Expert Opinion

pH-responsive shielding of non-viral gene vectors

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PEG shielding of non-viral vectors reduces undesired interactions with the extracellular environment. Combination with cell-binding domains enables *in vivo* targeting via specific attachment to the target cells. Pegylation, however, also interferes with effective intracellular nucleic acid delivery. Consistently triggered removal of the PEG shield after reaching the target cell would make non-viral vectors more compatible with the intracellular delivery steps. Physiological triggers may include changes in pH, enzyme concentration or redox potential. This review focuses on pH-sensitive shielding strategies that exploit the endosomal acidification process after endocytosis for deshielding of the delivery system.

Keywords: artificial viruses, bioresponsive, non-viral gene transfer, PEG shielding, smart delivery systems

Expert Opin. Drug Deliv. (2006) 3(5):563-571

1. Introduction

Systemic targeted *in vivo* delivery is a major challenge in the field of biomolecular therapeutics. Polycations and cationic lipids have shown potential in intracellular delivery of oligonucleotides (ODNs), small interfering RNAs (siRNAs) or plasmid vectors, but uncontrolled interactions with the extracellular environment and relatively low transfection efficiency limit their *in vivo* use. A common approach for reducing undesired interactions is to mask the particles with a hydrophilic polymer, such as PEG. Pegylation reduces the positive surface charge, reduces toxicity, prevents aggregation, protects from uptake by the mononuclear phagocytic system, increases circulation time, and, hence, improves systemic targeted gene transfer [1-5].

Regrettably, PEG shielding lowers the transfection efficiency [6-8] due to reduced cell-surface interactions. This hurdle has been partly overcome by inclusion of targeting ligands; but these targeted and pegylated carriers also do not reach the activity of the uncoated positively charged vectors [9,10]. This finding indicates the importance of the intracellular delivery steps subsequent to endosomal uptake. After endocytosis, the vectors are trapped within acidic vesicles of the endosomal/lysosomal system. Efficient cytosolic delivery requires endosomal escape. Direct interactions of positively charged particles with the inner endosome membrane may lead to membrane disruption through perturbation or fusion [11-13]. Additional endosomolytic moieties (either the carrier itself or linked domains) may be included to enhance gene transfer [14]. The different requirements outside and inside the target cell constitute the following dilemma: in the extracellular environment the surface charge and lytic activity must be shielded, but, after entering the target cell, the cationic surface charge and endosomal release functions should be re-exposed through deshielding of the particle. Therefore, it is obvious that dynamic non-viral gene delivery systems have to be developed that undergo bioresponsive changes after reaching the target cell. Differences in the pH of biological compartments can be exploited for the development of bioresponsive vectors. Importantly, endosomes and lysosomes exist at acidic pH values between 4.5 and 6.6, in contrast to the extracellular physiological pH around 7.4 [15]. As well as the pH gradient, changes in

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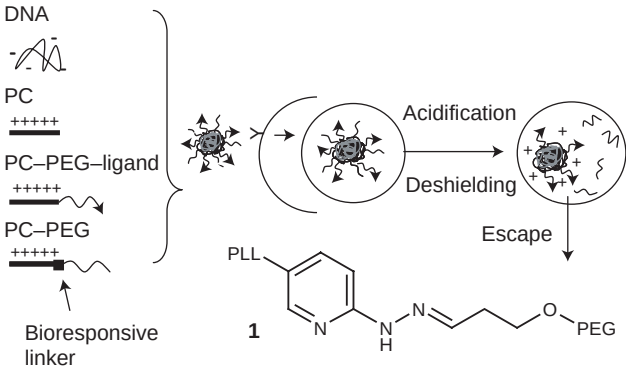

Figure 1. Schematic of complex formation and intracellular deshielding. Details are described in [19].
PC: Polycation; PEG: Poly(ethylene glycol); PLL: Poly-L-lysine.

the concentration of specific enzymes [16] or different redox potentials [17] are used as bioresponsive deshielding triggers for gene- and drug delivery [18].

2. Polycation-based delivery systems

2.1. Acid-triggered deshielding of polyplexes via cleavage of hydrazone-linked PEG

Walker *et al.* developed pH-labile shielded DNA–polycation polyplexes that show enhanced gene transfer *in vitro* and *in vivo* compared with stable shielded polyplexes [19]. In their strategy, the DNA-binding polycation poly-L-lysine (PLL) and the shielding polymer PEG were conjugated via pH-sensitive acyl- or pyridylhydrazone (HZN) bonds. For polyplex formation, the PEG conjugate was combined with polyethylenimine (PEI) for enhanced endosomal release [20] and either transferrin-linked PEI (Tf–PEI) or EGF-linked PEI (EGF–PEI) for targeting and receptor-mediated endocytosis (Figure 1). Suitable PLL–HZN–PEG conjugates should be stable under neutral conditions at 37°C but rapidly hydrolyse at pH 5. A conjugate that meets these criteria is the 2-pyridylhydrazone derivative 1 (Figure 1).

At 37°C and pH 7.4 conjugate 1 has a half-life of 1.5 h. At pH 5 it is rapidly hydrolysed, with ~ 90% degraded within 10 min. Polyplexes generated with PLL–HZN–PEG 1 remained shielded over 5 h at 37°C. At pH 5, deshielding of polyplexes was almost complete at the 1-h time point.

In cell culture experiments, stable shielded and labile shielded polyplexes were compared. Polyplexes contained ligand–PEI conjugates: Tf–PEI for uptake by K-562 and Neuro2A cells [21]; and EGF–PEI for HUH-7 and Renca-EGFR cells [22,23]. Remarkably, the shielded polyplexes generated from PLL–HZN–PEG 1 had transfection efficiencies that were comparable to the unshielded polyplexes, with up to a 250-fold (for Neuro2A) higher transfection efficiency compared with polyplexes with a stable thioether-linked PLL–PEG conjugate. These results led to the evaluation of systemic delivery in an *in vivo* tumour mouse model of bioreversibly shielded 1, compared with irreversible shielded EGF-targeted polyplexes. The bioreversibly shielded polyplexes mediated about one logarithmic magnitude higher luciferase expression than stably shielded polyplexes, with prevalent gene expression found in the subcutaneous HUH-7 tumour (> 10-fold higher compared with other organs).

2.2 Acid-triggered deshielding of polyplexes via cleavage of acetal-linked PEG

As well as hydrazones, chemical linkages that may possess pH-dependent hydrolysis include vinyl ethers [24], orthoesters [25,26] and acetals [27,28]. Murthy *et al.* linked PEG to an endosomolytic cationic backbone via acid-degradable acetal linkages and investigated the capability to deliver ODNs, peptides and other macromolecular drugs [27,28] (2, Figure 2). The endosomolytic backbone is a polymer of butyl methacrylate (membrane disruptive); dimethylaminoethyl methacrylate and a styrene derivative were PEG grafts that are linked via the acid-degradable acetal linkage. The PEG grafts can be terminated (R and R′) by methoxy, mannose, lactose (cell targeting) or fluorescein (covalent labelling for fluorescence microscopy studies). Therapeutic peptides or ODNs can be electrostatically complexed or covalently conjugated to these polymers. The backbone design is also flexible in terms of hydrophobicity and thus lytic activity can be controlled. Figure 2 shows a schematic diagram of these polymeric carriers.

The polymeric carrier should remain pegylated at pH 7.4 but rapidly depegylate when the pH drops in the endosome. The hydrolysis rates were measured at 37°C and revealed the desired properties. The capability to enhance endosomal escape of macromolecules was tested with grafted PEG–fluorescein as a model macromolecular drug, and PEG–lactose for hepatocyte targeting. Intracellular distribution of PEG–fluorescein was investigated by fluorescence microscopy. When PEG–fluorescein was delivered alone, only a punctuate fluorophore distribution was observed: PEG–fluorescein was able to enter the hepatocytes but was trapped in the endosome. In contrast, the bioresponsive polymeric delivery of PEG–fluorescein leads to a diffuse spreading throughout the cytoplasm, due to enhanced endosomal release after the acid-triggered depegylation/unmasking. Similar results were observed with electrostatically complexed rhodamine-labelled ODNs. Additionally, the investigators complexed antisense (AS)-ODNs with targeted bioresponsive polymers and analysed their ability to inhibit the inducible nitric oxide synthetase in macrophages. As a control, scrambled ODN and AS-ODN alone were delivered. Remarkably, the polymer/AS-ODN complex caused 80% inhibition of inducible nitric oxide synthetase, whereas the AS-ODN by itself only caused 25% inhibition.

2.3. pH-sensitive pegylated oligonucleotides

Although in the first two approaches the shielding agent was grafted onto the carrier, Oishi *et al.* linked PEG directly to the

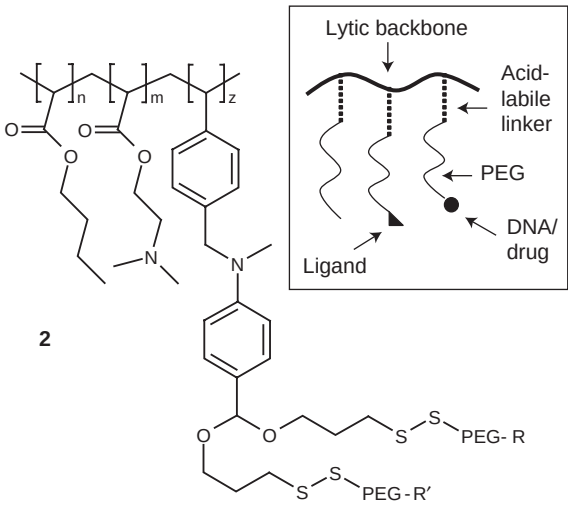

Figure 2. Illustration of the bioresponsive polymeric carrier. After endocytosis and acidification, the pH-sensitive linker hydrolyses, PEG dissociates from the polymer, and the endosomolytic backbone is exposed and enhances endosomal escape. Details are described in [27].
PEG: Poly(ethylene glycol).

AS-ODNs and siRNA via acid-labile β-thiopropionate linkages [29,30] (3, Figure 3). The anionic PEG–ODNs or targeted lactosylated PEG–ODNs (Lac–PEG–ODNs) were mixed with PLL and formed polyion complex micelles (PIC). An illustration of such a PIC micelle is shown in Figure 3.

Micelle formation and degradation characteristics looked auspicious for their use as a delivery vector [31]. A dual luciferase reporter assay in HUH-7 cells was carried out to investigate the AS efficiency of the pH-sensitive Lac–PEG–ODN PIC delivery system in comparison to a non-acid-cleavable linkage system (Lac–PEG–Mal–ODN/PLL PIC micelle). The pH-sensitive system achieved 65% inhibition, whereas the stable Lac–PEG–Mal–ODN PIC micelle showed only 27% inhibition of luciferase gene expression. This significant effect cannot be explained by a restored lytic activity after depegylation in the acidic endosomal compartment because no fusogenic functions are incorporated into this system. This enhancement is explained by an increase of the colloidal osmotic pressure in the endosome: after cleavage of the acid-labile linkage in the endosome, the released free PEG strands induce swelling and rupture of the endosome and consequently ease the transport of the ODN into the cytoplasm [32]. In contrast, the stable PIC micelles are not able to induce endosomal rupture and, moreover, the presence of the PEG strand may terminate the interaction of PEG–ODN with the target mRNA due to a steric hindrance effect. To study the effect of the polycation on the delivery system, PIC micelles of PLL or branched PEI 25 kDa were compared. Notably, the inhibition effect of the Lac–PEG–ODN/PEI system was significant lower. The investigators explain this activity reduction by the buffering effect of PEI: the decrease of the endosomal pH is prohibited and, hence, the acid-labile linkage of Lac–PEG–ODN is not adequately cleaved.

Jeong *et al.* reported PIC micelles composed of an anionic PEG–ODN conjugate containing an acid-cleavable phosphoramidate linkage [33] (4, Figure 3). This study group formed complexes with the cationic fusogenic peptide KALA, which is known to disrupt endosomes and used to enhance gene transfection [34]. By confocal microscopy of labelled ODNs in smooth muscle cells, it was observed that fluorescein-labelled micelles were distributed all over the cytoplasm, whereas cells treated with fluorescein-labelled ODN alone showed only marginal fluorescence in the cytoplasm. This is explained by limited uptake and poor endosomal release of the fluorescein–ODN alone. In case of the PEG–ODN/KALA micelles, the fusogenic action of KALA contributes to endosomal release. To analyse the efficiency of the PIC delivery system, the authors selected therapeutic AS-ODNs directed to c-myb, which suppresses muscle cell proliferation. The PIC micelles containing the AS-ODNs showed 70% inhibition in proliferation of smooth muscle cells. In addition, the effect of different core-forming polycations (KALA, branched PEI 25 kDa and protamine) was studied. Interestingly, not only did endosomolytic KALA and PEI show inhibition activity, but so did non-endosomolytic protamine.

This observation fits to the former hypothesis of Oishi *et al.* [29] and Murthy *et al.* [32] who claimed the acid-cleaved PEG strands were responsible for endosomal swelling and rupture, so that PEG–ODN/protamine micelles also mediate endosomal escape and inhibition efficiency. In contrast to the observation of Oishi *et al.*, the use of PEI did not reduce the AS activity of the delivery system.

2.4 Protective copolymers

When DNA is complexed with an excess of polycation the resulting particle is positively charged on the surface. These preassembled DNA complexes can be ionically coated by negatively charged molecules. Finsinger *et al.* synthesised protective copolymers (PROCOPs) consisting of PEG and different anionic peptides (polyglutamate and the fusogenic negatively charged influenza-derived peptide INF-7) that mediate the electrostatic interaction with the DNA particles [35]. As with the covalently coupled PEGs, this shielding coat also prevents aggregation and reduces undesired interactions and toxicity of the gene vectors. The PROCOP shielding coat was designed to dissociate from the polycation–DNA complex at the acidic pH of the endosomes: protonation of the glutamic acids (in the polyglutamate-, as well as in the INF-7 anchor) should reduce the electrostatic interactions between the coat and the particle.

The authors coated PEI, PLL and liposomes to investigate the PROCOP effect on transfection in different cell lines. At lower molar ratios of polycation nitrogen atoms to DNA phosphate (N:P), the polyglutamate–PROCOPs reduced the transfection efficiency of PEI 25 kDa on HepG2 cells, compared with uncoated polyplexes, probably due to a loss of

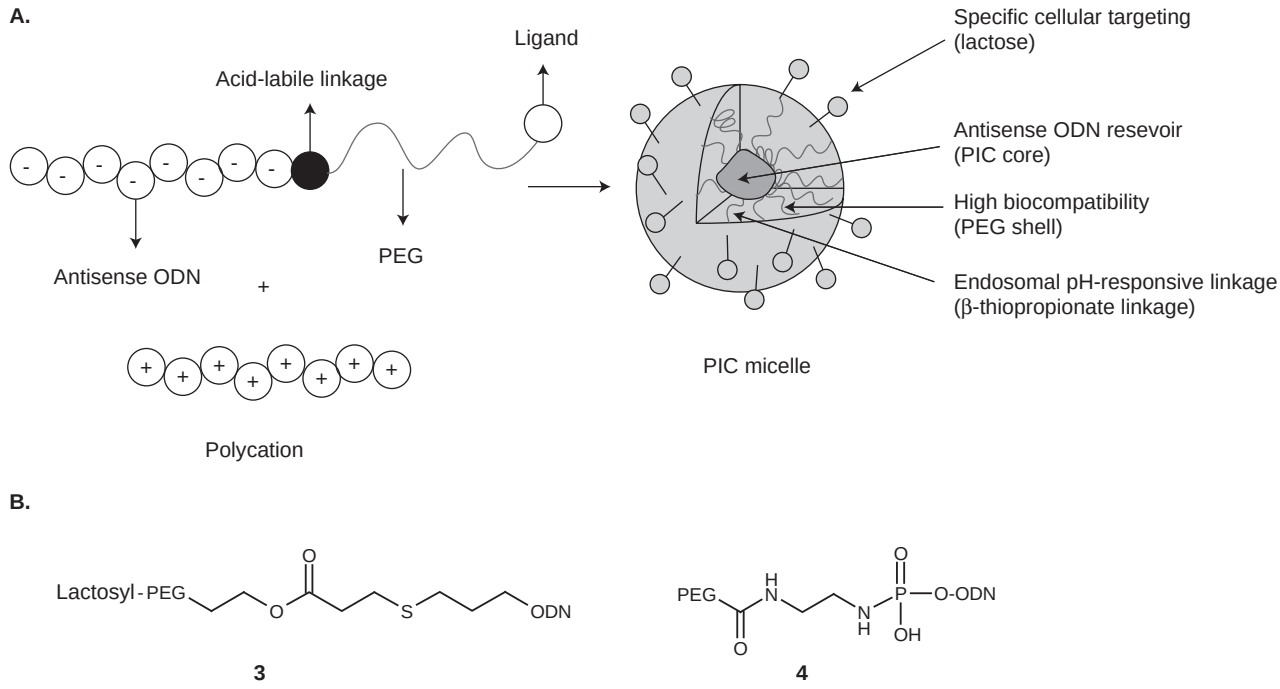

Figure 3. Schematic of PIC micelles and bioresponsive linkages. A. Concept of the PIC micelle. Reprinted with permission from [29]. **B.** Bioresponsive linkages: structure **3** described in [29], structure **4** described in [33].
ODN: Oligodeoxynucleotide; PEG: Poly(ethylene glycol); PIC: Polyion complex.

cell-binding capacity. However, at higher N:P ratios, where toxicity of PEI polyplexes is pronounced, the PROCOPs could enhance the transfection efficiency due to a better toxicity profile. It is known that PLL polyplexes show low transfection efficiency, probably due to a lack of endosomolytic activity. Membrane-disruptive INF-7–PROCOP significantly enhanced the transfection efficiency of PLL polyplexes on K-562 cells. The work proved that PROCOPs can control gene vector characteristics (size, interactions, toxicity) without affecting the gene transfer efficiency. Nevertheless, it has to be clarified that the ionic coating is stable enough for *in vivo* gene delivery.

The electrostatic coating strategy was further developed by Sethuraman *et al.* [36]. The investigators synthesised a pH-sensitive diblock copolymer containing PEG and poly(methacryloyl sulfadimethoxine) (PSD-*b*-PEG), which is negatively charged at pH 7.4, but electroneutral below pH 6.8. Thus, positively charged PEI–DNA complexes could be coated through charge–charge interactions with a PSD-*b*-PEG layer at physiological neutral pH. Under acidic conditions, the anionic sulfadimethoxine moieties get protonated and detach together with the conjugated PEG from the polyplex. In contrast to the previous described system, which is based on the pH-sensitive carboxylic group with a pK_a around 5, the PSD-*b*-PEG system shows a sharp transition between pH 6.8 and 6.9. The investigators deduce from this highly defined shift that, for example, the slightly acidic extracellular tumour matrix can be used to target the PSD system to the tumour cell: near the tumour tissue, the complex is depegylated and enables PEI to interact with the target cell. Indeed, the *in vitro* transfection efficiency (luciferase expression) at pH 6.6 is higher than at pH 7.4. Even though not all tumours are characterised by an acidic extracellular environment, this bioresponsive polyplex could be combined with targeting ligands to enhance the transfection efficiency due to endosomal deshielding. In this case, it may be advantageous that only slightly acidic conditions are necessary for deshielding because endosomes are not always strongly acidified. An illustration of the PROCOP concept is given in Figure 4.

3. Cationic lipid-based delivery systems

3.1 Acid-sensitive PEG–diorthoester lipids

Pegylation is also advantageous for *in vivo* application of cationic liposomes. For example, pegylation prevents opsonisation by the reticuloendothelial system, and prolongs circulating time in the systemic circulation [37]. Removal of the PEG shield after reaching the target cell is favourable to be compatible with the intracellular steps of gene delivery. To enforce this behaviour, Szoka *et al.* prepared various stabilised plasmid–lipid nanoparticles (SPLPs) consisting of a cationic lipid–phosphatidylethanolamine mixture and the pH-sensitive PEG–diorthoester–distearoylglycerol lipid (5, Figure 5) [25,26]. For this purpose, the investigators developed a liposome

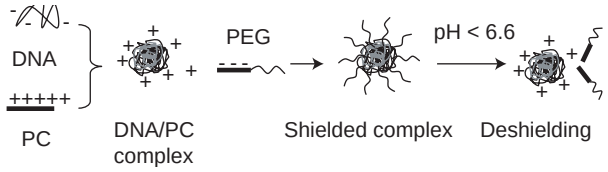

Figure 4. Bioreversible electrostatically coated polyplexes. Formation of shielded complexes through charge–charge interaction at neutral pH and deshielding after charge neutralisation under acidic conditions.
PC: Polycation; PEG: Poly(ethylene glycol).

formation method under alkaline conditions to reduce the degradation of compound 5 during the preparation. Preparation 5, containing SPLPs (PEG–diorthoester [POD]-SPLPs) showed similar characteristics to the stable pegylated controls. To determine the pH sensitivity, the mean diameter of the lipid particles was measured as a function of pH and time. At pH 5.3, POD–SPLPs broke down within 100 min and grew, whereas at pH 7.5 they did not collapse before 64 h. Stable pegylated SPLPs were not influenced by acidic conditions (diameter remained < 150 nm over 1 month).

Confocal microscopy studies revealed that stable, as well as pH-sensitive, pegylated SPLPs were internalised into CV-1 cells. This happens despite shielding of the positive charges; apparently the flexibility of the PEG chain and lipid layer allows surface binding [38]. When the CV-1 cells were transfected with a green fluorescent protein plasmid, only a few cells showed green fluorescent protein expression in case of the stable shielded SPLPs, but ~ 2% of the cells were transfected with the POD–SPLPs. Luciferase plasmid DNA transfection experiments showed greater transfection efficiency (up to three orders of magnitude) of POD–SPLPs, compared with pH-insensitive SPLPs. In comparison with PEI standard polyplexes, the SPLPs display reduced cytotoxicity and therefore represent an encouraging liposome formulation for drug/gene delivery. After Szoka *et al.*, Masson *et al.* presented the same method to generate bioresponsive liposomes for gene delivery [39]. PEG also was conjugated to lipids (by an orthoester linkage) and transfection levels on HeLa cells were higher, compared with stable shielded analogues.

Furthermore, a similar strategy to pH-responsive liposomes has been developed by Thompson *et al*. by conjugating PEG to lipids via an acid-labile vinyl ether bond [24]. Acid-triggered removal of the PEG coat destabilises the liposome and induces a lamellar-to-micellar phase transition, which enhances endosomal escape. Investigations for gene delivery purposes remain to be shown.

3.2 Diffusible PEG coating

As an alternative to the work described in the previous section, Ambegia *et al.* incorporated PEG domains by a diffusible hydrophobic anchor into SPLPs [40]. Ideally the diffusible PEG–diacylglycerols (PEG–DAGs) shield the particle during systemic circulation and diffuse out after administration to the target cells. The size of the PEG anchor determines its dissociation rate and, hence, can be used to tune the kinetics in a biocompatible way. Hydrophobic anchors varied in size from 14 to 18 carbon units (shorter acyl chains resulted in the production of particles with low stability). A fluorescence resonance energy transfer assay was carried out to investigate the PEG–lipid exchange rate *in vitro* and to finally prove the hypothesis [41]. Liposomes with the small C14 anchor (PEG–C14) reached a final degree of 72% fusion, whereas the other two anchors (C16 anchor: PEG–C16; C18 anchor: PEG–C18) only permitted, at most, 36% fusion in the observed 24-h time frame due to a slower dissociation from the liposomes. According to these deshielding kinetics, the PEG–C14 particles should behave in a different way *in vitro* and *in vivo* in comparison with liposomes with the larger PEG anchors. *In vitro*, the short anchor PEG–C14 exhibits the highest level of gene expression because the PEG coat that inhibits association and fusion with the cell/endosomal membrane is removed more quickly. *In vivo*, biodistribution and clearance were investigated: the longer the liposome remains pegylated, the longer the systemic circulation time. Indeed, increasing the length of the hydrophobic anchor increases the circulation half-time. Regarding biodistribution, pegylated particles should not accumulate in the lungs and organs of the reticuloendothelial system and thus can better reach the fenestrated distant tumour tissue. *In vivo* tumour model experiments support this hypothesis. The PEG liposomes with longer chains showed increased levels of tumour accumulation in comparison with the short anchor PEG–C14 liposomes, probably due to a decreased first-pass clearance as a result of the faster deshielding. None of these SPLPs accumulated in the lung to a high extent. As expected, gene expression at the tumour is lower for short-anchor PEGs due to a reduced accumulation in the target tissue.

3.3 Acid-triggered release of PEG–polycation copolymers

Similar to the PROCOP concept described in Section 2.4, Auguste *et al.* synthesised positively charged PEG copolymers for coating of liposomes through electrostatic interactions [42]. Although charge neutralisation of the PROCOP coat in an acidic environment triggers the dissociation of the PEG coat, in the case of the liposome the positive surface charge is enhanced by acidification, which initiates the dissociation of a cationic shielding polymer by charge repulsion. For this purpose, dimethylammonium propane (DAP) was incorporated into phosphatidylcholine/phosphatidylglycerol liposomes to cause a pH-dependent liposome charge. To prove the pH sensitivity of the liposomes, the investigators measured the electrophoretic mobility. Liposomes formulated with DAP exhibited mobility changes when the pH was changed from 7.4 to 5.5, demonstrating that it is possible to trigger an increase in cationic surface charge when the pH is shifted. This increased cationic charge should lead to a lower cationic

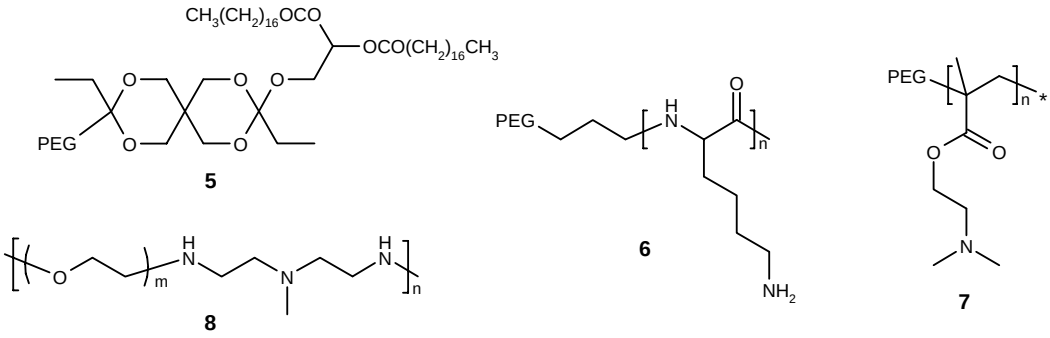

Figure 5. Bioresponsive structures applied in liposomes. 5: PEG–diorthoester–distearoylglycerol lipid. **6**: PEG–*b*–poly(L-lysine). **7**: PEG–*b*–poly(2-[dimethylamino]ethyl methacrylate). **8**: poly(PEG-3,30-diamino-*N*-methyldipropylamine).
PEG: Poly(ethylene glycol).

PEG polymer adsorption, suitable for endosomal deshielding of the liposome.

To investigate this hypothesis, different electrostatically modified PEG polymers (EMPEG) were synthesised (Figure 5): block copolymers with either lysine (EMPEG–Lys, 6) or a 2-(dimethylamino) ethyl methacrylate (EMPEG–DMAEMA, 7) residue and one alternating PEG–amine comb-graft copolymer (EMPEG–*N*–DP15, 8). PEG chain length and the ratio of PEG monomers to cationic anchors (PEG:CA) varied.

The adsorption measurements carried out with the coated liposomes demonstrated triggered release from the pH-dependent DAP liposomes. The investigators deduced that the capability of desorption under acidic conditions for EMPEG–Lys and EMPEG–DMAEMA depends on their PEG chain length but is independent of the PEG:CA ratio. With short PEG chains (45 monomers), no pH dependency was apparent; but with long PEG chains (113 monomers), the dissociation constants increase significantly for both EMPEG–Lys and EMPEG–DMAEMA when the pH is shifted from 7.4 to 5.5. It seems that a longer PEG chain is required for sufficient destabilisation of the electrostatic interactions and polymer desorption. In contrast, the alternating PEG–amine copolymers EMPEG–*N*–DP15 were not influenced by a change in pH. The observed triggered release of the PEG polycation copolymers looks encouraging but further biophysical parameters important for gene delivery (e.g., DNA encapsulation) have to be defined.

3.4 Bioresponsive carbamate-linked lipids

Another project that is in its infancy concerns the newly synthesised carbamate-linked lipids from Liu *et al.* [43,44]. Usually cationic lipids are synthesised with more or less stable linkers, such as amides, esters or ethers. In contrast, the carbamates are stable under neutral conditions but hydrolyse under acidic conditions. Therefore, lipoplexes that are formed from these lipids should be stable in the circulation system and prevent DNA from degradation, but unpack their payload after endocytosis and acidification. The group synthesised different lipids with variable carbon chain and amine head lengths and evaluated the liposome formulation. At that time, the synthesis of the carbamate-linked lipids is complete and the application as bioresponsive gene vectors *in vitro* and *in vivo* is in progress.

4. Endosomolysis by masking of a membrane-active agent

As previously mentioned in Section 1, endosomal escape is a limiting barrier in non-viral gene delivery. PEI takes advantage of the osmotic proton sponge effect, but polymers such as PLL possess no endosomolytic activity. One way to overcome this drawback is the incorporation of membrane-disruptive peptides in the vectors [45]. Thus, melittin, a cationic lytic peptide derived from bee venom, enhances the transfection efficiency of non-viral vectors [14]. Unfortunately, melittin displays high lytic activity at neutral pH. This has an unfavourable effect on the toxicity profile of the polyplexes when administered systemically because lytic activity is not restricted to the endosome. Rozema *et al*. showed how this problem may be solved: the lysines of the melittin were acylated with a dimethylmaleic anhydride derivative, which masks the lytic activity at neutral pH [46]. Following acidification, the maleamate shield is cleaved and the lytic activity of melittin is restored. This approach showed good results in the delivery of phosphorodiamidate morpholino ODNs (PMOs). PMOs are uncharged AS nucleotide analogues, which are endocytosed but are unable to escape from the endosome. The masked melittin and the PMOs were co-incubated with HeLa cells for the transfection experiments. Co-administration resulted in a 5- to 12-fold increase of AS efficiency in comparison to PMOs alone (dependent on the presence or absence of serum). These results are promising but for *in vivo* delivery, the membrane-disruptive peptide has to be associated with the vector.

5. Conclusion

The function of a delivery system is to overcome the biological barriers for reaching the intended/anticipated

target: the cytoplasm (e.g., siRNA and AS-ODNs) or the nucleus (for plasmid DNA). Shielding of particles is necessary so that vectors are not facing serious problems in the extracellular environment *in vivo*. For shielded particles, the presented data reveals that association and internalisation is not the limiting factor (or at least can be handled by the incorporation of targeting ligands), but subsequent intracellular delivery is a major barrier. The results show that reversibly shielded vectors have greater gene transfer efficiency than stable shielded polyplexes *in vitro* and *in vivo*, suggesting that intracellular delivery is positively affected by bioresponsive unmasking in the interior of the cell.

6. Expert opinion

At present, the theme in developing gene transfer vectors is to mimic nature's methods for delivery of nucleic acids. The first systems have been viral vectors. Many viral vectors escape immune surveillance, interact with cell membranes by receptor binding, internalise (via endocytosis), escape from endosomes, migrate to the nuclear envelope, enter the nucleus and finally take over cellular functions (a result of the long-lasting virus evolution). Until now, non-viral systems (cationic liposomes and cationic polymers) can only mimic a few parts of these delivery events.

Among the most widely used and promising non-viral vectors is PEI. It incorporates several of the required functions (DNA condensation, internalisation, endosomal buffering [which induces endosomal release] and dissociation from DNA) in only one polymer. Therefore, it looks at first sight to be an optimal all-in-one-device, a good starting point for developing inexpensive and easily producible gene transfer systems. However, on closer inspection, although the polymer has a broad range of functions, none of these are particularly in-depth. Even after refining with targeting devices, lytic peptides and shielding domains, *in vivo* transfection efficiencies of PEI polyplexes are still inferior to viral systems. This fact also counts for other non-viral systems. In the authors' opinion, this is mainly caused by the lack of bioresponsivity. Dynamic systems are necessary that sense their environment and assimilate (artificial viruses). The bioresponsive deshielding approaches that are presented in this review are steps towards such a nanodevice. The pH-dependent unmasking in the endosome could be compared with adenoviral properties. Nemerow *et al.* explored the adenoviral trafficking and discovered its protein VI as a membrane lytic factor associated with the adenovirus capsid [47]. Remarkably, the capsid reorganises under acidic conditions in the endosome, and the lytic protein IV is released and the virus escapes from the endosome.

As well as pH-responsive elements, other bioresponsive linkages (including disulfides and enzymatic degradable sequences) are the focus of ongoing investigations [17,48-51]. In addition, bioresponsivity goes beyond lytic and shielding functions: it should be a key feature of smart molecular therapeutics, which package their payload properly in the extracellular environment and release it after entering the cell in the bioactive form.

To form the ultimate therapeutic artificial virus, different strategies have to be compiled together and adapted to the specific characteristics and needs (e.g., variable intracellular pathways [52,53]) of the different biotherapeutics. Certainly, these systems will not be as simple to produce as, for example, the starting gene carrier PEI. This fact may indeed lead to criticism of non-viral systems: why not keep with viral vectors and optimise their developments only? There is a logical answer: viruses were designed by evolution for their propagation, and not for efficient and safe gene transfer. And their production is limited to the space of biological reactions, which is much more narrow than the diversity of chemical syntheses. The time will come where artificial systems outperform their natural counterparts [54,55].

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