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Recent Advances in Stimuli-responsive Poly(amidoamine) Dendrimer Nanocarriers for Drug Delivery

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ABSTRACT

Polyamidoamine (PAMAM) dendrimers have gained so much attention for drug and gene delivery since they have been introduced decades ago. The internal cavities and multivalent surface groups of PAMAM dendrimers have been widely used for small-molecule drugs/biomacromolecules encapsulation and conjugation. The involvement of PAMAM dendrimers in drug delivery systems (DDSs) has significantly improved drug pharmacokinetics and enhanced drug accumulation in pathological sites. The recent progress has been made on the design of stimuli-responsive PAMAM-based nano-sized vehicles that potentially deliver drugs in a spatially and/or temporally controlled fashion. Such smart dendrimer nanocarriers further the efficiency of targeted drug delivery, while decrease systemic exposure. In the review, we discuss recent advances in the design of PAMAM dendrimer-based DDSs that are able to release carried therapeutics only in response to a specific endogenous and exogenous stimuli.

Key words: *Polyamidoamine dendrimer, Stimuli-responsive, Drug and gene delivery, Intracellular controlled release, Disulfide.*

1. INTRODUCTION

The concept of stimuli-responsive strategy is based on the stimuli unique to pathological triggers or specific physiological (extracellular/intracellular) environment [1]. These stimuli that have been widely exploited for drug and gene delivery include endogenous biological variations such as pH value and redox in *in vivo* microenvironment and changes of exogenous conditions such as light and temperature.

Drug delivery has obtained much attention in the biomedical research area. Many drug delivery systems (DDS) have been developed to improve the therapeutic effect of drugs, including liposomes, element nanoparticles, polymer nanocarriers, micelles, solid lipid nanoparticles, quantum dots, and dendrimers. These nano-sized delivery vehicles can be categorized into encapsulates in which drugs are physically loaded into internal space of nanocarriers and drug nanoconjugates in which drugs are linked to their carriers via covalent bonds. Although these traditional drug-loaded nanoparticles are able to achieve temporally controlled release, improve pharmacokinetics, and to some extent promote drug accumulation to specific tissues, the effective access of drugs to pathological sites for action (in other words, the insufficient dose at disease sites) remains a challenge in the development of nanomedicines. To solve this challenge, various stimuli-responsive drug carriers have been developed (Table 1). The carriers are sensitive to one or more of endogenous and exogenous stimuli such as pH value, temperature, redox, and light state which are only existent at diseased sites or can be applied to specific sites.

Polymeric nanocarriers have proved their success for small molecules and biomacromolecules [2,3]. In contrast to other successful drug carriers (e.g., liposomes and micelles), polymer nanocarriers are much smaller, allowing for enhanced penetration into deep tumor areas, and their properties can be easily tailored according to needs. However, a broad range of molecular weight and random conformations of linear polymers make their pharmacokinetics and biodistribution relatively deviated. Conversely, dendrimers are hydrperbranched synthetic polymers

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Table 1: Preclinical studies of stimuli-responisve PAMAM-based nanomedicines for *in vitro* or *in vivo* therapy.

Application	Platform	Therapeutics	Stimuli	Disease	Status	Reference
Cancer therapy	G3NH_2-PEG1K	DOX	pH	Lung Adenocarcinoma	*In vitro*	[16]
Cancer therapy	G4NH_2-PEG1K	DOX	pH	Melanoma	*In vivo*	[42]
Cancer therapy and imaging	G4NH_2-SPIONP	DOX	pH	Melanoma	*In vivo*	[43]
Cancer therapy	RGD-modified G4NH_2-PEG5K	DOX	pH	Glioblastoma	*In vivo*	[49]
Anti-inflammation	G4OH	Ibuprofen	Enzyme	Fever and inflammation	*In vitro*	[51]
Anti-inflammation	G4OH	NAC	Redox	Neuroinflammation, stroke and cerebral palsy	*In vivo*	[59,60]
Cancer therapy	Hyperbranched PAMAM	siRNA	Redox	Liver Carcinoma	*In vivo*	[61]
Generic	Layer-by-layer PAMAM-PEI assembly	DNA plasmid	Redox	N.A.	*In vitro*	[65]
Generic	G3, G4, and G5NH_2-Phe/Ile	N.A.	Temper-ature	N.A.	*In vitro*	[70]
Cancer therapy	G5NH_2-PEG2K	RB, Pp IX	Light	N.A.	*In vitro*	[71]
Cancer therapy	G3NH_2-SNP	Phthalocyanine tetrasulfonates	Light	Breast cancer	*In vitro*	[72]

Gn denotes the number of generation (e.g., G3 is generation 3); surface group: NH_2 - Amine-terminated, -OH - Hydroxyl-terminated. SPIONP=Super-paramagnetic iron oxide nanoparticles, PEG1K=Polyethylene glycol 1000 Da, SNP=Silica nanoparticles, RGD=Arginyl-Glycyl-Aspartic acid, PEI=Polyethylimine, Phe=Phenylalanine, Ile=Isoleucine, N.A=Not applicable, PAMAM=Polyamidoamine, NAC=N-acetylcysteine, DOX=Doxorubicin

with high monodispersity and controllable architecture. Especially, a large amount of surface groups enable dendrimers to hold multiple functions at one time for improving delivery efficiency. Polyamidoamine (PAMAM) is the most popular and successful dendrimer as drug carriers [4]. Therefore, this review focuses on the recent development of stimuli-responsive PAMAM dendrimer nanocarriers. Major properties of PAMAM dendrimers regarding biomedical application are briefly introduced, and various examples of stimuli-responsive PAMAM dendrimer nanocarriers are provide in this review.

1.1. PAMAM Dendrimers

PAMAM dendrimer is a hyperbranched symmetric polymer. It is made up of a core molecule, building blocks, and terminal groups. The size, molecular weight, and surface groups increase predictably with increasing generation number. The hydrodynamic diameters of PAMAM dendrimers range from 1 to 10 nm, and their conformations shift from round disk to globular structure as the number of generation increases. PAMAM dendrimer represents one of the most promising nanocarriers for the delivery of small-molecule therapeutics and biomacromolecules [5-9], since they possess a variety of strengths listed in Figure 1: (i) Dendrimers can increase the aqueous solubility of hydrophobic therapeutics, (ii) internal cavity and surface reactive groups of PAMAM dendrimers can be used for therapeutics encapsulation and conjugation, respectively, (iii) PAMAM dendrimers can also be functionalized with ligands for various purposes, such as target dendrimers to pathological sites, gain access to the intracellular milieu, and modulate transport across epithelium, (iv) the relatively small size imparts PAMAM dendrimers to be able to diffuse into deep regions of tumors, and (v) highly monodispersity can ensure PAMAM dendrimers to have highly reproducible pharmacokinetics and pharmacodynamics.

1.2. Targeting Strategy of DDSs

One of the challenges in systemic therapy involves poor drug concentration in local disease sites, which reduces therapeutic efficacy and increases systemic toxicity. Passive and active targeting strategies have been widely employed to drive drugs to pathological tissues via systemic administration. In passive targeting strategy, both the properties of delivery systems and disease pathological nature promote the accumulation of drugs at the sites of interest and attenuate non-specific *in vivo* spreading of drugs. Nanocarriers (10-400 nm) are typically suitable for this purpose, since they can be designed to improve drug solubility, protect drugs from enzymatic degradation, release drugs in spatially/temporally controlled fashion, overcome drug resistance, and enhance their blood circulation residence and tissue

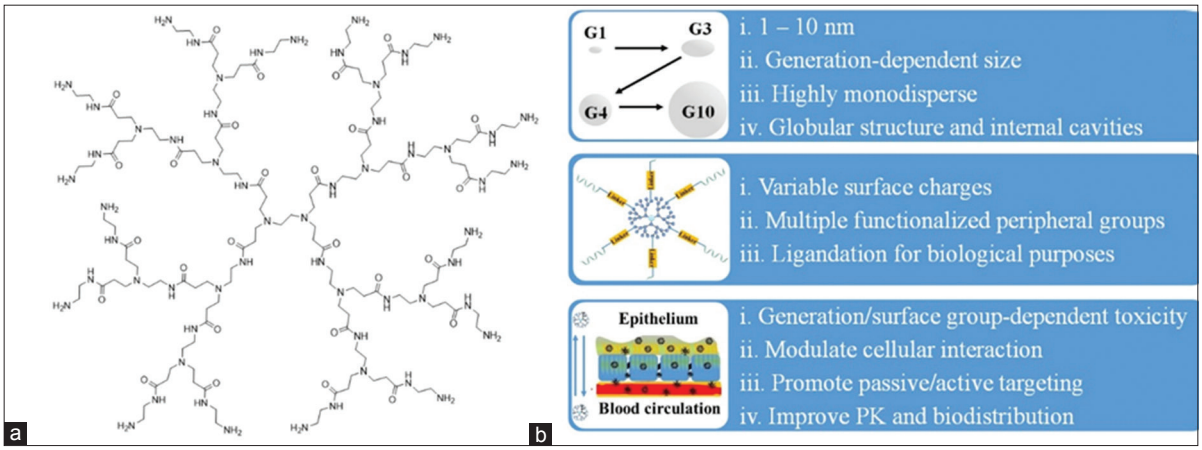

Figure 1: (a) Chemical structure of generation 2, NH_2-terminated polyamidoamine (PAMAM) dendrimers with ethylenediamine core molecule and 16 peripheral NH_2 groups, (b) chemical and biological benefits of PAMAM dendrimers as drug delivery nanocarriers.

biodistribution [10]. A passive process is known as enhanced permeability and retention (EPR) effect and has been widely accepted and applied in design of nanomedicines against tumors, chronic inflammations, and infections [11], since it was introduced by Maeda *et al*. [12]. The unique properties of solid tumors such as hypervasculature, defective vascular architecture, and impaired lymphatic drainage lead to the strong EPR effect of drug nanocarrier delivery systems. The systemic administration of polymer-based DDS can achieve 10-100 fold higher drug concentration in tumors due to the EPR effect compared to free drugs administered systemically [13,14].

Active targeting is another strategy to accumulate therapeutics at pathological sites in systemic administration. The rationale behind active targeting is the strong ligand-receptor binding affinity which enhances cellular uptake of delivery systems of interest. Due to rapid proliferation of tumor cells, many receptors are over-expressed on the surface of tumor cells compared to normal tissues which have been widely used as targets including folate receptor, vascular endothelial growth factor receptor, epidermal growth factor, prostate-specific membrane antigen, transferrin receptor, integrin receptor, and asialglycoprotein receptor [15]. Therefore, nanocarriers decorated with specific ligands on the surface can be readily recognized and internalized by cells at disease sites as the nanocarriers pass through nearby blood vessels and capillaries. Those ligands can be categorized as small molecules, sugars, peptides, proteins, and oligonucleotides (e.g., short interfering RNA-siRNA) [15]. This active targeting strategy has accomplished significant advance in targeted therapy of tumors.

One prerequisite to maximize the efficiencies of passive and active targeting strategies is sufficient residence time of DDS in systemic circulation as therapeutics are conveyed via bloodstream upon systemic administration. Regional administration is of great relevance to localize therapeutics into specific tissues. Local delivery has gained significant attentions as it imparts a high dose of drugs at local sites, fast action of drugs, ability to bypass the first-pass metabolism, and attenuated systemic exposure. The successful examples of local delivery include pulmonary delivery for lung disorders and cancers [16-18], ocular and nasal delivery for central nervous system diseases [19,20], intravaginal delivery for sexually transmitted diseases and infections [21-23].

The stimuli-responsive PAMAM dendrimer nanocarriers not only possess the major advantages of nanocarriers (e.g. passive/active targeting) but also are able to deliver and release therapeutics merely in response to environment change which can be found at pathological sites or applied externally. Therefore, such stimuli-responsive DDS hold the promise to promote targeted drug delivery (Figure 2).

2. PH-RESPONSIVE PAMAM DENDRIMER NANOCARRIERS FOR DRUG DELIVERY

2.1. Abnormal Extracellular and Intracellular pH Values in Tumor Microenvironment

Disorganized tumor vascular architecture is characterized by dilated and convoluted blood vessels, hyperbranched capillary network, and blind vascular endings. Fenestrations and lack of basal linings on vessel walls make tumor vasculature leaky [24]. These geometric abnormalities increase resistance to blood flow into tumor vessels and lower pressure difference between arterioles and venues in tumors. In addition, the lack of effective lymphatics in tumor regions elevates interstitial fluid pressure, which diverts blood flow away from tumor core to peripheral regions [25]. Due to nutrient deprivation and limited oxygen supply in the center of tumors, hypoxic region is thus formed in tumors [26]. Tumor cells in hypoxia region

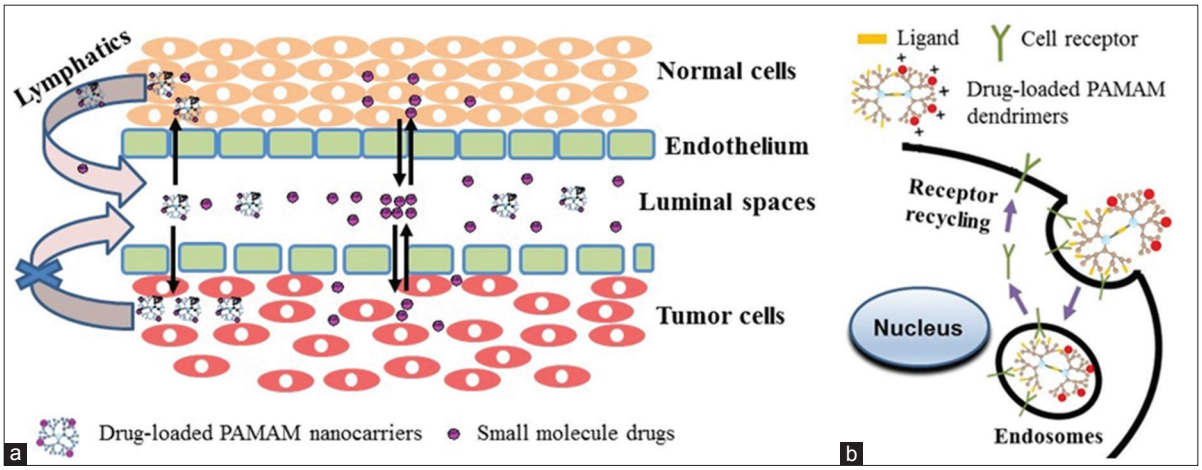

Figure 2: (a) Passive targeting of polyamidoamine (PAMAM) dendrimer nanocarriers to tumor tissues represented by enhanced permeability and retention effect. Small molecule drugs can easily diffuse between blood capillaries and interstitial space. Dendrimer nanocarriers easily extravasate into interstitial space, but are hard to diffuse back into blood capillaries. Dendrimer nanocarriers can be transported back to blood circulation via lymphatics in normal tissues, while the lack of effective lymphatic systems fails to drain PAMAM dendrimers, thus retaining dendrimer nanocarriers in tumor tissues, (b) Active targeting of PAMAM dendrimer nanocarriers to tumor cells using ligand-receptor coupling. Due to high binding affinity of ligands to their receptors, ligand-decorated PAMAM dendrimers can be internalized via receptor-mediated endocytosis at a faster pace and to a larger amount.

produce adenosine triphosphate for cell proliferation via a conversion of glucose to lactate. The inefficient clearance of acidic metabolites such as lactic acid and carbonic acid results in slightly acidic extracellular pH value (7.2-6.5) in solid tumors than that in normal tissues which have relatively basic extracellular pH [27]. The acidity is inversely proportional to the distance of tumor cells from nearest blood vessels [28]. The slightly acidic pH in tumor microenvironment impairs the penetration and distribution of cytotoxic chemotherapeutics in solid tumors because weakly basic anti-cancer drugs (e.g., doxorubicin [DOX]) diffuse faster in tumor matrix in non-ionic form, while protonated drug molecules in tumor regions tend to bind to interstitial proteins, and thus have decreased cellular uptake and tumor infiltration.

On top of acidic extracellular condition in the solid tumors, a significant pH drop from early endosomes (pH: 6.0-6.5) to lysosomes (pH: 4.5-5.0) in endocytic pathways can also be considered as a potential stimuli to trigger temporally and spatially controlled release of cytotoxic therapeutics. Lysosomes are terminal organelles in the endocytic pathway. Hydrolytic lysosomal enzymes specific to an array of targets are only functionally active under mildly acidic pH condition (between 5.0 and 4.5) for biomacromolecule digestion [29]. Therefore, protons are continuously pumped into lysosome lumen using metabolic energy to maintain the pH gradient across lysosomal membrane. In addition, polymeric nanocarriers including PAMAM dendrimers are well-known to be internalized by cells via various endocytic pathways such as clathrin-mediated endocytosis, caveolae-mediated endocytosis [30-32], non-specific adsorptive endocytosis [31,33,34] and micropinocytosis [32]. The great relevance of the acidic lysosomal pH in tumor chemotherapy is that prodrugs are designed to be dormant in the bloodstream and interstitial space of tumors, while are activated by high acidity in lysosomes upon cellular internalization as illustrated in Figure 3a [35]. Harmful adverse effects of cytotoxic chemotherapeutics can be potentially decreased by such an intracellularly triggered drug release.

2.2. Examples of pH-sensitive PAMAM Dendrimer Nanocarriers for Drug Delivery

Amine-terminated PAMAM itself is a pH-sensitive dendrimer due to an abundance of internal tertiary amines and peripheral primary amines. PAMAM dendrimers and polyethylene glycol (PEG)ylated counterparts have been used to solubilize a few hydrophobic drugs in a pH-dependent fashion such as 2-naphthol [36], nifedipine [37], nicotinic acid [38], and methotrexate [39]. The rationale behind pH-dependent solubilization is hydrophobic drugs can be efficiently encapsulated into internal cavities of PAMAM dendrimers at high pH while drugs are able to be released due to open structures conferred by repulsion of protonated amine groups [36,40]. This rationale can also be used to design pH-sensitive PAMAM dendrimer/drug encapsulates, which can maintain more stable drug encapsulates at neutral pH than at acidic pH [41].

PAMAM dendrimers have a large number of peripheral reactive groups which can be functionalized with various ligands for modulation of cellular interaction, tumor

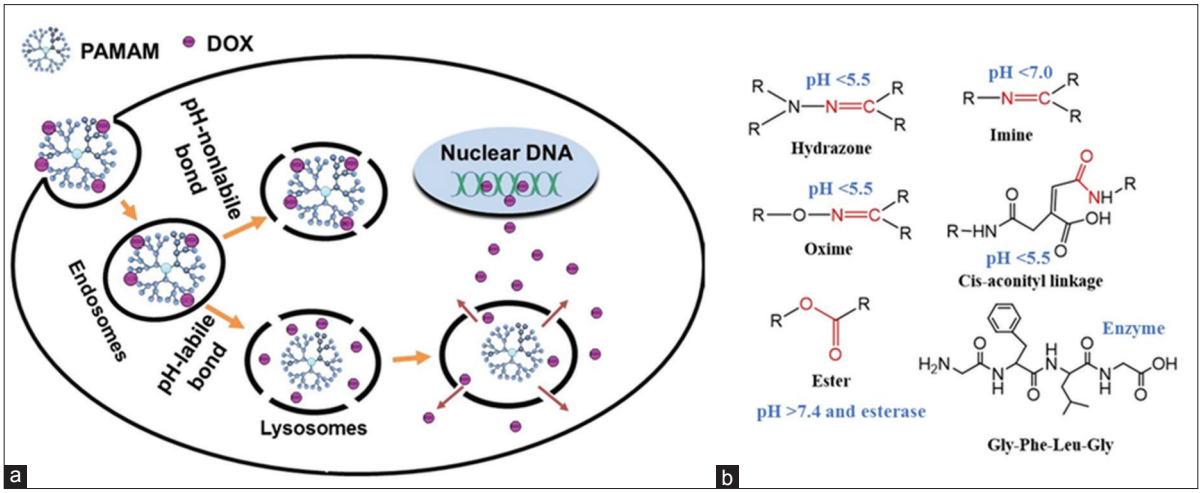

Figure 3: (a) Subcellular trafficking of polyamidoamine (PAMAM) dendrimer with doxorubiciin (DOX) conjugated via an acid-labile linker (PAMAM-DOX). PAMAM-DOX conjugates are internalized by cells on various endocytic pathways. The conjugates end up in acidic subcellular compartments (e.g. endosomes and lysosomes) where the cleavage of acid-labile linkers enables DOX to be released in protonated form. DOX eventually moves out of acidic compartments and then migrates to its target organelle — nucleus, (b) Common pH-sensitive linkers for drug conjugation to PAMAM dendrimers and their pH ranges with high sensitivity.

cell/tissue targeting, spatially controlled drug release, and prolonged residence in blood circulation. Those groups are particularly relevant for the conjugation of anticancer drugs to PAMAM dendrimers through the pH-responsive bonds as a high load of anticancer drugs can be bonded and the functions mentioned above can also be readily fulfilled in the meanwhile. A few acid-labile linkers have been explored in the development of PAMAM-bound anticancer drug conjugates, including imines, hydrazones, cis-aconitic anhydride, oximes, and ester. The chemical structures of these bonds and the pH range in which they are cleavable have been summarized in Figure 3b. The presence of these acid-sensitive spacers between anticancer drugs and PAMAM dendriemrs maintain the conjugated drug in extracellular fluid while enable drug release in endosomes and/or lysosomes upon endocytic internalization to tumor cells.

Lai *et al.*'s pioneer work already demonstrated the pH-responsive strategy is feasible for the intracellular delivery of anticancer drugs [5]. They conjugated DOX to carboxyl-terminated PAMAM dendrimer through acid-sensitive hydrazone bond and a stable amide bond as a control group. Only DOX in the conjugate with hydrazone bond reached its target organelle — nucleus, then killing tumor cells. The amide bond was not able to enable the release of DOX in endosomal/lysosomal environment due to its high chemical stability, and thus no cell kill was observed [5]. Another report verified the vital effect of endosomes/lysosomes on the intracellular acidity-triggered drug release [42]. Their studies showed DOX appeared only in perinuclear regions if endosomes/lysosomes were ruptured in the middle of the cellular internalization of these acid-labile PAMAM-DOX conjugates, while DOX was successfully freed from the conjugates and then migrated to target organelles as indicated by colocalization of DOX and nucleus as those acidic compartments were intact. Acid-labile PAMAM dendrimer-DOX conjugate was also attached to superparamagnetic iron oxide nanoparticles (SIONPs) [43]. The SIONPs can help carry the PAMAM-DOX conjugate to solid tumors upon EPR effect. Furthermore, the DOX/dendrimer-coated SIONPs have a potential to enhance magnetic resonance imaging contrast effect for *in vivo* cancer diagnosis. To efficiently deliver the pH-responsive PAMAM-drug conjugates to tumor sites upon EPR effect and/or active targeting through ligand-receptor coupling, PAMAM dendrimers need surface modification for long circulation in the bloodstream. The common strategy to protect PAMAM dendrimers from clearance by renal filtration and reticuloendothelial system is to coat dendrimer surface with a neutral and hydrophilic polymer — PEG. PEGylated PAMAM dendrimers are inert to serum protein binding and have reduced interaction with cell surface receptors [44,45], leading to a significantly prolonged residence of PEGylated nanoparticles in blood circulation and enhanced accumulation in tumor sites (mainly due to EPR effect) [17,46,47]. Based on the advantages PEGylation confers to dendrimer nanocarriers, Zhu *et al.* developed a series of PEGylated PAMAM dendrimers for intracellular delivery of DOX upon the cleavage of acid-labile cis-aconitic linkage in endosomes/lysosomes [48]. *In vivo* systemic biodistribution in this study showed 20-fold accumulation of acid-labile PAMAM-DOX as PEGylation degree increased, which can be ascribed to

the fact that larger carrier size at increased peripheral PEG density improves efficacy of EPR effect. Improved tumor targeting and intracellular release of DOX effectively inhibit melanoma (B16 cell line) and attenuate systemic toxicity in animal model, compared to DOX alone treatment group. Zhang *et al*. further attached Arg-Gly-Asp tripeptides (RGD) to the acid-sensitive PEGylated PAMAM-DOX conjugate for targeting cell adhesion protein intergrin $\alpha_v\beta_3$ which is overexpressed in glioblastoma [49]. This RGD-modified acid-sensitive PEGylated PAMAM-DOX showed enhanced *in vitro* cytotoxicity compared to free DOX and the conjugate without RGD due to higher cellular uptake upon RGD modification. When DOX was conjugated through acid-insensitive bond, no antitumor activity was found from the conjugate although similar biodistribution and pharmacokinetics were observed [49].

Drug delivery to specific tissues through the local route of administration is a more efficient strategy for drug retention in tumors, although only a few tissues (e.g., lungs) hold the accessibility for local delivery and potential toxicological challenges may exist in the tissues. A recent attempt combined the intracellularly triggered drug release with pulmonary delivery through oral inhalation to target lung cancers. In this study, the PEGylated PAMAM dendrimers with DOX conjugated through an acid-labile linker were formulated in solution type propellant-based pressurized metered-dose inhalers for deep lung delivery [16]. The pulmonary delivery of dendrimer-bound DOX holds potential advantages in maintaining high local drug concentration in lung tumor sites while decreasing a systemic side effect of DOX such as cardiomyopathy and myelosuppression [16].

Enzymes that are overexpressed in tumors can also be utilized to trigger the release of anticancer drugs or convert prodrugs into cytotoxic therapeutics either in tumor cells or at tumor sites. For example, cathespsin B is a thiol-dependent protease that resides more in lysosomes of tumor cells than in normal cells [50]. Cathespsin B is well-known to break down certain tetrapeptides such as Gly-Phe-Leu-Gly (GFLG) at acidic pH. From the perspective of steric crowding on dendrimer surface, the tetrapeptidyl linker may also provide sufficient spacing for enzymatic cleavage [51]. Kurtoglu *et al.* investigated *in vitro* release of GFLG-linked PAMAM-ibuprofen conjugate and Gly-Gly (GG)-linked counterpart in the presence of cathespsin B [51]. Over 40% ibuprofen was released from conjugate within 48 h upon the degradation of GFLG peptidyl spacer, while GG-linked dendrimer-ibuprofen conjugate was quite stable during measurement due to GG dipeptidyl spacer is not susceptible to enzymatic activity of cathepsin B. However, the GFLG peptidyl spacer has not been as widely used in PAMAM-drug conjugates as in other polymer-drug conjugates. In fact, PAMAM dendrimers should have also been explored for enzymatically cleavable cisplatin and DOX since huge success has been made in polymer conjugates of these drugs [35,52,53].

3. REDOX-RESPONSIVE PAMAM DENDRIMER NANOCARRIERS FOR DRUG DELIVERY

3.1. Redox Potential Change across Cellular Membrane

Gene therapy to treat cancers has grown significantly in past decades due to low toxicity and high treatment efficacy [54]. One major challenge for gene therapy is the limited delivery of genes to cytosol where they start to act on different intercellular compartments. Cationic polymers including PAMAM dendrimers have been widely employed to assemble polymer/gene complexes for intracellular delivery. The polymer/gene complexes are readily disintegrated in blood circulation and are then degraded by serum nucleases, which thus restricts the efficiency of gene delivery [55]. In other words, the carried genes are released in an uncontrolled fashion. A high redox potential difference (100-1000 fold) between the reducing intracellular environment and oxidizing extracellular space can be potential stimuli to trigger spatial (intracellular) release of gene [11]. Therefore, the rationale for redox-responsive PAMAM dendrimer nanocarriers is the high reducing capability inside cells. Glutathione (GSH) is a cysteine-containing tripeptide in cytosols, quenching reactive oxygen species such as free radicals, peroxides, lipid peroxides, and heavy metals [56]. GSH is also the essential component for many enzymes that need thiol-reducing equivalents to keep redox-sensitive active sites on enzymes [57]. As depicted in Figure 4, dendrimer nanocarriers for gene delivery with disulfide bonds in between are able to undergo disulfide cleavage via GSH pathway only upon cellular internalization. The efficiency of gene delivery to cytosol is therefore improved by promoting such an intracellular delivery, while systemic exposure can thus be lowered.

3.2. Examples of Redox-responsive PAMAM Dendrimer Nanocarriers for Drug Delivery

As stated above, intracellular environment is much more reductive than extracellular space. Such a redox potential difference can be used as stimuli to design redox-responsive PAMAM dendrimer nanocarriers for drug (especially gene) delivery. Disulfide bonds can be broken down in the intracellular reductive environment. Two major approaches have been used in the development of disulfide bond-containing PAMAM dendrimers: (i) Conjugation of therapeutics of interest to PAMAM dendrimers and (ii) insertion of disulfide bonds to backbones of PAMAM dendrimers or hyperbranched PAMAM.

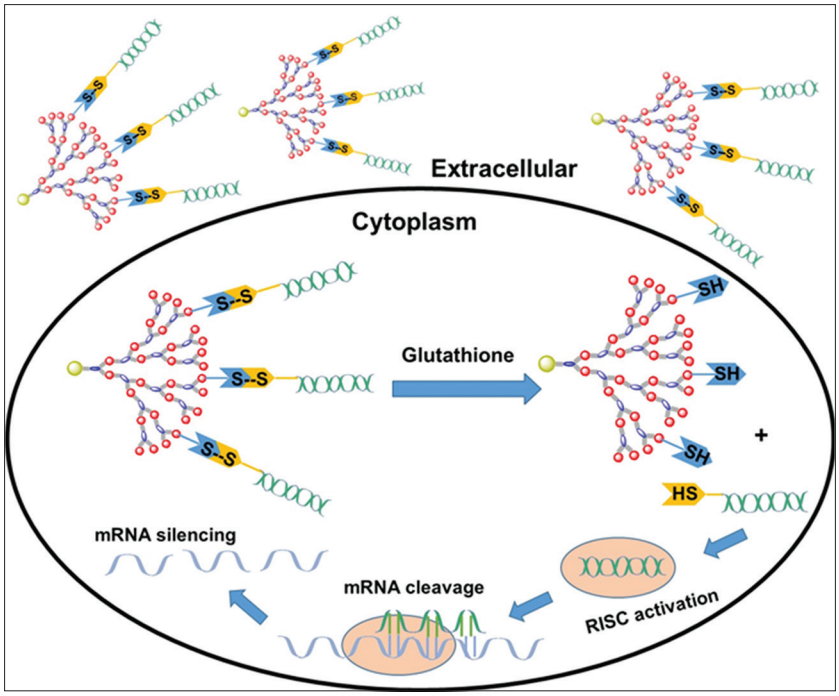

Figure 4: Cytosolic release of small interfering RNA (siRNA) from disulfide-containing polyamidoamine-siRNA conjugates by glutathione. Released siRNA is then activated in RNA-induced silencing complex (RISC). Messenger RNA (mRNA) is cleaved by activated siRNA, eventually leading to mRNA silencing.

N-acetylcysteine (NAC) is an anti-inflammatory agent widely used in the treatment of neuroinflammation, stroke and cerebral palsy [58]. The efficiency of free drug is significantly reduced by drug binding to serum proteins and low bioavailability [58]. Navath *et al*. conjugated high payload of NAC to PAMAM dendrimers bearing different surface termini via disulfide bonds [59,60]. NAC was released quickly (60% was at 1 h and over 80% at 5 h) at cytoplasm GSH level, while only a negligible amount of free NAC was observed at plasma GSH level [59]. Intracellular anti-oxidative activity of NAC was increased at an order of magnitude upon conjugation to PAMAM dendrimers through redox-sensitive bonds [60]. The further evaluation of the dendrimer-NAC conjugate was performed on an *in vivo* rabbit model with cerebral palsy. The dendrimer-NAC conjugate was able to suppress neuroinflammation and lead to significant improvement of motor function in the cerebral palsy-bearing newborn rabbit. In addition, the conjugation to dendrimer nanocarriers dramatically increased NAC ability to cross blood-brain barrier lead to enhanced bioavailability [58].

A few bioreducible PAMAM dendrimers or hyperbranched polymers have also been synthesized and used as gene delivery vehicle [61-63]. A disulfide bond-containing compound such as cystamine bisacrylamide is generally used as one of the monomers to construct the matrix of hyperbranched PAMAM. This type of hyperbranched dendrimer has three prominent advantages. That is, (i) The condensation of genes onto cationic hyperbranched dendrimers and buffer capacity of dendriplexes in endosomes/lysosomes can be tailored by adjusting the ratio of amino groups to other building blocks and/or changing amino monomers; (ii) the dendriplexes achieve spatial and temporal gene release by inserting stimuli-sensitive functional groups; (iii) cationic polymers show high toxicity after gene decomplexation. The disulfide bonds in backbone are degradable only via intracellular GSH pathways, which reduces the stability of dendriplexes in cytoplasm and the accumulation of cationic polymers inside cells [63]. Chen *et al.* developed a bioreducible hyperbranched PAMAM based on the copolymerization of bisacrylamide monomer and triamine monomer that binds PAMAM and DNA together in bloodstream, while releases DNA/RNA quickly upon cellular internalization. The content of disulfide bonds determined degradation products, ease of dendriplex disassembly, polycation cytotoxicity, and dendriplex transfection activity. The cytotoxicity and transfection activity improved as the content of disulfide bonds increased, and the gene transfection activities of all bioreducible dendriplexes were significantly higher than those of non-reducible counterparts and PEI-based polyplexes (PEI: 25 kDa) [64]. Zou *et al.* further assembled the similar disulfide-based reducible dendriplex into a layer-by-layer (LbL) DNA delivery vehicle. The alternating deposition of anionic plasmid DNA and cationic hyperbranched PAMAM on substrate showed a sustained release of plasmid DNA at intracellular

GSH concentration upon the disassembly of layers which is caused by both detachment of dendriplexes from underlying layer and cleavage of intramolecular disulfide bonds. In contrast, the non-degradable PAMAM/plasmid DNA complexes resulted in a bulk release [65]. The controlled release of DNA is strongly affected by the kinetics of disulfide degradation since it determines the rate at which each layer flake off LbL structure. In addition, a single dendriplex remains as a reservoir that promotes sustained release upon degradation of reducible PAMAM. Bioreducible hyperbranched PAMAM was also evaluated for *in vivo* transfection efficiency. Parmar *et al.* conjugated siRNA to N-acetylgalactosamine-modified reducible PAMAM. The conjugates with hepatocyte targeting ligand can effectively accumulate in the liver and showed robust gene silencing activity in *in vivo* studies (gene knockdown efficiency: ca. 60-80%) [61].

4. TEMPERATURE-RESPONSIVE PAMAM DENDRIMER NANOCARRIERS FOR DRUG DELIVERY

4.1. Enhanced Sensitivity of Cancer Cells to Temperature Changes

Tumor cells have been proved more sensitive to heat energy than normal cells be [66]. Cancer therapy that combines hyperthermia with radiation or chemotherapy thereby has gain much attention in the past decades, due to the progressive advances of temperature-monitoring technology and understanding of hyperthermia on cancer biology. Super-paramagnetic iron oxide (SPIO)-containing liposomes and polymeric nanoparticles could provide a unique way for localization of SPIO into targeted tumor tissues (due to EPR effect) and subsequent heat damage of tumor cells [11,67].

4.2. Examples of Temperature-responsive PAMAM Dendrimer Nanocarriers for Drug Delivery

Two major approaches to impart PAMAM dendrimers with thermosensitivity have been reported in the literature. That is, dendrimers are generally modified on the surface with monomers which can be polymerized into thermo-sensitive polymers, such as N-isopropylacrylamide (NIPAM). Alternatively, such monomers can also be inserted into internal branches of dendrimers for thermosensitivity. However, all thermo-sensitive dendrimers developed based on PAMAM adopted the first strategy. Haba *et al.* reacted NIPAM with carboxyl-terminated PAMAM dendrimer on the surface. The resulting PAMAM-NIPAM conjugate had low critical solution temperature (LCST) at ca. 30°C [68]. The PEGylation of PAMAM-NIPAM on surface further increased LCST to 35°C [40]. Furthermore, the PEGylated PAMAM-NIPAM dendrimer has improved drug payload, enabled prolonged drug release, and enhanced biological activity efficiency such as biodistribution and pharmacokinetics [69]. However, temperature-sensitive nanocarriers with LCST above 37°C is necessary from the perspective of drug delivery. Tono's work developed a series of PAMAM dendrimers with peripheral phenylalanine residues which has LCSTs range from 32 to 60°C. Among these dendrimers, the one with the ratio of phenylalanine to isoleucine = 44:20 was imparted with appropriate LCST (42°C) for drug delivery application [70].

5. PHOTOSENSITIVE PAMAM DENDRIMER NANOCARRIERS FOR DRUG DELIVERY

Photodynamic therapy was approved in the 1990s in the treatment of superficial tumor and age-related muscular degeneration. Photosensitive drugs generate singlet oxygen after light irradiation, resulting in oxidative damage on mitochondria in cells [40]. Photosensitive drugs are activated only after irradiation and singlet oxygen are ephemeral and thereby affecting only a specific area. Kojima *et al.* synthesized PEG-grafted PAMAM and polypropylenimine dendrimers for encapsulating two model photosensitizers — rose bengal (RB) and protoporphyrin IX (PpIX). Both complexes were stable at physiological condition and light irradiation induced *in vitro* toxicity comparable to free RB or PpIX on Hela cells [71]. A novel PAMAM-coated silica nanoparticle was recently reported for photodynamic therapy [72]. Generation 3 amine-terminated PAMAM dendrimers were grafted to the surface of porous hollow silica nanoparticles (PHSNPs), followed by the attachment and encapsulation of aluminum phthalocyanine tetrasulfonates (APTSs) [72]. The abundance of amino groups on surface and high porosity of PHSNPs can significantly increase APTS payload in the nanoconstruct. The PAMAM-coated PHSNPs photosensitive nanoconstruct were more potent therapeutically than free APTSs against MCF-7 cells (a human breast adenocarcinoma cell line), partly due to higher cellular internalization of the nanoconstructs.

6. CONCLUDING REMARKS AND FUTURE DIRECTIONS

The in-depth understanding on the disparities between normal tissues and pathological tissues and advance in materials synthesis allow us to design targeted DDSs that are only responsive to special environmental changes. PAMAM dendrimer is one of the ideal nanocarriers to accomplish such a design. This review discussed the preparation, *in vitro* assessment, and *in vivo* performance of many examples of PAMAM-based stimuli-responsive nanocarriers for drug delivery. These studies firmly verify the use of various endogenous and exogenous stimuli in combination with targeted drug delivery can make a significant advance in drug delivery field. However, the application of such a stimuli-responsive mechanism

remains under infancy and in the conceptual phase. Therefore, further in-depth study in therapeutic efficiency and application of stimuli-responsive nanocarriers should be performed. The future studies may focus the following few aspects:

6.1. Standardized Synthesis

The synthesis should focus on the reproduction of the PAMAM-based drug conjugates and encapsulates with standardized methodologies since the addition of drug molecules to pre-existing PAMAM dendrimers follows a distribution profile and subsequently results in undefined mixture of dendrimer-drug conjugates [73]. Furthermore, the isolation and separation of this undefined mixture are almost impossible. This may cause non-reproductive/less reproductive pharmacokinetics which is a communal disadvantage of all polymer-bound drug conjugates.

6.2. In vivo Evaluation

In vitro evaluation of stimuli-responsive PAMAM dendirmers has been widely reported, which indicates the high efficacy of these smart systems on cell models. However, only a few *in vivo* evaluations focused on pH-sensitive PAMAM-DOX conjugates and one on disulfide-containing PAMAM-siRNA conjugates. Due to the heterogeneity of tumors, the *in vivo* evaluation of such stimuli-responsive dendritic systems should focus on the drug accumulation and penetration into deep tumor regions, which can be optimized through the modulation of surface chemistry, drug payloads, and ligand attachments.

6.3. Routes of Administration

The major challenge of cancer chemotherapy is to concentrate sufficient drug molecules in tumor sites, which requires DDSs to efficiently cross an abundance of extracellular and intracellular barriers *in vivo*. Systemic delivery (intravenous and intraperitoneal injection) of drugs and their delivery systems is primary route of administration from the perspectives of almost all clinicians and researchers. However, regional administration as a potential route for local diseases has showed advantages regarding high local dosage, fast drug action and decreased systemic exposure [16,58,74]. In addition, the regional administration can deliver drugs to the vicinity of pathological sites, which may significantly improve targeting efficiency. Although EPR effect and ligand-based active targeting strategy have been used to enhance drug accumulation in disease sites, tumor heterogeneity and *in vivo* clearance mechanisms decrease their efficiency [75]. Therefore, stimuli-responsive dendrimer nanocarriers in combination with local delivery is a promising strategy to enhance therapeutic efficacy, while to decrease systemic toxicity.

6.4. Toxicological Issues

Extremely low toxicity is indispensable for nanocarrier-based DDS. Acute toxicity of PAMAM dendrimers and PEGylated counterparts have been well investigated such as hemolysis and protein binding [76,77]. However, the long-term toxicity of PAMAM dendrimers after carried drugs are released remains unclear. For instance, carcinogenesis, mutagenesis, and long-term immune responses due to its non-degradability.

6.5. Clinical Trial

PAMAM dendrimers have not been as successful as liposomes and N-(2-Hydroxypropyl) methacrylamide in clinical translation, although they have gained high medical expectations [73]. Only a few PAMAM-based DDSs have been found under clinical trials [78]. None of them is related to stimuli-responsive PAMAM dendrimer systems. Therefore, the clinical test is definitely the future direction of these novel stimuli-responsive PAMAM-based DDSs.

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