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REVIEW ARTICLE

# Invading target cells: multifunctional polymer conjugates as therapeutic nucleic acid carriers

**Ulrich LÄCHELT (✉), Ernst WAGNER**

Pharmaceutical Biotechnology, Center for System-based Drug Research, and Center for NanoScience, Ludwig-Maximilians-Universität Munich, Butenandtstrasse 5-13, D-81377 Munich, Germany



**Abstract** Polymer-based conjugates are an interesting option and challenge for the design of nano-sized drug-delivery systems, as they require advanced conjugation chemistry and precise engineering. In the case of nucleic acid therapy, non-viral carriers face several biological barriers during the delivery process, namely 1) protection of the cargo from extracellular degradation, 2) avoidance of non-specific interactions with non-targeted tissues, 3) efficient entry into the target cells, 4) intracellular trafficking to the site of action and 5) cargo release. To take on these obstacles, multifunctional conjugates can act as "smart polymers" with microenvironment-sensing dynamics to facilitate the separate delivery steps. Synthesis of defined polymer architectures with precise functionalization enables structure-activity relationships to be investigated and the integration of key functions for efficient delivery. Thus bioresponsive polymer conjugates, which are equipped with molecular devices responding to the certain microenvironments within the delivery pathway (e. g. pH, redox potential, enzymes) can be assembled. This review focuses on the modular engineering and conjugation of multifunctional polymeric structures for the utilization as "tailor-made" nucleic acid carriers.

**Keywords** conjugate, DNA, gene transfer, polymer, RNA, targeting

## 1 Introduction

The therapeutic usage of nucleic acids has generated great expectations, since this class of biopolymers offers a tremendous array of functionalities, versatile modes of action and medicinal applications [1,2]. Classical gene therapy in terms of the substitution of defect genes by nucleic acid delivery was the first application which reached medical reality. In addition, the silencing of target genes via RNA interference with small interfering RNA (siRNA) and blocking RNA by antisense oligonucleotides have served as "molecular adjusting screws" for the regulation of gene expression. Moreover, synthetic long dsRNA that mimicks the viral RNA intermediates present in cells bearing infections can act as immunostimulatory, antiproliferative and pro-apoptotic agents. Examples include poly-inosine-cytosine dsRNA, poly(I:C), a dsRNA analog composed of mismatched poly(inosine) and poly(cytosine) strands, that in combination with epidermal growth factor (EGF) receptor-targeted carriers show a promising approach for cancer treatment [3–5], and small conditional RNAs which form long dsRNA only in target cancer cells and in this way selectively mediate cell death [6].

Aptamers are DNA or RNA based single stranded oligonucleotides that bind molecular targets with high affinity and specificity which are comparable to those of antibodies [7–13]. Aptamers' targets range from small molecules like ATP [9] to large macromolecular compounds like cell-surface proteins [8,12]. Therefore they are a promising tool for diagnostic, therapeutic or targeting purposes [11,13]. A polyethylene glycole (PEG) modified aptamer that specifically binds to the vascular endothelial growth factor (VEGF) has been approved by the FDA and has been marketed for the treatment of neovascular age-related macular degeneration. Further eight aptamers are currently being evaluated in clinical trials [13].

This review focuses on polymer-based carriers for therapeutic nucleic acids with intracellular target sites, especially plasmid DNA and siRNA, since their applicability strongly depends on capable delivery systems.

Although gene delivery by viral vectors is quite efficient in general, non-viral carriers have significant advantages such as flexibility in transportable payloads, safety,

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E-mail: ulrich.laechelt@cup.uni-muenchen.de

immunogenicity, ease of manufacture and scale-up and cargo capacity [14]. Unfortunately the efficient delivery of therapeutic nucleic acids to (and into) target cells is sophisticated and hampered by several hurdles. Systemic administered nucleic acids are cleared from circulation or degraded by nucleases in a relatively short period of time. Therefore effective carrier systems should encapsulate the nucleic acids, protect them against rapid elimination, enable them to be circulated in the blood stream and finally allow accumulation in the target tissue. Non-specific interactions with non-targeted tissues and blood constituents should be avoided. Furthermore they have to facilitate the cellular uptake and intracellular delivery to the target site.

Since delivery devices are subjected to a multitude of demands, several functional modules can be integrated in order to enhance their efficiency. If possible, conjugation of different targeting ligands should be carried out in a precise fashion. Defined structures and precise conjugation allow for the study of more accurate structure-activity and structure-toxicity relationships, ensure reproducibility and fulfill the demands on pharmaceutical products for a higher leverage than polydisperse products.

## 2 Protecting the payload: polymeric backbones

Free nucleic acids are rapidly removed from systemic circulation via renal excretion, hepatic elimination or non-specific nucleases. Therefore the payload has to be encapsulated, complexed or bound to molecular carrier systems in order to prolong the circulation time, protect the cargo from enzymatic degradation and enable sufficient delivery in the target tissues.

Many different types of cationic polymers have been used to develop self-assembling polyion complexes (polyplexes) due to electrostatic interactions of the negatively charged nucleic acid backbone and the positively charged polymers. Polyplexes typically are 50–200 nm in size and possess positive surface charges. As a result of the particle sizes, the nucleic acids are expected to avoid renal elimination and cellular uptake is improved due to electrostatic interactions with the negatively charged plasma membranes [15]. Some of the most extensively investigated cationic polymers for nucleic acid delivery are poly(*L*-lysine) (PLL), poly (ethylenimine) (PEI), poly(2-(dimethylamino)ethyl methacrylate) (PDMAEMA), poly(amidoamine) (PAMAM) dendrimers and chitosan (Fig. 1).

PLL is a biodegradable cationic polymer, which contains only primary amines permanently charged under physiological conditions. It efficiently binds nucleic acids, but unfortunately its transfection efficiency is relatively low and due to its charge and non-specific interactions it produces significant toxicity. Chitosan is a linear polysaccharide derived from chitin by deacetylation. It is a biodegradable and biocompatible cationic polymer and has gained increasing attention as scaffolding for nucleic acid carrier devices [16].

PEI is one of the most extensively investigated polymers for nucleic acid therapy [17,18]. Its high charge density, which increases upon endosomal acidification, and endosomal membrane destabilization makes it an efficient carrier for DNA and siRNA delivery. PEIs are available as linear or branched polymers. Linear PEI (LPEI) is prepared by hydrolysis of propionamides from poly(2-ethyl-2-oxazoline) [4]. Branched PEIs (BPEIs) are obtained by a polymerization reaction of aziridine [19], which results in a highly branched polyamine network capable of nucleic acid complexation. Unfortunately the in vivo use of PEI especially for systemic applications is limited by its significant toxicity. The polydispersity of BPEI as a result of the fabrication procedure is unfavorable. Werth et al. reported the fractionation of commercially available branched 25-kDa PEI by gel permeration chromatography [20]. A fraction of low molecular weight PEI (PEI F25-LMW) was identified, which showed superior transfection efficacy and low toxicity in various cell lines. These findings make it clear that precision and monodispersity of polymeric compounds are crucial for the study of structure-activity and –toxicity relationships as well as for the rational design of safe and capable nucleic acid delivery systems. Furthermore the multiplicity of primary, secondary and tertiary amines in BPEI complicates a precise functionalization of the polymeric backbone at defined locations.

PDMAEMA is a synthetic polymer containing side chains with tertiary amino groups capable of condensing DNA and facilitating cellular uptake [21]. However PDMAEMA exhibits considerable cytotoxicity and van de Wetering et al. found a clear correlation between transfection efficiency and toxicity of PDMAEMA/pDNA complexes [22]. The preparation of copolymers based on 2-(dimethylamino)ethyl methacrylate has been shown to be a good approach for the achievement of better transfection/toxicity ratios [23]. Xu et al. used consecutive atom transfer radical polymerizations (ATRPs) to prepare well-defined pentablock copolymers (PBPs) composed of PDMAEMA, PEG and poly(2-hydroxyethyl methacrylate) (PHEMA) with the molecular configuration PHEMA-*b*-PDMAEMA-*b*-PEG-*b*-PDMAEMA-*b*-PHEMA [24]. The PBPs effectively bound plasmid DNA and formed PBP/pDNA nanoparticle complexes in the range of 100–160 nm. The PBPs exhibited remarkable transfection efficiencies while being much less toxic than PEI or PDMAEMA homopolymers.

PAMAM dendrimers are recognized as a unique class of synthetic nanostructures. The use of dendrimers or dendritic polymers is intended to mimic the globular shape of natural DNA-histone complexes in regards to size, surface charge and flexibility in order to achieve good

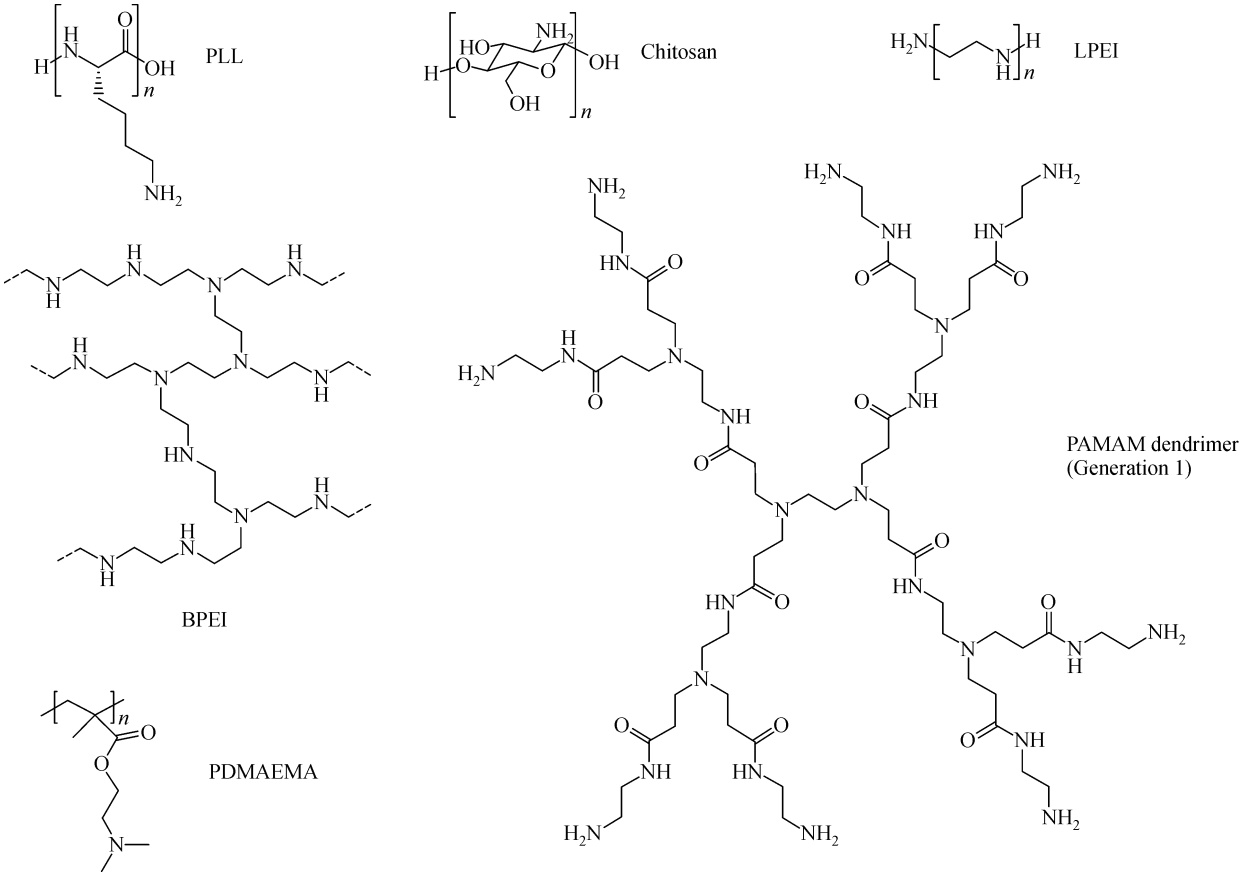


**Fig. 1** Frequently used cationic polymers for nucleic acid complexation and delivery

DNA complexation and high transfection efficiencies [25]. Advantages of dendrimeric structures are their highly branched spatial orientation and the possibility of achieving high molecular weights along with narrow molecular weight distributions by relatively simple synthetic procedures [25]. Furthermore dendrimers and dendrons allow the precise control of size, shape and placement of functional groups [26].

Another strategy toward precise and sequence-defined polymers is derived from classical solid-phase supported peptide synthesis. Hartmann et al. developed a solid-phase supported synthesis of linear poly(amidoamines) by alternating the coupling of succinic acid and polyamines [27]. This approach is thought to allow the build-up of sequence defined polymers with precisely positioned functionalities. The synthesis of block copolymers composed of monodisperse poly(amidoamines) with an oligopeptide [28] or polyethylene glycol (PEG) [28,29] has also been shown. The concept was recently extended by the design of novel artificial Fmoc-protected polyamino acids containing short protected forms of triethylentetramine or tetraethylenpentamine [30]. These building blocks can be assembled into homo- or hetero-poly(amidoamine) polymers by standard Fmoc based peptide synthesis, or optionally in combination with standard amino acids and peptide sequences. Sequence-defined linear and branched structures were synthesized.

Unfortunately, conventional polyplexes often show low stability under physiologic conditions. Non-specific interactions with proteins and electrolytes in body fluids and extracellular matrixes cause polyplex unpacking and dissociation [31] due to counter anion exchange. Thus enhanced stability of the carrier system during blood circulation is an important issue for efficient delivery. However, besides stabilization of the polyplex, the controlled disassembly of the carrier and cargo at the target site also has to be taken into consideration. Strong complexation and high stability of the polyplexes do not necessarily correlate with gene transfer efficiency [32]. Itaka et al. explored intracellular trafficking and pDNA release of LPEI, BPEI and PLL polyplexes using confocal microscopy and fluorescence resonance energy transfer (FRET) [33]. Both LPEI/pDNA and BPEI/pDNA polyplexes showed rapid endosomal escape, whereas no endosomal escape was observed for PLL/pDNA polyplexes. However, LPEI/pDNA polyplexes rapidly disintegrated and released their cargo within the cytosol, whereas pDNA complexed with BPEI remained in a

condensed state even after 24 h. The endosomal escape and disassembly kinetics correlated with the transfection efficiency of the transfecting agents used. LPEI was found to be much more effective than BPEI. Therefore both extracellular stability as well as payload release after internalization have to be provided by the delivery device. Several approaches for the stabilization of polyplexes based on the crosslinking of linear cationic polymers have been reported [34–36]. Concerning the issue of controlled cargo release the stabilization can be accomplished with the use of bioreducible disulfide crosslinks which degrade intracellularly. Also for complex dendrimer-like structures, crosslinking can be advantageous in order to enhance the stability of the polyplex. Russ et al. reported lateral stabilization of pseudodendrimers composed of oligoethylenimine and hexanediol diacrylate diesters with bifunctional bioreducible crosslinkers [37]. They showed clear improvements in the stability of the polyplex as well as enhanced gene transfer efficacy both in vitro and in vivo.

## 3 Increasing the specificity: shielding and targeting

The positive charge of most nucleic acid carriers mediates complexation with DNA and RNA and facilitates cellular uptake of the negatively charged cargo. However the ionic interactions are non-specific and charged particles can cause unintended interaction with non-target cells.

Shielding the surface charge with hydrophilic polymer coatings leads to higher solubility, reduced aggregation with serum proteins, improved biocompatibility, decreased elimination and prolonged systemic circulation [32,38]. Hydrophilic substructures that have been used for surface charge masking include polyethylene glycol (PEG) [39–45], polyhydroxymethacrylamide (pHPMA) [34,46,47], hyaluronan [48,49] and the plasma protein transferrin, which combines both an intrinsic stealth and targeting effect [50,51].

Attachment of polyethylene glycol ("PEGylation") has been widely used on polymer- [39–45], liposome- [52–55] and virus-based [56,57] delivery devices. A distinction is drawn between pre- and post-PEGylation according to the time of PEG attachment, namely before (pre-) or after (post-) polyplex formation. The major limitation of pre-PEGylation is that the hydrophilic portions of the copolymers such as PEI-PEG and PLL-PEG appear to hinder proper DNA condensation and particle formation [58,59]. The major drawback of post-PEGylation strategies is the requirement of an additional time-consuming synthesis step. Furthermore the degree of surface PEGylation is not well defined and removal of the PEGylation reagent from the nanoparticles is difficult. Kursa et al. described a simple and quick method for the formation of PEG shielded transferrin-receptor targeting PEI/DNA complexes by mixing blends of PEG-PEI and Tf-PEG-PEI in various ratios with DNA [60]. With this pre-PEGylation strategy the degree of shielding and targeting domains in the complex can easily be varied and the properties of the carrier modulated. Furthermore it has been shown for the first time, that freeze–thawed polyplex vectors have systemic targeting activity toward disseminated tumors, which is a major step forward in the development of nonviral vectors as pharmaceutical products. Since then, improved protocols for cryoformulation of PEI polyplexes have been developed [61,62].

The specificity and efficacy however influence each other and have to be balanced: high shielding prevents non-specific and specific interactions as well as internalization and can lower endosomal escape, polyplex dissociation, cargo release and overall efficacy. Zhang et al. synthesized a set of PEG-PEI conjugates with different PEG chain lengths and varying degrees of PEGylation [63]. The copolymer structure strongly influences particle size, surface charge, cytotoxicity and transfection efficiency. Particle size decreased with increasing molecular weight (MW) and with the number of PEG blocks. Zeta potential decreased with increasing degrees of PEGylation and with MW of the PEG chains. Polymers with high density PEGylation had low transfection efficiency, possibly due to steric hindrance.

To overcome this "PEG dilemma" and to maintain high efficiency, either a compromise in the optimum for shielding has to be found or a programmed deshielding has to be integrated into the delivery devices [64]. It has been shown by several groups that reversible PEGylation through linkages responding to environmental conditions, such as pH, redox potential or the presence of certain enzymes can enhance transfection efficiency and are superior to irreversible PEGylation. Acid labile linkages used for reversible PEGylation are hydrazones [41,64,65], acetals [66,67] and orthoesters [68]. Knorr et al. reported the synthesis of an acetal based PEGylation reagent for the pH-sensitive shielding of oligoethylenimine (OEI) based polyplexes [66,67]. The reversibly conjugated PEG-OEI copolymers remained stable at pH 7.4, whereas deshielding occurred at pH 5 within 15–30 min. Irreversible PEGylated OEI analogs showed reduced transfection efficiencies compared to non-PEGylated OEI, whereas the acid-labile PEGylated polymers mediated significantly increased efficiencies over the non-PEGylated counterpart [66]. Takae et al. used disulfide linkages of thiol-modified PEG and the polycationic polymer P[Asp(DET)], which was based on poly(aspartamide) with flanking diethylenetriamine [44]. The bioreducible PEG-SS-P[Asp(DET)] showed higher gene transfection efficiency and more rapid onset of gene expression than the irreversible PEGylated counterpart PEG-P[Asp(DET)]. Another approach for programmed deshielding of nanoparticles is enzyme mediated cleavage. Hatakeyama et al. reported the development of a PEG-peptide-lipid conjugate, where the peptide serves as a predetermined breaking point via

enzymatic degradation. The peptide linker between PEG and the lipid is cleaved by a matrix metalloproteinase (MMP), which is overexpressed in tumor tissues [69]. In vitro studies with the corresponding liposome gene carriers showed dependency of transfection efficiency from MMP expression. Tumor accumulation was observed in vivo.

Since surface decoration with hydrophilic polymers prevents non-specific interactions as well as intended interactions with target cells, shielding is commonly combined with the attachment of homing ligands that specifically bind to receptors expressed primarily on the target cells. Figure 2 exemplifies the combination of shielding and targeting with its intended selectivity for target cells. Serious efforts have been made to selectively target tumor cells, since nucleic acid therapy raises hopes for improved treatment and specificity might reduce the adverse side-effects commonly associated with cancer therapy [70]. In many cases, tumor cells have unique surface antigens or receptors that are not found on normal cells to a large extent.

The delivery system ideally does not randomly interact with non-target cells but only specifically with target cells. Non-specific interactions can be prevented by surface shielding such as PEGylation. Specificity for target cells is provided by surface decoration with targeting ligands homing to specific target antigens or receptors.

Ligands that have been investigated for targeting purpose include antibodies [71–74], native ligands like proteins [3–5,75,76], peptide hormones [77], cholesterol [78] and vitamins [79–83], as well as artificial ligands such as small-molecule receptor agonists and antagonists [84–87], oligosaccharides [88–90], oligopeptides [91–94] and aptamers [12].

The epidermal growth factor receptor (EGFR) is an attractive and widely used target for tissue-specific delivery of nucleic acids. EGFR is a transmembrane glycoprotein belonging to the receptor tyrosine kinase family. It is overexpressed in a variety of human tumors [95], which enables selective targeting of certain malignancies. EGFR can be targeted by attachment of the native ligand EGF [3–5,75], monoclonal antibodies [73] or synthetic targeting peptides like GE11 [94]. However the natural ligand EGF has tumor promoting properties due to activation of EGFR, which is unfavorable in cancer treatment. In contrast GE11 does not cause strong EGFR activation and has a much lower mitogenic activity than EGF and therefore is advantageous [94,96]. The uptake of GE11 is however less efficient than that of the native EGF ligand. Administration of poly(I:C)/PEI-PEG-EGF polyplexes was found to be a promising approach for the treatment of several types of EGFR overexpressing cancers such as glioblastoma multiforme, breast cancer and adenocarcinoma tumors [3–5]. EGFR-targeted poly(I:C) induced rapid apoptosis of the target cells in vitro and in vivo. de Bruin et al. analyzed and compared the effects of EGF-PEG-PEI and non-targeted polyplexes with respect to the internalization into EGFR overexpressing HuH7 cells [75]. Uptake kinetics and internalization dynamics were investigated and clearly showed that EGFR targeting by EGF-polyplexes leads to faster and more efficient internalization. After 5 min, 50% of the targeted polyplexes were internalized in contrast to the untargeted ones, where only about 20% entered the cell after 20 min. With the use of single-particle tracking, a three-phase dynamic of the internalization process, which was independent of the presence of targeting ligands, was revealed. Phase I was

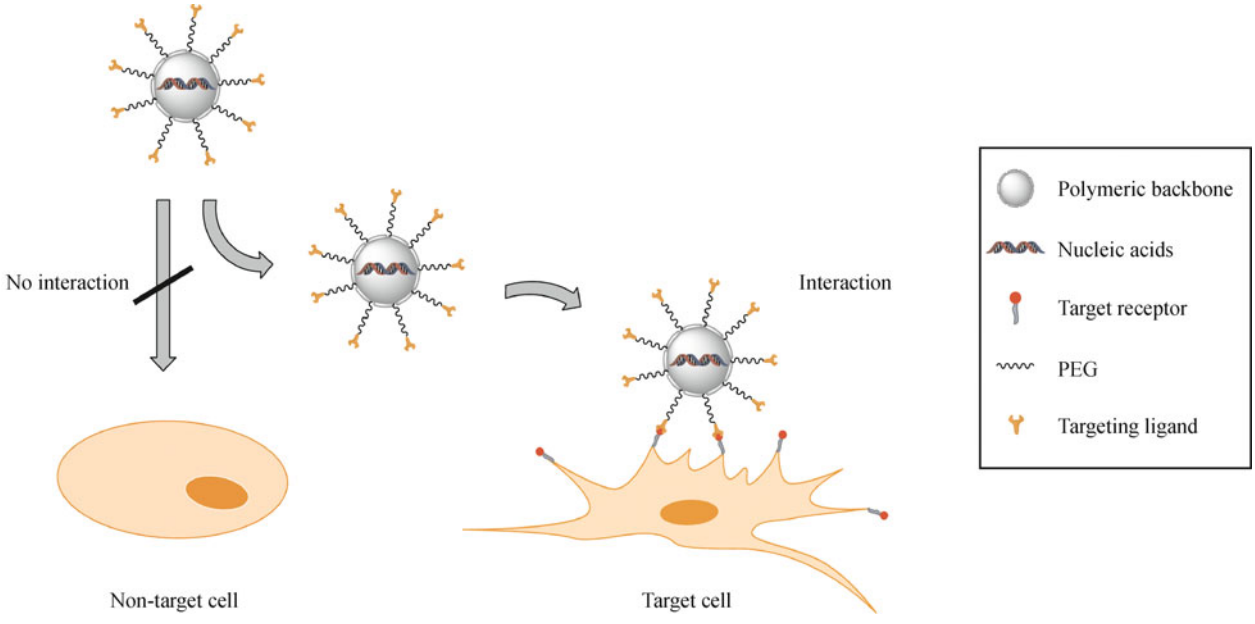


**Fig. 2** Selectivity of targeted nucleic acid carriers

characterized by a slow, directed, actin cytoskeleton-mediated motion of the particles after attaching to the plasma membrane. Phase I ended with subsequent internalization into the cell. Phase II showed increased velocity and random motion due to diffusion in the cytoplasm. Finally Phase III was characterized by the fast active transport of the polyplex-containing endosomes along microtubules. For EGFR targeted polyplexes, the analysis revealed a shortened Phase I and strongly accelerated internalization.

Folate receptor (FR) is a membrane associated protein that binds vitamin B9, folic acid, with nanomolar affinity and mediates its cellular uptake via endocytosis [97].

The folate receptor is overexpressed in many human tumors, whereas normal tissues generally express low to moderate amounts [70,98]. Therefore folic acid constitutes a high-affinity ligand with ideal properties for targeting purposes. Attachment of folic acid to carrier systems allows for high-affinity binding to the folate receptor and subsequent receptor-mediated endocytosis of folate-associated molecules [79–82,97]. The successful delivery of plasmid DNA encoding for a gene silencing construct with the use of folic acid-modified PEI was reported [99]. Zhang et al. used the noncovalent attachment of folic acid-conjugated oligodeoxynucleotides to the sense strand of siRNA via nucleic acid base-paired interactions to achieve specific cellular uptake and gene silencing in vitro [83].

Transferrin (Tf), a 79 kDa iron binding glycoprotein, has been extensively studied as a ligand for tumor targeting. Expression of the transferrin receptor (Tf-R) is elevated in numerous types of cancer cells due to their high metabolism and a need for iron [100–102]. Davis et al. reported the first human clinical trial to systemically deliver siRNA in a targeted fashion for the treatment of patients with refractory solid cancers. The delivery device consisted of self-assembled cyclodextrin polymer-based nanoparticles together with PEG- and PEG-Tf-conjugated adamantine [103,104]. In addition to the native ligand Tf [100,103–107], artificial targeting ligands like T7 (HAIYPRH) [93,108] or B6 (GHKAKGPRK) [109] can be utilized to home the drug delivery devices.

Another interesting approach is the combination of different ligands targeting different receptors to achieve a synergistic extension of internalization [106,110] or enhanced specificity for target cells [73]. It is known that gene transfer of some viruses takes part in a double-receptor mediated pathway. Thus some viruses need the involvement of two different receptors. For adenovirus, both the coxsackievirus-adenovirus receptor (CAR) and integrins αvβ3 or αvβ5, which recognize the peptide sequence RGD of the adenovirus penton, are needed [111,112]. Li et al. used PEI modified with two different ligands for dual-targeting to achieve enhanced efficiency of the gene transfer. The two peptides YC25 and CP9 which bind fibroblast growth factor (FGF) receptors and integrins were covalently attached to branched PEI and an improvement of gene-transfer efficiency both in vitro and in vivo was demonstrated [110]. Related observations were made by Nie et al. using dual-targeted PEGylated PEI/DNA polyplexes (Fig. 3). In these dual targeted polyplexes, a division of tasks was observed: a bicyclic RGD containing peptide (attached via a 3.4 kDa PEG spacer) was found to be mostly responsible for attachment to DU145 prostate carcinoma cells, whereas a TfR binding peptide B6 (attached via a 2 kDa PEG spacer) was found to mediate cellular uptake [113].

## 4 Widening the bottleneck: endosomal escape

After successful receptor-mediated uptake the internalized nucleic acid carriers are caged within endosomes and have to escape these compartments to reach their target site. Endosomal escape represents one of the major bottlenecks of nucleic acid delivery. The application of PLL as a nucleic acid delivery device is strongly limited by its poor endosomal escape performance. Experiments with fluor-

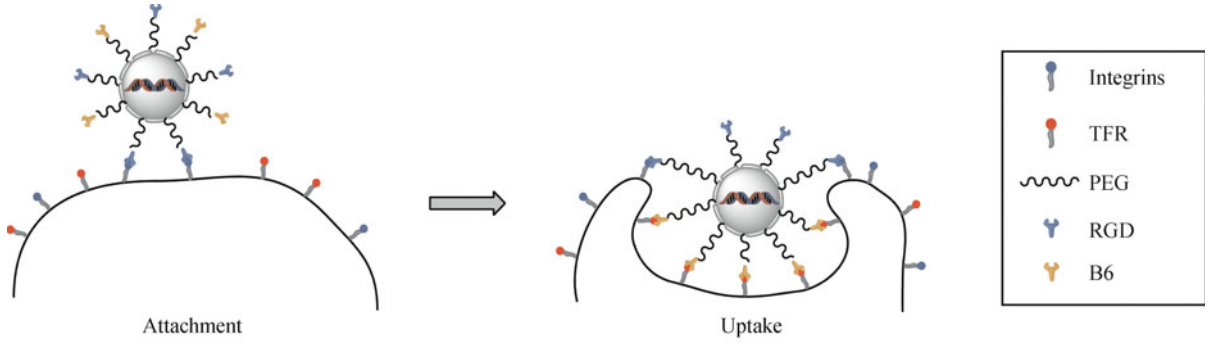


**Fig. 3** Synergy of dual-targeting approach mimicking the biphasic cell entry of natural viruses. Attachment of the dual-targeted polyplexes is primarily achieved by RGD peptide targeting integrins on the cell surface, whereas TfR binding B6 mediates internalization and uptake [113]

escently labeled DNA/PLL complexes showed an almost exclusive accumulation of the DNA in the intracellular vesicles like endosomes or lysosomes. The addition of chloroquine or glycerol to serve as endosome disruptive agents considerably increased the transfection efficiency in several cell lines [114]. Curiel et al. reported that the transfection efficiency of Tf-PLL conjugates can be powerfully augmented by co-treatment of the target cells with replication-incompetent adenovirus particles [115]. Notably, this augmentation depends on the adenoviral mechanism of endosome disruption as part of their entry mechanism.

Compared to PLL, PEI possesses acceptable endosomal escape and transfection efficiency. It has been hypothesized that the efficiency of PEI has its source in its buffering capacity below physiologic pH [17]. PEI buffers the acidic conditions within endosomes and captures protons like a sponge ("proton sponge effect"). As a result more protons are actively transported into the endosomes together with the corresponding chloride counter ions. By implication of the developing osmotic pressure, water flows into the endosomes and causes swelling and rupture that provides endosomal escape [17]. Kichler et al. investigated the importance of endosomal acidification during PEI-mediated DNA transfer with the use of proton pump inhibitors such as bafilomycin A1 and concanamycin A during transfection [18]. PEI-mediated transfection in the presence of bafilomycin A1 resulted in a 7–74 fold decrease in the expression of the reporter gene depending on the cell line used. It has been shown in erythrocyte interaction and lysis assays that PEI binds but does not strongly destabilize the erythrocyte membranes, even at acidic pH. This data implies that the transfection efficiency of PEI largely relies on its ability to capture protons within endosomes. Because of the critical step of endosomal escape the use of endosome-disruptive agents or cell-penetrating-peptides can be advantageous for efficient nucleic acid delivery. Meyer et al. used dimethyl maleic anhydride (DMMAn) to mask melittin, a lytic peptide from bee venome, by making use of pH sensitivities. PLL conjugated with DMMAn modified melittin showed pH-triggered endosomolytic activity and enhanced transfection efficiency along with a decrease in the toxicity in comparison to the unmasked melittin conjugates [43,116,117].

Several approaches of non-viral nucleic acid delivery use virus-derived substructures, since viruses in general possess high transfer efficiencies, although they also have to face the challenge of endosomal escape. Wagner et al. described the use of fusogenic peptides derived from the influenza virus hemagglutinin subunit HA-2 to enhance endosomal escape. They combined PLL as a nucleic acid packaging module, PLL-modified transferrin or a synthetic trigalactose ligand as a TfR or asialoglycoprotein receptor binding module and PLL-bound influenza peptides as endosomolytic modules to form an "artificial virus", which was then used for the delivery of nucleic acids [118,119]. HIV derived TAT peptide, a basic sequence of 10 amino acids (GRKKRRQRRR) within the transactivator of transcription (TAT) protein has been utilized for nucleic acid delivery [120–122]. However Roy et al. hypothesized that the cationic nature of the TAT peptide hinders exposition on the polyplex surface. They showed, that the length of PEG linkers between the cationic polymer backbone and the TAT peptide influences its availability and thus transfection efficiency [120].

## 5 Putting the bricks together: multifunctionalization of polymers

Many demands are made on nucleic acid carriers for efficient delivery. Multiple functionalities need to be integrated into the carriers to enhance their capabilities (Fig. 4), but unfortunately most commonly used polymeric backbones contain a relatively low number of distinct conjugation sites. The integration of different coexisting functionalities such as shielding, (multiple) targeting and endosomolytic domains in a precise and regioselective fashion is a challenging problem. Several approaches for the functionalization of amine containing polymers use classical carbonyl chemistry with linkers providing activated carboxylic groups (e.g. anhydrides, *N*-hydroxysuccinimide (NHS) esters or carboxylic groups activated with carbodiimides) to form amide bonds. Roy et al. used maleimide-PEG-NHS, a heterobifunctional linker with amine- and thiole-reactive termini, for the conjugation of cell-penetrating TAT peptides to PEI [120]. Fella et al. reported the synthesis of an amine-reactive PEGylation reagent for pH-reversible PEI polyplex shielding [65]. The reagent is composed of PEG coupled to an NHS ester via an acid-labile pyridylhydrazone linkage (PEG-HZN-NHS). Furthermore a bifunctional pH-sensitive reagent was synthesized offering an additional thiole-reactive ω-2-pyridyldithio functionality (OPSS-PEG-HZN-NHS) which allows for the coupling of targeting ligands. Pun et al. reported cyclodextrin (CD) modification of linear and branched PEI [123] which allows for host-guest interactions with different adamantine (Ad) conjugated domains such as PEG, targeting ligands and cell-penetrating peptides. Wang et al. used an impressive approach to integrate multiple functionalities in a nucleic acid carrier system and to generate a huge combinatorial library of polyplexes in a high-throughput fashion. Using a digital microreactor, CD-PEI was combined with Ad-PEG, Ad-PEG-RGD, Ad-PEG-TAT and Ad-PAMAM dendrimers in various ratios and subsequently characterized in order to screen for optimal compositions [121]. Although PEI provides no orthogonal or distinct conjugation sites the self-assembling polymers along with a microfluidic mixing procedure have been shown to generate defined structures allowing for the study of composition-activity relationships.

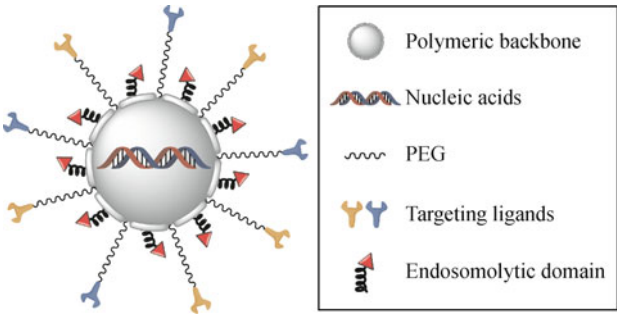


**Fig. 4** Multifunctional nucleic acid carrier. A polymeric backbone encapsulating (complexing) nucleic acids is equipped with diverse functionalities addressing the separate delivery steps: 1) PEGylation for surface-shielding, prevention of non-specific interactions with non-target cells and long systemic circulation. 2) Targeting ligands for the homing to target cells, specific interactions and cellular uptake. Use of different ligands can cause dual- (plural-) targeting effects. 3) Endosomolytic domains facilitating endosomal escape and delivery to the cytosol.

## 6 Future prospects

In summary, modification with functional transport domains "breathes life into polycationic carriers" [116]. To broaden the possibilities of precise multi-functionalization, sequence defined synthesis strategies are desirable [27,30]. Enabling the use of site-specific conjugation approaches [124,125] like click-chemistry or a priori coupling of ligands to the polymeric backbone would further promote the development of defined nucleic acid carrier systems and help better fulfill the demands on pharmaceutical products.

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