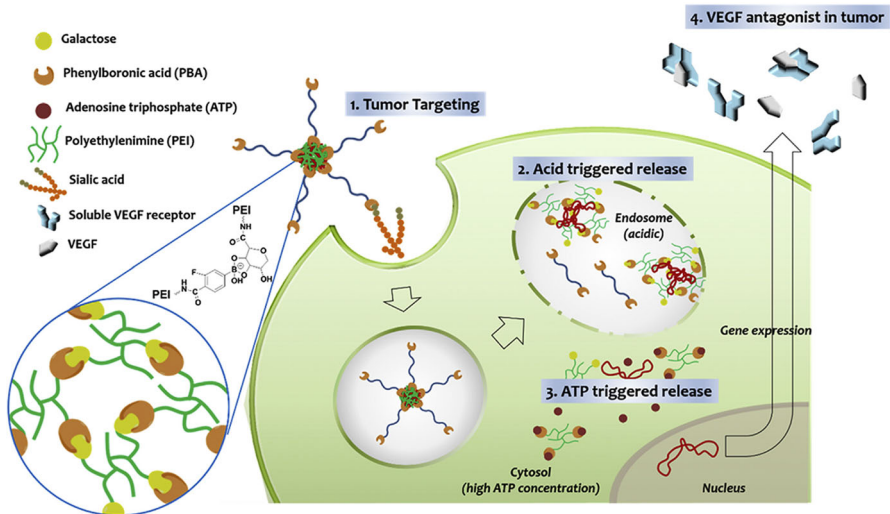
**Fig. 8** Drug delivery strategy of MMP-responsive nanocarrier. Reproduced with permission from Zhu et al. [61]

target tumor for *in vivo* gene transfection. Triggered de-shielding of the PEG covering layer induced by overexpressed levels of proteolytic enzymes led to cleavage of the enzyme-responsive linkage.

#### 2.4.2 ATP-Responsive Polymers

Adenosine triphosphate (ATP) is an essential biomolecule for cell energy metabolism and has an intracellular concentration range of 1–10 mM, which is much greater than that of ATP in the extracellular environment (below 0.4 mM) [57]. The huge difference in ATP levels between the extracellular and intracellular environment is the key point in the design of ATP-responsive carriers, which have been receiving considerable attention recently. ATP-responsive polymers used for drug delivery present drug-release behaviors associated with the surrounding ATP levels and have also been used for gene delivery [63].

Jinhwan Kim et al. [64] reported a cationic polymer PBA-PEG-Cross-PEI, composed of phenylboronic acid (PBA), sugar-installed PEI, and polyethylene glycol (PEG), which was utilized as a tumor-specific gene delivery carrier (Fig. 9). The interaction between PBA and sugar was dependent on the dual stimuli of intracellular pH and ATP, facilitating the release of gene payloads more effectively. This dual stimuli-responsive system delivered DNA at a high transfection efficiency, with low cytotoxicity at the cellular level and in animal models. Effective release of pDNA was observed at acidic pH or with 5 mM ATP, due to separation of Cross-PEI into low-MW PEI. Stimuli-responsive, cleavable Cross-PEI revealed a much higher gene transfection efficiency than non-degradable PEI 25k.



**Fig. 9** Schematic illustration of anti-angiogenic gene delivery mediated by PBA-PEG-Cross-PEI vector with multi-step: 1 tumor targeting by PBA moiety, 2, 3 intracellular pH and ATP dual-responsive gene release, 4 expression of soluble VEGF antagonist for antiangiogenic tumor growth inhibition. Reproduced with permission from Kim et al. [64]

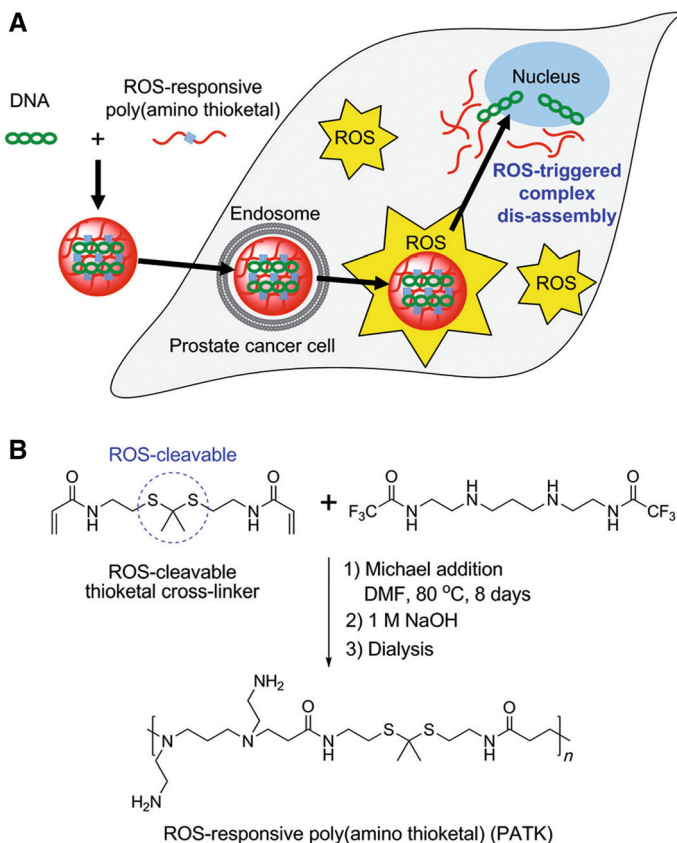
Furthermore, anticancer drug and gene co-delivery based on complexes of PEI with an adenosine triphosphate (ATP)-responsive aptamer duplex (ARAD) was generated by the Guan-Hai Wang group [65]. In this work, an effective method to fabricate ATP-responsive nanocarriers for anticancer drug and gene co-delivery based on complexes of PEI with an adenosine triphosphate (ATP)-responsive aptamer duplex (ARAD) was developed. With increasing amounts of ARAD, the PEI/ARAD complex exhibited good transfection efficiency, and the release of Dox could be controlled by a switch in the ATP concentration in the environment. These nanocarriers, based on cationic polymers and aptamers, represent a new approach for ATP-responsive co-delivery of drugs and genes.

### 2.4.3 ROS-Responsive Polymers

Reactive oxygen species (ROS) are regarded as important signaling molecules in transduction and metabolism. ROS generally refer to a class of oxygen chemical species produced in cells. ROS include hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), singlet oxygen ( $^1\text{O}_2$ ), superoxide ( $\text{O}_2^-$ ), and hydroxyl radicals ( $\text{HO}\cdot$ ), which may transform from one to another as by-products of electron transfer reactions [66, 67]. Compared with normal cells, there are high levels of ROS in cancer cells. The intracellular concentration of ROS in cancer cells was used as a stimulus in gene delivery. Several ROS-responsive polymers have been explored in gene delivery applications, including those containing thioether, selenium/tellurium, thioketal, polysaccharide, aminoacrylate, peroxalate ester, and polyproline [54].

Min Suk Shim and Younan Xia [68] have developed a novel ROS-responsive thioketal-based PATK polymer as a gene delivery agent targeting prostate cancer cells (Fig. 10). Plasmid DNA was delivered to the nucleus of a cancer cell using ROS-cleavable poly(amino thioketal) (PATK). After the DNA/PATK polyplexes were internalized in the cancer cell and escaped from the endosome, the polyplexes disassembled upon ROS exposure via cleavage of the thioketal linkages. ROS-triggered degradation of PATK facilitated the intracellular release of DNA from the particle, leading to enhanced gene transfection. In the comparison of B-PEI and non-degradable poly(amine), the transfection efficiency of PATK was significantly higher than that of the other cells.

The self-assembly system of responsive macromolecules based on biological stimuli is significant to the development of intelligent polymers. Therefore, the direct use of existing species as a source of stimulus will be an important factor in stimulating the self-assembly of responsive polymers. At present, many types of self-assembly systems have been developed, including biological macromolecule enzymes, small metabolites (ATP,  $\text{CO}_2$ ), and reactive molecules (hydrogen peroxide, singlet oxygen). These studies have greatly promoted the development of stimulus-responsive polymer self-assembly, and the application of stimuli-responsive polymers has more closely linked polymer science and biochemistry, molecular biology, cell biology, and other disciplines, thus opening up a milestone development path for the stimulus-responsive polymer field. However, researchers have not yet developed an effective strategy for detection and application of other biological-related molecules, such as carbon monoxide (CO), hormones, and



**Fig. 10** **a** Illustration of intracellular delivery of plasmid DNA to the nucleus of a cancer cell using ROS-cleavable PATK. After the DNA/PATK polyplexes have been internalized by the cancer cell and escaped from the endosome, ROS-triggered degradation of the PATK facilitates the intracellular release of DNA from the particle, leading to enhanced gene transfection. **b** Synthetic scheme of ROS-responsive PATK. Reproduced with permission from Shim and Xia [68]

vitamins. Therefore, we not only need to continue to improve the existing biological stimulus-responsive assembly systems, but also need to focus on the above biological-related species for stimulus-responsive macromolecular self-assembly system design and development.

### 3 External Stimuli-Responsive Systems

#### 3.1 Ultrasound

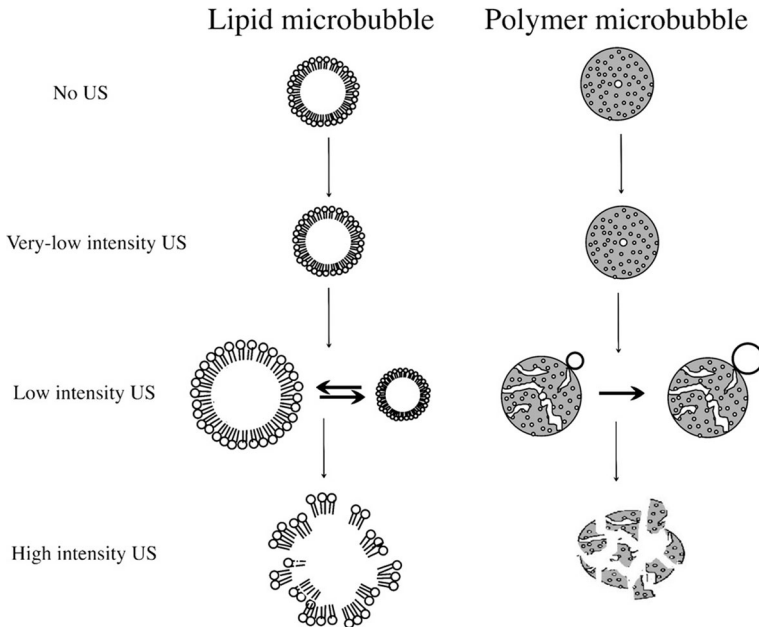
Ultrasound (US) is known to have many advantages to gene transfer systems to achieve efficient, safe, and targeted gene delivery [69, 70]. Extensive studies have revealed that ultrasound has been successfully used to deliver nucleic acids into various cells *in vitro* as well as *in vivo* [69, 71–73].

In general, in the design of US-responsive microbubbles (MBs), there are two main properties of this type of vector that have to be addressed. Ultrasound contrast agents (UCAs) not only greatly improve the resolution and sensitivity of clinical ultrasound imaging, but also are indispensable to activating the therapeutic ability [74]. Widely used UCAs can be divided into two categories: heavy gases, such as sulphur hexafluoride and nitrogen, and volatile liquids, such as perfluoropropane and perfluorohexane. The volatile liquids can change from liquid to gas phases as the temperature is increased, and this phase change is associated with the ultrasound physical effect. No matter what type of UCAs are used, the microbubble-core has to be strongly sensitive to ultrasound, so it can act as an active reflector and become a source of methods to improve gene transfection efficiency.

The design of the polymer also needs to be optimized so that it can undergo ultrasound-induced vibration and disintegration. Moreover, the shell of the microbubble must not only encapsulate the ultrasound contrast agents and nucleic acids, but also must avoid gas leakage and triggering the reticuloendothelial system (RES), which will act to eliminate the nanoparticles.

However, different materials of the microbubble shell exhibit quite different behaviors in response to ultrasound [75]. The shell of liposome bubbles can generate different phenomena based on the various amplitudes of ultrasound (Fig. 11). For very-low intensity US ( $MI < 0.5-0.01$ ), the microbubble compression and expansion are uniform. The size changes linearly with the acoustic pressure. For low intensity US ( $0.1 < MI < 0.3$ ), the microbubble becomes more resistant to compression than to expansion and that causes non-linear oscillations and backscatter at a variety of frequencies (harmonics). This phenomenon is called stable or non-inertial cavitation. For high intensity US ( $MI > 0.3-0.6$ ), the microbubbles are crushed. This is called inertial cavitation that induces micro-streams/microjets around the bubbles. The obvious contrast is with a polymeric shell of microbubbles, microbubbles will not oscillate dynamically at low ultrasound intensity because of the stiff shell. When exposed to low intensity US, shell defects or cracks will form, through which the encapsulated gas can escape. As the US intensity increases, the microbubbles are also crushed. In addition, non-inertial and inertial cavitation can also enhance transient cell membrane permeability. Based on the above-mentioned factors, different materials of the microbubble shell play a key role in ultrasound imaging results and cavitation effects.

Several studies have demonstrated that the ultrasound energy provides a highly efficient mechanism for perturbing cell membranes and increasing their permeability through the cavitation and sonoporation effect [76, 77]. Depending on the intensity of the ultrasound, the mechanism of the microbubble to form a pore on the cell membrane is different. Non-inertial cavitation uses the oscillation of pushing and pulling forces. However, inertial cavitation oscillates fiercely and transforms on the micron scale, eventually disintegrating the bubble [78, 79]. Furthermore, microbubble collapse affects the plasma membranes, leading them to become permeable temporarily through formation of transient pores that are induced by shockwaves (Fig. 12) [80, 81]. This phenomenon is known as sonoporation, and such transient pores have been shown to be beneficial for enhanced uptake of genes from the extracellular environment [82–89]. Particularly in the case that



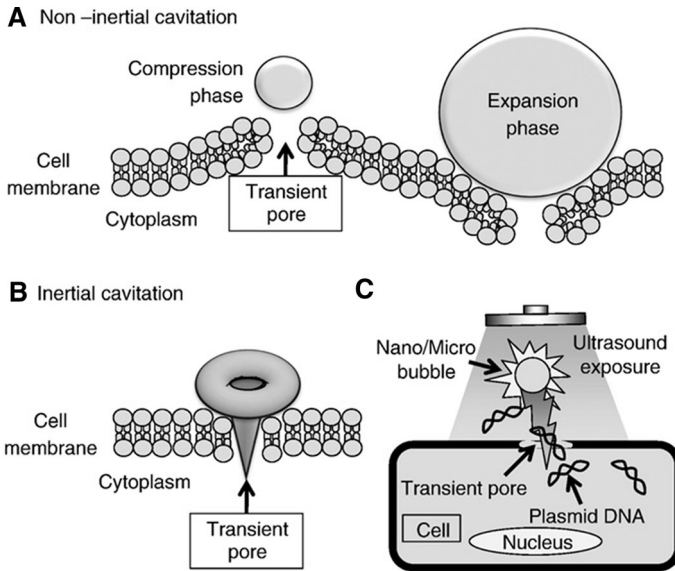
**Fig. 11** Schematic representation of lipid (left column) and polymer (right column) microbubble interaction with ultrasound of increasing intensity (top to bottom). Reproduced with permission from Hernot and Klibanov [72]

microbubbles contain volatile liquid as an ultrasound contrast agent, the vaporization of a superheated emulsion droplet into gas bubbles will occur after ultrasound exposure. This phenomenon is termed acoustic droplet vaporization (ADV). However, the physical mechanisms involved in ADV are not clearly understood. Some studies indicate that the phase transition occurs before inertial cavitation events [90]. Meanwhile, ADV has unique advantages. Firstly, the ADV-triggered bubble formation can promote tumor aggregation and liberate an encapsulated gene for tumor treatment [91, 92]. Secondly, ADV can act as secondary cavitation nuclei that both damages cells and provides significant contrast enhancement in ultrasound imaging [93]. Hence, the characteristics of ADV make it a promising technique for gene delivery, and it is a technique that can be used as an ultrasonic imaging agent.

According to the various types of ultrasound contrast agents, ultrasound-triggered gene delivery microbubbles fall into two parts.

Gas microbubbles are currently the most common and earliest used contrast agents in ultrasonography [72]. For example, Shuai et al. [94] prepared a novel siRNA-encapsulated ultrasound-responsive microbubble (MB) that achieved excellent simultaneous cancer treatment and therapeutic monitoring. Moreover, the novel method, termed hetero-assembling strategy, was used for preparing siRNA/MBs. This method not only has high siRNA encapsulation efficiency and protects siRNA from ribonucleases, but also achieves local ultrasound imaging potential, and it prolongs the imaging duration time. Specifically, cationic micelles of diblock copolymer poly(ethylene glycol)-*b*-poly(L-Lysine) (mPEG-*b*-PLys) were used to

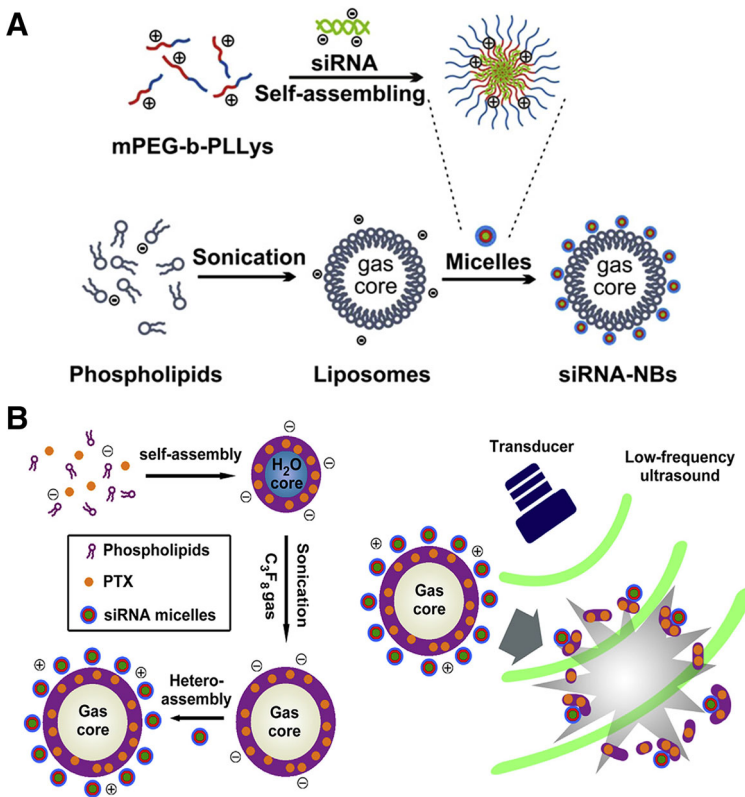




**Fig. 12** Scheme showing the pore formation in the cell membrane by oscillating or disrupting microbubble. **a** The pushing and pulling behavior (non-inertial cavitation) of the microbubble and **b** the collapse of microbubbles (inertial cavitation) cause rupture of the cell membrane creating pore allowing trans-membrane flux of fluid and macromolecules such as plasmid DNA and oligonucleotides (**c**). Reproduced with permission from Suzuki et al. [79]

encapsulated the siRNA and the microbubble of phospholipids was loaded with  $C_3F_8$  gas (Fig. 13a) [95]. Additionally, formation of siRNA/MBs using this strategy is easier to control in comparison with other systems. With low-frequency (1 MHz) US applied to the tumor region after intratumoral injection of XIAP-siRNA/MBs, the small positively charged siRNA-loaded nanoparticles are broken from the siRNA/MBs, the nanoparticles further deliver siRNA to deep locations of tumor tissue and may cross the cancer cell membrane. Most importantly, siRNA micelles are easier to delivery into cytoplasm by “sonoporation”. Consequently, significant improvements in XIAP gene silencing and cleaved caspase-3 activation were achieved at low doses of siRNA (40  $\mu$ g siRNA per mouse) in nude mice. Using a similar principle, a nanobubble (PTX-NBs/siRNA) carrying both the anti-cancer drug paclitaxel (PTX) and BCL-2 siRNA was obtained (Fig. 13b) [96]. These US-sensitive gas-cored hetero-nanoassembly (PTX-NBs/siRNA) MBs also showed excellent hepatocellular carcinoma (HCC) therapy efficacy. With the aid of low-frequency US, meanwhile, the small nanoparticles may aggregate better on the tumor tissue and facilitate cellular uptake because of the increasing capillary endothelium and emergent temporary holes in cancer cell membranes. All this may contribute to the enhanced co-delivery efficacy of PTX and BCL-2 siRNA by avoiding the conventional endocytosis pathway [97].

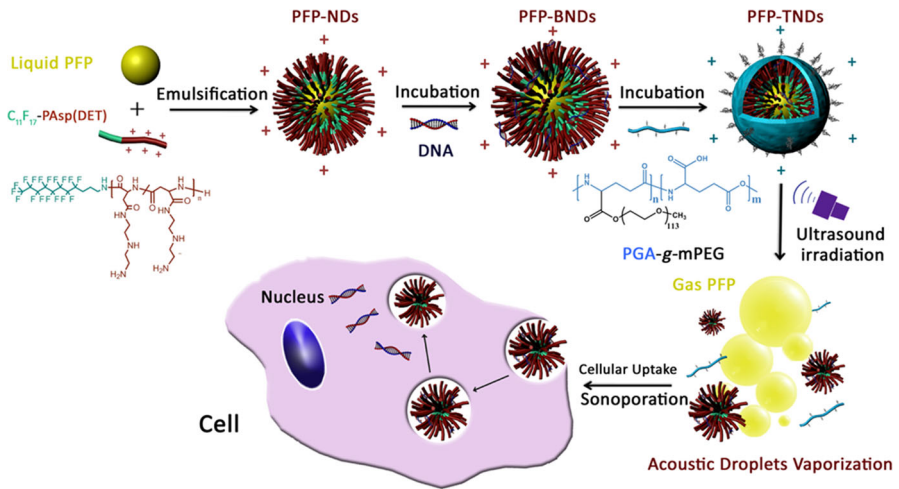
Furthermore, a number of examples that encapsulated gas to achieve excellent performance were reported. Jin et al. [98] developed a cationic MB by modifying PEI with stearic acid and coating it onto the phospholipid shell of MBs that



**Fig. 13** **a** Illustrative preparation of siRNA-loaded nanobubbles complexes using positively charged siRNA micelles and negatively charge gas-cored liposomes. Reproduced with permission from Yin et al. [95]. **b** Illustrative preparation and principle of the destruction under external low-frequency US force of PTX-NBs/siRNA by hetero-assembly of positively charged siRNA micelles and negatively charged PTX-loaded gas-cored liposomes (PTX-NBs). Reproduced with permission from Yin et al. [96]

contained C<sub>3</sub>F<sub>8</sub> gas. The modifications of stearic-PEI not only significantly increased the DNA-loading capacity but also effectively protected the DNA from degradation by coupling it to the cationic MBs via electrostatic interactions. Moreover, this work revealed various interesting information that clarified the relationship between DNA transfection efficiency and multiple ultrasound factors. For example, transfection efficiency increased approximately linearly with MB concentration, but cell viability did not. In addition, acoustic intensity and transfection efficiency was highly nonlinear. This finding provided a tremendous reference value and showed that optimal conditions may need to be carefully adjusted to obtain high transfection efficiency.

However, only a few studies used ADV to enhance gene transfection because ADV requires higher ultrasound intensity to trigger compared to the gas microbubble. Liu et al. investigated a method for gene delivery that used ultrasound-triggered phase-transition cationic nanodroplets (Fig. 14) [99]. For the structure of these cationic nanodroplets, a new type of cationic polymer, C<sub>11</sub>F<sub>17</sub>-



**Fig. 14** Illustrative preparation of US-triggered phase-transition cationic nanodroplets and the process of US-assisted gene transfection. Reproduced with permission from Gao et al. [99]

poly[*N*-[*N'*-(2-aminoethyl)]aspartamide] [C<sub>11</sub>F<sub>17</sub>-PAsp(DET)], was synthesized to efficiently load the perfluoropentane (PFP) resulting from the hydrophobic perfluorinated segment in the polymer and also to provide a hydrophilic cationic segment for DNA binding. Specifically, DNA in the electrostatic complex was also protected from enzymatic degradation [69, 100, 101]. Then, the anionic polymer poly(glutamic acid)-*g*-MeO-poly(ethylene glycol) (PGA-*g*-mPEG) was selected to modify the surface of the nanodroplets to enhance the biocompatibility and serum stability. Moreover, the liquid-phase vehicles had a long circulation time in vivo and also had a markedly enhanced acoustic impedance for ultrasonography [102, 103]. Therefore, with the aid of US, the gene transfection efficiency of PFP-Ternary nanodroplets was significantly enhanced, and the cellular uptake by sonication was significantly increased by approximately 3.5-fold over that of the same sample without US irradiation. This result confirms that the US-triggered phase-transition cationic nanodroplets can be used as efficient theranostic agents for targeting gene delivery.

Although the emphasis of most ADV system is on drug delivery, it is the same principle for gene delivery. For example, Yang et al. [104] designed a new type of triple-stimuli responsive (ultrasound/pH/GSH) biodegradable nanocapsules. They used DOX-loaded PMAA with disulfide cross-linking to form the wall and encapsulate the liquid perfluorohexane. The PMAA material has unique characteristic for drug delivery, such as high drug content (36 wt%) and drug loading efficiency (93.5 wt%), uniform nanoscale and biodegradability. Therefore, the nanocapsules entered the tumor tissue via the EPR effect [105], as well as enhanced the US imaging signal through ADV and triggered the drug release quickly in the low pH tumor environment. Therefore, the triple-stimulation DOX-loaded nanocapsules achieved excellent biocompatibility and tumor treatment. Meanwhile, the ADV system is not just promoted for drug/gene delivery, it can also act as an HIFU

therapy enhancer. For example, Yang et al. [106] also used the ADV effect to improved ultrasound imaging and a synergistic agent for enhanced HIFU ablation of a tumor. The DSPE-PEG-folate/PFH nanoemulsion acted as an HIFU therapy enhancer, and it can be vaporized to generate sufficient microbubbles via the ADV mechanism and achieved excellent treatment effect, which is very favorable for enhancing the capabilities of ultrasound imaging and therapy.

Overall, US-triggered gene transfection systems have brought many advantages. In order to enhance the gene transfection, there are two ways to improve the US-triggered system. The ultrasound contrast agents should easily trigger evaporation, such as encapsulation gas or low boiling point liquid perfluorocarbons, thus generating cavitation more effectively. Also, the shell material of microbubbles or nanobubbles should possess proper hardness, in order to protect the UCAs for extending the circulation time and effectively crush under the ultrasound field. However, there has been limited development of ultrasound-responsive polymeric nanoparticles for gene delivery, since the mechanism of cavitation and sonoporation is still not clearly understood. On the other hand, the physical responsive properties of various polymer shells of microbubbles triggered by ultrasound are not yet clearly defined. In summary, ultrasound as a tool for enhanced gene transfection efficiency still has huge advancement potential.

### 3.2 Magnetic Field

Magnetic field (MF) is one of best options for an external physical stimulus to trigger or enhance therapies against cancer because it barely has any physical interaction with the body. It is well known that magnetic nanoparticles, such as iron oxide nanoparticles, that are able to provide a real-time response to a brief triggering impulse, can serve as contrast probes for MR imaging, as well as act as the therapeutic agent or as the delivery platform.

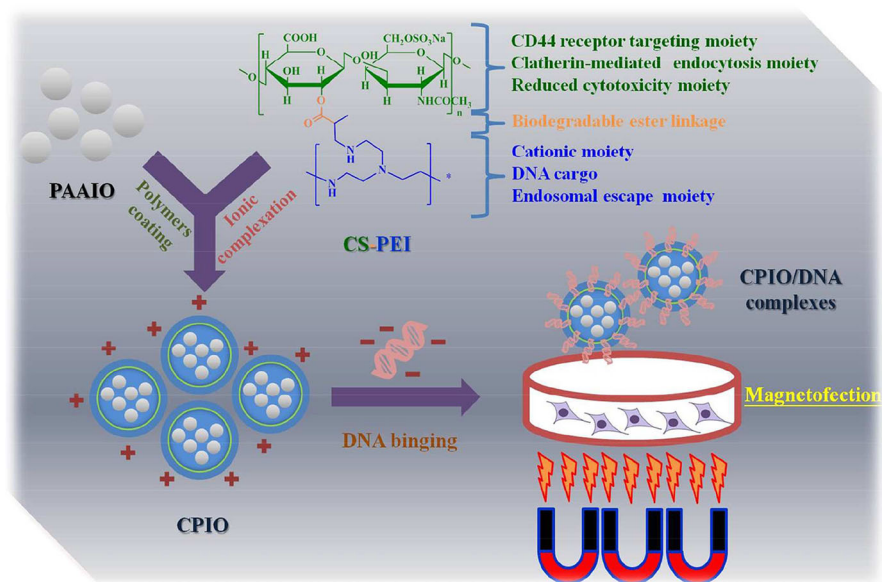
There are two types of significant applications using magnetic fields. Firstly, magnetic hyperthermia is a well-known therapy method that is triggered by an oscillating magnetic field, which is able to induce heating of superparamagnetic located inside the tumor site to ablate cancer cells. This method is usually combined with anti-cancer drugs that trigger the release of chemotherapeutic agents loaded onto theranostic magnetic nanoparticles [107, 108]. However, this strategy has rarely been applied in gene delivery, since such trigger release by hyperthermia did not show significant improvement in gene transfection and might cause serious cell death. Secondly, magnetic tumor targeting is a unique strategy to enhance tumor accumulation of therapeutic nanoparticles in the targeted tumor region to improve therapeutic efficacy of cancer treatments. Therefore, the active targeting of magnetic fields in gene transfection will be described specifically hereinafter.

It is well known that nanoparticles that play a role in a magnetic field absolutely contain metallic elements. Maghemite ( $\text{Fe}_3\text{O}_4$  or  $\gamma\text{Fe}_2\text{O}_3$ ) are commonly use in forming magnetic nanoparticles. Because individual superparamagnetic (SPIO) nanoparticles are incapable of loading nucleic acid, surface modification becomes a common strategy to make them a gene carrier. At present, most research has used several polymers, including organic cationic polymers and inorganic metals, to coat

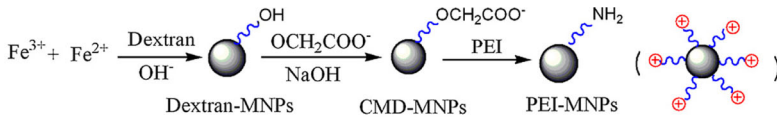
the magnetic nanoparticles. For instance, PEI, *N*-acylated chitosan, poly(lysine), cationic dendrimers, polymethacrylate derivatives, cationic liposomes, gold, silica, and carbon have been used [109]. These materials offer a great opportunity to realize magnetofection (magnetic nanoparticle-guided gene transfection). Since Mah's group [110] first reported magnetic nanoparticles achieving target-specific enhanced transfection both in vitro and in vivo, the potential of magnetic tumor targeting to improve the delivery of nucleic acids in transgene expression has been extensively studied.

For example, Wang et al. developed the chondroitin sulfate-polyethylenimine copolymer-coated superparamagnetic iron oxide nanoparticles (CPIOs) for miRNA-encoding plasmid DNA delivery [111] (Fig. 15). The CPIOs/DNA showed effective magnetofection under the magnetic field just within 20 min incubation in 293T, CRL-582, and U87 cells compared with the PEI/DNA and the commercial magnetofection reagent (PolyMag/DNA). Also, CPIOs showed the least cytotoxicity against the three cell lines. In vivo studies showed the CPIOs/pMIRNA-128 could accumulate to the tumor site more effectively and expressed higher miR-128 with the assisted magnetic field.

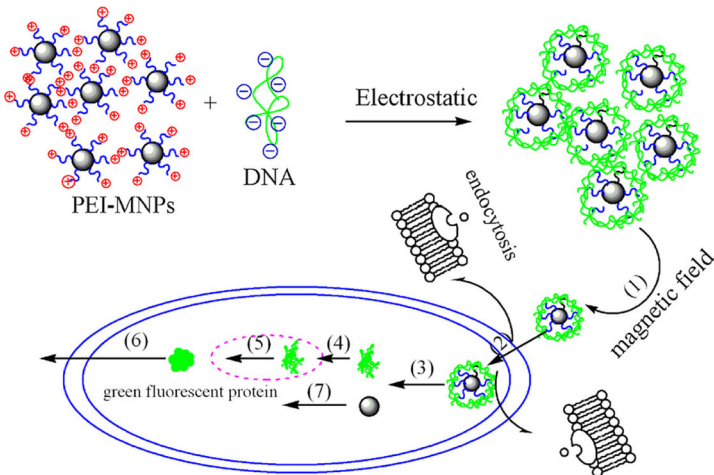
Using a similar pattern, Zheng et al. [112] also investigated the surface modification of magnetic  $\text{Fe}_3\text{O}_4$  nanoparticles as a gene transfection system. The carboxymethyl dextran (CMD) and PEI were modified on the magnetic  $\text{Fe}_3\text{O}_4$  nanoparticle surface (Fig. 16). In the study, the nanoparticles exhibited excellent stability over the entire pH and NaCl concentration. Moreover, the PEI-CMD-MNPs successfully transfected BHK21 cells with the green fluorescent protein (GFP) gene,



**Fig. 15** A schematic illustration of chondroitin sulfate-polyethylenimine (CP)-coated superparamagnetic iron oxide nanoparticles (CPIO) mediated magnetofection. Reproduced with permission from Lo et al. [111]



Stage 1. Synthesis route for the magnetic gene vectors



Stage 2. Gene delivery, expression and green fluorescent protein detected

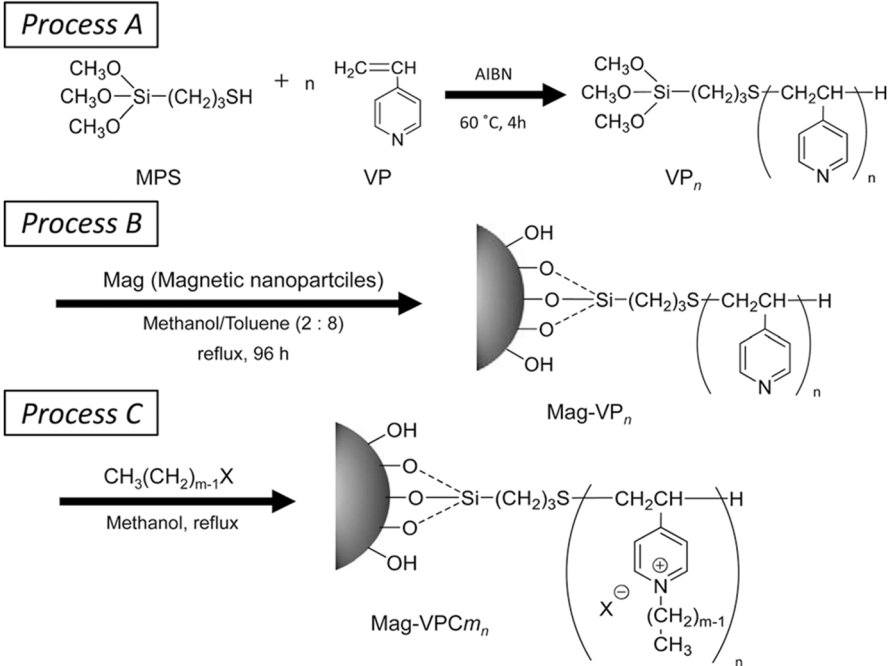
(1) Magnetic gene vectors suspension is added to cells in culture. (2) Endocytosis. (3) Intracellular release. (4) Nuclear entry. (5) Expression. (6) Detected by fluorescence microscope. (7) Exclusion.

**Fig. 16** Schematic diagram illustrating the process for the synthesis of magnetic gene vectors and their performance in gene delivery. Reproduced with permission from Zheng et al. [112]

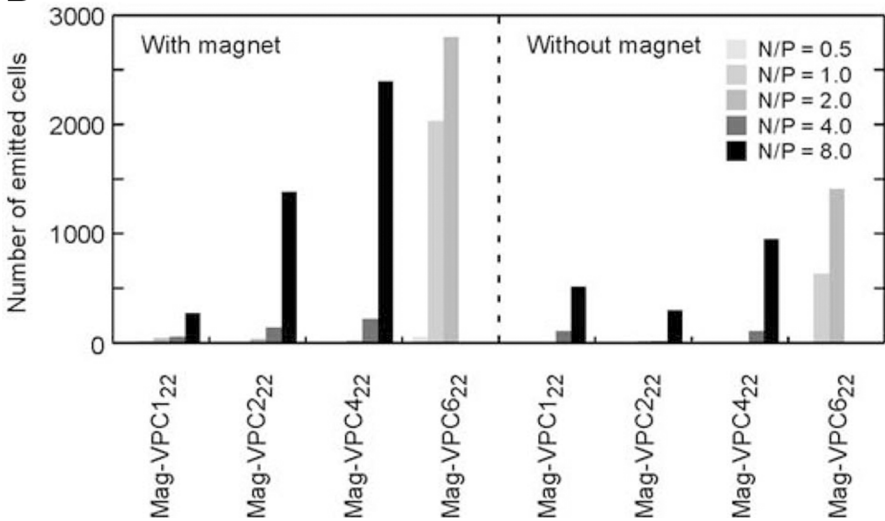
and the gene expression efficiency of the PEI-CMD-MNPs after 13 h of magnetofection transfection was much higher than that of a standard transfection in absence of magnetic field.

In addition, Takafuji et al. [113] investigated the effects of surface chemical structures of magnetic carriers on the transfection efficiency because the cationic properties and hydrophobic moieties play a key role in determining the transfection efficiency [114, 115]. The poly(vinyl pyridine halide)-grafted magnetic (Mag-VPCm) nanoparticles were synthesized so that the chemical structure could be accurately and facily tuned using various alkyl halides (Fig. 17a). The magnetic nanoparticles were then encapsulated by both electrostatic interactions and hydrophobic effects. With the aid of a magnetic field, the gene transfection efficiency of the carriers with specific surface modifications was significantly enhanced (Fig. 17b). As expected, the different length of the alkyl chain on the Mag-VPCm<sub>n</sub> shows different transfection efficiencies by 1 h with magnetic plate. A higher transfection efficiency was obtained with longer alkyl chains. This investigation provides guiding suggestions for the materials used to encapsulate superparamagnetic iron oxide to improve the gene transfection efficiency.

**A**



**B**



**Fig. 17 a** Preparation procedure of Mag-VPC<sub>m</sub><sub>n</sub>. **b** Transfection efficiencies of pmxGFP to HEK293 cells with Mag-VPC<sub>m</sub><sub>22</sub>. The bar graphs show the number of GFP expression cells with magnet (*left*) and without magnet (*right*). Reproduced with permission from Takafuji et al. [113]

Despite significant progress in magnetic-triggered gene transfection in recent years, gaps in biocompatibility continue to prevent translation from the bench to the bedside. Thus, the modification of materials on the SPIO surface should have good biocompatibility and efficient genetic load capacity, and the biomimetic materials might be a promising tendency to overcome biosafety problem compared to the organic polymer. Overall, the external magnetic field has to be strong enough to compel the complexes at the desired site. However, the use of a magnetic field has unique natural advantages and will give rise to personalized medicines in theranostic applications.

### 3.3 Light Response

Light is another appealing stimulus for gene delivery that has undergone a veritable explosion of interest in recent years. The use of light as an external stimulus offers a number of advantages because light is easy to apply and is a clean energy and, importantly, is both spatially and temporally controllable. According to the wavelength coverage, light can be divided into three main regions: ultraviolet (10–400 nm), visible, or near-infrared (NIR) regions (650–900 nm). These three main regions of the light spectrum can be used to trigger drug or gene release from appropriately designed nanocarriers. Because not all wavelengths of light are suitable for use as triggers, most of the present research is focused on UV and NIR. The response to light in light-cleavable polycationic gene carriers is usually introduced via a linker that can be cleaved or chemically decomposed upon irradiation. As a result, the gene is liberated and a high level of gene-transcription is achieved.

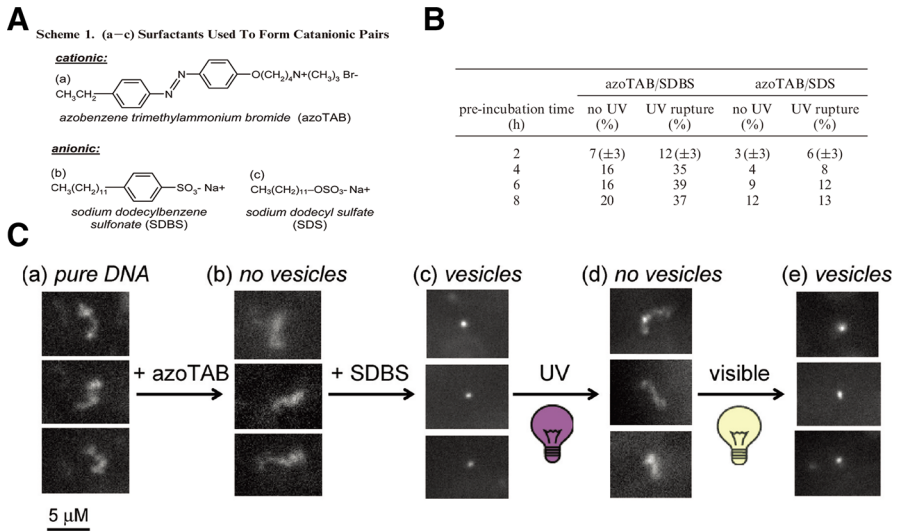
#### 3.3.1 UV

In recent years, significant progress has been made by using azobenzene derivatives and an *o*-nitrobenzyl group that are capable of photoisomerization upon UV irradiation.

The polymers containing azobenzene groups have attracted much interest for their reversible *trans*–*cis* isomerization upon UV/visible irradiation [116–118]. When an azobenzene surfactant is exposed to 350 nm UV light, the hydrophobic *trans* isomer transforms to the hydrophilic *cis* isomer. In addition, the *cis* isomer can be converted back to the *trans* form upon exposure to 436 nm visible light or through thermal relaxation. Consequently, the *trans* isomer can condense the gene and stably assemble the vesicles. Then, UV exposure rapidly liberates the gene through a change in molecular size or enlargement of intermolecular spaces, leading to high gene transfection efficiency. It should be noted that the reversible complex formation was just demonstrated above in a simple buffer, and the situation in the internal environment is likely to be more complex.

For example, Liu et al. [119] prepared a novel complex of azobenzene-containing cationic surfactant and DNA as a light-responsive gene delivery carrier. To form light responsive cationic vesicles, an aqueous solution of the cationic azobenzene-trimethylammonium bromide (azoTAB) surfactant species was mixed with an

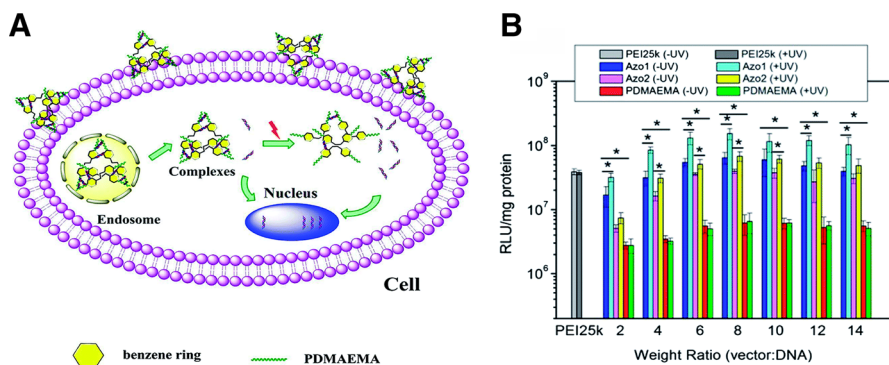




**Fig. 18** **a** Surfactants used to form catanionic pairs. **b** Percent transfection efficiencies of NIH 3T3 cells with eGFP-coded DNA encapsulated in photoresponsive catanionic vesicles. Both without and with UV-induced vesicle rupture following various pre-incubation times. **c** Fluorescent microscope images of conformational changes in T4-DNA molecules in aqueous solution. Reproduced with permission from Liu et al. [119]

aqueous solution of either of two conventional anionic surfactants, sodium dodecylbenzenesulfonate (SDBS) or sodium dodecylsulfate (SDS) (Fig. 18a). In the study, a fluorescent probe was used to visual the reversible complex formation (Fig. 18c). The complexes decomposed after UV exposure, and the fluorescent probe dispersed in the solution, while the fluorescent probe was changeover after visible light exposure. In addition, the light-responsive vesicle at a much lower surfactant concentration (0.0028 wt%) induced a low cytotoxicity effect compared to a previous study in which the surfactant concentration range of 0.1–1 wt% totally. Moreover, the transfection efficiencies of azoTAB/SBDS vesicles under UV illumination (365 nm) reached a much higher percentage compared to the group without UV illumination after incubation for 8 h (Fig. 18b). This result demonstrated that the reversible formation of cationic vesicles by UV illumination can dramatically enhance the transfection efficiency.

Li et al. [120] fabricated light-responsive azobenzene-centered polycationic vectors (Azo-PDMAEMA) for gene delivery (Fig. 19a). The structure of the vectors comprised a middle azobenzene moiety and two terminal blocks of poly[2-(dimethylamino)ethyl methacrylate]. This nanocomplex was capable of condensing plasmid DNA and exhibited *trans* to *cis* isomerization under UV–visible irradiation. In an *in vitro* gene transfection assay against HepG2 cells, the Azo1 (Azo-PDMAEMA<sub>150</sub>) increased by multiple orders of magnitude with UV irradiation (Fig. 19b). This primarily occurred because the light-responsive Azo-PDMAEMA underwent a *trans* to *cis* photoisomerization that induced the formation of less compact complexes, and the longer hydrophobic azobenzene groups enhanced the



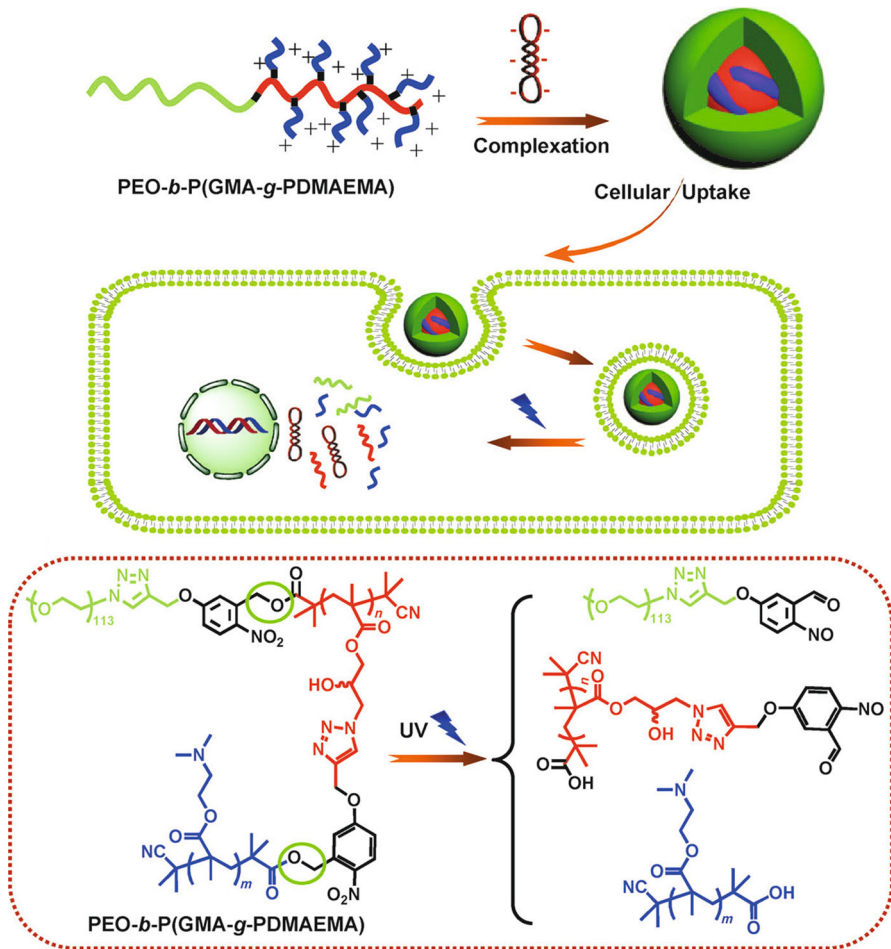
**Fig. 19** **a** The mechanism of the UV light stimuli-induced enhancement in gene transfection. **b** In vitro gene transfection efficiency of the three kinds of cationic polymers/pDNA complexes in comparison with that of PEI25k at various weight complexation ratios in COS-7 cells. Reproduced with permission from Li et al. [120]

interaction of complexes with the cell membrane. Based on this, cell uptake and transfection efficiency will continue to improve.

Furthermore, an *o*-nitrobenzyl linker also has been employed as a useful tool for the light-responsive gene transfection [121–124]. Distinct from azobenzene derivatives, the *o*-nitrobenzyl group can be cleaved upon UV irradiation (365 nm), which leads to effective DNA release and excellent biodegradability of the loaded materials.

For example, Lee et al. [124] developed a UV-degradable polymer gene carrier. It was synthesized by cross-linking low molecular weight branched polyethylenimine (bPEI-2k) molecules using UV-cleavable *o*-nitrobenzyl urethane (NBU) as the linker molecule. The results showed that the gene expression was increased along with the prolonged UV exposure time. Hence, the exact timing of the UV degradation plays an important role in gene transfection efficiency. Using a similar method, Deng et al. [122] elevated the poly( $\beta$ -amino ester)s (PBAEs) with UV trigger-responsive backbones in the polymer that can be rapidly cleaved and promote intracellular DNA release, as well as reduce material toxicity upon external UV illumination. The nitrobenzene moiety was placed in each repeating unit of the PBAEs so that the polymer exhibited a fast response to external UV irradiation and reduced material toxicity. However, the properties of the PBAEs were different from the above case in that 2 min of irradiation was sufficient to trigger intracellular DNA release towards maximal transfection. Hu et al. [123] also designed a light-degradable gene delivery carrier (Fig. 20). This vector was based on PEO-*b*-P(GMA-*g*-PDMAEMA) neutral–cationic brush block copolymers containing nitrobenzyl ester linkages. The results demonstrated that the polyplexes could condense DNA and had good transfection efficiency at a high *N/P* ratio. Furthermore, the UV irradiation protocol could further enhance the gene transfection efficiency with light-responsive degradation of the polymeric vector.

Even though UV-triggered gene release vectors have a certain role in promoting transfection efficiency, the UV irradiation itself has a side-effect. Since UV light



**Fig. 20** Schematic illustration for the side-chain and backbone photodegradable neutral–cationic brush block copolymer and proposed mechanism for the photoinitiated degradation of the PEO-*b*-P(GMA-*g*-PDMAEMA) brush block copolymer. Reproduced with permission from Hu et al. [123]

possesses the intrinsic limitation of shallow penetration depth and harm to the health organization, visible light or near-infrared light might be more favorable for the design of next-generation non-viral polymeric gene-delivery vectors.

### 3.3.2 NIR

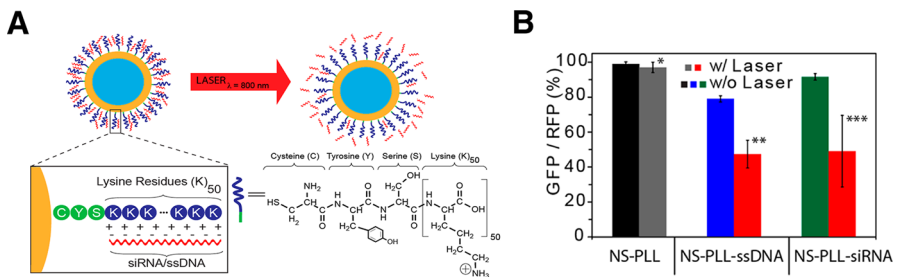
Compared to ultraviolet light, near-infrared light has better transmission through tissue due to its lower absorption and scattering in tissue (penetrating into the body approximately 10 cm) and causes less damage to cells due to its lower energy per photon.

For near-infrared light-triggered gene release, there are two strategies in general use. One strategy consists of covalently attaching the gene therapeutic to the gold

surface. The second strategy consists of covalently attaching a “carrier” to the gold surface and then loading the “cargo” gene therapeutic molecule onto the carrier, typically by weaker, non-covalent bonds. Wijaya et al. [125] presented a smart DNA liberation system that could load and selectively release two different DNA oligonucleotides from two different gold nanorods. DNA was loaded onto the nanorods directly via thiol conjugation. Using the different wavelengths of the NIR ( $\lambda_{800}$  nm or  $\lambda_{1100}$  nm) absorption of gold nanorods according to the different longitudinal surface plasmon resonance peaks, two different DNA oligonucleotides conjugated to each of the NRs were released selectively by irradiation at specific wavelengths. The release efficiently achieved  $\sim 50$ – $80\%$  without any side effects from either the NIR or the gold nanorods. Hence this concept will be a powerful method for gene delivery strategies.

Huschka et al. [126] used the second strategy to design a gold nanoshell (NS)-based therapeutic oligonucleotide delivery vehicle (Fig. 21a). The PLL peptide (cysteine–tyrosine–serine–lysine<sub>50</sub>) was used as a nucleic acid receptor to attach the Au nanoshell surface through both Au-thiol binding and electrostatic binding. Therefore, the negatively charged phosphate backbone of the siRNA/ssDNA (red) was electrostatically attached to the cationic PLL peptide. The poly-lysine nanoshell complex was successfully used as a non-viral gene delivery vector that could controllably release antisense oligonucleotide ssDNA and siRNA to silence the target reporter GFP gene and downregulate GFP protein expression in vitro with 800 nm laser irradiation. Notably, the complexes obtain  $\sim 47$  and 49% downregulation of the targeted GFP expression by NS-PLL-ssDNA and NS-PLL-siRNA, respectively (Fig. 21b). Neither the poly-L-lysine nanoshells nor the laser treatment hardly showed any significant evidence of cytotoxicity.

Recently, Jayakumar et al. [127] reported unprecedented NIR-to-UV upconversion nanoparticles (UCNs) used for gene expression. As described above, light-triggered polymers are mostly active to UV irradiation and few respond to visible and NIR light except for some inorganic substances. However, UV irradiation was limitations. Consequently, the emergence of the upconversion nanoparticles was able to perfectly solve this hurdle. The nanoparticle can act as nanotransducer to absorb NIR light and locally emit UV light that was found to be relatively safe for



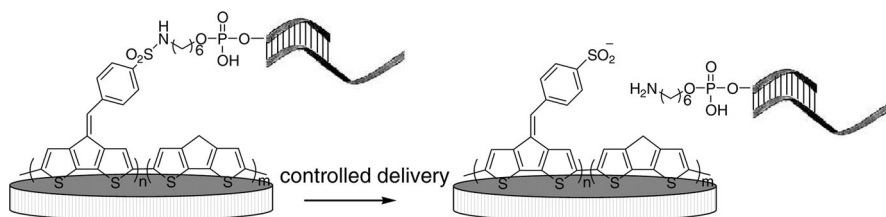
**Fig. 21** **a** Au-nanoshell poly-L-lysine (NS-PLL)-based therapeutic RNAi oligonucleotide delivery system. **b** Downregulation of green fluorescent protein (GFP) in H1299 GFP/RFP cell line by antisense ssDNA and siRNA using nanoshell poly-L-lysine (NS-PLL) delivery vectors. Reproduced with permission from Huschka et al. [126]

cells to triggering the DNA release. Meanwhile, a mesoporous silica shell was employed on the surface, which efficiently adsorbed the DNA and also protected the DNA from harsh biological conditions. Compare to conventional systems, the UCNs can be used for light-controlled gene expression in deeper tissue. The results demonstrated that light activation in both agarose-based tissue models and in animal models after using NIR irradiation, the UCNs successfully activated photocaged nucleic acids. Consequently, this technique has enormous potential for controlled and specific gene delivery/knockdown, and it redefines light-triggered responsive nanoparticles.

In designing the polymer for light-responsive nanoparticles, the photochromic group should be involved so that the polymers are responsive to the light either by physical or chemical phenomena. Light-to-heat transduction mediated by NIR irradiation of gold nanorods could cause a controllable local temperature rise to release the drug or gene, which could also enhance the permeability of the cellular membrane and then improve the gene transfection efficiency. However, the response of the polymer to light illumination is still slow in most cases. In addition, the gene transfection efficiency still has great room for improvement and the safety problem hinders it from further applications.

### 3.4 Electrical Field Response

As stated above, in order to enhance gene transfection efficiency from a non-invasive external trigger, much effort has been invested in ultrasound, magnetic and light stimuli-responsive materials. An electric field has advantages similar to light, such as flexible equipment that allows precise control and accurate remote control. In that way, electrically responsive delivery systems are prepared from polyelectrolytes (polymers which contain relatively high concentration of ionizable groups along the backbone chain), which are similar to the pH responsive polymers, and the polymer electrolytes include synthetic, as well as natural polymers. The synthetic polyelectrolytes include hyaluronic acid, chondroitin sulfate, agarose, carbomer, xanthan gum, and calcium alginate, and the synthetic polymers are generally acrylate and methacrylate derivatives, such as partially hydrolyzed polyacrylamide, polydimethyl aminopropyl acrylamide [128]. The principle of electro-responsive materials is that they can change their shape and transform electrical energy into mechanical energy. For example, electro-responsive hydrogels will swell or bend so that they are able to successfully trigger molecular or drug release from the polymeric bulk materials and implantable electronic delivery devices. Therefore, electrical field is widely used in on-demand drug release systems [129–134]. However, it is rarely used in the gene transfection field. Therefore, remote-controlled gene delivery is one of the promising applications in the electrical field. For example, Gautier et al. developed a method, different from a vector delivery system, to detect genetic disorder and to release the normal gene at a specific location [135]. The monomer 4-cyclopenta[1,2-*b*;3,4-*b'*]dithiophene-4-ylidene-methyl-*N,N*-diethylbenzenesulfonamide ( $M_1$ ) was modified on the oxidation of cyclopentadithiophene (CPDT) units surface to capture the gene (Fig. 22). In response to an electronic stimulus, the arylsulfonamide groups would be cleaved in



**Fig. 22** Electrochemically controlled DNA delivery. Reproduced with permission from Gautier et al. [135]

a physiological environment. The reported results demonstrate the ability to electrochemically liberate DNA from a conducting polymer by cleavage of a covalent bond. Therefore, this would allow us to program different DNA delivery profiles by a series of timed pulses of potential applied to the different biologically active spots under external electrical stimulus.

Moreover, Hook et al. [136] investigated an electro-responsive platform for the controlled transfection of mammalian cells. This platform was highly doped p++ silicon that was modified by plasma polymerization of allylamine (ALAPP). Then, a high density of poly(ethylene oxide) was grafted and formed a layer resistant to biomolecule adsorption and cell attachment. Electro-stimulated preferential adsorption of DNA to the ALAPP surface occurred, and subsequent desorption could be performed by the application of a negative bias. Therefore, HEK 293 cells were used to demonstrate that this solid phase transfection could feasibly transfect cells on the ALAPP regions with an efficiency of up to 25%, which compared favorably with literature values.

Above all, the controlled gene delivery based on electro-responsive is still in infancy. Many shortages has not been solved yet, such as slow response of the material, need a controllable voltage source and not easy to work in the physiological environment.

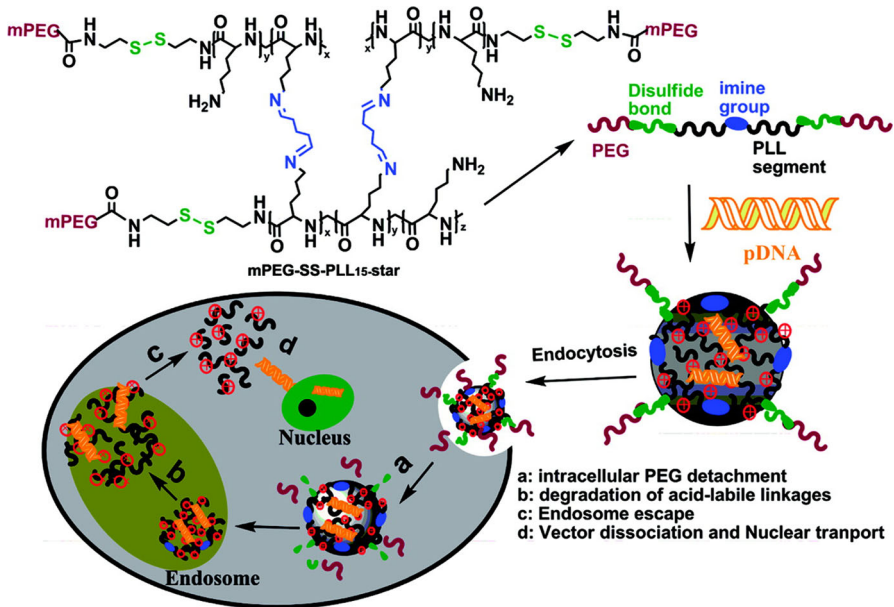
#### 4 Combination of Triggers Response

Because the human body is a complex environment, multi-responsive nanocarriers are a comparatively new development in the field of environmentally responsive gene delivery systems. Such systems in general can orthogonally respond to two different stimuli. Very often these multi-responsive systems are created by the combination of two monomers having responsiveness to different stimuli. Responsive-polymeric nanocarriers (Table 1) in the gene delivery field usually combine triggers including pH/redox [137–139], pH/ATP [64], pH/ROS [140], pH/temperature [141], pH/magnetic field [142, 143], redox/magnetic field [144, 145], pH/light [146], light/redox [147].

On the one hand, combining different internal stimulations has been extensively researched. For example, An et al. [137] structured a dual-responsive carrier by a 4-arm poly(ethylene glycol)-*b*-poly(disulfide histamine) copolymer. This dual-

**Table 1** Examples of stimuli-responsive materials for controlled nucleic acid delivery

Dual-stimuli	Materials	Achieved properties	References
pH	4-arm poly(ethylene glycol)- <i>b</i> -poly(disulfide histamine) copolymer	Rapidly dissociated to liberate DNA in intracellular reducing environment	[137]
Redox	mPEG-SS-PLL <sub>15</sub> -glutaraldehyde star cationer	Marked surface charges increase in tumor microenvironment mPEG was cleavable in intracellular reducing environment to augment intracellular uptake	[138]
ATP	Small molecule ammonium salt was modified into the mesoporous silica nanoparticles PBA-PEG-Cross PEI	Accelerates the release of genetic payload in low pH Delivery specific nucleic acids into the target tumor sites	[139]
ROS	PPDDBP	PBA and sugar were disrupted by either acidic endosomal pH or intracellular ATP	[64]
Temperature	PEEP- <i>b</i> -PDMAEMA	pH responsiveness for endosomal escape and effective ROS responsiveness for improved targeting in vitro model Self-assembles into aggregates with different particle size and morphology in aqueous solution in various pH media	[140]
Magnetic field	PDEA-MNPs Iron oxide magnetic nanoparticle core coated PEI, siRNA, and CTX	Reproducible temperature-responsive behavior with a low critical solution temperature (LCST) Positively charged at acidic pH, negatively charged at neutral or alkaline pH The PEI block was removable in acidic condition	[142]
Light	DSPE-PEG <sub>2000</sub> -PPP	Magnetic resonance imaging and active targeting delivery The internalization function of CPPs (positively charged) is quenched by a pH-sensitive inhibitory peptide (negatively charged), which is linked via a photo-cleavable group	[143]
Redox	Magnetic field Redox-sensitive PEI/SPIO nanocomplex SSPEI-SPIO nanoparticle	Redox-responsive pDNA release MR imaging agent Redox-triggered gene release MR imaging agent	[144]
Light	GNR-DSPEI-PEG-RGD	Gene release from the complex triggered by photothermal effect of NIR irradiation at 808 nm Degradability of disulfide cross-linked short PEIs	[145]
			[147]

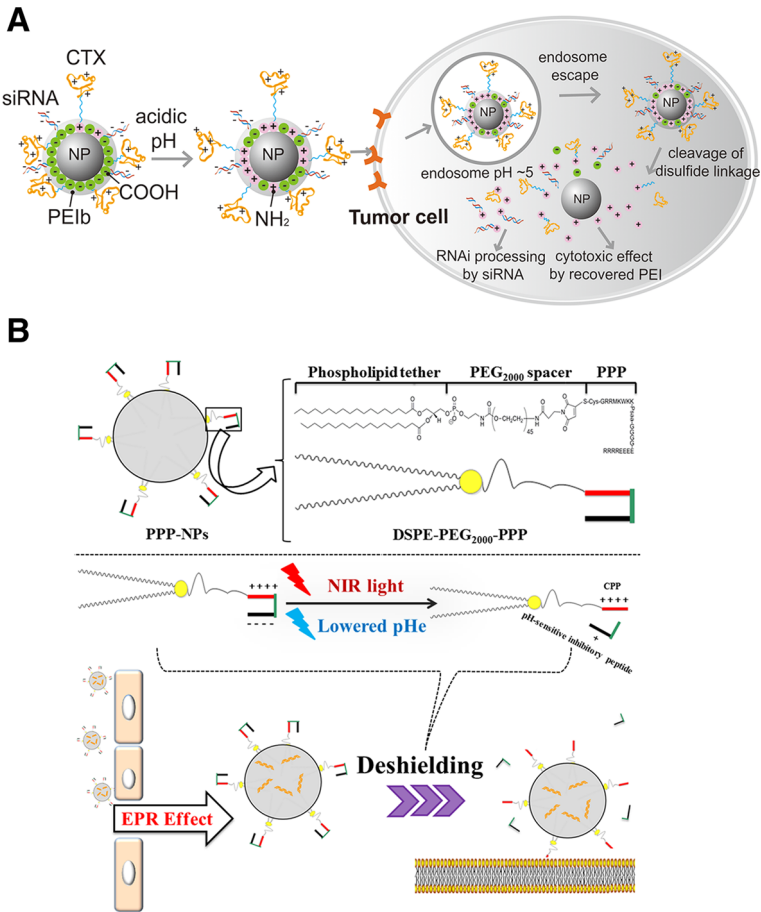


**Fig. 23** Schematic diagram illustrating mPEG-SS-PLL<sub>15</sub>-star cationic copolymer for pDNA encapsulation and the intracellular stimulus-responsive pDNA release. Reproduced with permission from Cai et al. [138]

responsive carrier could rapidly dissociate to liberate DNA in the presence of 10 mM glutathione and also revealed good pH-responsiveness in which the surface charge markedly increased with reduced pH values in the range of 7.4–6.3. These properties are suitable for gene transfection by enhancing the cellular uptake. Meanwhile, the copolymer possessed other unique characteristics, including nanoscale size, colloidal stability, intracellular gene release, and pH-responsive surface charges. The results showed that efficient transfection activities in MCF-7 and HepG2 cancer cells were obtained, and low cytotoxicity was observed. Another similar dual-responsive system was obtained by Cai et al. [138] mPEG-SS-PLL<sub>15</sub>-glutaraldehyde star (mPEG-SS-PLL<sub>15</sub>-star) cationic copolymer was used (Fig. 23). The PEG shell was designed to be removed in the presence of a tumor-relevant glutathione (GSH) concentration so that nanoparticles were capable of accumulating at the tumor site, and acid-induced dissociation could accelerate the release of genetic material. This copolymer had high DNA binding affinity and superior cellular viability compared with PEI and exhibited huge potential for further evaluation as a future gene delivery vector. Consequently, gene payloads were targeted and released effectively.

On the other hand, some investigations have combined internal and external stimulation in one responsive nanocarrier. For example, Hyejung et al. [143] prepared a nanovector that was composed of an iron oxide magnetic nanoparticle core coated with three different functional molecules: polyethylenimine (PEI), siRNA, and chlorotoxin (CTX) (Fig. 24a). This nanovector had pH-responsiveness in that the primary amine group of PEI was blocked with citraconic anhydride at





**Fig. 24 a** Schematic diagram illustrating the intracellular uptake, extracellular trafficking, and processing of NP-PEIb-siRNA-CTX in tumor cells. Reproduced with permission from Mok et al. [143]. **b** Schematic illustration of the PPP-NPs. The siRNA-loaded NPs comprise a spherical PLGA core that is coated with a phospholipid-linked PEGylated photo- and pH responsive polypeptide (PPP) ligand. Reproduced with permission from Yang et al. [146]

physical conditions and also had magnetic targeting specificity that was able to accumulate the vector at the tumor site. Therefore, the superparamagnetic iron oxide nanoparticles successfully enhanced cytotoxicity and gene silencing in cancer cells. Similarly, Huang et al. and Li et al. used the redox-sensitive PEI to contain and cover the SPIO [144, 145]. They also showed a higher transfection efficiency and excellent MR imaging effect using a dual-responsive polymeric carrier.

Yang et al. [146] developed polymer nanoparticles (NPs) modified with light- and pH-responsive polypeptides (PPPs) (Fig. 24b). The DSPE-PEG<sub>2000</sub>-PPP and DSPE-PEG<sub>2000</sub>-CPP were used to load the siRNA by a double-emulsion technique. In PPPs, the internalization function of CPPs (positively charged) was quenched by a pH-sensitive inhibitory peptide (negatively charged), which was linked via a light-

cleavable group. The results showed that the PPP-NPs could selectively accumulate at tumor sites and were internalized into tumor cells by NIR light illumination and the lowered pH at the tumor site after intravenous injection.

Furthermore, several studies have synthesized triple multi-stimuli sensitive copolymers that may have huge potential to be utilized as gene delivery systems [148, 149], which we have not discussed in detail in this review. It is noteworthy that on the basis of the delicate design, the dual- or multi-stimuli sensitive copolymers should be designed in the desired range of the extra- or intra-cellular environmental and could respond to the stimulus simultaneously. For example, the pH and redox responsiveness can be used in combination due to the coexistence of a pH gradient and an oxidative environment in certain pathological conditions. Despite the advantageous of the multi-stimuli responsive systems, the preparation of multifunctional polymers appear too complicated, which might impede the clinical translation of these systems.

## 5 Conclusion

Gene therapy holds the promise of treating numerous diseases including cancers, viral infections, and genetic diseases. To ensure that the gene can reach the target location, many non-viral vectors have been reported during the past decades. There is continuous progress in gene transfection efficiency that can be used as theranostic tools, and smart stimuli-responsive have been extensively explored. Although internal stimulation (e.g., pH, redox potential, and ROS), external stimulation (e.g., US, light, and magnetic field) and dual-stimulation, as described in the present review, have overcome most barriers for non-viral nucleic acid carrier pathways, gene transfection efficiency still has huge potential for improvement. Moreover, the potential toxicity and systemic clearance have not been fully determined for human use. Considering all the challenges faced by gene delivery vectors for clinical translation, a number of triple- or multiple-stimulation delivery systems, developed through engineering new biocompatible and biodegradable stimuli-responsive copolymers, have appeared. The more stimulation that is used in the gene delivery system, the larger advantages in breaking through the extracellular and intracellular hurdles to enhance the degree of gene targeting. Additionally, the large potential of the smart polymeric nanocarriers are capable of encapsulating a broad range of chemotherapeutic agents, such as medicines and specific bioactive compounds, and therefore, it can be used in other diseases and not just primarily focus on cancer therapy.

Taken together, stimuli-responsive materials hold great potential as a new platform for gene therapies, and the translation of these laboratory-based processes to industry-scale techniques is the ultimate goal. Therefore, we should focus on extensive preclinical evaluation in relevant orthotopic animal models in the near future. One thing we can be sure of is that there will be no effort spared in the scientific community to continuously improve stimuli-responsive nanocarriers for triggered gene release due to the promise of this approach to bring non-viral gene therapy a step closer to the clinic.

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## References

1. Ragusa A, Garc a I, Penad s S (2007) Nanoparticles as nonviral gene delivery vectors. *IEEE Trans Nanobiosci* 6:319–330
2. Pack DW, Hoffman AS, Pun S, Stayton PS (2005) Design and development of polymers for gene delivery. *Nat Rev Drug Discov* 4:581–593
3. Mintzer MA, Simanek EE (2009) Nonviral vectors for gene delivery. *Chem Rev* 109:259–302
4. Kumar SR, Markusic DM, Biswas M, High KA, Herzog RW (2016) Clinical development of gene therapy: Results and lessons from recent successes. *Mol Ther Methods Clin Dev* 3:16034
5. Tani K (2016) Current status of *ex vivo* gene therapy for hematological disorders: A review of clinical trials in Japan around the world. *Int J Hematol* 104:42–72
6. Davis ME, Zuckerman JE, Choi CHJ, Seligson D, Tolcher A, Alabi CA, Yen Y, Heidel JD, Ribas A (2010) Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature* 464:1067–U1140
7. Huang S, Shao K, Kuang Y, Liu Y, Li J, An S, Guo Y, Ma H, He X, Jiang C (2013) Tumor targeting and microenvironment-responsive nanoparticles for gene delivery. *Biomaterials* 34:5294–5302
8. Xu P, Li S-Y, Li Q, Van Kirk EA, Ren J, Murdoch WJ, Zhang Z, Radosz M, Shen Y (2008) Virion-mimicking nanocapsules from pH-controlled hierarchical self-assembly for gene delivery. *Angew Chem Int Ed* 47:1260–1264
9. Kim M-H, Na H-K, Kim Y-K, Ryoo S-R, Cho HS, Lee KE, Jeon H, Ryoo R, Min D-H (2011) Facile synthesis of monodispersed mesoporous silica nanoparticles with ultralarge pores and their application in gene delivery. *ACS Nano* 5:3568–3576
10. Huh YM, Lee ES, Lee JH, Yw Jun, Kim PH, Yun CO, Kim JH, Suh JS, Cheon J (2007) Hybrid nanoparticles for magnetic resonance imaging of target-specific viral gene delivery. *Adv Mater* 19:3109–3112
11. Zhang G, Liu J, Yang Q, Zhuo R, Jiang X (2012) Disulfide-containing brushed polyethylenimine derivative synthesized by click chemistry for nonviral gene delivery. *Bioconjug Chem* 23:1290–1299
12. Thomas CE, Ehrhardt A, Kay MA (2003) Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet* 4:346–358
13. Chen DJ, Majors BS, Zelikin A, Putnam D (2005) Structure-function relationships of gene delivery vectors in a limited polyclonal library. *J Control Release* 103:273–283
14. Kaur S, Prasad C, Balakrishnan B, Banerjee R (2015) Trigger responsive polymeric nanocarriers for cancer therapy. *Biomater Sci* 3:955–987
15. Dehousse V, Garbacki N, Colige A, Evrard B (2010) Development of pH-responsive nanocarriers using trimethylchitosans and methacrylic acid copolymer for siRNA delivery. *Biomaterials* 31:1839–1849
16. Schafer FQ, Buettner GR (2001) Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 30:1191–1212
17. Thornton PD, Mart RJ, Ulijn RV (2007) Enzyme-responsive polymer hydrogel particles for controlled release. *Adv Mater* 19:1252–1256
18. Reineke TM (2016) Stimuli-responsive polymers for biological detection and delivery. *ACS Macro Lett* 5:14–18
19. Li J, Yu X, Wang Y, Yuan Y, Xiao H, Cheng D, Shuai X (2014) A reduction and pH dual-sensitive polymeric vector for long-circulating and tumor-targeted siRNA delivery. *Adv Mater* 26:8217–8224
20. Midoux P, Pichon C, Yaouanc JJ, Jaffres PA (2009) Chemical vectors for gene delivery: A current review on polymers, peptides and lipids containing histidine or imidazole as nucleic acids carriers. *Br J Pharmacol* 157:166–178

21. Guo C, Chen W, Lin S, Li H, Cheng D, Wang X, Shuai X (2012) Synthesis and characterization of polycation block copolymer Poly(L-lysine)-*b*-poly[*N*-(*N'*, *N'*-diisopropyl-aminoethyl)aspartamide] as potential pH responsive gene delivery system. *Polymer* 53:342–349
22. Knorr V, Allmendinger L, Walker GF, Paintner FF, Wagner E (2007) An acetal-based PEGylation reagent for pH-Sensitive shielding of DNA polyplexes. *Bioconjug Chem* 18:1218–1225
23. Mignet N, Richard C, Seguin J, Largeau C, Bessodes M, Scherman D (2008) Anionic pH-sensitive pegylated lipoplexes to deliver DNA to tumors. *Int J Pharm* 361:194–201
24. Bennis JM, Choi JS, Mahato RI, Park JS, Kim SW (2000) pH-sensitive cationic polymer gene delivery vehicle: *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) comb shaped polymer. *Bioconjug Chem* 11:637–645
25. Midoux P, Monsigny M (1999) Efficient gene transfer by histidylated polylysine pDNA complexes. *Bioconjug Chem* 10:406–411
26. Durmaz YY, Lin Y-L, ElSayed MEH (2013) Development of degradable, pH-sensitive star vectors for enhancing the cytoplasmic delivery of nucleic acids. *Adv Funct Mater* 23:3885–3895
27. Lee H, Jeong JH, Park TG (2002) PEG grafted polylysine with fusogenic peptide for gene delivery: High transfection efficiency with low cytotoxicity. *J Control Release* 79:283–291
28. Li W, Nicol F, Szoka FC Jr (2004) GALA: A designed synthetic pH-responsive amphipathic peptide with applications in drug and gene delivery. *Adv Drug Deliv Rev* 56:967–985
29. Stayton PS, El-Sayed MEH, Murthy N, Bulmus V, Lackey C, Cheung C, Hoffman AS (2005) 'Smart' delivery systems for biomolecular therapeutics. *Orthod Craniofac Res* 8:219–225
30. Felber AE, Castagner B, Elsabahy M, Deleavey GF, Damha MJ, Leroux JC (2011) siRNA nanocarriers based on methacrylic acid copolymers. *J Control Release* 152:159–167
31. Peddada LY, Garbuzenko OB, Devore DI, Minko T, Roth CM (2014) Delivery of antisense oligonucleotides using poly(alkylene oxide)-poly(propylacrylic acid) graft copolymers in conjunction with cationic liposomes. *J Control Release* 194:103–112
32. C-b Wu, J-y Hao, X-m Deng, Liu Y (2008) Random branched poly(hydroxyetheramine): A novel polycation with proton sponge effect and high density of discrete charge. *Polym Bull* 60:635–645
33. Kim HJ, Miyata K, Nomoto T, Zheng M, Kim A, Liu X, Cabral H, Christie RJ, Nishiyama N, Kataoka K (2014) siRNA delivery from triblock copolymer micelles with spatially-ordered compartments of PEG shell, siRNA-loaded intermediate layer, and hydrophobic core. *Biomaterials* 35:4548–4556
34. Sanjoh M, Hiki S, Lee Y, Oba M, Miyata K, Ishii T, Kataoka K (2010) pDNA/poly(L-lysine) polyplexes functionalized with a pH-sensitive charge-conversional poly(aspartamide) derivative for controlled gene delivery to human umbilical vein endothelial cells. *Macromol Rapid Commun* 31:1181–1186
35. Tangsangasakri M, Takemoto H, Naito M, Maeda Y, Sueyoshi D, Kim HJ, Miura Y, Ahn J, Azuma R, Nishiyama N, Miyata K, Kataoka K (2016) siRNA-loaded polyion complex micelle decorated with charge-conversional polymer tuned to undergo stepwise response to intra-tumoral and intra-endosomal pHs for exerting enhanced RNAi efficacy. *Biomacromolecules* 17:246–255
36. Asayama S, Sudo M, Nagaoka S, Kawakami H (2008) Carboxymethyl poly(L-histidine) as a new pH-sensitive polypeptide to enhance polyplex gene delivery. *Mol Pharm* 5:898–901
37. Boylan NJ, Kim AJ, Suk JS, Adstamongkonkul P, Simons BW, Lai SK, Cooper MJ, Hanes J (2012) Enhancement of airway gene transfer by DNA nanoparticles using a pH-responsive block copolymer of polyethylene glycol and poly-L-lysine. *Biomaterials* 33:2361–2371
38. Khalil IA, Hayashi Y, Mizuno R, Harashima H (2011) Octaarginine- and pH sensitive fusogenic peptide-modified nanoparticles for liver gene delivery. *J Control Release* 156:374–380
39. Plank C, Oberhauser B, Mechtler K, Koch C, Wagner E (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems. *J Biol Chem* 269:12918–12924
40. Yuan Q, Yeudall WA, Yang H (2010) PEGylated polyamidoamine dendrimers with bis-aryl hydrazone linkages for enhanced gene delivery. *Biomacromolecules* 11:1940–1947
41. Guk K, Lim H, Kim B, Hong M, Khang G, Lee D (2013) Acid-cleavable ketal containing poly(beta-amino ester) for enhanced siRNA delivery. *Int J Pharm* 453:541–550
42. Cheng R, Feng F, Meng F, Deng C, Feijen J, Zhong Z (2011) Glutathione-responsive nano-vehicles as a promising platform for targeted intracellular drug and gene delivery. *J Control Release* 152:2–12

43. Wen Y, Zhang Z, Li J (2014) Highly efficient multifunctional supramolecular gene carrier system self-assembled from redox-sensitive and zwitterionic polymer blocks. *Adv Funct Mater* 24:3874–3884
44. Takemoto H, Ishii A, Miyata K, Nakanishi M, Oba M, Ishii T, Yamasaki Y, Nishiyama N, Kataoka K (2010) Polyion complex stability and gene silencing efficiency with a siRNA-grafted polymer delivery system. *Biomaterials* 31:8097–8105
45. Jung S, Lee SH, Mok H, Chung HJ, Park TG (2010) Gene silencing efficiency of siRNA-PEG conjugates: Effect of PEGylation site and PEG molecular weight. *J Control Release* 144:306–313
46. Mok H, Lee SH, Park JW, Park TG (2010) Multimeric small interfering ribonucleic acid for highly efficient sequence-specific gene silencing. *Nat Mater* 9:272–278
47. Matsumoto S, Christie RJ, Nishiyama N, Miyata K, Ishii A, Oba M, Koyama H, Yamasaki Y, Kataoka K (2009) Environment-responsive block copolymer micelles with a disulfide cross-linked core for enhanced siRNA delivery. *Biomacromolecules* 10:119–127
48. Manickam DS, Li J, Putt DA, Zhou QH, Wu C, Lash LH, Oupicky D (2010) Effect of innate glutathione levels on activity of redox-responsive gene delivery vectors. *J Control Release* 141:77–84
49. Schwerdt A, Zintchenko A, Concia M, Roesen N, Fisher K, Lindner LH, Issels R, Wagner E, Ogris M (2008) Hyperthermia-induced targeting of thermosensitive gene carriers to tumors. *Hum Gene Ther* 19:1283–1292
50. Kurisawa M, Yokoyama M, Okano T (2000) Gene expression control by temperature with thermo-responsive polymeric gene carriers. *J Control Release* 69:127–137
51. Lee SH, Choi SH, Kim SH, Park TG (2008) Thermally sensitive cationic polymer nanocapsules for specific cytosolic delivery and efficient gene silencing of siRNA: Swelling induced physical disruption of endosome by cold shock. *J Control Release* 125:25–32
52. Choi SH, Lee SH, Park TG (2006) Temperature-sensitive pluronic/poly(ethylenimine) nanocapsules for thermally triggered disruption of intracellular endosomal compartment. *Biomacromolecules* 7:1864–1870
53. Beckert L, Kostka L, Kessel E, Krhac Levacic A, Kostkova H, Etrych T, Lachelt U, Wagner E (2016) Acid-labile pHPMA modification of four-arm oligoaminoamide pDNA polyplexes balances shielding and gene transfer activity in vitro and in vivo. *Eur J Pharm Biopharm* 105:85–96
54. Zintchenko A, Ogris M, Wagner E (2006) Temperature dependent gene expression induced by PNIPAM-based copolymers: Potential of hyperthermia in gene transfer. *Bioconjug Chem* 17:766–772
55. Mura S, Nicolas J, Couvreur P (2013) Stimuli-responsive nanocarriers for drug delivery. *Nat Mater* 12:991–1003
56. Liang J, Liu B (2016) ROS-responsive drug delivery systems. *Bioeng Transl Med* 1:239–251
57. Molla MR, Prasad P, Thayumanavan S (2015) Protein-induced supramolecular disassembly of amphiphilic polypeptide nanoassemblies. *J Am Chem Soc* 137:7286–7289
58. Rozema DB, Blokhin AV, Wakefield DH, Benson JD, Carlson JC, Klein JJ, Almeida LJ, Nicholas AL, Hamilton HL, Chu Q, Hegge JO, Wong SC, Trubetskoy VS, Hagen CM, Kitas E, Wolff JA, Lewis DL (2015) Protease-triggered siRNA delivery vehicles. *J Control Release* 209:57–66
59. Thambi T, Park JH, Lee DS (2016) Stimuli-responsive polymersomes for cancer therapy. *Biomater Sci* 4:55–69
60. Huang S, Shao K, Kuang Y, Liu Y, Li J, An S, Guo Y, Ma H, He X, Jiang C (2013) Tumor targeting and microenvironment-responsive nanoparticles for gene delivery. *Biomaterials* 34:5294–5302
61. Zhu L, Perche F, Wang T, Torchilin VP (2014) Matrix metalloproteinase 2-sensitive multifunctional polymeric micelles for tumor-specific co-delivery of siRNA and hydrophobic drugs. *Biomaterials* 35:4213–4222
62. Veiman KL, Kunnapuu K, Lehto T, Kiisholts K, Parn K, Langel U, Kurrikoff K (2015) PEG shielded MMP sensitive CPPs for efficient and tumor specific gene delivery in vivo. *J Control Release* 209:238–247
63. Mo R, Jiang T, Gu Z (2014) Enhanced anticancer efficacy by ATP-mediated liposomal drug delivery. *Angew Chem Int Ed Engl* 53:5815–5820
64. Kim J, Lee YM, Kim H, Park D, Kim J, Kim WJ (2016) Phenylboronic acid-sugar grafted polymer architecture as a dual stimuli-responsive gene carrier for targeted anti-angiogenic tumor therapy. *Biomaterials* 75:102–111

65. Wang G-H, Huang G-L, Zhao Y, Pu X-X, Li T, Deng J-J, Lin J-T (2016) ATP triggered drug release and DNA co-delivery systems based on ATP responsive aptamers and polyethylenimine complexes. *J Mater Chem B* 4:3832–3841
66. Balazadeh S, Jaspert N, Arif M, Mueller-Roeber B, Maurino VG (2012) Expression of ROS-responsive genes and transcription factors after metabolic formation of H<sub>2</sub>O<sub>2</sub> in chloroplasts. *Front Plant Sci* 3:234
67. Chung MF, Chia WT, Wan WL, Lin YJ, Sung HW (2015) Controlled release of an anti-inflammatory drug using an ultrasensitive ros-responsive gas-generating carrier for localized inflammation inhibition. *J Am Chem Soc* 137:12462–12465
68. Shim MS, Xia Y (2013) A reactive oxygen species (ROS)-responsive polymer for safe, efficient, and targeted gene delivery in cancer cells. *Angew Chem Int Ed Engl* 52:6926–6929
69. Christiansen JP, French BA, Klibanov AL, Kaul S, Lindner JR (2003) Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles. *Ultrasound Med Biol* 29:1759–1767
70. Shimamura M, Sato N, Taniyama Y, Yamamoto S, Endoh M, Kurinami H, Aoki M, Ogihara T, Kaneda Y, Morishita R (2004) Development of efficient plasmid DNA transfer into adult rat central nervous system using microbubble-enhanced ultrasound. *Gene Ther* 11:1532–1539
71. Azuma H, Tomita N, Kaneda Y, Koike H, Ogihara T, Katsuoka Y, Morishita R (2003) Transfection of NFκB-decoy oligodeoxynucleotides using efficient ultrasound-mediated gene transfer into donor kidneys prolonged survival of rat renal allografts. *Gene Ther* 10:415–425
72. Hernot S, Klibanov AL (2008) Microbubbles in ultrasound-triggered drug and gene delivery. *Adv Drug Deliv Rev* 60:1153–1166
73. Pichon C, Kaddur K, Midoux P, Tranquart F, Bouakaz A (2008) Recent advances in gene delivery with ultrasound and microbubbles. *J Exp Nanosci* 3:17–40
74. Unger EC, Hersh E, Vannan M, Matsunaga TO, McCreery T (2001) Local drug and gene delivery through microbubbles. *Prog Cardiovasc Dis* 44:45–54
75. Bloch SH, Wan M, Dayton PA, Ferrara KW (2004) Optical observation of lipid- and polymer-shelled ultrasound microbubble contrast agents. *Appl Phys Lett* 84:631
76. Miller DL, Pislaru SV, Greenleaf JF (2002) Sonoporation: Mechanical DNA delivery by ultrasonic cavitation. *Somat Cell Mol Genet* 27:115–134
77. Simpson DH, Chin CT, Burns PN (1999) Pulse inversion Doppler: A new method for detecting nonlinear echoes from microbubble contrast agents. *IEEE Trans Ultrason Ferroelectr Freq Control* 46:372–382
78. Newman CM, Bettinger T (2007) Gene therapy progress and prospects: Ultrasound for gene transfer. *Gene Ther* 14:465–475
79. Suzuki R, Oda Y, Utoguchi N, Maruyama K (2011) Progress in the development of ultrasound-mediated gene delivery systems utilizing nano- and microbubbles. *J Control Release* 149:36–41
80. Mitragotri S (2005) Innovation—healing sound: The use of ultrasound in drug delivery and other therapeutic applications. *Nat Rev Drug Discov* 4:255–260
81. Tachibana K, Uchida T, Ogawa K, Yamashita N, Tamura K (1999) Induction of cell-membrane porosity by ultrasound. *Lancet* 353:1409
82. Deng CX, Sieling F, Pan H, Cui J (2004) Ultrasound-induced cell membrane porosity. *Ultrasound Med Biol* 30:519–526
83. Hallow DM, Mahajan AD, McCutchen TE, Prausnitz MR (2006) Measurement and correlation of acoustic cavitation with cellular bioeffects. *Ultrasound Med Biol* 32:1111–1122
84. Juffermans LJM, Dijkmans PA, Musters RJP, Visser CA, Kamp O (2006) Transient permeabilization of cell membranes by ultrasound-exposed microbubbles is related to formation of hydrogen peroxide. *Am J Physiol Heart Circ Physiol* 291:H1595–H1601
85. Mehier-Humbert S, Bettinger T, Yan F, Guy RH (2005) Ultrasound-mediated gene delivery: Kinetics of plasmid internalization and gene expression. *J Control Release* 104:203–211
86. Pan H, Zhou Y, Izadnegahdar O, Cui JM, Deng CX (2005) Study of sonoporation dynamics affected by ultrasound duty cycle. *Ultrasound Med Biol* 31:849–856
87. Sundaram J, Mellein BR, Mitragotri S (2003) An experimental and theoretical analysis of ultrasound-induced permeabilization of cell membranes. *Biophys J* 84:3087–3101
88. van Wamel A, Kooiman K, Harteveld M, Emmer M, ten Cate FJ, Versluis M, de Jong N (2006) Vibrating microbubbles poking individual cells: Drug transfer into cells via sonoporation. *J Control Release* 112:149–155

89. Zarnitsyn VG, Prausnitz MR (2004) Physical parameters influencing optimization of ultrasound-mediated DNA transfection. *Ultrasound Med Biol* 30:527–538
90. Fabiilli ML, Haworth KJ, Fakhri NH, Kripfgans OD, Carson PL, Fowlkes JB (2009) The role of inertial cavitation in acoustic droplet vaporization. *IEEE Trans Ultrason Ferroelectr Freq Control* 56:1006–1017
91. Rapoport NY, Kennedy AM, Shea JE, Scaife CL, Nam KH (2009) Controlled and targeted tumor chemotherapy by ultrasound-activated nanoemulsions/microbubbles. *J Control Release* 138:268–276
92. Zhang M, Fabiilli ML, Haworth KJ, Fowlkes JB, Kripfgans OD, Roberts WW, Ives KA, Carson PL (2010) Initial investigation of acoustic droplet vaporization for occlusion in canine kidney. *Ultrasound Med Biol* 36:1691–1703
93. Wang CH, Kang ST, Yeh CK (2013) Superparamagnetic iron oxide and drug complex-embedded acoustic droplets for ultrasound targeted theragnosis. *Biomaterials* 34:1852–1861
94. Wang P, Yin T, Li J, Zheng B, Wang X, Wang Y, Zheng J, Zheng R, Shuai X (2016) Ultrasound-responsive microbubbles for sonography-guided siRNA delivery. *Nanomedicine* 12:1139–1149
95. Yin T, Wang P, Li J, Zheng R, Zheng B, Cheng D, Li R, Lai J, Shuai X (2013) Ultrasound-sensitive siRNA-loaded nanobubbles formed by hetero-assembly of polymeric micelles and liposomes and their therapeutic effect in gliomas. *Biomaterials* 34:4532–4543
96. Yin T, Wang P, Li J, Wang Y, Zheng B, Zheng R, Cheng D, Shuai X (2014) Tumor-penetrating codelivery of siRNA and paclitaxel with ultrasound-responsive nanobubbles hetero-assembled from polymeric micelles and liposomes. *Biomaterials* 35:5932–5943
97. Cavalli R, Bisazza A, Trotta M, Argenziano M, Civra A, Donalizio M, Lembo D (2012) New chitosan nanobubbles for ultrasound-mediated gene delivery: Preparation and in vitro characterization. *Int J Nanomed* 7:3309–3318
98. Jin Q, Wang Z, Yan F, Deng Z, Ni F, Wu J, Shandas R, Liu X, Zheng H (2013) A novel cationic microbubble coated with stearic acid-modified polyethylenimine to enhance DNA loading and gene delivery by ultrasound. *PLoS One* 8:e76544
99. Gao D, Xu M, Cao Z, Gao J, Chen Y, Li Y, Yang Z, Xie X, Jiang Q, Wang W, Liu J (2015) Ultrasound-triggered phase-transition cationic nanodroplets for enhanced gene delivery. *ACS Appl Mater Interfaces* 7:13524–13537
100. Lentacker I, De Geest BG, Vandenbroucke RE, Peeters L, Demeester J, De Smedt SC, Sanders NN (2006) Ultrasound-responsive polymer-coated microbubbles that bind and protect DNA. *Langmuir* 22:7273–7278
101. Lentacker I, De Smedt SC, Demeester J, Sanders NN (2006) Microbubbles which bind and protect DNA against nucleases. *J Control Release* 116:E73–E75
102. Chin LS, Lim M, Hung TT, Marquis CP, Amal R (2014) Perfluorodecalin nanocapsule as an oxygen carrier and contrast agent for ultrasound imaging. *Rsc Adv* 4:13052–13060
103. Min HS, Son S, Lee TW, Koo H, Yoon HY, Na JH, Choi Y, Park JH, Lee J, Han MH, Park R-W, Kim I-S, Jeong SY, Rhee K, Kim SH, Kwon IC, Kim K (2013) Liver-specific and echogenic hyaluronic acid nanoparticles facilitating liver cancer discrimination. *Adv Funct Mater* 23:5518–5529
104. Yang P, Li D, Jin S, Ding J, Guo J, Shi W, Wang C (2014) Stimuli-responsive biodegradable poly(methacrylic acid) based nanocapsules for ultrasound traced and triggered drug delivery system. *Biomaterials* 35:2079–2088
105. Maeda H, Wu J, Sawa T, Matsumura Y, Hori K (2000) Tumor vascular permeability and the EPR effect in macromolecular therapeutics: A review. *J Control Release* 65:271–284
106. Zhou Y, Wang Z, Chen Y, Shen H, Luo Z, Li A, Wang Q, Ran H, Li P, Song W, Yang Z, Chen H, Wang Z, Lu G, Zheng Y (2013) Microbubbles from gas-generating perfluorohexane nanoemulsions for targeted temperature-sensitive ultrasonography and synergistic HIFU ablation of tumors. *Adv Mater* 25:4123–4130
107. Kim D-H, Nikles DE, Johnson DT, Brazel CS (2008) Heat generation of aqueously dispersed CoFe<sub>2</sub>O<sub>4</sub> nanoparticles as heating agents for magnetically activated drug delivery and hyperthermia. *J Magn Magn Mater* 320:2390–2396
108. Koning GA, Eggermont AMM, Lindner LH, ten Hagen TLM (2010) Hyperthermia and thermosensitive liposomes for improved delivery of chemotherapeutic drugs to solid tumors. *Pharm Res* 27:1750–1754
109. Shubayev VI, Pisanic TR 2nd, Jin S (2009) Magnetic nanoparticles for theragnostics. *Adv Drug Deliv Rev* 61:467–477

110. Mah C, Fraites TJ, Zolotukhin I, Song SH, Flotte TR, Dobson J, Batich C, Byrne BJ (2002) Improved method of recombinant AAV2 delivery for systemic targeted gene therapy. *Mol Ther* 7:106–112
111. Lo Y-L, Chou H-L, Liao Z-X, Huang S-J, Ke J-H, Liu Y-S, Chiu C-C, Wang L-F (2015) Chondroitin sulfate-polyethylenimine copolymer-coated superparamagnetic iron oxide nanoparticles as an efficient magneto-gene carrier for microRNA-encoding plasmid DNA delivery. *Nanoscale* 7:8554–8565
112. Zheng SW, Liu G, Hong RY, Li HZ, Li YG, Wei DG (2012) Preparation and characterization of magnetic gene vectors for targeting gene delivery. *Appl Surf Sci* 259:201–207
113. Takafuji M, Kitaura K, Nishiyama T, Govindarajan S, Gopal V, Imamura T, Ihara H (2014) Chemically tunable cationic polymer-bonded magnetic nanoparticles for gene magnetofection. *J Mater Chem B* 2:644–650
114. Agopian K, Wei BL, Garcia JV, Gabuzda D (2006) A hydrophobic binding surface on the human immunodeficiency virus type 1 Nef core is critical for association with p21-activated kinase 2. *J Virol* 80:3050–3061
115. Li QG, Lindman K, Wadell G (1997) Hydropathic characteristics of adenovirus hexons. *Arch Virol* 142:1307–1322
116. Jochum FD, zur Borg L, Roth PJ, Theato P (2009) Thermo- and light-responsive polymers containing photoswitchable azobenzene end groups. *Macromolecules* 42:7854–7862
117. Nagasaki T, Wada K, Tamagaki S (2003) Photo-enhancement of transfection efficiency with a novel azobenzene-based cationic lipid. *Chem Lett* 32:88–89
118. Hamada T, Sato YT, Yoshikawa K, Nagasaki T (2005) Reversible photoswitching in a cell-sized vesicle. *Langmuir* 21:7626–7628
119. Liu YC, Le Ny AL, Schmidt J, Talmon Y, Chmelka BF, Lee CT Jr (2009) Photo-assisted gene delivery using light-responsive catanionic vesicles. *Langmuir* 25:5713–5724
120. Li Y, Yang J, Sun L, Wang W, Liu W (2014) UV light-triggered unpacking of DNA to enhance gene transfection of azobenzene-containing polycations. *J Mater Chem B* 2:3868
121. Kim MS, Diamond SL (2006) Controlled release of DNA/polyamine complex by photoirradiation of a solid phase presenting *o*-nitrobenzyl ether tethered spermine or polyethylenimine. *Bioorg Med Chem Lett* 16:5572–5575
122. Deng X, Zheng N, Song Z, Yin L, Cheng J (2014) Trigger-responsive, fast-degradable poly(beta-amino ester)s for enhanced DNA unpacking and reduced toxicity. *Biomaterials* 35:5006–5015
123. Hu X, Li Y, Liu T, Zhang G, Liu S (2014) Photodegradable neutral-cationic brush block copolymers for nonviral gene delivery. *Chem Asian J* 9:2148–2155
124. Lee H, Kim Y, Schweickert PG, Konieczny SF, Won YY (2014) A photo-degradable gene delivery system for enhanced nuclear gene transcription. *Biomaterials* 35:1040–1049
125. Wijaya A, Schaffer SB, Pallares IG, Hamad-Schifferli K (2009) Selective release of multiple DNA oligonucleotides from gold nanorods. *ACS Nano* 3:80–86
126. Huschka R, Barhoumi A, Liu Q, Roth JA, Ji L, Halas NJ (2012) Gene silencing by gold nanoshell-mediated delivery and laser-triggered release of antisense oligonucleotide and siRNA. *ACS Nano* 6:7681–7691
127. Jayakumar MKG, Idris NM, Zhang Y (2012) Remote activation of biomolecules in deep tissues using near-infrared-to-UV upconversion nanotransducers. *Proc Natl Acad Sci USA* 109:8483–8488
128. Anal AK (2007) Stimuli-induced pulsatile or triggered release delivery systems for bioactive compounds. *Recent Pat Endocr Metab Immune Drug Discov* 1:83–90
129. Nair M, Guduru R, Liang P, Hong J, Sagar V, Khizroev S (2013) Externally controlled on-demand release of anti-HIV drug using magneto-electric nanoparticles as carriers. *Nat Commun* 4:1707
130. Qiu Y, Park K (2012) Environment-sensitive hydrogels for drug delivery. *Adv Drug Deliv Rev* 64:49–60
131. Servant A, Leon V, Jasim D, Methven L, Limousin P, Fernandez-Pacheco EV, Prato M, Kostarelos K (2014) Graphene-based electroresponsive scaffolds as polymeric implants for on-demand drug delivery. *Adv Healthc Mater* 3:1334–1343
132. Servant A, Methven L, Williams RP, Kostarelos K (2013) Electroresponsive polymer-carbon nanotube hydrogel hybrids for pulsatile drug delivery in vivo. *Adv Healthc Mater* 2:806–811
133. Weaver CL, LaRosa JM, Luo XL, Cui XT (2014) Electrically controlled drug delivery from graphene oxide nanocomposite films. *ACS Nano* 8:1834–1843



134. Ying X, Wang Y, Liang J, Yue J, Xu C, Lu L, Xu Z, Gao J, Du Y, Chen Z (2014) Angiopep-conjugated electro-responsive hydrogel nanoparticles: Therapeutic potential for epilepsy. *Angew Chem Int Ed Engl* 53:12436–12440
135. Gautier C, Cougnon C, Pilard J-F, Casse N, Chenais B (2007) A poly(cyclopentadithiophene) matrix suitable for electrochemically controlled DNA delivery. *Anal Chem* 79:7920–7923
136. Nicolau DV, Hook AL, Thissen HW, Hayes JP, Voelcker NH (2005) Development of an electro-responsive platform for the controlled transfection of mammalian cells. *Proc. SPIE* 5651, *Biomedical Applications of Micro- and Nanoengineering II*, p 418
137. An K, Zhao P, Lin C, Liu H (2014) A pH and redox dual responsive 4-arm poly(ethylene glycol)-block-poly(disulfide histamine) copolymer for non-viral gene transfection in vitro and in vivo. *Int J Mol Sci* 15:9067–9081
138. Cai X, Dong C, Dong H, Wang G, Pauletti GM, Pan X, Wen H, Mehl I, Li Y, Shi D (2012) Effective gene delivery using stimulus-responsive cationic copolymer designed with redox-sensitive disulfide and acid-labile imine linkers. *Biomacromolecules* 13:1024–1034
139. Li Y, Hei M, Xu Y, Qian X, Zhu W (2016) Ammonium salt modified mesoporous silica nanoparticles for dual intracellular-responsive gene delivery. *Int J Pharm* 511:689–702
140. Gupta MK, Lee SH, Crowder SW, Wang X, Hofmeister LH, Nelson CE, Bellan LM, Duvall CL, Sung H-J (2015) Oligoproline-derived nanocarrier for dual stimuli-responsive gene delivery. *J Mater Chem B* 3:7271–7280
141. Liu X, Ni P, He J, Zhang M (2010) Synthesis and micellization of pH/temperature-responsive double-hydrophilic diblock copolymers polyphosphoester-block-poly[2-(dimethylamino)ethyl methacrylate] prepared via ROP and ATRP. *Macromolecules* 43:4771–4781
142. Sun H, Zhu X, Zhang L, Gu X, Wang J, Li J, Zhang Y (2013) PDEA-coated magnetic nanoparticles for gene delivery to Hep G2 cells. *Biotechnol Bioprocess Eng* 18:648–654
143. Mok H, Veiseh O, Fang G, Kievit FM, Wang FY, Park JO, Zhang M (2010) pH-sensitive siRNA nanovector for targeted gene silencing and cytotoxic effect in cancer cells. *Mol Pharm* 7:1930–1939
144. Huang RY, Chiang PH, Hsiao WC, Chuang CC, Chang CW (2015) Redox-sensitive polymer/SPIO nanocomplexes for efficient magnetofection and MR imaging of human cancer cells. *Langmuir* 31:6523–6531
145. Li D, Tang X, Pulli B, Lin C, Zhao P, Cheng J, Lv Z, Yuan X, Luo Q, Cai H, Ye M (2014) Theranostic nanoparticles based on bioreducible polyethylenimine-coated iron oxide for reduction-responsive gene delivery and magnetic resonance imaging. *Int J Nanomed* 9:3347–3361
146. Yang Y, Xie X, Yang Y, Li Z, Yu F, Gong W, Li Y, Zhang H, Wang Z, Mei X (2016) Polymer nanoparticles modified with photo- and pH-dual-responsive polypeptides for enhanced and targeted cancer therapy. *Mol Pharm* 13:1508–1519
147. Wang F, Shen Y, Zhang W, Li M, Wang Y, Zhou D, Guo S (2014) Efficient, dual-stimuli responsive cytosolic gene delivery using a RGD modified disulfide-linked polyethylenimine functionalized gold nanorod. *J Control Release* 196:37–51
148. Guo W, Wang T, Tang X, Zhang Q, Yu F, Pei M (2014) Triple stimuli-responsive amphiphilic glycopolymers. *J Polym Sci Part A Polym Chem* 52:2131–2138
149. Klaukherd A, Nagamani C, Thayumanavan S (2009) Multi-Stimuli Sensitive Amphiphilic Block Copolymer Assemblies. *J Am Chem Soc* 131:4830–4838