Top Curr Chem (Z) (2017) 375:26
DOI 10.1007/s41061-017-0112-0

REVIEW

History of Polymeric Gene Delivery Systems

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Received: 26 November 2016 / Accepted: 24 January 2017 / Published online: 8 February 2017

Abstract As an option for genetic disease treatment and an alternative for traditional cancer chemotherapy, gene therapy achieves significant attention. Nucleic acid delivery, however, remains a main challenge in human gene therapy. Polymer-based delivery systems offer a safer and promising route for therapeutic gene delivery. Over the past five decades, various cationic polymers have been optimized for increasingly effective nucleic acid transfer. This resulted in a chemical evolution of cationic polymers from the first-generation polycations towards bioinspired multifunctional sequence-defined polymers and nanocomposites. With the increasing of knowledge in molecular biological processes and rapid progress of macromolecular chemistry, further improvement of polymeric nucleic acid delivery systems will provide effective tool for gene-based therapy in the near future.

Keywords Gene delivery · Gene therapy · Polymers · Polyplexes · Polymeric gene delivery system · Transfection

This article is part of the Topical Collection"Polymeric Gene Delivery Systems"; edited by Yiyun Cheng.

Communicated by: Yiyun Cheng.

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1 Introduction

Gene therapy is a disease treatment approach that introduces exogenous nucleic acids into specific cells for modulating the gene expression [1]. Benefiting from the advances of nucleic acid research in biology and chemistry, the therapeutic nucleic acids already expanded from plasmid DNA (pDNA) to messenger RNA (mRNA), small interfering RNA (siRNA), microRNA (miRNA), antisense oligonucleotides, or even genome editing systems [2]. For instance, systemic delivery of CRISPR/Cas9 mRNA, guide RNA and repair template DNA for therapeutic genome editing in mice was recently reported [3].

Since 1989, numerous clinical trials of gene therapy have been approved worldwide [4]. However, success in clinical trials was hampered by numerous extracellular and intracellular barriers (Fig. 1) for gene delivery vectors, such as nucleic acid packaging, serum stability, targeting to specific cells, cellular internalization, endosomal escape, efficient intracellular unpackaging, and transport to specific organelles [5–7]. Viral vectors have evolved to overcome these barriers, but coming with some fundamental drawbacks, like toxicity [8], immunogenicity [9], limited nucleic acid capacity [10] and difficult production upscale [11]. Synthetic cationic polymers came out as a new opportunity for better safety and easier large scale manufacture [6, 7] with efficient condensation of larger nucleic acids into nanoscale particles called polyplexes and effective transfection of gene into cells [5, 12]. Meanwhile, benefiting from the flexibility in chemical synthesis, a large variety of cationic polymers with different cationic backbones was designed for improved nucleic acid delivery [5, 6, 12]. It is also feasible to synthesize virus-inspired multifunctional polymeric gene delivery systems by incorporating all kinds of functional units into the polyplexes, such as targeting ligands, shielding domains, endosomolytic units and nuclear localization signals, to conquer the biological hurdles [5, 12, 13]. In summary, nucleic acid transfer by polymeric materials may have great advantages in gene therapy. Nevertheless, only few clinical trials with polymeric carriers were performed [14–25], because of their previous lower efficiency in gene delivery as compared with viral vectors. Therefore, great efforts remain to be taken to improve further polymeric gene delivery systems for effective human genetic therapy.

In this review, we outline the historical evolution of polymer-based gene delivery systems during the past 50 years. The strengths and weaknesses, as well as structure–activity relations of developed cationic polymers will be discussed in the following sections.

2 First-Generation Polycations: DNA Binding, Compaction, and Targeting

Our learning curve of polymeric gene delivery systems initiated from the first-generation polycations (Fig. 2). In 1962, Smull and Ludwig reported that cationic proteins could significantly enhance the infectivity of poliovirus RNA, which opened the door for polycation-based gene transfection [26]. In 1965, Pagano and

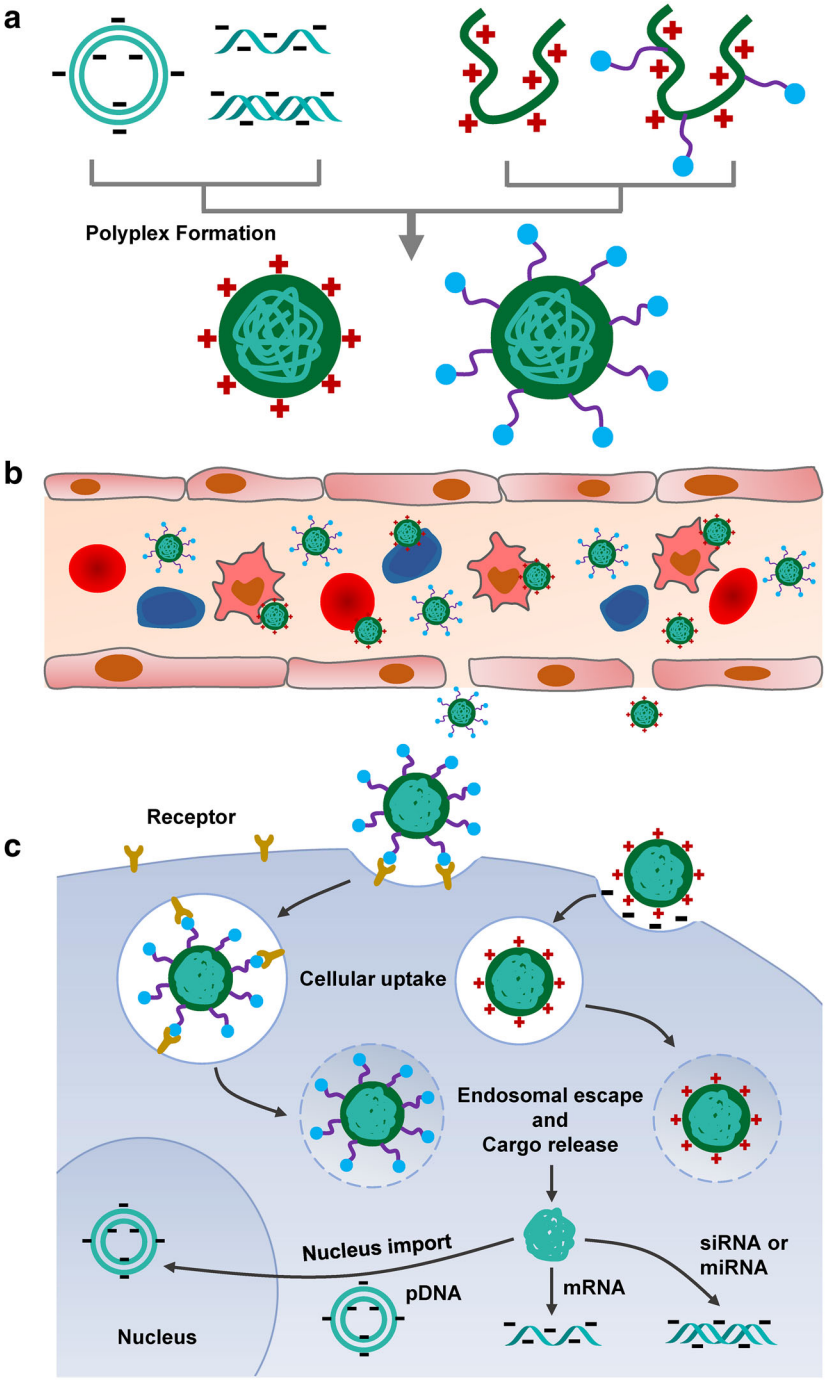

Fig. 1 Barriers in the nucleic acid delivery pathway of polyplexes. **a** Formation of stable polyplexes. **b** Avoidance of rapid clearance and unspecific interactions with blood components. **c** Cellular barriers

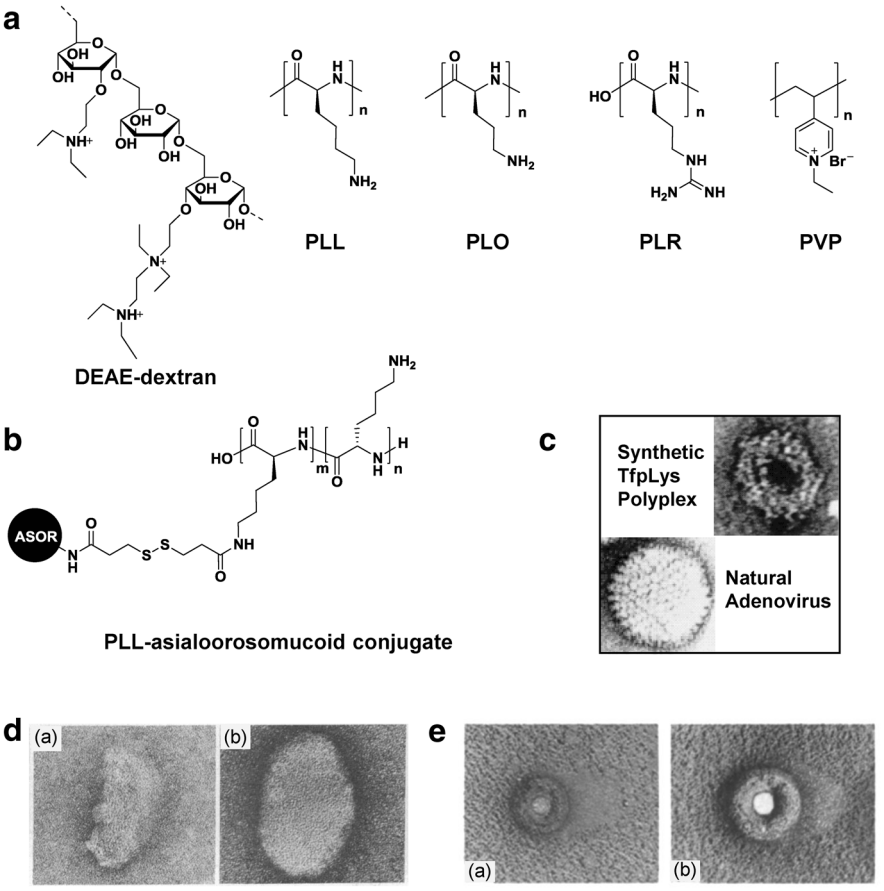

Fig. 2 First-generation polycations for gene delivery. **a** *DEAE-dextran* diethylaminoethyl-dextran; *PLL* poly-L-lysine; *PLO* poly-L-ornithine; *PLR* poly-L-arginine; *PVP* poly(*N*-alkyl-4-vinylpyridinium) bromide. **b** First targeted gene delivery polymer by Wu and Wu 1987, PLL-asialoorosomucoid (ASOR) conjugate. **c** Compaction of pDNA by transferrin-polylysine by Wagner 1990 and 1991 into donut-like nanoparticles (*top*) with 100 nm size, comparable with natural adenovirus (*bottom*) dimension. **d** Compaction of T4 DNA with poly (ethylene oxide) by Laemmli 1975. Adapted from [32] with kind permission from National Academy of Sciences and Professor U. K. Laemmli. **e** Compaction of T7 DNA with spermidine by Chattoraj 1978 into donut-like nanoparticles. Adapted from [33] with kind permission from Elsevier

colleagues found that diethylaminoethyl (DEAE) dextran (Fig. 2a) could strongly enhance poliovirus RNA transfection [27]. Similar findings on SV40 viral DNA were demonstrated subsequently [28]. Besides DEAE dextran, other cationic polymers, such as polyornithine, polylysine, and polyarginine (Fig. 2a), also had the potential for transferring nucleic acids into cells. Their efficiency for exogenous DNA transfection was evaluated in comparison by Farber and Butel in 1975; as a result, polyornithine presented the best performance [29]. In the early 1990s Kabanov & Kabanov designed poly(*N*-alkyl-4-vinylpyridinium) salts (Fig. 2a) as transfection agents [30, 31]. Our understanding of the interaction between cationic

polymers and negatively charged nucleic acids was further deepened by the finding that cationic polymers not only bind nucleic acids, but also spontaneously condense them into compact structures (Fig. 2c–e) with specific shapes and sizes which resemble natural viruses [32, 33]. Most recent research using pair correlation microscopy by Hinde and Gaus showed that polymeric nanoparticles with same surface modifications, but different shapes presented different moving rates through the diverse cellular obstacles, which may be regarded as a reference for the role of polyplex shape in gene delivery [34].

A milestone in the progress of polymer-based gene delivery came out in 1987 with the report of receptor-mediated gene transfection by George and Catherine Wu [35, 36]. They first introduced asialoorosomucoid (ASOR), a ligand for asialoglycoprotein receptor specifically expressed on hepatic cells, into polylysine and combined this conjugate with DNA to form receptor-targeted polyplexes (Fig. 2b). They could verify asialoglycoprotein receptor-mediated DNA transfection via enhanced cellular internalization of plasmid DNA into hepatocytes both in vitro and in vivo into livers upon intravenous administration in rat. The CAT marker gene expression was transient. Gene transfer in combination with partial hepatectomy, which during liver regeneration triggers intensive hepatocyte proliferation, resulted in transgene integration and prolonged gene expression after 11 weeks [37, 38]. Using the receptor-targeted pDNA complexes, partial correction of genetic liver defects in experimental animals was possible [39, 40]. Following on this breakthrough, other receptor targeted gene delivery systems were developed as reviewed in [5, 41]. Published in 1990, Birnstiel, Wagner and colleagues investigated transferrin-protamine and transferrin-polylysine for their transferrin receptor-specific nucleic acid transfer efficiency into tumor and hematopoietic cells [42–44]. In 1993 Pam Davis and colleagues demonstrated gene transfer into respiratory cells by targeting the polymeric immunoglobin receptor [45].

Although the incorporation of ligands into polyplexes improved the gene delivery into specific cells, the efficiency of polylysine-based gene delivery was still moderate [46]. For example, Cotten and colleagues found intensive uptake of transferrin-polylysine/DNA complexes into human transferrin receptor overexpressing K562 leukemia cells, but insignificant gene expression [43]. Luthman and Magnusson had established in 1983 that the lysosomotropic anti-malaria drug chloroquine could significantly enhance DEAE-dextran mediated gene transfection [47]. Consistently, transferrin-polylysine mediated targeted DNA transfer into K562 cells was strongly enhanced by this treatment [43]. In those days the detailed mechanism of the chloroquine enhancement was unclear; hypothetical modes of action ranged from block of lysosomal acidification and thus rescue of endolysosomal DNA from enzymatic degradation, to dissociation of DNA complexes by chloroquine, facilitating intracellular release of DNA [48, 49].

The effectiveness of chloroquine indicated that some biological barriers hindered the highly effective transfection of polyplexes. The effectiveness of chloroquine depended on the natural endosomal acidification process mediated by the vacuolar ATP-dependent vATPase [48–50]. vATPase actively pumps protons into the early endosomes or late endosomes and lysosomes and keeps an acidic microenvironment in the early endosomes (pH 5.9–6.5) or late endosomes and lysosomes (pH 5.0)

[51, 52]. Unprotonated weakly basic chloroquine can freely diffuse into the cells, but is trapped as protonated form in the acidifying organelles endosomes and lysosomes. With continuous protons uptake by vATPase, the protonated chloroquine keep gathering in the vesicles coming with continuous internalization of chloride counter ions and water, consequently leading to the osmotic swelling and membrane disruption of endosomes or lysosomes.

Realizing the bottleneck of endo-lysosomal entrapment, more active measures for cytosolic delivery were followed, similar to those which natural viruses use in their cellular infection process. In 1991 Curiel and Cotten introduced inactivated adenoviruses [53, 54] to enhance nucleic acids transfection by co-incubation with receptor-targeted gene transfection systems. Later on also rhinoviruses were used for this purpose [55]. It was assumed that the viruses promoted the nucleic acids transfer through enhancing the endosomal escape of the co-delivered polyplexes with their nature-derived endosome membrane destabilization domains. Further refinements were carried out by Wagner et al. via direct attachment of whole inactivated adenoviruses (Fig. 3) via various couplings including a biotin-streptavidin linkage [56, 57]. A series of related virus-linked systems were subsequently developed presenting high efficacy in vitro and in vivo [58–70]. The exceptionally efficient gene transfer activity of AVET (adenovirus-enhanced transferrinfection) even in case of "difficult-to-transfect" primary cells and primary tumor cell isolates, resulted in the first polymer-based human gene therapy trial in 1994 [14, 15]. In this study, GMP production of autologous melanoma vaccines transfected with IL-2 pDNA was performed for each individual melanoma stage 4 patient.

For endosomal escape, instead of whole viruses, proteins were tested. The group of Lee enhanced cytosolic delivery of pDNA by a sulfhydryl-activatable listeriolysin O/protamine conjugate [72]; Gottschalk et al. introduced a prefringolysin O into polyplexes via a biotin-streptavidin linkage [73]. The group of Wels designed recombinant multidomain proteins including receptor-binding, toxin derived endosomal escape, and pDNA binding motifs [74–76]. Influenza virus hemagglutinin subunit HA2-derived synthetic fusion peptides named INF (Fig. 4) were incorporated by

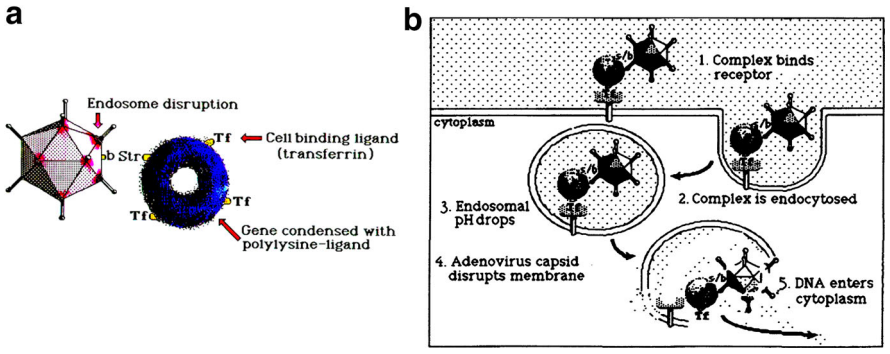

Fig. 3 Adenovirus enhanced transferrinfection (AVET). **a** Chemically (8-methoxypsoralen/UV light) inactivated biotinylated adenovirus particles attached to streptavidine-polylysine/transferrin-polylysine/pDNA polyplexes. Reproduced with kind permission from Dr. Matthew Cotten. **b** Receptor-mediated uptake and endosomal escape of AVET. Adapted from [71] with kind permission from Elsevier

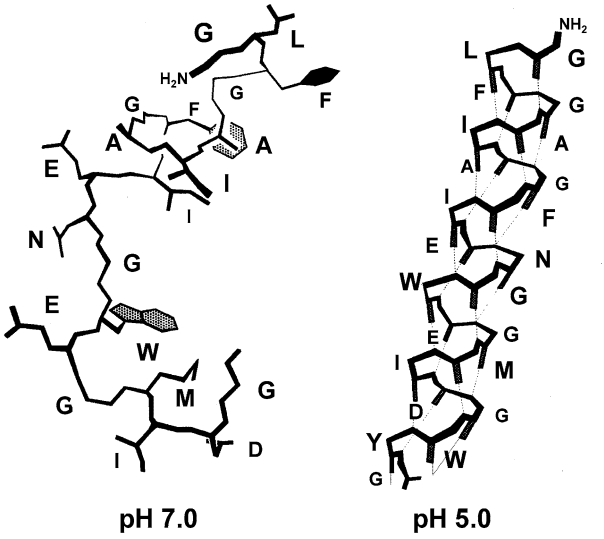

Fig. 4 *N*-Terminal sequence of the influenza virus hemagglutinin subunit HA-2 at neutral and acidic pH. Adapted from [79] with kind permission from the American Society for Biochemistry and Molecular Biology

Plank and Wagner into receptor-targeted polylysine/pDNA polyplexes, which could also strongly enhance gene transfer [77–81]. As part of this series, Plank developed in 1992 the first synthetic "artificial virus" based on polylysine conjugated with a synthetic INF peptide and a synthetic tetra-antennary galactose ligand for the hepatocyte asialoglycoprotein receptor [78]. As endo-lysosomal entrapment remains a substantial bottleneck also for the majority of novel nucleic acid nanoparticle types, analogous active peptide modifications are still relevant [82–84]. Also, artificial amphipathic endosomolytic peptides such as GALA and KALA, GALA-INF, EGLA, JTS1, or H5WGY have been introduced into polyplexes by the group of Szoka [85, 86] and other labs [79, 87–90]. Photochemical internalization (PCI) presents an alternative method developed by Berg and Hogset to enhance gene transfer by loading intracellular vesicles with a photosensitizer followed by photoinduced endosomal release of co-internalized polyplexes [91–93].

3 Second-Generation Polycations: Proton Sponges or Endosomolytic Polymers

The next step in development of polymeric gene delivery systems included polymers with inherent endosomal escape characteristics, to avoid the need of additional agents. Since the early 1990s, the lab of Frank Szoka developed

polyamidoamine (PAMAM) dendrimers [85, 94, 95], which were found particularly effective in pDNA transfer also in the absence of chloroquine or fusion peptides. These polyamine dendrimers (Fig. 5a) are "proton sponges", displaying at neutral pH only moderate protonation of nitrogens, which continuously increases with endosomal acidification. At demonstrated in subsequent mechanistic studies [96], this increased density of positive charge density leads to an influx of chloride and water into the endosome. As a consequence of the "proton sponge effect", the endosome bursts presumably due to the elevated osmotic pressure and the membrane destabilizing effect of positively charged polymer domains. The classical

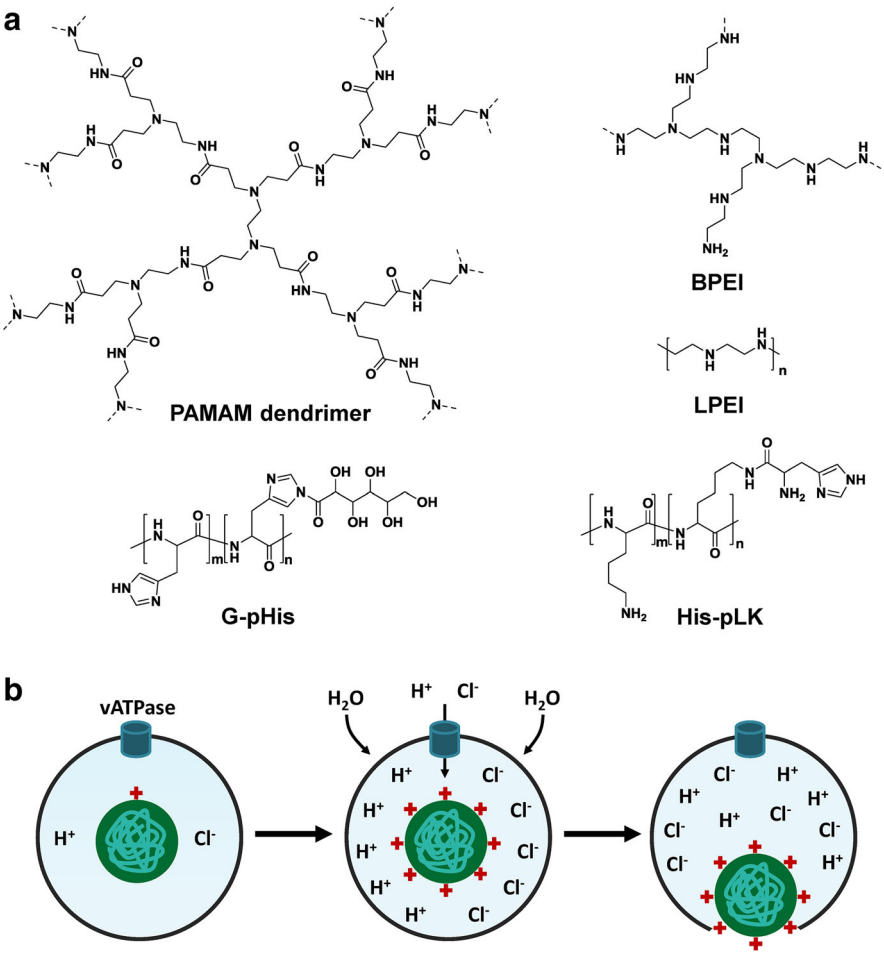

Fig. 5 Proton sponge polymers. **a** Polyamidoamine (PAMAM) dendrimers, branched polyethylenimine (BPEI), linear polyethylenimine (LPEI), gluconic acid-modified polyhistidine (G-pHis) and histidylated polylysine (His-pLK). **b** Schematic illustration of the proton-sponge hypothesis. The protonation of polymers with proton-sponge effect mediates the influx of protons, chloride counterions and water into endolysosomes, resulting in the increased osmotic pressure and endolysosomal swelling and then, facilitated by interaction of cationized polymer with phospholipid membrane, the rupture of endolysosomes

gene delivery polymers such as polylysine or polyarginine could not meet this criterion because of their complete protonation at neutral pH, enabling only nucleic acid binding, but not endolysosomal buffering.

Jean-Paul Behr and coworkers had previously demonstrated the positive transfection characteristics of the cationic lipo-spermine DOGS [97, 98]. They hypothesized a favorable endosomal buffering effect by the ionizable spermine head group, and, therefore, screened a series of commonly available cationic polymers having proton-sponge characteristics. Polyhistidine has buffer capacity, but no sufficient cationization at physiological pH for effective nucleic acid binding. The hypothesis-driven search resulted in the discovery of an abundantly available and powerful transfection polymer, polyethylenimine (PEI) (Fig. 5a) [99–102]. Owing to the high transfection efficiency both in vitro and in vivo and only moderate cytotoxicity, clinical trials in localized applications with modified PEI and derivatives were approved and performed [103]. A huge diversity of branched PEI (BPEI) and linear PEI (LPEI) with different molecular weights can be produced. As benefit from this chemical flexibility, one may tune the stability, transfection efficacy and cytotoxicity of PEI polyplexes [104–106]. Because of the necessity of nucleic acid unpackaging at the targeting organelles, it might be contra-productive to keep a high polyplex stability for the whole transfection process. Itaka and colleagues found that, compared with poly-L-lysine (PLL) formed DNA polyplex, both LPEI and BPEI based polyplexes were able to mediate endosomal escape, but LPEI polyplexes presented higher efficiency in gene transfection and expression compared with BPEI polyplexes. This was attributed to the higher stability of BPEI polyplexes with limited payload unpacking [105]. The less stable LPEI polyplexes also exhibited better nucleus delivery in non-dividing cells. The intranuclear transfer of BPEI polyplexes however correlated with the breakdown of nuclear membrane, which was more efficient for dividing cells that were in or before mitosis [107–109].

Although the detailed mechanism of proton sponge effect by PAMAM dendrimer, PEI and other similar transfection polymers remains under debate [13, 96, 106, 110–116], significant proof sustains the proton sponge hypothesis at least in slightly modified form (Fig. 5b). The effect of proton sponge strongly depended on the vATPase-mediated proton internalization into endo-lysosomes. Bafilomycin, a specific inhibitor for vATPase, hindered the transfection, while the transfection was enhanced by chloroquine that becomes protonated itself. Free PEI, unattached to polyplexes, but co-delivered with the polyplexes, promotes the endosomal escape [116]. Delayed acidification process, chloride gathering, and accidental disappearance of vesicles have been verified. Some new mechanistic models of the proton sponge are also reported [13, 114, 115]. Benjaminsen and Andresen found that the proton sponge of PEI did not notably change the pH of lysosomes, which is not contradictory, but a potent evidence for the PEI cationization. Some critical calculations presented that the osmotic pressure deriving from the proton sponge effect was not powerful enough to rupture endosomes. Furthermore, some cationic polymers with proton sponge effect do not mediate nucleic acid transfection [117]. Even introducing polyethylene glycol (PEG) molecules to the PEI polyplex surface could block the transfection activity,

which is recovered by removing acid-cleavably linked PEG in endo-lysosomes [118–120]. Besides the osmotic effect of proton sponge, these reports are in agreement with the hypotheses that higher cationization of the PEI molecules in protonation-dependent way results in the direct endosomal membrane interaction and permeabilization. Such lytic effects have been found during the studies of high-density polycations, for example in erythrocyte membrane destabilization in the absence of osmotic pressure. Presumably synergistic effects exist depending both on the increasing osmotic pressure and direct destabilizing membrane contact. More recently, a thermosensitive nanoparticle swelling was applied for cytosolic siRNA delivery. siRNA pluronic/PEI2 K nanocapsules of 100 nm size at 37 °C during transfection into cells undergo subsequent swelling to a size of 400 nm upon cooling to 15 °C, which mechanically induced endosomal disruption [121].

Based on the hypothesis of the proton sponge, some buffering units with a pK_a around 6 have been introduced into polymers to enhance their buffering ability, yielding polymers such as histidinylated polylysine or imidazole-containing biopolymers [122, 123] (Fig. 5a). The positive effects of these buffering units on gene delivery efficiency were reported by many studies [124–128].

Polyethylenimine, as the classical proton sponge polymer, presents many favorable properties on nucleic acid transfection, but also displays obvious disadvantages: it is not degradable and significantly toxic in a molecular weight-dependent manner [129, 130]. The cytotoxicity of PEI contains the defects on cell surface and mitochondria or nucleus membranes, inducing apoptosis and necrosis, or also the inhibition of ATP synthesis in mitochondria [131]. In addition to the direct cytotoxicity, many cationic polymers such as PEI and polylysine are recognized by the innate immune system and induce complement activation both in vitro and in vivo [132]. For instance, this led to strong anaphylactic reactions in pigs upon intravenous administration of PEI [133].

Amphiphilic cationic polymers (Fig. 6) present a second class of carriers with endosomal escape capacity. Allan Hoffman and collaborators synthesized pH-sensitive amphiphilic polymers containing hydrophobic alkyl groups and hydrophilic acidic carboxyl groups, which could enhance the disruption of lipid membranes because of the increased hydrophobicity when protonated in acidic pH. They further polymerized a functional vinyl monomer, pyridyl disulfide acrylate (PDSA) with butyl acrylate and methacrylic acid to generate the redox-sensitive and pH-dependent membrane disruption polymers to enhance cytosolic delivery of functional molecules such as antisense oligonucleotides. However, these amphiphilic polymers themselves suffered from inefficient DNA binding ability because of the lack of a cationic backbone. To promote the DNA binding ability of amphiphilic polymers, Rozema and colleagues synthesized amphiphilic cationic polyvinyl ethers. They introduced amino groups into these polymers to form stable DNA complexes, simultaneously incorporated alkyl groups containing one to four carbons to disrupt the membrane. The membrane-disruptive activity was found to be dependent on the carbon chain length. The DNA transfection efficiency of these polyvinyl ethers depended on their membrane-disruptive activity. The polyvinyl ethers containing butyl groups obtained highest nucleic acid transfection efficiency compared with the ones containing shorter alkyl groups [134–137].

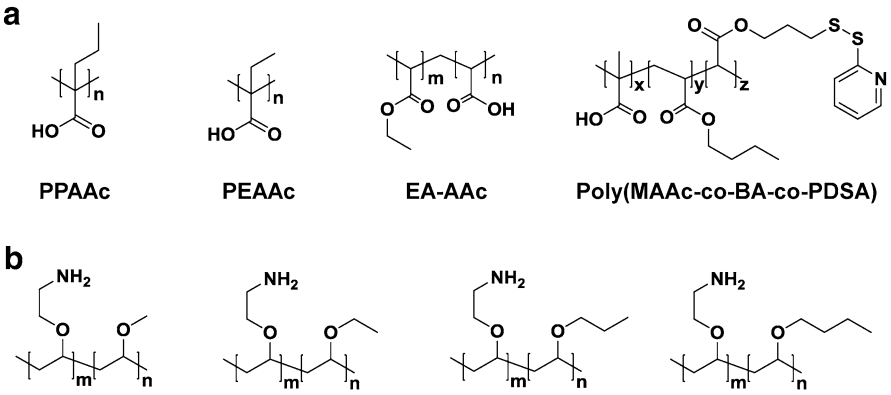

Fig. 6 Amphiphilic polymers. **a** PPAAc, poly(propyl acrylic acid); PEAAc, poly(ethyl acrylic acid); EA-AAc, random 1:1 copolymers of ethyl acrylate (EA) and acrylic acid (AAc); Poly(MAAc-*co*-BA-*co*-PDSA), poly(methacrylic acid-*co*-butyl acrylate-*co*-pyridyl disulfide acrylate). **b** Amphiphilic cationic polyvinyl ethers containing methyl, ethyl, propyl or butyl groups (from *left* to *right*)

To sum up, the first two generations of polymers provided efficient gene transfer reagents, deepening also our understanding of gene delivery. It, however, was critically required to reduce unspecific effects and the cytotoxicity of cationic polymers. Therefore, as will be outlined in the following sections, biocompatible polymers such as nature-derived polymers, biodegradable polymers, or nanoparticle-shielding polymers remained to be synthesized, ideally in pure form with maximum precision.

4 Third-Generation Polycations: Biodegradable and Biocompatible

The research of cationic polymers has been dominated by three aims: higher transfection efficiency, improved biocompatibility, and refined pharmaceutical precision. Learning from the lessons of first-generation polymers, multiple efforts were taken to improve the biocompatibility of polymer-based gene delivery systems.

Nature-derived polymers are advantageous templates for the generation of biodegradable and increasingly biocompatible polymers [138]. Chromosomal proteins such as HMG1 [139, 140] or histones [141] with or without modifications were already used for nucleic acid transfection. Cationic forms of collagen including gelatin (thermally denatured collagen, extracted under acidic conditions) and atelocollagen (derived from native collagen, with the terminal telo-peptides enzymatically removed) were also investigated as gene delivery vectors [142, 143]. Atelocollagen/siRNA polyplexes presented effective systemic siRNA delivery into bone metastatic tumor. The previously mentioned DEAE-dextrans are also nature-derived polymers based on carbohydrates.

Many researchers have reported that chitosan (poly-D-glucosamine, generated by deacetylation of chitin) (Fig. 7a) and derivatives showed great potential as nucleic

acid delivery carriers [144, 145]. Further chemical modification of chitosan such as PEGylation or methylation could effectively improve the colloidal stabilization and formation of DNA polyplexes [146, 147]. Other favorable modifications contains histidinylation for facilitating endosomal escape [148], targeting ligands, or thiolation for improvement in cellular internalization [145, 149].

Cyclodextrins (CD) including alpha-, beta-, or gamma forms are degradable carbohydrate-based polymers with favorable biocompatibility. They were used to modify other cationic polymers. Beta-CD is also well known for its ability to form a CD-adamantane host–guest interaction. For example, introduction of beta-CD into LPEI and BPEI (Fig. 7b) decreased the toxicity of polyplexes, but kept the transfection efficiency high [150]. Using the host–guest interaction between beta-CD and adamantane, adamantly-PEG was inserted into PEI polyplexes, and the resulting PEGylated PEI polyplexes were used for systemic nucleic acid delivery to the liver [151]. Ping and Li recently reported low molecular weight PEI-CD based

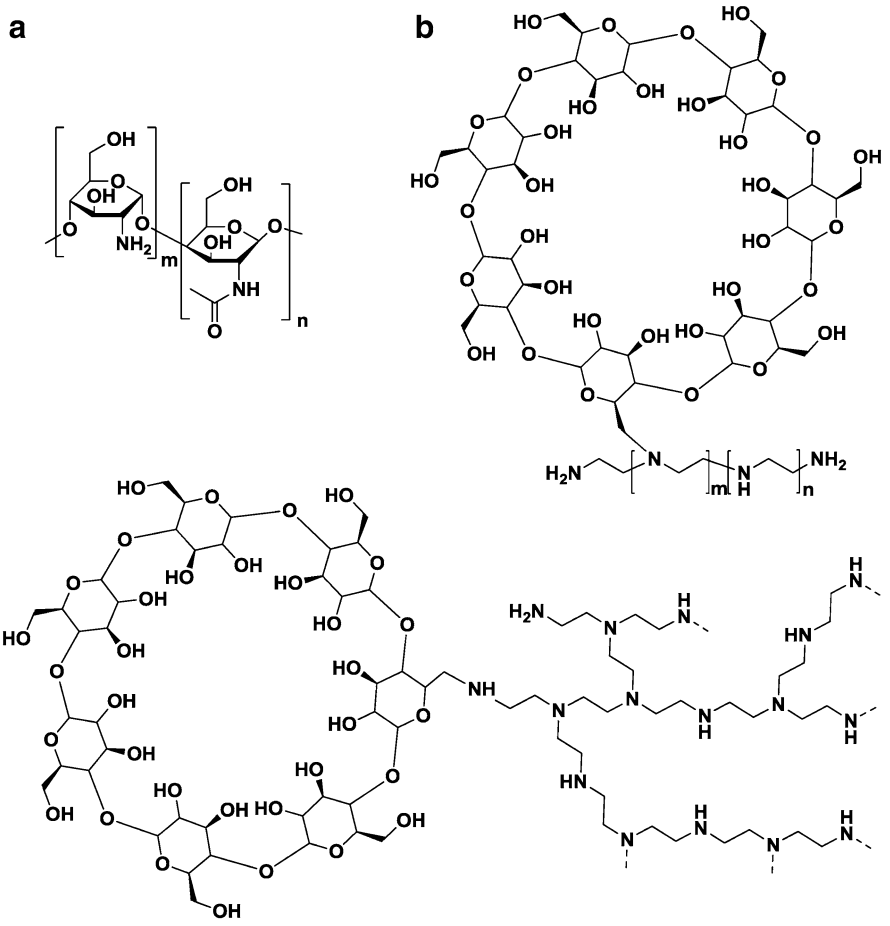

Fig. 7 Nature-derived cationic polymers. **a** Chitosan. **b** β-Cyclodextrin modified linear and branched PEI

pDNA polyplexes containing an adamantly-modified peptide ligand for fibroblast growth factor receptors (FGFRs) and adamantly disulfide-connected PEG molecules for shielding [152].

Hwang and Davis synthesized linear cationic β-cyclodextrin-based polymers (β-CDPs) via amidine group cross-linkages. The CD modified polycations presented better biocompatibility than the one lacking CD modifications [153]. Furthermore, the CD units on these polycations was applied to introduce the receptor-targeting serum protein transferrin through CD-adamantane host–guest interaction [154]. Davis and colleagues further improved this concept into PEG-shielded, transferrin receptor-targeted therapeutic siRNA polyplexes [155]. With these polyplexes (Fig. 8) they evidenced for the first time that intravenous administration of siRNA can mediate gene silencing in metastatic tumors of humans as published in 2010 [22]. Polycationic CDs could be also synthesized by direct incorporation of heptakis-pyridylamino moieties [156]. To tune the cationic modification degree of polymers, Ooya and Harashima synthesized biocleavable polycationic polyrotaxanes with monocationic CDs cascaded on degradable linear polymer chains, which unraveled enhanced gene transfection efficiency [157].

Synthetic biodegradable polymers are another direction to increase the biocompatibility of polyplexes. We already learned that polycations such as polylysine and PEI present highly molecular weight dependent cytotoxicity. For instance, PEI with a low molecular weight (MW) of 800 exhibits low cellular toxicity but also low transfection efficiency, whereas linear PEI 22 K or branched PEI 25 K are more powerful, but have remarkable cytotoxicity, and PEI 800 K has high cytotoxicity and moderate transfection activity. To combine the advantages of low molecular weight polymers (low cytotoxicity) and high molecular weight polymers (high transfection efficiency), biodegradable polymers were designed and generated by assembling low molecular weight polymers into high molecular weight conjugates by bioreversible linkages.

One direction has been to synthesize various biodegradable cationic conjugates with low molecular weight oligoethylenimine (OEI) via bioreversible linkages [158], for example, ketals [159, 160], imines [161], disulfides [162, 163], ester bonds [164, 165], polyglutamic acid amide, and other amides [166–169] (Fig. 9). Introduction of bioreversible linkages such as hydrolyzable ester bonds in intra- or extracellular locations, endosomally degradable imine or acetal bonds, or bioreducible disulfide bonds in cytosol into cationic polymer backbone obviously increased biocompatibility and maintained or even promoted nucleic acid transfection efficiency.

Another direction is to synthesize new polymers with cleavable backbones. Sung Wan Kim and collaborators performed pioneering work published in 2000 by replacing the amide bonds in polylysine with ester bonds; consequently, nontoxic and biodegradable analog poly(alpha-(4-aminobutyl)-l-glycolic acid) (Fig. 10a) was generated [170]. Although the transfection efficiency was enhanced up to twofold compared with polylysine, it remained moderate, probably due to the lack of effective endosomal escape ability. More effective hyperbranched network-type poly(amino esters) named as n-PAE (Fig. 10b) and poly(beta-amino esters) were synthesized through Michael addition of amines to acrylate esters [171, 172]. n-PAE

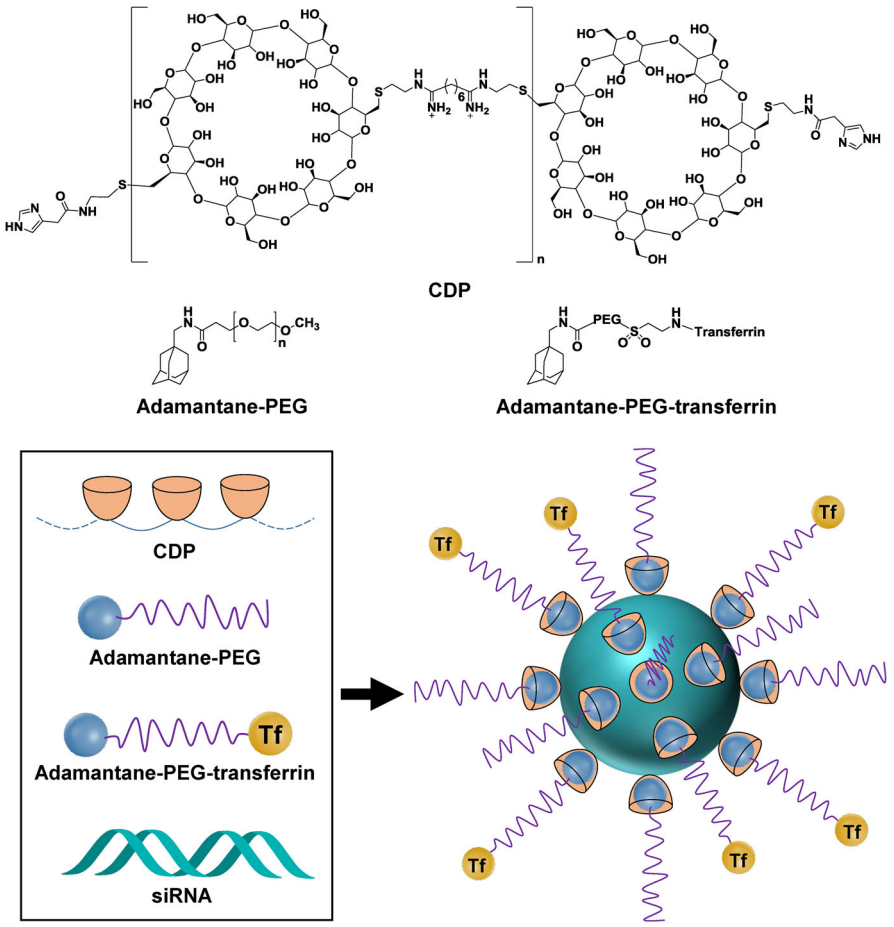

Fig. 8 Schematic illustration of a PEG-shielded, transferrin receptor-targeted therapeutic siRNA delivery system based on cyclodextrin-based polymers and cyclodextrin/adamantane host–guest interactions

presented comparable transfection efficiency and endosomal escape ability to PEI, but a far higher biocompatibility [171]. Linear beta-amino ester cationic polymers were also optimized by reacting 4-aminobutanol with 1,4-butanediol diacrylate or 1-6-hexanediol diacrylate and exhibited effective gene transfection [172]. To find better gene delivery polymers, the laboratory of Robert Langer set a next conceptual milestone around 2001. Based on a combinatorial Michael addition approach, they added a variety of primary amines or secondary diamines to diverse bisacrylates (Fig. 10c) and obtained a library including 2350 biodegradable cationic polymers. With a large-scale screen on transfection efficiency, some interesting candidates were identified [173–175]. This approach was further applied to other library chemistries. For example, introduction of aliphatic hydrocarbon-associated epoxides, combined with a high throughput screen, resulted in a series of hydrophobic

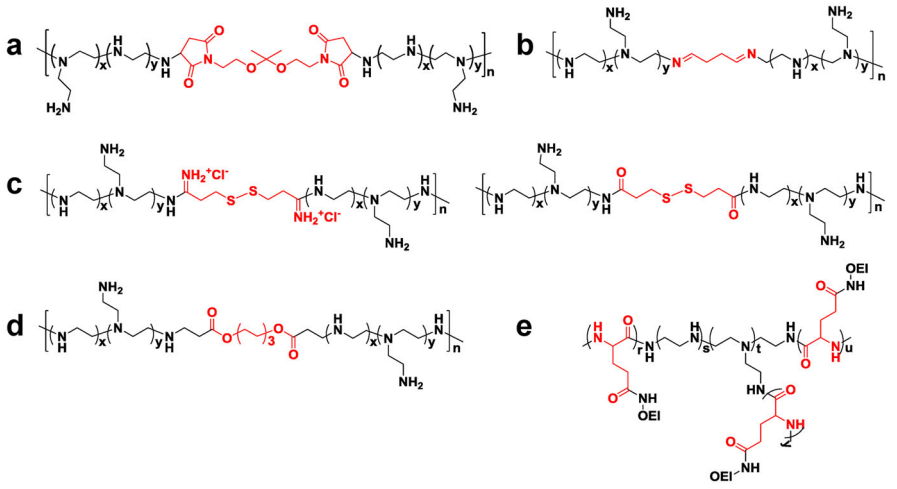

Fig. 9 Biodegradable polymers based on oligoethylenimine. **a** Ketals [159, 160]. **b** Imines [161]. **c** Disulfide bonds [162, 163]. **d** Ester bonds [164, 165]. **e** Polyglutamic acid amides [166]. Bioreversible cross-linkages are labeled in *red*

oligocationic polymers termed lipidoids, which were used for siRNA transfection [176, 177]. Also in polylactides, the ester bonds present a biodegradable character. Cheng and colleagues synthesized cationic polymeric nanocapsules from tertiary amine- and allyl-functionalized polylactide and a bis-(thiol) crosslinker, applying thiol-ene chemistry. They used these carriers for pDNA and siRNA delivery [178–180]. The group of Kataoka introduced small protonatable oligoamines onto the degradable polymeric amide backbone of poly(aspartate) via the amidation of the side chain of polyaspartic acid (Fig. 10d) [114, 181–183].

Michael addition, similarly to studies by Langer and colleagues, was also used by the groups of Engbersen, Kim and Feijen [184, 185] for the synthesis of disulfide bond-based biodegradable polymers (Fig. 11a). As amine reactants, small oligoethylenimines such as triethylene tetramine were reacted with cystamine bisacrylamide, generating effective and biodegradable PEI analogs. With a different synthetic route, Lu and collaborators first generated defined bioreducible oligomers (Fig. 11b) including triethylene tetramine, histidines, and disulfide-forming cysteines by solid-phase-assisted synthesis followed by oxidative disulfide formation [186].

In parallel to the development of novel nature-derived or biodegradable polymers, the increasing knowledge of the relevant transfection mechanisms resulted in the modification of existing effective polymers. For example, PEI has limited transfection efficiency for siRNA. Simple modifications of 10% nitrogens of a 25 kDa branched PEI by succinylation [187], or 20% nitrogens with tyrosine residues [188], or pyridylthiourea grafting [189] made this PEI molecule a potent siRNA delivery polymer. Introduction of uncharged hydrophilic moieties such as PEG molecules [118, 190–192], carbohydrates [150, 193], poly[*N*-(2-hydroxypropyl) methacrylamide] (pHPMA) [194], hyaluronic acid [195, 196], hydroxyl

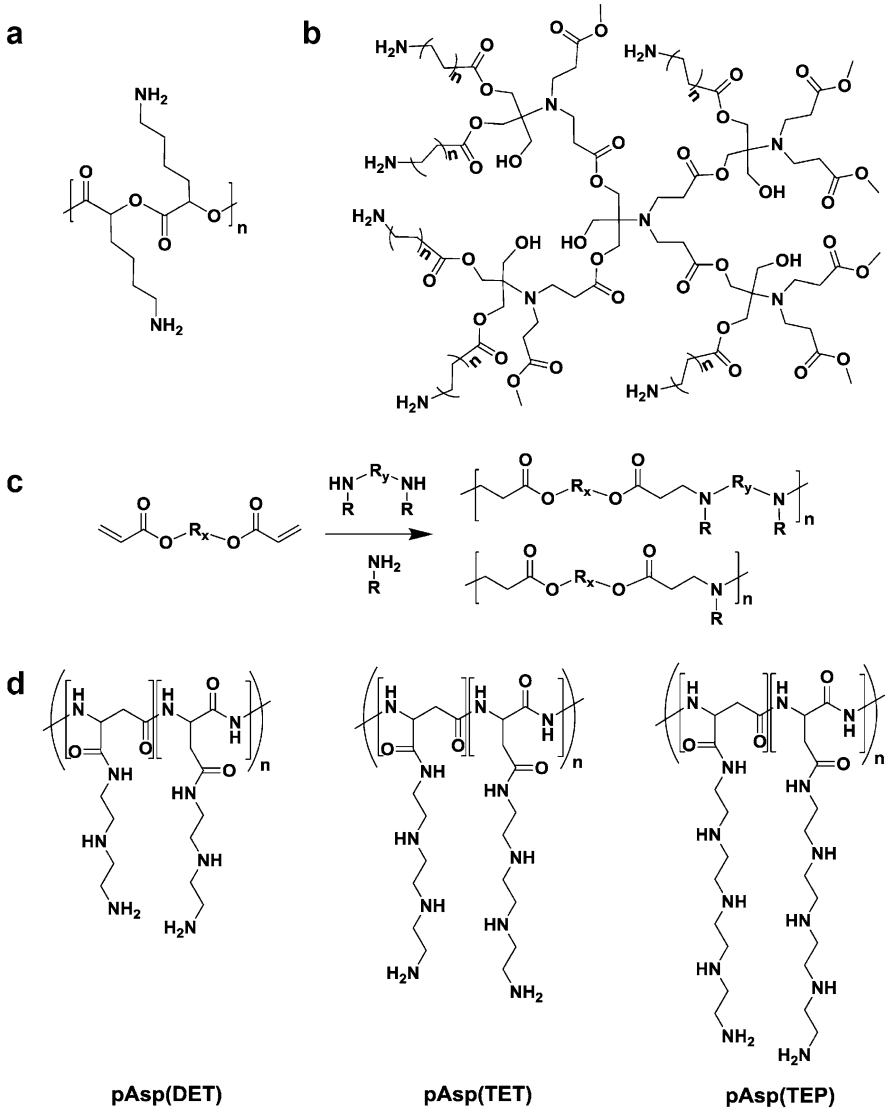

Fig. 10 Biodegradable cationic poly(amino esters). **a** Poly(α-(4-aminobutyl)-L-glycolic acid). **b** Hyperbranched network-type poly(amino ester) (n-PAE). **c** Combinatorial design and generation of β-amino polyesters by Michael addition. **d** Poly(aspartic acid) amidated with different oligoamines containing diethylenetriamine (DET), triethylenetetramine (TET), or tetraethylenepentamine (TEP)

ethyl starch [197] or polysarcosine [198] strongly decreased the toxicity and increased the biocompatibility of the synthesized polycations. PEG-pLys based DNA polyplexes were the first ones used in epithelia of cystic fibrosis patients in human clinical trials [17, 199].

As thought before, PEG reduced non-specific cellular interactions in a stealth effect by hindering protein adsorption. Recently, Schöttler and Wurm found that

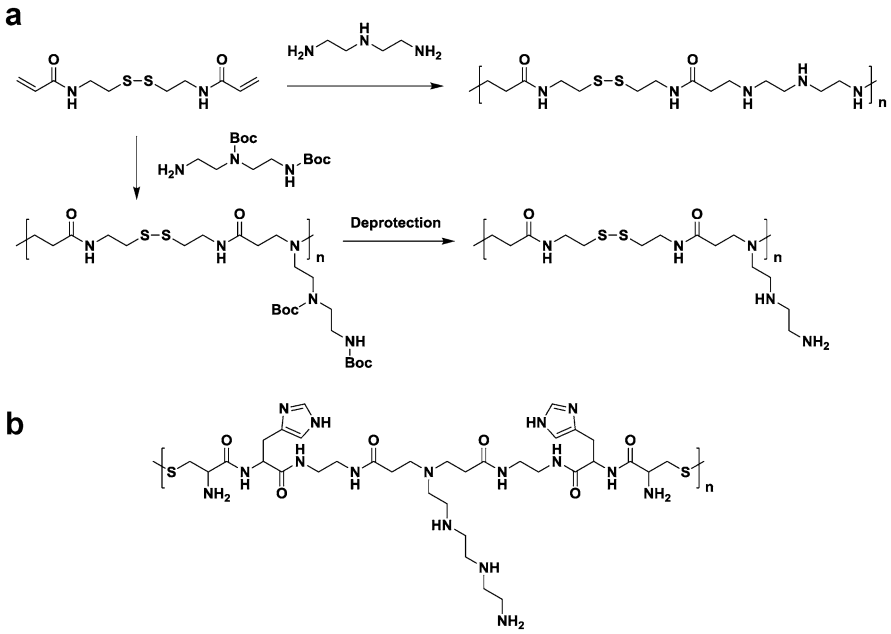

Fig. 11 Bioreductive disulfide-based cationic polymers. **a** Cationic polymers synthesized by Michael addition to cystamine bis-acrylamide. **b** Defined cationic polymers generated by solid phase-assisted synthesis and subsequently cross-linked by thiol oxidation

besides reducing protein adsorption, PEG and poly(ethyl ethylene phosphate) (PEEP) could also regulate the composition of protein corona by enriching clusterin proteins around nanocarriers, which are distinct proteins required to reduce non-specific cellular internalization, and PEEP presented higher clusterin enrichment than PEG [200]. This finding may contribute to the future design of shielding polymers, for example, the screen of more potent shielding polymers by identify their functions on clusterin enrichment.

In general, several directions have been explored to identify more biocompatible polymers, for example, development of nature-derived polycations, generating and optimizing new degradable polymers, and modifications of well-established polymers with shielding units.

5 Pharmaceutical Precise Polymers

One always must keep in mind that the final purpose of our design and optimization of polymers is for clinical application. Therefore, pharmaceutical precision of polymers is equally significant to transfection efficiency and biocompatibility. The pharmaceutical precision requests the generated polymers to be defined and reproducible in size, topology (dendritic, branched, hyperbranched, linear, comb), number, and coupling sequence and sites of subunits.

All these elements obviously play a vital role in polymer transfection efficiency. Therefore, in addition to pharmaceutical precision, synthesis of chemically precise polymers also has great significance for the investigation of structure–activity relations. Several of the previously mentioned polymer generations suffer from the lack of chemical precision: polymer conjugates frequently stand for heterogenous mixtures in macromolecule sizes, topologies, and chemical conjugation sites.

Dendrimers are one class of well-defined polymers with globular morphology. They are synthesized by precise step-wise coupling of branching sites to a molecular core, resulting in a duplication of surface chains after each coupling. Commonly used dendrimers include polyamidoamine (PAMAM), dendritic polylysine, polypropylenimine (PPI) (Fig. 12a), and their derivatives [85, 201–204]. The transfection efficiency and cellular toxicity of dendrimers are basically generation-dependent. Several efforts were made to increase the biocompatibility. For example, incorporation of transferrin, a protein ligand, into dendrimers, improved the biocompatibility and sustained the transfer efficiency of in vivo nucleic acid delivery [205]. Haag and collaborators applied well-tolerated dendritic oligoglycerol cores modified with oligoamine shells for effective siRNA delivery [206]. Russ and colleagues used a low molecular weight branched PEI or a PPI dendrimer core modified with various oligoamines such as oligoethylenimine via biodegradable hexanediol diacrylate ester linkages. This pseudo-dendrimer presented a more defined structure than randomly cross-linked polymer preparations, higher biocompatibility, and efficient in vivo DNA transfection and expression [207–211].

Nature provides us great hints how to pursue precisely sequence-defined macromolecular architecture. Natural viruses with diverse functional microdomains have been created based on defined protein sequences translated from the genetic sequence information stored in the viral nucleic acids. The functional domains in

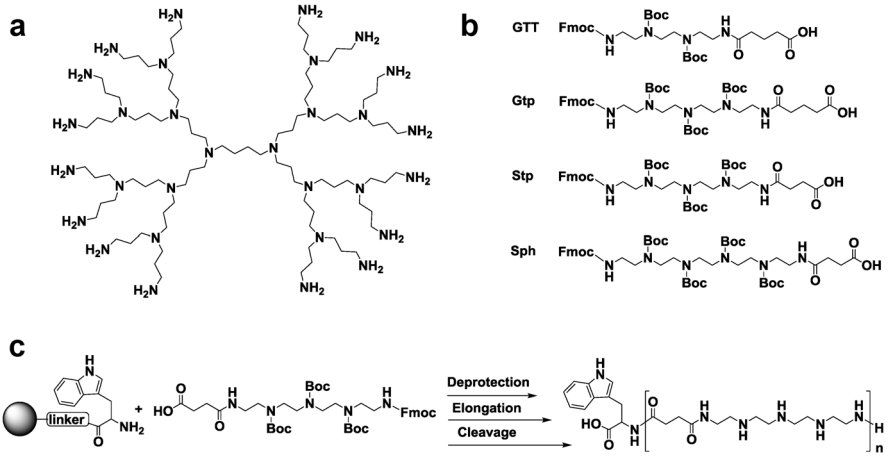

Fig. 12 Defined polymers. **a** Polypropylenimine (PPI) dendrimer. **b** Artificial oligoamino acid building blocks (glutaryl-triethylene-tetramine, glutaryl-tetraethylene-pentamine, succinyl-tetraethylene-pentamine, succinyl-pentaethylene-hexamine, presented in fmoc/tBoc-protected form). **c** Schematic illustration of the linear assembly of Stp building blocks

viruses were optimized by natural selection and evolution. With the great progress of macromolecular chemistry based on solid-phase-assisted synthesis, generation of oligonucleotide and peptide sequences is routine. To apply this analogous sequence-based generation and evolution process to artificial polymer optimization, some requirements need to be met. For example, the identification of novel functional building blocks for specific delivery processes, which could be artificial moieties or nature-derived amino acids or lipids. These building blocks must be assembled in defined macromolecular sequences, and the sequences evaluated by relevant bioassays for gene delivery. The obtained structure–activity relationships can be used for further improvements of novel polymers with several rounds of evolutional selection. Many investigators have already carried out several elements of such a "chemical evolution" process.

Such sequence-defined structure–activity relationships were first investigated for peptide-based transfection reagents, such as the requirements for sequence and numbers of gene binding amino acids including arginine, lysine, or ornithine in defined branched or linear peptide structures, the effect on polyplex stability of cysteines, improvements of nucleic acid delivery with endosomal-buffering histidines [126, 186, 212–214]. Laura Hartmann and Hans Börner applied solid-phase-assisted peptide generation by assembling completely artificial building blocks such as protected di-acids and diamines into poly(aminoamides), which can be used for DNA polyplex formation [215, 216]. Based on this concept, Schaffert and colleagues designed novel artificial amino acid building blocks (Fig. 12b). These oligoamino acids protected by tBoc and Fmoc contain several repeats of the effective aminoethylene motif mediating the proton sponge effect in PEI [217, 218]. These novel oligoamino acid building blocks can be assembled into a large variety of sequences and topologies using classical peptide synthesis methodology [219, 220].

The simple questions on structure–activity relations can be explained by the precise chemical design. For instance, linear assembly of Stp building blocks (succinyl tetraethylene pentamine including three protonatable nitrogens per unit) were implemented to evaluate the effect of increasing molecular weight on linear oligo(ethanamino) amides (Fig. 12c) [221]. Exceeding a critical length of more than 10 Stp repeats, containing 31 protonatable nitrogens, the linear oligo (ethanamino) amides can efficiently form polyplexes with DNA and mediate nucleic acid transfection. With an optimized length of 30 Stp repeats, containing 91 protonatable nitrogens, the transfection efficiency was enhanced up to sixfold compared to a standard linear PEI of 22 kDa, which includes about 500 (±200) protonatable nitrogens. Importantly, this oligomer containing 30 Stp repeats presented better biocompatibility, with a 10-fold lower cytotoxicity than linear PEI.

6 Polymer-Containing Nanocomposites

For nucleic acid delivery, in addition to pure polymer-based polyplexes also lipid-based lipoplexes [222], combined lipopolyplexes, and various nanocomposites have been designed as reviewed in the following.

For example, polymer-based transfection has been enhanced by physical targeting using a magnetic field. In this process, termed "magnetofection" [223, 224], cationic magnetic nanoparticles are associated with the pDNA or other nucleic acid cargo, which then is further compacted by cationic polymers or other delivery carriers. An appropriately placed magnetic field results in faster and more intensive accumulation of nucleic acids in the target cells. Magnetofection has shown enhanced gene transfer activity in numerous applications, including a clinical phase I study in cats with fibrosarcoma [225]. Immunostimulatory gene therapy with two intratumoral GM-CSF pDNA magnetofection treatments resulted in enhanced fraction of recurrence-free patients and did not show any treatment related toxicity.

Kostarelos and colleagues have introduced single-walled or multi-walled carbon nanotubes (CNT) as nanocarriers for nucleic acid delivery, as reviewed in [226]. Cationic groups have either been directly incorporated by chemical modifications, by polymer conjugation, or indirectly by physical attachment. CNTs may provide the nanocomposite a defined structure and shape. Also, polymer-modifed graphene oxide (GO) has been extensively investigated as carrier for pDNA and siRNA [227–230]. Zhang and colleagues demonstrated that PEGylated reduced GO (rGO) presented higher ssRNA loading and delivery capacity than the PEG-GO analog [231]. PEI-PEG-rGO and PEI-PEG-GO were developed as pDNA transfection agents or a nanohybrid theranostic for simultaneous doxorubicin (via MMP2-cleavable linker) delivery and gene transfer, respectively [230, 232]. Using the NIR optimal absorbance, photothermally enhanced pDNA or siRNA delivery was obtained [227, 232]. First systems toward therapeutic in vivo applications in myocardiac tissue [229] or neurons [233] have already been established.

Kim et al. [234] combined polymer complexes with gold nanoparticles (AuNPs). Unimolecular siRNA polyion complexes (uPICs) were generated by charge-matched polyionic complexation. A single siRNA ($\sim$40 negative charges was complexed with a single copolymer molecule of poly(ethylene glycol)-*b*-poly(L-lysine) with the $DP_{PLL} = \sim 40$ ($\sim$positive charges) and a thiol group at the ω-end of PEG (PEG-PLL-SH). Subsequently, the formed uPICs were coupled to 20 nm AuNP via thiol-gold coordinated bonding, resulting in a siRNA uPIC-AuNP nanoarchitecture with a homogeneous size of 38 nm. Upon intravenous administration these siRNA complexes displayed enhancing blood circulation time, efficiently accumulated in a subcutaneous HeLa-Luc cervical cancer and achieved significant luciferase gene silencing in the tumor mouse model. Analogous subsequent work introduced cRGD for active targeting of tumor and tumor endothelial integrins, resulting in therapeutic antitumoral effects [235].

Bein and colleagues generated core–shell mesoporous silica nanoparticles (MSNs) with increased pore size for high-capacity loading with siRNA. To inhibit premature release of siRNA from loaded MSNs, the pores were capped with a multifunctional lipo-oligomer which also enhanced endosomal escape of internalized MSNs. This process resulted in highly efficient marker gene silencing [236].

The Kataoka lab developed an inorganic/organic hybrid micelle system for siRNA delivery. They combined siRNA with a calcium phosphate (CaP) core and poly(ethylene glycol)-*block*-charge-conversional polymer (PEG-CCP) shell. With

this system, effective silencing in spontaneous pancreatic tumors in transgenic mice after intravenous injection was demonstrated [237].

7 Virus-Inspired Multifunctional Dynamic Polymers

As mentioned above, for effective gene transfection into cells, numerous extracellular and intracellular barriers must be overcome, such as nucleic acid packaging, serum stability, targeting to specific cells, cellular internalization, endosomal escape, efficient unpackaging, and subsequent transport into specific organelles (Fig. 1). Significant advances were made by the design and synthesis of block copolymers with two or more functional delivery domains. For example, PEG-PEI copolymers can condense pDNA by electrostatic interaction, partly shield the polyplexes with PEG molecules, promote cellular association and internalization via residual cationic charges, and to some degree enhance endosomal escape through the additional protonation of PEI. However, this type of polymer cannot meet all requirements for effective gene delivery, because PEG shielding reduces cell association and endosomal escape via positive surface charges. In fact, some delivery goals work against each other, for instance, optimization of polyplex stability is opposed to intracellular nucleic acid release, and optimized shielding is opposed to the optimal endosomal escape [118].

Inspired by natural viruses with distinct structural and functional domains to promote the step-wise transfection, first "synthetic virus" prototypes were designed [77, 78, 114, 118, 238–242]. This research direction presents a significant conceptual progress over the former design of standard polyplexes. For example, introduction of various tailor-made functional transfection domains into the nanoparticle at different sites (shell or core), with temporally and spatially programmed activation of the functional subdomains at the targeting cellular compartments is conceived. The second point, spatio-temporal control, demands the introduction of chemical sensing moieties, which could mediate cleavage of chemical bonds and/or changes of conformations and physical characteristics responding to the distinct biological microenvironment. Based on this concept, dynamic and programmed nanoparticles should be generated [242].

A large number of targeting ligands has been applied in polymer-based gene delivery. They were incorporated into the polyplexes by several methods, for example, as separate polymers co-integrated into prepared polyplexes [243], by coupling to a preformed polyplex [190], or by direction incorporation into a core-forming polymer [244]. A library of possible targeting ligands may include large proteins such as antibodies and transferrin, or small peptides, carbohydrates, vitamins, and chemical ligands. Targeted polyplexes have been successfully applied in vivo to target various organs and tissues, such as brain, lung, liver, and tumors [5, 41]. Transferrin, as one of the most frequently used targeting ligand, has been already applied in clinical investigations. In the 1990s, within the very first polymer-based ex vivo human gene therapy, transferrin was used as targeting ligand in an interleukin-2 gene-modified cancer vaccine, due to its effective targeting to transferrin receptor on primary melanoma cells [14, 15]. Recently, transferrin was

applied to target cationized cyclodextrin/PEGylated siRNA polyplexes to tumors in an in vivo human clinical trial [20, 22].

Usually, targeting ligands, especially smaller ligands, were introduced by direct conjugation with shielding domains such as PEG. PEGylation can also counteract undesired biological reaction of cationic polymers. For example, the interaction of polylysine and other polycations with the innate immune system, and the activation of the complement system can be greatly reduced [132]. Moreover, cationic nanoparticles without shielding domains could ionically interact and aggregate with blood cells and serum, resulting in opsonization by a serum corona and the loss of targeting ability or triggering in vivo toxicity.

Polyplexes with shielding domains against unspecific interactions may suffer from inefficient transfection into target cells. In this regard, PEG domains with acid-induced detachment abilities were introduced to PEI polyplexes and can significantly enhance the in vitro and in vivo nucleic acid transfection efficiency, when compared with analogous polyplexes with stable shielding [118]. One can also hypothesize that a stable PEG shielding effect will reduce the interactions between cationic polymers and the endo-lysosomal compartment membrane, whereas this problem is solved with removable PEG molecules (Fig. 13a, b). Commonly used acid-sensitive bonds [119] between PEG or other shielding domains and cationic polymers include acetals [160, 245, 246], pyridyl-hydrazones [118, 120], and dialkylmaleic acid monoamides [135, 247].

A virus-like polyplex should have chemically dynamic characteristics [248]. Xu and Shen designed a ter-polymer (PCL/PDEA/PEG) to package pDNA into the core of virion-mimicking nanocapsules [249]. They used the pH-responsive building block poly[2-(*N*,*N*-diethyl)aminoethyl methacrylate] (PDEA), which is protonated and cationic at pH below 6. Poly(ε-caprolactone) (PCL) was used as a hydrophobic, capsule–forming module and PEG as a capsule-shielding module. At pH 5, the ter-polymer cationic micelles undergo, with pDNA, a pH-sensitive hierarchical self-assembly into a pDNA containing nanocapsule, which retains pDNA internally also at physiological pH 7. Upon endocytosis, at endosomal pH the PDEA block binds and protects pDNA against degradation, whereas the PCL ester block probably hydrolyzes in endo-lysosomes. After endosomal escape, at neutral cytosolic pH the PDEA unit becomes uncharged again, and pDNA is released from this block. Upon intravenous administration in SKOV3 tumor-bearing mice, pEGFP nanocapsules mediate efficient eGFP gene transfer in the distant subcutaneous tumors [249].

In the 2007 work of Wolff and Rozema, the acidic microenvironment in the endo-lysosomes not only helps to remove the hydrophilic shielding molecules from polyplexes, but also makes the hydrophobic lytic polymer units exposed to the endosomal vehicle membrane, and then facilitates the endosomal escape of polyplexes into the hepatocyte cytosol [135, 250]. Similarly, Meyer and collaborators [251, 252] reported around 2008 that with PEGylation and introduction of endosomally activated endosomolytic melittin peptides they could convert inactive polylysine-based polyplexes into highly efficient siRNA transfection reagents (Fig. 13c). Pun and colleagues recently generated a novel block copolymer called VIPER (virus-inspired polymer for endosomal release) [253] (Fig. 13d). VIPER contains a cationic hydrophilic block (DMA) to condense nucleic acids and a pH-

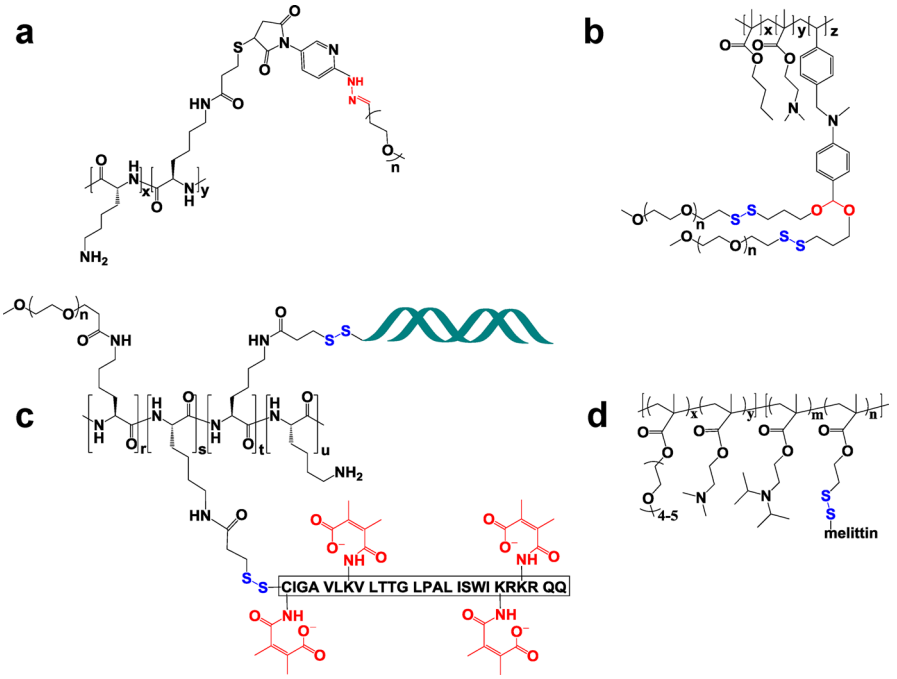

Fig. 13 Dynamic polymers. **a** PEGylated polymers with endosomal pH-cleavable pyridylhydrazone bonds. **b** PEGylated polymers with endosomal pH and cytosol redox cleavable bonds. **c** PLL conjugates with disulfide attached siRNA and pH-reversible masked lytic peptide melittin. **d** Block copolymer VIPER (virus-inspired polymer for endosomal release) with a polycationic dimethylaminoethyl (DMA) block and a pH-sensitive diisopropylaminoethyl (DPA) block. Acid-labile bonds are labeled in *red* and bioreducible bonds in *blue*

sensitive block (DPA), which is hydrophobic at physiological pH, but turns out to be hydrophilic in the acidic endosomal environment. This known sharp phase transition of the DPA block around pH 6 [254] can destabilize the VIPER self-assembled micellar structures, resulting in the display of melittin peptide buried in the hydrophobic core and enhanced endosomal release through disrupting endolysosomal membrane, further mediating efficient in vivo DNA gene transfer to solid tumors [253]. The DPA building block was also recently applied by Haijun Yu and Yaping Li as component of pH-sensitive micelleplexes, used for anti-PD L1 siRNA-enhanced photodynamic cancer immunotherapy [255].

Moreover, also the Warburg effect-induced acidic microenvironment [256] around the tumor (pH 6.2–6.9) [257] has been applied for an extracellularly acid-sensitive PEG deshielding. Sun and Wang [258] synthesized an acid-sensitive bridged copolymer for in vivo siRNA delivery, where the PEG molecules will be extracellularly detached from the copolymers response to the acidic pH of the tumor microenvironment, resulting in the exposure of cationic nona-arginine (R9) and enhanced cellular uptake, finally mediating efficient gene silencing and inhibition of tumor growth with better biocompatibility.

In addition to the acidic pH in the endo-lysosomes, the bioreductive microenvironment of the cytosol can also be used to activate the transfection functions [72, 259–261]. For example, disulfide bonds within the polyplexes have been shown to increase extracellular nanoparticle stability. Reductive cleavage in the cytosol by glutathione can trigger beneficial intracellular nucleic acid unpackaging [262, 263]. Sterically accessible disulfide bonds were found also to be extracellularly reduced [264, 265] in a process that is mediated by protein disulfide isomerases (PDI) on the cell surface. Therefore, it is possible to detach the disulfide-coupled PEG molecules via the bioreducing cleavage on the cell membrane [152, 266–268].

Although our understanding of intracellular biological processes significantly increased, one must keep in mind that great hurdles persist, for example, the inefficient delivery of DNA into the cellular nucleus, and longer persistence of gene expression. Appropriate nuclear localization functional domains were evaluated for their effect on gene transfection, however still need to be verified [269–271]. In addition, the nanoparticle size in relation to the nuclear pore needs to be considered. Recent work demonstrated that only sub-50 nm PEI polyplexes locate to the inner side of the nuclear pore without damaging the nuclear envelope [272].

Incorporation of various functional units, such as targeting ligands, shielding domains, endosomolytic units, and nuclear localization signals as mentioned above may help to conquer the diverse biological barriers, but also makes polymers become very sophisticated virus-like gene carriers [239]. To fulfill all these required functions, macromolecular chemistry suffers from a new challenge: orthogonal conjugation chemistries with defined conjugation sites and precise cationic polymers with low or no polydispersity must be available. Actually, polymer chemists frequently conjugate functional domains into polymers in a rather random way, making defined and reproducible production of polymer conjugates with more than three functional domains practically impossible. However, as reviewed above, with solid phase-assisted synthesis technology, based on the previous knowledge of conventional polymers, it is feasible to synthesize more sophisticated multifunctional and precise sequence-defined nucleic acid carriers. For example, targeting ligands such as peptide sequences [128, 273], methotrexate [274], or folic acid [83] were attached to precise PEG shielding domains and sequence-defined oligoamine domains with different topologies such as two-arm or four-arm architectures (Fig. 14). In addition to the direct conjugation of targeting and shielding functional units into oligomers during the same solid phase-assisted generation process, it is also feasible to couple them to cationic oligomer core via site-specific native chemical peptide ligation reaction [275].

Various further modifications on oligomers can improve the nucleic acid transfection efficiency. For example, introduction of terminal cysteines can enhance the bioreversible stability of polyplexes [83, 218, 276], incorporation of tyrosine residues improved the siRNA transfection efficiency of novel oligomers [277], analogously as has been reported for the conversion of PEI into efficient siRNA transfection polymers [188]. Also, histidines, enhancing endosomal escape, and transfection efficiency of diverse polymers [125] were incorporated into novel cationic oligomers with or without shielding domains and targeting ligands and could promote their endosomal escape [128]. Meanwhile, with incorporation of

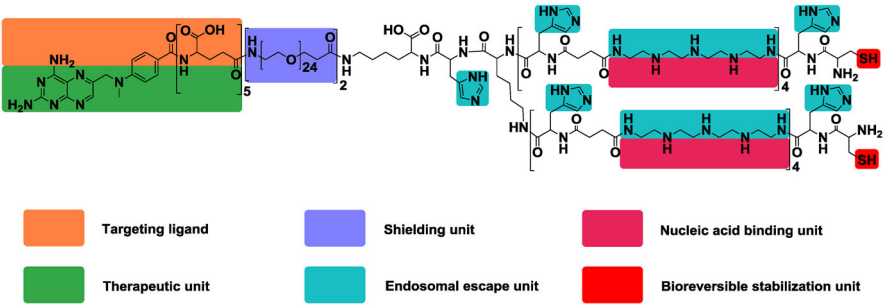

Fig. 14 Multifunctional sequence-defined oligomer. Oligomer contains a dual-functional methotrexate ligand for targeting and therapy, PEG for shielding, artificial oligoamino acid Stp for nucleic acid binding and endosomal escape, terminal cysteines for bioreversible polyplex stabilization, and histidines for endosomal escape. The functions of the individual units are indicated in color: *two colors* present a dual function

specific numbers of the aminoethylene unit, Lächelt and colleagues [128] could finely tune the proton sponge effect of the oligoamine microdomains. The microdomain with even protonatable nitrogens showed preferential buffering at pH 5–6 in endosomes, whereas with odd protonatable nitrogens, the buffering ability was around pH 7. These findings were well consistent with prior reports by Kataoka and colleagues. They designed degradable cationic polymers via the amidation of the side chain in polyaspartic acid with various small defined oligoamines [114, 182].

Synthesis of precise sequence-defined oligomers provides a great progress for the chemical evolution of polymeric gene delivery systems. However, screening and identification of promising carrier candidates and further optimization of polyplexes present a new challenge. One selection strategy first applied in 2001 by Langer and collaborators [173] was the high-throughput semi-automated screening method. With this strategy, they could screen libraries of a variety of novel polymers on selected cell lines and identify the most interesting candidates [175, 176]. It will be important to identify the most relevant criteria for in vivo effectiveness and develop corresponding screening assays. Very significant in this respect, Whitehead and colleagues recently reported the identification of four criteria, which are necessary to predict the in vivo activities of siRNA lipidoid nanoparticles [278].

8 Conclusions and Perspectives

From the first-generation cationic polymers to the multifunctional sequence-defined gene delivery system, the evolution of polymers experiences a period of 50 years. Within this time span, several breakthroughs in conception and chemistry were obtained (Table 1) deriving from our better understanding on the biological process of gene delivery and the great progress in macromolecular synthetic chemistry. The efficacy of polymeric gene delivery vectors, however, is still moderate, and clinical applications of these vectors remain limited. Therefore, further chemical

Table 1 Breakthroughs in conception and chemistry of polymer-based gene delivery

Year	Polymer	Significance	Investigator	References
1962	Histone and protamine	Cationic proteins could enhance the infectivity of poliovirus RNA	Smull and Ludwig	[26]
1965	DEAE dextran	First reported polycation mediating enhanced poliovirus RNA transfection	Vaheri and Pagano	[27]
1975	Poly(ethylene oxide) and polylysine	Condense nucleic acids into compact structures	Lämmli	[32]
1987	PLL-asialoorosomucoid (ASOR) conjugate	First targeted gene delivery polymer	Wu and Wu	[35, 36]
1990	Transferrin-protamine and transferrin-polylysine	First transferrin receptor-specific nucleic acid transfer	Birnstiel, Wagner et al.	[42–44]
1992	Polylysine conjugated with a synthetic INF peptide and a synthetic tetra-antennary galactose ligand	First polymer-based synthetic "artificial virus"	Plank, Wagner et al.	[78]
1993	Polyamidoamine (PAMAM) dendrimer	Defined polymer with proton sponge effect	Haensler and Szoka	[85]
1994	AVET (adenovirus enhanced transferrinfection)	First polymer-based ex vivo human gene therapy trial	Stingl, Wagner et al.	[14, 15]
1995	Polyethylenimine (PEI)	Abundantly available and powerful proton sponge transfection polymer	Boussif, Behr et al.	[99]
2001	Biodegradable cationic polymers	Library chemistry based on Michael addition approach	Lynn, Langer et al.	[173]
2007	Dynamic polyconjugates	In vivo targeted delivery of siRNA to hepatocytes	Rozema, Wolff et al.	[135]
2010	Transferrin-receptor-targeted therapeutic siRNA delivery system based on cyclodextrin-based polymers and cyclodextrin/adamantane host–guest interactions	First report that systemic administration of siRNA could mediate gene silencing in humans	Davis, Ribas et al.	[22]
2011	Polyamino acids	Solid phase synthesis of sequence-defined polyaminoamides	Schaffert, Wagner et al.	[217]
2016	VIPER	Virus-inspired polymer for endosomal release of gene transfer polyplexes	Cheng, Pun et al.	[253]

improvement of polymeric carriers is necessary. It is also predictable that the rapid advances in nanotechnology and microscopy will further deepen our understanding of nanoscale particles for nucleic acid delivery. With the continuous efforts, it is

reasonable to believe that in near future polymeric gene delivery system will play a significant role in human nucleic acid and gene therapy.

Acknowledgements Peng Zhang appreciates receiving China Scholarship Council fellowships as support to his PhD studies at Ludwig-Maximilians-Universität München, Germany. We also acknowledge the financial support by the DFG Excellence Cluster Nanosystems Initiative Munich (NIM) and SinoGermanCenter Grant GZ995.

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