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# Polymeric delivery systems for nucleic acid therapeutics: Approaching the clinic

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ABSTRACT

Gene therapy using nucleic acids has many clinical applications for the treatment of diseases with a genetic origin as well as for the development of innovative vaccine formulations. Since nucleic acids in their free form are rapidly degraded by nucleases present in extracellular matrices, have poor pharmacokinetics and hardly pass cellular membranes, carrier systems are required. Suitable carriers that protect the nucleic acid payload against enzymatic attack, prolong circulation time after systemic administration and assist in cellular binding and internalization are needed to develop nucleic acid based drug products. Viral vectors have been investigated and are also clinically used as delivery vehicles. However, some major drawbacks are associated with their use. Therefore there has been substantial attention on the use of non-viral carrier systems based on cationic lipids and polymers. This review focuses on the properties of polymer-based nucleic acid formulations, also referred as polyplexes. Different polymeric systems are summarized, and the cellular barriers polyplexes encounter and ways to tackle these are discussed. Finally attention is given to the clinical status of non-viral nucleic acid formulations.

## 1. Introduction

Increasing knowledge about genetic causes of disease has driven the development of nucleic acids as attractive strategies for therapeutic intervention. Nucleic acids can either restore deficient functional protein production or supress protein synthesis by inhibiting gene expression. However, the therapeutic applicability of nucleic acids is limited due to rapid degradation by nucleases, excretion or uptake by non-target tissues, immune activation by binding to Toll-like receptors, and inefficient cellular uptake due to their relatively large size and hydrophilic nature. Therefore, carrier systems that provide protection, guide nucleic acid tissue delivery and intracellular trafficking are required to nucleic acids as drugs. Various carrier systems including viral vectors, and natural and synthetic lipids and polymers have been investigated.

*Abbreviations:* AAV, adeno-associated virus; ABC, accelerated blood clearance phenomenon; ASGPR, asialoglycoprotein receptor; ASOR, asialoorosomucoid; AVET, adenovirus-enhanced transferrinfection; BBB, blood-brain-barrier; BPEI, branched PEI; CAG, Cys-Ala-Gly; CD, Cyclodextrin; CDP, cyclodextrin-based polymer; CFDA, China Food and Drug Administration; CFTR, cystic fibrosis transmembrane conductance regulator; Chol, cholesterol; DEAE, diethylaminoethyl dextran; DMAE, dimethylaminoethanol; DNase, deoxyribonuclease; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; dsRNAs, double-stranded RNAs; ECM, extracellular matrix; EMA, European Medicines Agency; EPR, enhanced permeability and retention effect; FGF, fibroblast growth factor; FR, folate receptor; GAGs, glycosaminoglycans; GI, gastrointestinal; HA, hyaluronic acid; HES, hydroxyethyl starch; HMW, high molecular weight; hTf, human transferrin protein; IL-2, Interleukin-2; LMW, low molecular weight; LNPs, lipid-based nanoparticles; LPEI, linear PEI; LPL, lipoprotein lipase; miRNA, micro RNA; mRNA, messenger RNA; PAMAM, polyamidoamine dendrimers; PAsn, N-substituted polyaspartamides; PBAs, phenylboronic acids; pDNA, plasmid DNA; PEG, poly(ethylene glycol); PEGMA, poly (ethylene glycol) methyl ether methacrylate; PEI, polyethylenimine; pHPMA, poly(*N*-(2-hydroxypropyl)methacrylamide); PLL, poly-L-lysine; PLO, poly-L-ornithine; PLR, poly-L-arginine; PLys, PEG-poly(lysine); PRRs, pattern recognition receptors; pSar, polysarcosine; RA, rheumatoid arthritis; REDV, Arg-Glu-Asp-Val; RGD, ArgGly-Asp; RISC, RNA-induced silencing complex; RNAa, RNA activation; RNase, ribonuclease; RRM2, ribonucleotide reductase; SARS-CoV-2, novel coronavirus; siRNA, small interfering RNA; TLRs, toll-like receptors; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; β-CDPs, cationic b-cyclodextrin-based polymers.

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Additionally, inorganic particles are explored as carrier systems.

This review focusses on polymeric delivery systems for nucleic acids, also highlighting some of the contributions of professor Sung Wan Kim to this field.

## 2. Therapeutic nucleic acids

Therapeutic nucleic acids can act at different stages of the gene expression process. Approaches to modulate gene expression can be divided into two main categories: *(i) increase or correct gene expression,* and *(ii) silence or block gene expression.*

### 2.1. Gene therapy

Gene therapy is a therapeutic modality to treat genetic diseases by transferring one or more therapeutic nucleic acids into patient's cells, correcting or substituting a defective gene. Effective strategies for clinical gene therapy are based on *in vivo* gene delivery to target cells or tissues, or *ex vivo* gene-modification of autologous cells (*i.e.* hematopoietic stem cells) that are transplanted back into the patient. Whereas the majority of therapeutic nucleic acids interferes in the DNA-mRNA-protein axis, the increased knowledge on the various RNA species that regulate gene expression but also other cellular processes has offered a range of new points of intervention (Fig. 1).

(i) *Increasing and correcting gene expression using gene expression constructs*

When a complete gene needs to be expressed, the therapeutic nucleic acid is usually supplied in the form of plasmid DNA (pDNA) as this enables cheap manufacture and long-term transcription into messenger RNA (mRNA) for efficient protein production. *In vivo* this requires a vector carrying the pDNA into the target cells of the patient. Among the various viral based vector systems, adeno-associated virus (AAV) vectors have demonstrated the greatest clinical success for *in vivo* gene delivery [1]. Given their array of serotypes and capsid variants, these vectors can target a wide variety of tissues and cell types. An important first step in gene-based drug development was the approval of the first gene therapy product Glybera, an AAV vector for the treatment of lipoprotein lipase (LPL) deficiency, by the European Medicines Agency (EMA) in 2012 [2]. Additionally, the use of AAV vectors has been successful in the treatment of ocular diseases, neurological disorders and hemophilia B [1].

Viral vectors represent the most efficient delivery vehicles since viruses have evolved in nature for the purpose of nucleic acid transfer into host cells. Despite their high transfer capacity, viral-based vectors also exhibit significant limitations, such as immunogenicity, limited transgene carrying capacity, restricted cell tropism, and complex analytics and difficult large-scale pharmaceutical production. Additional technical and biosafety issues for gene transfer systems are nuclear delivery of genes into off-target cells and long-term incorporation of DNA that may disrupt the natural host genome.

An interesting alternative approach for pDNA is mRNA. The cellular delivery of mRNA to its site of action does not require transport to and transcription in the nucleus. Furthermore, without the barrier for nuclear translocation synthetic delivery systems come into play that can circumvent many of the challenges that viruses face. For example, an optimized version of the synthetic polymer polyethyleneimine (PEI) has been successfully applied for the delivery of mRNA. Li et al. developed a mRNA-based intranasal vaccination system with PEI for HIV-1 treatment [3]. It was shown that this delivery vehicle overcame the biological barriers in the nasal epithelium by reversibly opening the tight junctions, enhancing the paracellular delivery of mRNA and achieving strong anti-HIV immune responses. Formulation of mRNA into lipid-based nanoparticles (LNPs) enabled efficient *in vivo* delivery of mRNA encoding human anti-HER2 antibody (trastuzumab). Treatment of tumor-bearing mice with trastuzumab mRNA LNPs reduced the volume of HER2-positive tumors and improved animal survival [4]. Furthermore, all currently investigated SARS-Cov2 mRNA vaccines are lipid-based [5,6]. Finally, small nucleic acids can be used to correct or enhance gene expression as well. For example, exon-skipping oligonucleotides can ensure that mutated exons can be omitted from a mRNA, which can provide therapeutic benefit for example in Duchenne's muscular dystrophy [7,8].

(ii) *Silencing and blocking gene expression using antisense and RNAi nucleic acids*

Gene expression can also be inhibited at the mRNA level. Antisense nucleic acids inhibit gene expression by binding to complementary target mRNA, resulting in RNase-dependent degradation or translational repression of the target mRNA which supresses the translation of the target protein. A second class of therapeutic nucleic acids regulating

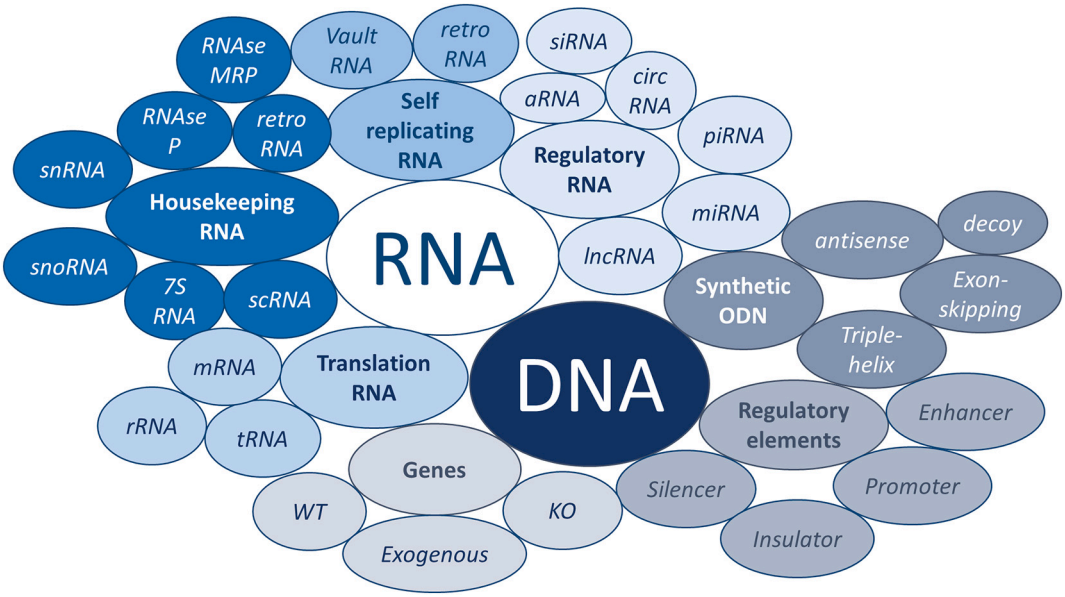


**Fig. 1.** Gene expression regulating RNAs and DNAs that constitute possible targets for therapeutic intervention.

gene expression are small interfering RNAs. These small non-coding RNAs include double stranded small interfering RNA (siRNA) and micro RNA (miRNA). When siRNA is delivered into the cytosol, one of the two 20–30 bases long RNA strands is degraded. The remaining strand of the siRNA duplex, the guide strand, is selectively retained in the multiprotein RNA-induced silencing complex (RISC). The siRNA guide strand directs the siRISC to the target mRNA through complementary base pairing, resulting in gene knockdown of the target RNA (Fig. 2) [9]. An analogous process is the posttranscriptional regulation of gene expression by miRNAs which are small non-coding endogenous RNA molecules (~22 nucleotides long) that regulate cellular fate and function mostly by translational repression but sometimes also mRNA cleavage. Some miRNAs have been shown to regulate cell proliferation and apoptosis processes that are important in cancer treatment. For example, miR-182-mediated down-regulation of BRCA1 has been shown to impact DNA repair and sensitivity to PARP inhibitor, thus may impact breast cancer therapy [10]. miR-19b promotes tumor growth and metastasis *in vivo* via targeting p53. Therefore, it is possible that miR-19b antagomirs could be developed as therapeutic agents against tumor development [11]. Moreover, different studies have shown that several miRNAs are directly involved in human cancers, including lung, breast, brain, liver, colon cancer, and leukemia [12]. Additionally, some miRNAs may function as oncogenes (oncomiRNAs) or tumor-suppressor *miRNAs* [12]. OncomiRs are negatively regulating tumor suppressor genes and/or genes that control cell differentiation or apoptosis, whereas tumor-suppressor miRNAs inhibit these processes. Overexpression of oncomiRs and loss or underexpression of tumor-suppressor miRNAs in cancer promote neoplasm development. miRNA therapy could therefore be a powerful therapeutic approach. Cancer development can potentially be suppressed by introducing tumor-suppressing miRNAs or antisense miRNA antagonists (antogomirs) that inhibit oncomiRNAs and tumor-promoting activity.

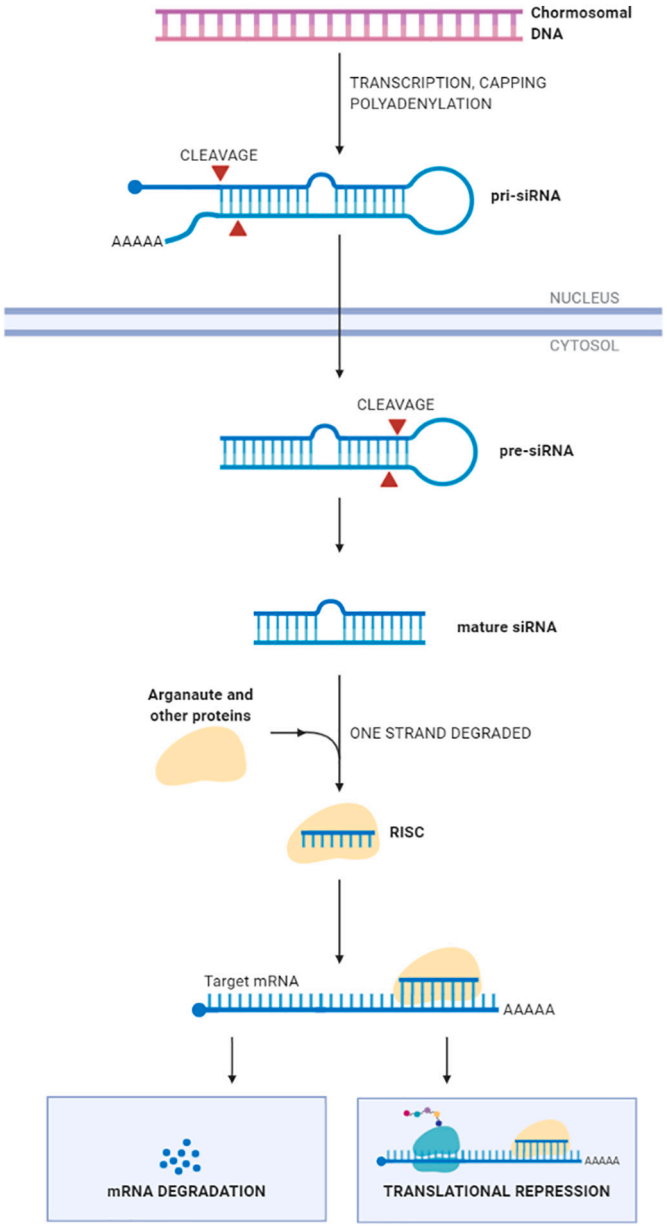


**Fig. 2.** RNA interference. mRNA is formed after removal of introns from the pre mRNA, a process that is described as splicing. Endogenous siRNA precursors are self-complementary and form a hairpin-like structure. Mature siRNA is formed after multiple cleavage steps and incorporated into RNA-induced silencing complex (RISC), resulting in translational repression and/or degradation of target RNA.

In a surprising twist, it recently emerges that promoter-targeted short double-stranded RNAs (dsRNAs) can also induce target gene expression at the transcriptional or epigenetic level. It has been suggested that RNA activation (RNAa) is an evolutionarily conserved mechanism of gene regulation and can be activated by siRNAs or miRNAs [13]. These developments underline that nucleic acids still hold many surprises on the many ways they can regulate cellular processes.

### 2.2. Challenges in nucleic acid delivery

Nucleic acids offer excellent opportunities for therapeutic applications when the delivery problem can be solved. All nucleic acids exceed the size of conventional small drugs. Additionally, they are easily degraded by nucleases present in physiological fluids, leading to limited biological stability. Importantly the hydrophilic nature and overall strong negative charge restricts their passage through lipid cell membranes into target cells. Finally, even when limited intracellular uptake occurs, this likely results in localization of nucleic acids in the early endosomes and lysosomes where they are degraded by endolysosomal enzymes. All these factors contribute to a poor therapeutic efficiency and limit the development of nucleic acid therapy. Therefore, the complexation and protection of nucleic acids with appropriate vehicles are required for successful delivery and to exploit the therapeutic potential of these drug candidates.

Oligonucleotides may form the only exception. Like small molecules they can be chemically synthesized which allows a wide variety of chemical modifications to be introduced, which can stabilize them against degradation, allow transport over cellular membranes, improve binding to complementary nucleic acid target sequences, and reduce immunogenicity. For example, equipping an siRNA with a tri-antennary *N*-acetyl galactosamine results in specific uptake by the asialoglycoprotein receptor (ASGPR) on hepatocytes [14]. This strategy has recently reached the market as Givosiran, an siRNA to treat hepatic porphyria [15].

### 2.3. Nanoparticle-based gene delivery system

Various types of (cationic) formulation materials, including natural and synthetic lipids and polymers, have been explored to facilitate complexation of the nucleic acid payload by electrostatic interaction with positively charged excipients into nanoparticles that have an appropriate size to be internalized by cells and to guide intracellular trafficking of exogenous nucleic acids. Over the years, a great variety of cationic polymers have been used for the development of polyplex formulations, where charge density, pKa, molecular weight, branching, biodegradability and membrane interaction have emerged as important factors in success of the polymeric excipients.

## 3. Polyplex delivery hurdles

For efficient nucleic acid delivery, two seemingly incompatible qualities need to be combined into the polyplex: protection and stability outside the target cell and degradation of the carrier and release of intact nucleic acid after internalization. These processes are discussed in more detail in the next sections.

### 3.1. Extracellular delivery barriers

For nucleic acid delivery, both local and systemic administration approaches have been considered. Local administration includes direct

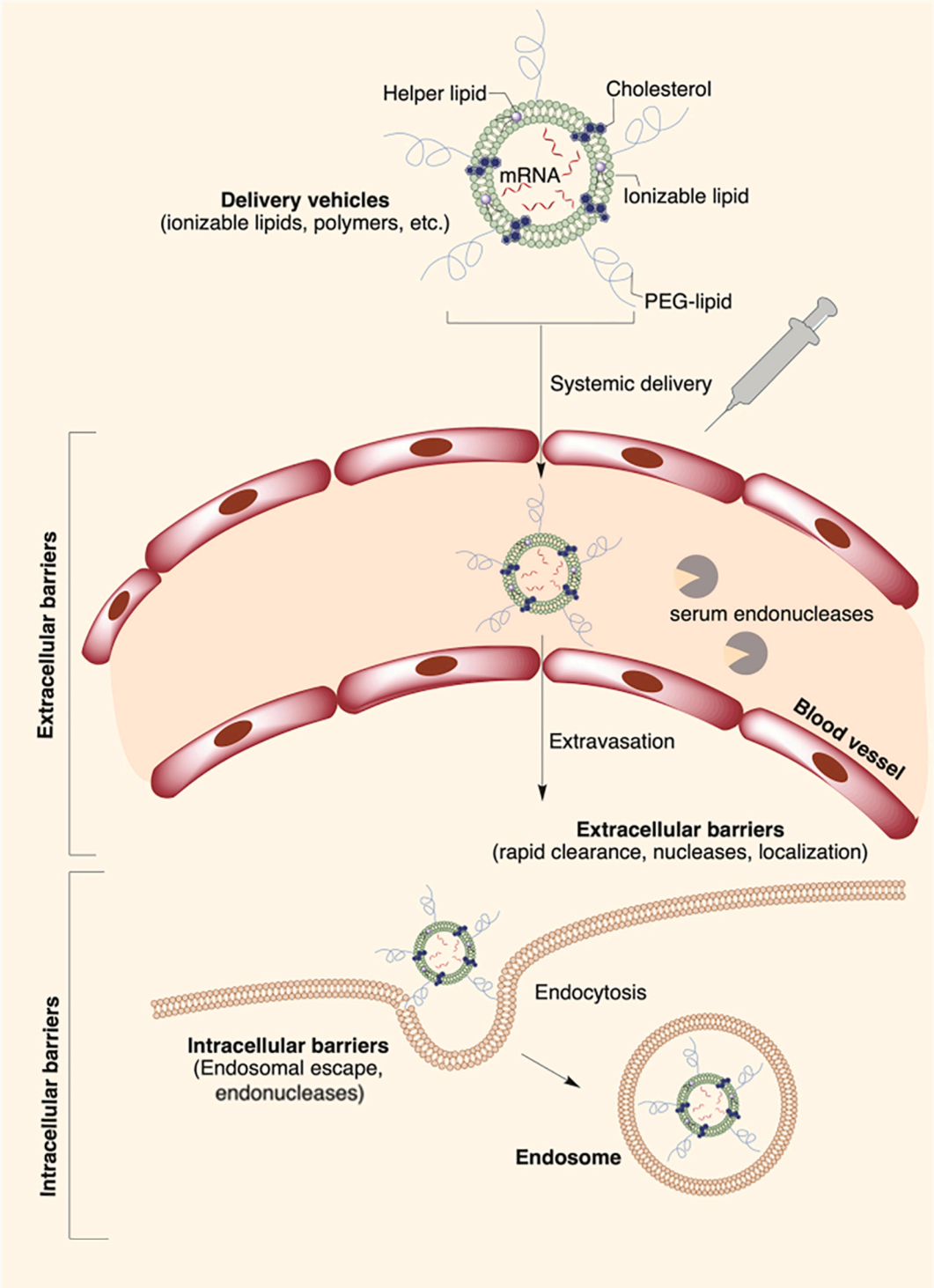


**Fig. 3.** Schematic representation of extra-and intracellular barriers. Delivery vehicles are used to protect nucleic acids (NAs) (*i.e.* mRNA) against degradation by extracellular nucleases and shield their negative charge. The delivery vehicle must cross the negatively charged cell membrane in order to reach the cytoplasm which creates another barrier for the negatively charged NAs. Upon cellular entry, the vehicle is entrapped within endosomes. Commonly used delivery vehicles are lipid nanoparticles (LNP), consisting of (i) an ionizable lipid or polymeric material, to encapsulate NAs; (ii) a helper lipid that resembles the lipids in the cell membrane; (iii) cholesterol to stabilize the lipid bilayer of the LNP; and (iv) polyethylene glycol (PEG) to improve stability, and reduce protein adsorption. Adapted from [44].

injection (for example into the skin, retina, or tumor) and topical delivery. Additionally, several drug delivery systems have been investigated for nasal and pulmonary delivery [6]. These administration routes are limited to interaction with the local tissue and lead to a very limited systemic exposure. Also classical routes for reaching systemic circulation, *i.e.*, oral and intestinal administration, are associated with significant barriers including degradation in the gastrointestinal (GI) tract and virtually absent transport over the GI epithelium, resulting in an extremely low or absent bioavailability. Because of these barriers, intravenous administration remains the main route for delivery of nucleic acids to many tissues. However, even after intravenous drug administration extracellular barriers are considerable (Fig. 3). Nucleic acids are rapidly cleared from the blood by the kidneys when their size is <30 kDa whereas larger nucleic acids are generally cleared by the mononuclear cells in liver and spleen [16]. As a result, the nucleic acids would not reach the target cells. Therefore, nanoparticles can help to protect nucleic acids against enzymatic degradation and prolong their circulation kinetics.

Polyplex formation is based on ionic interactions between polyanionic nucleic acids and multivalent cations [17,18]. This process allows the preparation of nanoparticles that contain the nucleic acids in compacted and protected form. A critical issue is polyplex stability in blood and other biological fluids. After systemic administration, both serum proteins and extracellular matrix (ECM) act as extracellular barriers to polyplex-mediated delivery because of their ability to unpack polyplexes [19].

To get stable polyplexes that protect the loaded nucleic acid against nuclease triggered degradation, mostly an excess of cation polymer is used. This results in the formation of polyplexes with an overall positive charge. Upon intravenous administration, such particles interact with negatively charged blood components (*i.e.* with blood cell membranes and plasma proteins). Serum proteins bind to positively charged particles, leading to structural reorganization, aggregation and/or dissociation of the delivery vehicle [19–22]. An additional problem is nucleic acid degradation by nucleases present in serum, which leads to a loss of biological activity. To induce nuclease resistance, studies with tight associations between the delivery vehicle and DNA have been performed. However, these studies neglect that the delivery vehicle needs to be capable of disassembly within the cell [23]. Other consequences that have an important impact on the fate of administered polyplexes are activation of the immune system, self-aggregation into larger microstructures, and binding to erythrocytes and other blood components. Association of the delivery vehicle with erythrocytes reduces transfection efficiency and leads to rapid clearance from the circulation *via* liver and spleen. In order to prevent these kinds of interactions and to obtain better circulation properties, there is a need for stable package of polyplexes, for example by covalently cross-linking the core of polymeric systems and shielding of the positive polyplex surface charge. The predominant strategy to sterically shield the delivery vehicles from blood components is to utilize PEGylated components, discussed later in more detail (Section 5.1) [24–26]. A stabilized and shielded nanoparticle is able to reach vascularized target sites *via* systemic circulation, and can target certain cancer tissues by passive targeting strategies exploiting the so called EPR (enhanced permeation and retention) effect [27]. However, binding to target cells and uptake might be limited. For example, nanocarriers with a positively charged surface show efficient internalization triggered by electrostatic interactions with the negatively charged cell membranes *in vitro*, but have limited applicability *in vivo* because of rapid elimination and self-aggregation or aggregation with components in the bioenvironment.

Polyplexes have different sizes, varying from ten to up to several hundreds of nanometres. Larger size polyplexes contain multiple copies of the nucleic acids [28,29]. The polyplex size is a critical parameter for systemic administration, bearing a big impact on the biodistribution and pharmacokinetics. Nanoparticles with a small diameter (< 6 nm) are rapidly cleared by the kidneys [30]. In contrast, larger particles with a diameter of up to 400 nm, can accumulate in highly vascularized solid tumors and impaired lymphatic drainage systems, through a process also referred to as the enhanced permeability and retention (EPR) effect [31]. The EPR effect has been extensively explored in nanocarrier-assisted cancer therapy. However, the extent of passive accumulation and the size threshold of the porous tumor vasculature is depending on the type of cancer and can be heterogeneous [32]. It also critically depends on a long circulation time which is difficult to achieve even for PEGylated polyplexes. To explain, the circulation half-lives for pegylated polyplexes in mice is 1–5 h [26,33–37], whereas pegylated liposomes [26,33,34,38–41] and polymeric nanoparticles (excluding polyplexes) [33,42] have half-lives of 12–24 h.

Additional challenges are the accessibility *via* the bloodstream of some tissues since the blood vessel structure in many tissues is extremely tight. For example, the blood-brain-barrier (BBB) cannot be passed by passive processes. Endogenous proteins and nanoparticles make use of efficient intra-and transcellular delivery processes to cross such barriers, which could also be used for therapeutic approaches in nanoparticle transport. Adding ligands to the surface of polyplexes may enhance target cell binding and receptor-specific cellular internalization. Important factors to take into consideration when selecting target receptors and corresponding ligands are abundance, selectivity of expression and function. There is a need for a sufficient number of receptors on the surface of target cells, that should be selectively expressed on the tissue of interest, and these receptors should of course be involved in processes of endocytosis and transcytosis. The performance of targeted delivery might be influenced by affinity of the ligand for the receptor, concentration of competing endogenous natural ligands and nanoparticle size. Furthermore, incorporation of multiple receptor ligands for different receptors can be considered [43]. This allows to involve in subsequent stages of the delivery process, for example: transcytosis across the endothelial barrier, followed by cellular uptake within the target tissue.

Delivery vehicles are used to protect nucleic acids (*i.e.* mRNA) against degradation by extracellular nucleases and shield their negative charge. Upon cellular entry of the delivery vehicle loaded with the therapeutic nucleic acid of interest, it is entrapped within the endosome form which need to be escaped in order to reach the cytoplasm and even the nucleus for pDNA. This is discussed in more detail in the next section. Commonly used delivery vehicles are lipid nanoparticles (LNP), consisting of (i) an ionizable lipid or polymeric material, to encapsulate NAs; (ii) a helper lipid that resembles the lipids in the cell membrane; (iii) cholesterol to stabilize the lipid bilayer of the LNP; and (iv) polyethylene glycol (PEG) to improve stability, and reduce protein adsorption [44].

### 3.2. Intracellular barriers

After arrival in the target tissue nucleic acids still need entry across the cell membrane and transfer into the cytosol to be active. The most widely studied transduction vehicles for the internalization of proteins into the cell are the antennapedia peptides, the herpes simplex virus VP22 protein, and HIV TAT protein [45]. This mechanism of docking and fusing is used by some types of viruses to infect host cells. Transfer efficacy and cytotoxicity are closely related since cell membrane integrity is important for cell survival.

Nonenveloped viruses and protein toxins use an alternative process for cell entry. They are engulfed into endolysosomal compartments and subsequently escape from these vesicles into the cytosol. Nanoparticles are usually internalized into primary endosomes [46]. Subsequent vesicle transport through the cytosol and sorting in various compartments, like recycling to cell surface or transcytosis, trafficking into organelles (*i.e.* Golgi System and endoplasmic reticulum), or maturation into lysosomes, are influenced by receptor and vesicle type, nanoparticle size and ligands, and receptor crosslinking [43]. Importantly, enzymatic degradation in the lysosome must be avoided to ensure an efficient

delivery of the loaded nucleic acids into the cytosol/nucleus. Many viruses and toxins have the ability to either escape early out of endosomes, or avoid endolysosomal sorting.

Even after arrival in the cytoplasm, several challenges remain. Intracellularly released plasmid DNA (pDNA) has to be transported to the nucleus, whereas the targets of mRNA, siRNA and miRNA are located in the cytosol. Cytosolic mobility and nuclear entry are size dependent: oligonucleotides generally distribute over the cytosol, whereas larger nucleic acids (>250 bp) have a substantial lower mobility. Passage of the nuclear membrane can be a major hurdle as this process is highly restricted and dependent on the correct molecular signals [47,48]. Experiments have shown that direct injection of naked transgene pDNA into the nucleus resulted in a more efficient gene expression compared to cytosolic injection. Moreover, pDNA is guided by direct transport or microtubular vesicle transport for efficient delivery. Several interesting studies have investigated delivery of DNA into the nucleus DNA by active transport domains [49,50]. Despite partial successes, to date no efficient solution exists for intranuclear delivery of polyplexes or encapsulated cargo [43].

## 4. Cationic polymers

### 4.1. First generation polycations

The first generation polycations include a variety of permanently charged polymers usually consisting of repetitions of one single monomer. In 1965, it was found that diethylaminoethyl (DEAE) dextran could strongly enhance the transfection of various infectious viral nucleic acid constructs (poliovirus RNA and SV40 viral DNA) (Fig. 4) [51]. Later, other cationic polymers such as poly-L-lysine (PLL), poly-L-ornithine (PLO), and poly-L-arginine (PLR), showed the potential for nucleic acid delivery into cells. In 1975, Farber and Butel evaluated the efficiency of these polymers for exogenous DNA transfection and their studies showed that polyornithine had the best performance [52]. The positively charged polyornithine forms a complex with DNA, thereby shielding it from nuclease attack and degradation, and it may also neutralize the negatively charged groups of DNA protecting it from rejection by target cells. However this property is shared by the other poly-amino acids that were tested. Only recently a more detailed investigation points to the structural differences between the two amino acids that affect cellular and subcellular processing [53]. The switch to polymer-based transfection of plasmid-based gene constructs showed a

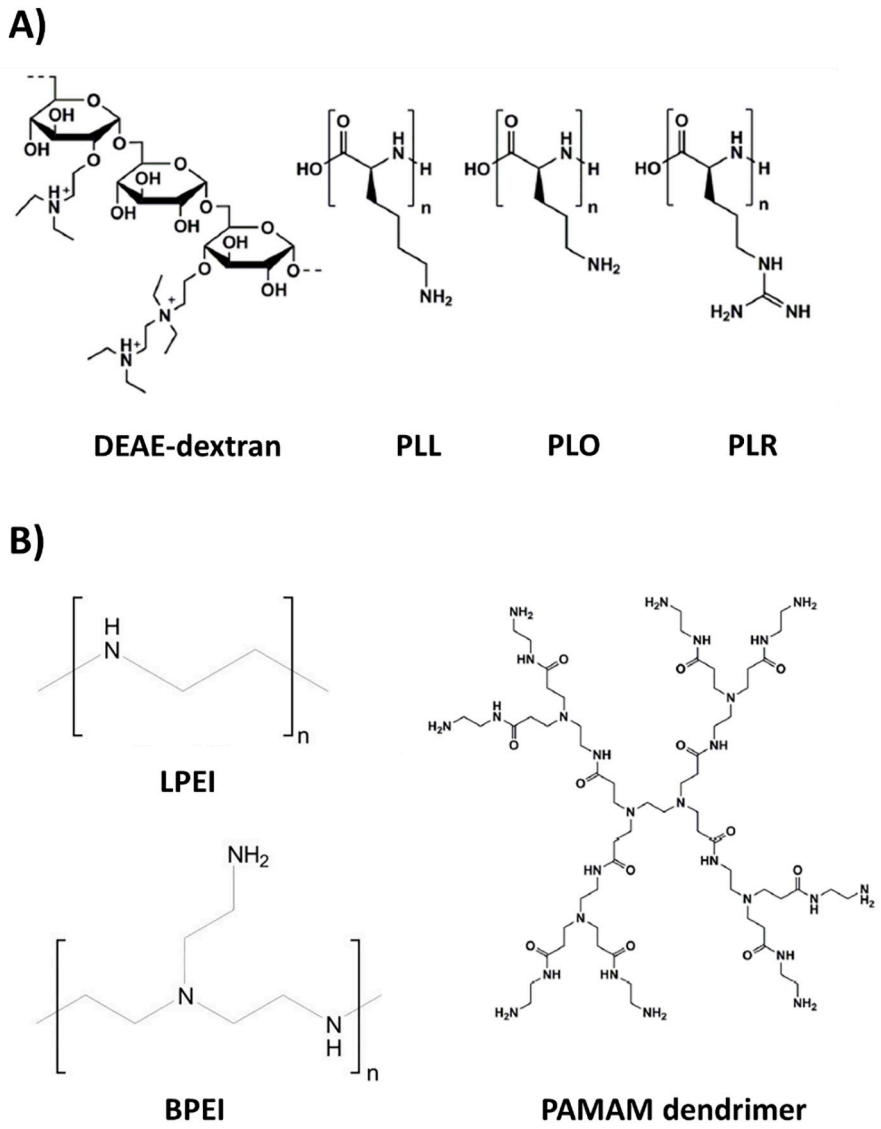


**Fig. 4.** Polymers for nucleic acid delivery. A) First generation polymers. DEAE-dextran, diethylaminoethyl-dextran; PLL, poly-L-lysine; PLO, poly-L-ornithine; PLR, poly-L-arginine. B) Second generation polymers. Polyethylenimine in linear (LPEI) or branched (BPEI) form, and poly(amidoamine) PAMAM dendrimer [43,68].

moderate transfection efficacy compared to the delivery of infectious viral genomes. Different approaches were taken for polyplex improvement. In 1987, Wu and Wu reported receptor-mediated gene transfection by covalently coupling ASOR, a ligand for asialoglycoprotein receptor that is expressed on hepatic cells, to PLL. Next, they combined the PLL-ASOR conjugate with pDNA to form receptor-targeted polyplexes and investigated receptor-mediated delivery to hepatocytes both *in vitro* and *in vivo*. Importantly, enhanced cellular uptake by endocytosis and receptor specificity was convincingly demonstrated [54]. Following this milestone, many other ligand-PLL-based strategies were developed, including the first human clinical gene therapy studies with pDNA polyplexes [55]. In this study the authors developed a human melanoma vaccine consisting of irradiated autologous tumor cells transfected with a human IL-2 gene construct, using transfection complexes consisting of inactivated adenovirus linked to the human IL-2 plasmid, and polylysine-modified transferrin. This receptor-mediated, adenovirus-augmented gene delivery system has been termed AVET (adenovirus-enhanced transferrinfection) [56,57].

Another approach to improve the transfection efficiency of polyplexes focussed on the use of excipients. In 1983, Luthman and Magnusson demonstrated that the anti-malaria drug chloroquine significantly enhanced pDNA/DEAE-dextran transfection [58]. In another study, Cotten and Birnstiel demonstrated that gene transfer into K562 cells using polylysine-transferrin polyplexes, was strongly enhanced by this treatment [59]. It is assumed that chloroquine accumulates in lysosomes and reduces the activity of lysosomal nucleases, thus protecting pDNA in the polyplexes from degradation [60]. The natural endosomal acidification process is mediated by the vacuolar ATP-dependent vATPase that generates an acidic luminal microenvironment of pH 5.9–6.5 in early endosomes and of pH 5.0 in late endosomes and lysosomes, by actively pumping protons into the endosomes and lysosomes. The unprotonated form of the weakly basic drug chloroquine can freely diffuse across the membranes, but is entrapped in its protonated form in acidifying endosomes and lysosomes. A continuous vATPase activity results in accumulation of protonated drug in the vesicles and influx of chloride counterions and water consequently leading to osmotic swelling and membrane destabilization of endolysosomes. Furthermore, chloroquine may facilitate intracellular release of DNA by dissociation of polyplexes [61].

Besides the use of chloroquine more natural approaches have been investigated to trigger release of polyplexes form endosomes. It is known that viruses such as adenoviruses and rhinoviruses promote nucleic acid transfer by destabilizing the endosomal membrane. Additionally, different kinds of proteins and peptides were investigated to promote endosomal escape and enhance gene transfer. Wagner and co-workers demonstrated that synthetic influenza-virus derived peptides increase membrane disruption activity and gene transfer [62]. The group of Szoka designed a cationic amphipathic peptide KALA that binds to DNA, causes membrane disruption, and mediates nucleic acid delivery and transfection [63]. Szoka and collaborates also developed the GALA peptide and conjugated it to positively charged polymers for DNA delivery [64].

### 4.2. Second generation polyplexes

Polyamidoamine (PAMAM) dendrimers were developed in the 1990s as carriers with improved properties compared to polymers of the first generation (Fig. 4). These polyamine dendrimers were found to be effective in pDNA transfer in the absence of chloroquine or fusion peptides. Characteristic for PAMAM dendrimers is that they display a high amine density with a pKa around 6. This pKa value around the pH of an acidifying endosome is suggested to lead to proton of the amines which in turn results in an enhanced influx chloride anions into the endosome. As a consequence, the osmotic pressure increases and the endosome will burst. This mechanism is called the ‘proton sponge effect’ and was first proposed in 1995 by Behr and colleagues, but the hypothesis is debated [65,66]. In other studies it has been suggested that positively charged polymer domains have a destabilizing effect on the endosomal membrane which contributes to the endosomal escape of polyplexes [51,67].

Behr et al. screened a series of cationic polymers with proton-sponge characteristics which led to the discovery of polyethylenimine (PEI) as a powerful transfection polymer [65]. PEI has a high transfection efficiency both *in vitro* and *in vivo* at dosages where the polymer displays limited cytotoxicity. However, toxicity of PEI can be argued. Depending on the molecular weight and cationic charge density, PEI as well as other frequently used polycationic macromolecules evaluated as gene delivery systems display cytotoxic effects *in vitro* [69]. In order to reduce toxicity of polycations the use of low molecular weight PEI [70] also studied by Sung Wan Kim and collaborators in combination with cholesterol [71] and PEGylation have been proposed [72,73]. These polymers indeed showed a better cytocompatibility in their free form and complexed with nucleic acid than high molecular weight PEI.

A large variety of branched PEI (BPEI) and linear PEI (LPEI) with different molecular weights are commercially available, which allows tuning of polyplex stability, transfection efficacy, and cytotoxicity [74]. Because after extracellular protection nucleic acid unpackaging has to take place intracellularly for functional delivery, high polyplex stability does not necessarily correlate with high transfection. Intracellular trafficking studies performed by Itaka and collaborators showed that, compared with PLL, both LPEI and BPEI were able to mediate endsosomal escape, but LPEI presented higher efficiency in gene transfection and expression compared to BPEI likely due to the lower stability [75]. Interestingly LPEI displays a better nuclear delivery of pDNA in non-dividing cells, whereas BPEI polyplex based transfection is more efficient in dividing cells in or before mitosis. Apparently LPEI based polyplexes can even be transported in intact nuclei whereas BPEI relies on breakdown of the nuclear envelope [51].

Polymers like PEI and PAMAM are promising for nucleic acid transfection, but also display some disadvantages. They are not degradable and still display a certain degree of toxicity which is correlated to their molecular weight. PEI can trigger apoptosis and necrosis by generating defects in cell surface and mitochondria or nuclear membranes. PEI cytotoxicity also includes the inhibition of mitochondrial ATP synthesis. Additionally, PEI and other cationic polymers such as PAMAM and PLL are recognized by the innate immune system and induce complement activation *in vitro* [76]. As pointed out in more detail in the next section, PEGylation of polyplexes might reduce protein adsorption on their surfaces and be an approach to inhibit complement activation.

In summary, the first two generations of polycations are efficient transfection reagents, primarily *in vitro*, and have increased the knowledge about gene delivery and the underlying mechanisms contributing to functional delivery. However, critical problems as cytotoxicity leading to undesired side-effects upon *in vivo* administration of cationic polymers remain. Therefore, polymer systems should be generated with improved precision and reduced cytotoxicity.

## 5. Lessons learned: the importance of shielding, targeting and trafficking

The work on the first generations of polymeric delivery systems have taught important lessons on the importance of shielding the polyplex surface, increasing cell type specificity by attaching targeting ligands and guiding the intracellular trafficking of polyplex and payload. This section discusses the approaches taken to improve these aspects.

### 5.1. Shielding domains

The surface character of a nanoparticle is of great importance for its behaviour after systemic administration. In cell culture transfections, the positive charge of cationic polyplexes can be advantageous for binding to negatively charged cell surfaces and subsequent

internalization. However, the positive surface charge of polyplexes leads to undesired effects in the extracellular space. Acute toxicity is triggered by dissociation and aggregation of polyplexes and blood cells, and unspecific interactions with blood components and non-target cells [76]. These reactions can be reduced by coating the polyplexes with hydrophilic macromolecules to shield the surface charge from the exterior environment, and reduce unwanted interactions and thus enhance their circulation kinetics [77–79]. The most commonly used and investigated shielding agent is poly(ethylene glycol) (PEG). This polymer strongly binds three water molecules per repeating ethylene oxide units to form a hydrated shell covering the nanoparticles and thereby increase their colloidal stability and sterically reduce interactions with serum proteins and cells, and extends circulation lifetime of polyplexes [19,37]. For example, PEGylation of PEI affects polyplex stability in a dose-dependent manner after intravenous administration in mice. Administration of a high dose PEG-PEI showed a more favourable organ deposition and significantly lower acute *in vivo* toxicity [80]. Furthermore, pH-triggered removal of the PEG shield within the endosome might be advantageous. Reversibly shielded polyplexes enhance gene expression *in vitro* and *in vivo*, compared to stably shielded polyplexes [81].

However, an important drawback of PEG is the formation of PEG specific antibodies, particularly after repeated administration of PEGylated nanoparticles [82]. This immunogenic response is known as the 'accelerated blood clearance (ABC) phenomenon' and results in increased clearance and reduced efficacy of PEGylated nanocarriers [83]. Therefore, alternative hydrophilic polymers were investigated for shielding, including poly(*N*-(2-hydroxypropyl)methacrylamide) (pHPMA), hydroxyethyl starch (HES), poly(oxozalines), hyaluronic acid (HA) and polysarcosine (pSar) [84–86]. The latter polymer has been demonstrated to have both neglectable complement activation and immunogenicity in various *in vivo* models [87] and aggregation of nanoparticles surface-grafted with this polymer in human serum is not observed [88]. Additionally, pSar shielding strongly reduces aspecific binding and cellular uptake of polyplexes. Furthermore, *in vivo* biodistribution studies demonstrated that pSar-shielded polyplexes clearly enhance blood circulation time compared to unshielded polyplexes and similar to PEG-shielded polyplexes [89].

Different pre-or postintegration strategies for the coating of polyplexes will hydrophilic polymers exist. For pre-integration, block copolymers of a hydrophilic and uncharged block and a cationic block such as PEGpLys and PEGpDMAEmA [90–92], pHPMA-pTMAEM [93], or PEG-pAsp(DET) [94,95] were used for the preparation of polyplexes. The block copolymers are able to form core/shell polymeric micelles with a cationic core that is neutralized by the loaded nucleic acid, and an outer hydrophilic polymer shell. Importantly, the polyplex characteristics are controlled by the length and ratio of hydrophilic polymer to cationic polymer within the block copolymer [96,97]. The first step in postintegration strategies is the formation of non-shielded polycation/nucleic acid polyplexes, followed by incorporation of the hydrophilic coat *via* either covalent coupling [26,98], or noncovalent association of PEG [99–101]. In this manner, the compaction of nucleic acids is not compromised by the shielding polymers. However, drawbacks of this strategy might appear in scaling-up, for example less chemical control on the coat attachment as compared to the use of block copolymers, possibly requesting improved polyplex purification methods for removal of nonincorporated reagents.

Polyplex shielding offers great advantages for systemic delivery in gene therapy. Shielding reduces nanoparticle aggregation, strongly improves biocompatibility, and significantly extends the blood circulation time, however not to the extent as has been observed for correspondingly modified polymeric and liposomal drugs, as discussed in Section 3.1. Polyplex stability and circulation time can be further improved by covalent cross-linkage with disulphide bonds and decationization, respectively [102–104]. Furthermore, polyplex shielding from unspecific extracellular interactions may reduce intracellular efficacy in transfecting the target cells ("PEG dilemma") [105,106]. For example, PEGylated PEI polyplexes strongly reduced their transfection activity, which was recovered by introducing endosomal pH-sensitive bonds for PEG attachment [81].

### 5.2. Targeting ligands

Polyplexes composed of cationic polymers and nucleic acid and prepared at a high N/P (charged nitrogen groups of the polymer and negatively charge of phosphate groups of the nucleic acids) attach to the cell surface *via* electrostatic interactions between the opposite charges of polyplexes and cell membranes [18,107]. This non-specific cellular attachment may lead to internalization of the polyplexes by other cell types than the desired ones. For efficient gene delivery, polyplexes are therefore modified with certain ligands and peptides that assist in their interaction receptors present on target cells. (Table 1) [31]. Several small chemical compounds (vitamins, folate), carbohydrates, peptides (among which ArgGly-Asp (RGD), Arg-Glu-Asp-Val (REDV) and Cys-Ala-Gly (CAG)), proteins (such as transferrin), antibodies have been explored to modify gene carriers. It has been demonstrated that targeting delivery can enhance cellular recognition and uptake, especially by cancer and endothelial cells [108–110]. Transferrin, an iron transport protein that targets the transferrin receptor that is commonly expressed on growing tumor cells, has been utilized as a targeting ligand to enhance the endocytosis of polyplexes and has been used in several human clinical studies. For example, in the first polymer-based human gene therapy study for anticancer vaccination, transferrin-polylysine was used for the delivery of interleukin-2 (IL-2) pDNA polyplexes into

**Table 1**
Examples of receptors and ligands used in targeted polyplexes.

| Target tissue | Receptor | Targeting ligand | Polymer |
|---|---|---|---|
| Tumor | Transferrin R | Transferrin, B6 peptide, T7 peptide | pLys, protamine, dend pLys, PEI, PEG-PAMAM, PEG-PEI, PEG-CD, PEG-STP |
| | EGF R | EGF, EGF peptide, GE11 peptide, anti-EGF AB, TGF-$\alpha$ | pLys, PEI, PEG-PEI, PAMAM, PEG-STP |
| | HER2/neu | Anti-Her2 AB | pLys, PEI |
| | Folate R | Folate, MTX | PEG-pLys, PEG-PEI, PEG-STP |
| | Integrin | RGD peptide | pLys, PEG-PEI, PEI, PAE |
| | CD13 | NGR peptide | PEI/PEG |
| | CXCR4 | Plerixafor (AMD) | PEG-polyAMD |
| Brain | Transferrin R | Transferrin | PEG-PAMAM |
| | Lactoferrin R | Lactoferrin | PEG-PAMAM |
| | GABA(B) R | RVG29 peptide | PEG-PAMAM |
| Liver | ASGP R | ASOR, tri/tetrameric galactose, lactose-$\alpha$-CD, tri-NAG | pLys, PEI, PAMAM, PEG-masked DPC |
| | Hepatocyte | Malaria protein CS | pLys |
| | LDL R family | RAP | pLys |
| Lung | Polymeric Ig R | Antisecretory component AB | pLys |
| | Airway cells | Surfactant A, B | pLys |
| | PECAM | Anti-PECAM AB | PEI |
| | $\beta$2-R | Clenbuterol | PEI |
| | IPI | Iloprost | PEI |
| | Insulin R | insulin | PEI |
| | Lactoferrin R | Lactoferrin | PEI |

Abbreviations: AB, antibody; ASGP, asialoglycoprotein; ASOR, asialoorosomucoid; CD, cyclodextrin; dend pLys, dendritic polylysine; DPC, Dynamic PolyConjugate; Ig, immunoglobulin; LDL, low-density lipoprotein; NAG, *N*-acetyl galactosamine; PAMAM, poly(amido amine); PEG, polyethylene glycol; PEI, polyethylenimine; PPI, polypropylenimine; pLys, polylysine; R, receptor; RAP, receptor-associated protein; STP, Stpbased sequence-defined oligomer. Adapted from [43].

patient derived primary melanoma cell cultures [55]. More recently, the first in-human Phase I clinical trial involving siRNA-mediated gene silencing through systemic administration of a targeted nanoparticle delivery system was conducted. Transferrin was used for *in vivo* targeting of PEGylated cationized siRNA polyplexes to transfect solid cancers of patients [111]. These nanoparticles consisted of a cyclodextrin-based polymer (CDP) with human transferrin protein (hTf) displayed on the exterior of the particle as targeting ligand, and a PEG-coat to enhance nanoparticle stability and reduce aspecific binding to non-target cells. siRNA designed to reduce the expression of the M2 subunit of ribonucleotide reductase (RRM2), an established anti-cancer target, was loaded in the nanoparticles. The amounts of intracellularly-localized nanoparticles correlated with dose levels of the nanoparticles administered and a reduction in both the specific RRM2 mRNA and protein when compared to pre-dosing tissue was observed. Another frequently applied ligand targeting of both soluble drugs and drug loaded nano particles, including polyplexes, is folate [112,113]. The folate receptor (FR) is overexpressed on a large number of tumors (*i.e.* breast, lung, kidney, ovarian) and can consequently serve as target for selective delivery of anti-cancer treatment [114]. For example, folic acid was used as cell targeting ligand for receptor-mediated siRNA delivery *in vitro* and *in vivo* [115]. The nanosized polyplexes were stable *in vivo* and showed receptor-specific cell targeting in receptor-positive tumors. Moreover, folate-targeted nanoparticles serve as potential for rheumatoid arthritis (RA) therapy. RA is an inflammatory disease and the pathogenesis is characterized by the activation of macrophages [116].

Ligand expression on the polyplex surface enhances uptake by endocytosis, and intracellular fate can be modulated by the selected ligands and their density at the polyplex surface to achieve multivalent binding [117]. Active targeting of gene carriers using ligands is supposed to be a good approach, but it has to be considered that efficient attachment and binding of the carriers to the cell membrane do not guarantee the completion of the delivery process.

### 5.3. Endosomal escape and the proton sponge hypothesis

Endosomal escape is considered to be a major limitation for the intracellular delivery of polyplexes [118,119]. When nanoparticles reach their target cell, they are internalized through endocytosis, which is an efficient process for entry into the intracellular environment. However, the majority of nanoparticles remains entrapped inside the endosomes and are trafficked toward late endo- and lysosomes where they are degraded by digestive enzymes. To avoid enzymatic degradation, polyplexes should find a way to escape the endosome. The addition of chloroquine, which accumulates in the acidic environment of the endolysosomes, had beneficial effects on inducing endosomal escape (as discussed in Section 4.1) [61]. Later, it was discovered that several cationic polymers with high buffer capacity below physiological pH such as polyethylenimine (PEI), were able to mediate high transfection efficiencies without the need of adding membrane-disruptive agents. A hypothesized mechanism for the enhanced endosomal escape is an altered osmolarity which leads to vesicle swelling and subsequent endosome disruption (Fig. 5). This hypothesis is known as 'the proton sponge hypothesis' and is an intensively studied strategy for the endosomal escape of nanoparticles. In 1997, Jean-Paul Behr and colleagues described the essence of the proton sponge hypothesis as the endosomal accumulation of protons brought in by ATPase that is coupled to an influx of chloride anions which will lead to a large increase in the ionic concentration within the endosome resulting in subsequent osmotic swelling and bursting of the endosome [120]. This process of swelling and bursting results in endosomal escape of the originally entrapped polyplexes. In accordance with the proton sponge hypothesis, the incorporation of histidine residues provides buffer capacity and greatly enhances the transfection efficiency of polymers such as PLL. For example, professor Sung Wan Kim and collaborators designed pH-sensitive PLH-g-PLL polyplexes that enhanced transfection efficiency [121]. Alternatively, viral strategies have been explored to trigger

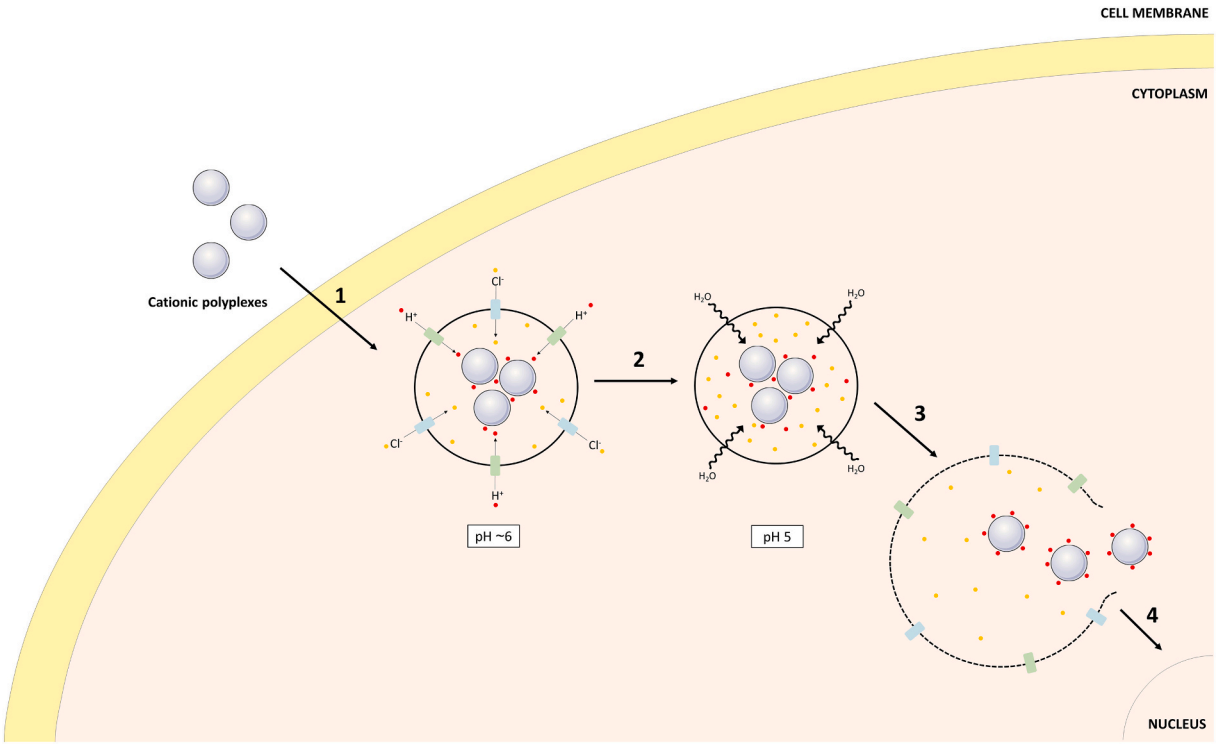


**Fig. 5.** Schematic illustration of the proton sponge hypothesis. The cationic complexes are enclosed in an endosome after endocytosis (1). Due to acidic endosomal buffering the protons are continued to pump in the vesicle resulting in $Cl^-$ influx and increase in the osmolarity inside the endosomal vesicle (2). The osmolarity increase results in an influx of water and osmotic vesicle swelling (3). Endosomal membrane disruption and polyplex release into the cytoplasm leading to nuclear uptake of DNA (4).

endosomal escape of polyplexes. Viruses express membrane-destabilizing domains which promote endosomal escape. In some studies it was demonstrated that the presence of non-infectious viral particles such as inactivated adeno- or rhinovirus particles, enhanced transfection efficiency of receptor-targeted polylysine (PLL) [122,123]. The viruses particles enter the cell *via* their natural endocytosis pathway and consequently interact with the internalized polyplexes, facilitating endosomal escape of the polyplexes and their localization in the cytosol. Instead of using whole virus particles, synthetic virus-derived or artificial membrane active peptides can be incorporated into the polyplexes [124,125]. Furthermore, approaches using triggers such as pH cleavable polymers and irradiation have been used to induce endosomal escape. To mention, pH-triggered membrane destabilization by hydrophobic polymers with lipid domains, and photoinduced endosomal escape using a photosensitizer resulted in an effective release of endocytic material into the cytosol to strongly improve polyplex transfection efficiency [126,127].

Altogether, effective endosomal escape exploiting proton-sponge polymers is caused by osmotic swelling of the endosomes and destabilization of the endosomal membrane. Furthermore, various alternative approaches have been explored to enhance or induce endosomal escape. Interesting future directions would be to quantify endosomal escape efficiency, thereby eliminating interference of subsequent intracellular barriers. Importantly, barriers that precede endosomal escape should still be taken into account.

### 5.4. Nuclear delivery and retention

Nuclear delivery of pDNA-based gene expression constructs is an additional challenge beyond the barriers previously described. Transfection studies have proved that this process successfully takes place, but the involved mechanisms are not clearly understood. Timing and location of the nucleic acid release from cationic polymers varies between different polyplexes, both free and complexed nucleic acids have been observed in different cellular compartments, including the nucleus [75,128–130]. Following cytosolic delivery, the pDNA has to be delivered to the pores of the nuclear envelope of nondividing cells. For nondividing cells, cytosolic delivery is followed by the transport of pDNA through the pores of the nuclear envelope. However, passive diffusion of polyplexes and pDNA in the cytosol is limited and transport needs to be regulated *via* either endosome trafficking along microtubules or active transport within the cytosol. At the nuclear envelope, the polyplex or pDNA has to be transported across the nuclear membrane by active transport mechanisms or alternative pathways. Cell cycle studies indicated that this is an important barrier and that polymer transfection is depends on cell cycle. It has been demonstrated that transfection is enhanced (up to >100-fold) in the G2/M phase of the cell cycle when the nuclear envelope disintegrates. After nuclear entry, the pDNA payload has to be retained and actively expressed. Nuclear retention could be improved by the incorporation of peptides such as histone H3 tails [131], NLS peptides [132–135], or other cell division-responsive peptides [50] into polyplexes. It should be noted that although an attractive strategy, attachment of an NLS to pDNA does not always result in enhanced transfection nor in its nuclear import [136,137].

## 6. New polymer designs

### 6.1. Natural polymers

Natural polymers are perceived to have an improved biodegradability and reduced cytotoxicity profile which is not by definition true since also synthetic polymers have been designed that show tailorable biodegradability and good biocompatibility. Natural polymers investigated for gene therapy include chitosan, collagen, gelatin, and their modified derivatives [138,139].

Besides collagen and gelatin, also other protein based carriers such as chromosomal proteins HMG1 and histones, with or without modifications, have been used for nucleic acid transfection [140–142]. Cationized gelatin was shown to be able to facilitate delivery of siRNA into the kidney *via* administration through the ureter [143,144]. Atelocollagen, a soluble form of collagen, was found effective in siRNA delivery into bone metastatic tumors after systemic administration [145].

Chitosan (poly-D-glucosamine, obtained by deacetylation of natural polymer chitin) and its derivates are among the most investigated naturally derived polymeric gene carriers. The transfection efficiency of chitosan/DNA complexes is dependent on several factors, including the degree of deacetylation and molecular weight of the chitosan, plasmid concentration, charge ratio of amine (chitosan) to phosphate (DNA), serum concentration, pH, and cell type [146]. Due to its mucoadhesive property, chitosan is also very successful in oral and nasal delivery of nucleic acid based drugs. Modification of chitosan by methylation and PEGylation has shown to improve formation and colloidal stability of pDNA/chitosan polyplexes. Additional favourable modifications include histidinylation for improved endosomal escape [147] and targeting ligands or thiolation for enhanced cellular uptake [148,149].

Cyclodextrin (CD) conjugates including alpha-, beta-, or gamma forms are cyclic oligosaccharides that are able to form host-guest complexes with hydrophobic compounds [150]. Covalent conjugation of beta-CD which is able to form complexes with conjugate of cholesterol with LPEI and BPEI reduced the toxicity of pDNA polyplexes without compromising their transfection efficiency [151]. Incubation of these PEI polyplexes with adamantane-PEG, resulted in the formation of PEGylated PEI polyplexes that were successfully used for systemic gene transfer into the liver [152]. In another study, low molecular weight PEI-CD conjugates were used for the formation of pDNA polyplexes that were subsequently modified with an adamantane peptide ligand specific for the fibroblast growth factor (FGF) receptor and adamantane-disulfide-linked PEG for shielding [51]. Hwang and Davis synthesized linear cationic β-cyclodextrin-based polymers (β-CDPs) which showed better biocompatibility than the unmodified polycations. Furthermore, the CD groups on these polycations were used to introduce transferrin *via* adamantane host–guest interaction [153]. These polyplexes were evaluated in clinical trials, demonstrating that intravenous administration of siRNA mediated gene silencing in metastatic tumors [111,154]. It was shown that the degree of cationic modification is critical for nucleic acid polyplex formation and can be tuned. Polycationic polyrotaxane polymers, with monocationic CDs assembled to degradable linear polymer chains were synthesized and showed enhance DNA transfection [155].

### 6.2. Biodegradable polymers

The design and use of biodegradable synthetic polymers is another direction to increase polyplex biocompatibility and/or decrease cytotoxicity, and to avoid accumulation of the polymer in the body. Importantly, degradability can also be used as a tool to trigger the release of pDNA or siRNA into the cytosol after cellular internalization of polyplexes based on these polymers [156,157]. The cytotoxicity of polycations (*i.e.* PLL and PEI) is highly dependent on molecular weight. For example, PEI with a low molecular weight (LMW) of 800 (less than 20 monomer units) presents low cellular toxicity but also low transfection efficiency, while high molecular weight (HMW) LPEI 22 kDa or BPEI 25 kDa are more powerful but significantly more toxic. Therefore, novel strategies aimed at combining the advantages of LMW polymers (low cytotoxicity) and HMW polymers (high transfection efficiency). Various biodegradable PEI based polymers containing ester bonds, disulfides, ketals, imines, polyglutamic acid amide, and other amides have been synthesized [51].

Modification of the cationic polymer backbone by introduction of either hydrolysable ester bonds, endosomally degradable acetal or imine bonds, as well as bioreducible disulfide bonds greatly reduced cytotoxicity and maintained or even enhanced nucleic acid transfection

efficiency. In 2000, Kim and collaborators reported on the synthesis of biodegradable poly(alpha-(4-aminobutyl)-L-glycolic acid) by replacing the amide bonds in polylysine with ester bonds. Transfection activity was enhanced compared to PLL but still remained moderate, which might be attributed to the lack of effective endosomal escape. Importantly poly(alpha-(4-aminobutyl)-L-glycolic acid) was substantially less toxic than pLys [158]. Following this, more effective hyperbranched network-type poly(amino esters) termed n-PAE were synthesized by Michael addition of amines to acrylate esters. N-PAE showed transfection efficiency and endosomal escape properties similar to PEI, but a substantially better biocompatibility. Langer and colleagues synthesized a library of more than 2000 biodegradable cationic polymers by reaction of a series of primary amines and secondary diamines via Michael addition with a variety of bisacrylates [159,160]. Interesting candidates were identified using a high throughput screening for transfection efficiency. The Michael addition strategy was used by other groups for the generation of disulfide-based biodegradable polymers. Engbersen and collaborators showed in a number of papers that polyplexes based on these polymers showed high transfection efficiency of both pDNA and siRNA combined with a low cytotoxicity. Cationic charged groups inside the core of the nanogels induce efficient loading of nucleic acids by electrostatic interactions in aqueous solution. Proton buffering functionalities in the nanogel promote endosomal escape after cellular uptake of the nanogels. Due to the relatively high concentration of glutathione in the cytoplasm the disulfide linkages in the nanogel network become rapidly cleaved by the thiol-disulfide exchange reaction, resulting in rapid disassembly of the nanogel-cage and release of the free payload [161–164]. Bioreducible PEI oligoamines were synthesized by reacting cystamine bis-acrylamide with small oligoethylenimines. These polymers showed enhanced levels of gene expression combined with no or only very low cytotoxicity [161,163]. These results demonstrate that bioreducible polymers are promising for safe and efficient gene delivery.

### 6.3. Decationized polymers

Toxicity of cationic polymers is a complex process and occurs at many levels. Polycations can compromise the cell membrane integrity, disrupt cellular homeostasis by interaction with cellular polyanions, change the genomic expression profile, and induce the activation of oncogenes or apoptosis [104,165]. Accordingly, a new class of gene delivery polyplexes was developed as an alternative for conventional polycation-based systems. Unlike polycation-based systems, the structure of decationized polyplexes is based on neutral polymers. Particularly these polyplexes are composed of a disulfide crosslinked poly (hydroxypropyl methacrylamide) (pHPMA) core that stably encapsulates and retains nucleic acids (such as pDNA and siRNA), surrounded by a PEG shell. Decationized polyplexes are prepared by a three-step process. The first step is charge-driven condensation with pDNA/siRNA using the pHDP-PEG polycation precursor. This is followed by stabilization of polyplexes by interchain disulfide crosslinking, yielding cationic pHDP-PEG polyplexes. Finally, the cationic dimethylaminoethanol (DMAE) side groups are removed by hydrolysis and decationized pHP-PEG polyplexes are generated. Nucleic acids are physically entrapped in the polymer-based particles due to the presence of disulfide crosslinks, whereas previously developed disulfide-containing polyplexes require electrostatic interactions for pDNA entrapment. Stabilization of the polyplexes by disulfide crosslinks also prevents their disassembly in the bloodstream, thereby ensuring polyplex stability in the circulation. Conversely, disulfide bonds are rapidly cleaved in the intracellular environment, resulting in the destabilization of polyplexes and release of the nucleic acid content. Moreover, the PEG-coat effectively ensures the colloidal stability of the polyplexes, avoids the formation of aggregates and reduces protein binding, resulting in improvements of the circulation kinetics and tumor accumulation *in vivo* [104]. Additionally, PEGylation enables the introduction of targeting ligands that can be used to improve cellular uptake and internalization.

Decationized polyplexes have important advantageous features when compared to conventional polyplexes. In general, decationized polyplexes have a size of ~120 nm and a slightly negative zeta-potential (−5 mV) [165]. They also show an excellent colloidal stability, meaning that the particles remain suspended in a solution and show triggered pDNA release in a reductive environment [166]. Another characteristic is the very low degree of non-specific cell uptake, which is important for targeted therapies. *In vitro* studies with folate-targeted decationized polyplexes have shown that cellular uptake of targeted decationized polyplexes was significantly higher in folate receptor overexpressing cell lines (HeLa and OVCAR-3), when compared to their nontargeted counterparts [167]. Folate was used as targeting ligand because of its high binding affinity to its receptor, which is overexpressed in many tumors. In contrast, a similar uptake for both targeted and nontargeted decationized polyplexes was observed in a nonexpressing folate receptor cell line (A549). Furthermore, transfection studies using OVCAR-3 cells showed higher transfection efficiency for folate-targeted systems because of improved cellular uptake. Importantly, the nontargeted cationic system showed the highest cellular uptake, mainly due to nonspecific interaction between the positively charged polyplexes membrane anionic components, which is not favourable for highly specific targeted delivery which requires low uptake by nontarget cells. These results suggest that folate coupling to decationized polyplexes have potential for targeted gene therapy.

Importantly, decationized polyplexes have an excellent safety profile. As shown by various *in vitro* tests, decationized polyplexes do not interfere with cell viability or induce membrane destabilization [166,167]. Furthermore, upon incubation of the neutral polymer pHP-PEG used for the formation of decationized polyplexes with HUVEC cells, excellent cytocompatibility was observed. The safety was further demonstrated by a remarkable low teratogenicity and mortality activity of this polymer in a *in vivo* nanotoxicity assay, in great contrast with its cationic counterpart [104,168].

The applicability of decationized polyplexes for systemic administration was evaluated by determining the stability of decationized polyplexes in human plasma and it was demonstrated that these polyplexes had a stable size distribution for 48 h, whereas their cationic counterpart showed some degree of aggregation [104]. Additionally, polyplexes were evaluated for biodistribution and tumor accumulation by noninvasive optical imaging based on the combination of computed tomography and fluorescence molecular tomography [169], when administered systemically in A431 tumor bearing mice. In the same study it was reported that decationized polyplexes exhibited an increased circulation time and higher tumor accumulation, when compared to cationic polyplexes. Furthermore, histological analysis of tumor sections showed that decationized polyplexes induced transgene expression *in vivo*.

Despite these important advantages, the *in vivo* efficiency of decationized polyplexes needs further optimization. The circulation half-life could be increased by improving the stability or by modification of the polymer structure (PEG-coating and molecular weight). Another important challenge is the identification of functionalities that can be introduced in the particles to overcome cellular barriers for transfection. In particular, endosomal escape and nuclear localization should be improved to increase transfection efficiency without adversely affecting their safety profile. The flexibility of these systems enables the generation of optimized decationized polyplexes that have great potential for systemic gene delivery applications.

## 7. Hybrid systems: nanomaterial-based delivery systems for oncolytic adenovirus

Adenoviruses have been long and widely recognized as a promising gene therapy vector owing to their high gene transfer efficiency, facile production at a high titer, and no risk of insertional mutagenesis [170].

For cancer gene therapy, ONYX-015, an oncolytic adenovirus that is currently being marketed in China as Oncorine, was the first oncolytic virus to be tested in humans and reached commercialization in 2005 [171,172]. Although there are several advantageous attributes of adenoviruses for cancer therapy, there are several inherent limitations that must be overcome to fully exploit the therapeutic potential of oncolytic adenoviruses in clinic. For local delivery application of oncolytic adenoviruses, coxsackie adenovirus receptor (CAR)-dependent internalization, nonspecific viral shedding to normal tissues from injected site, and poor and short intratumoral retention of the administered viruses are major therapeutic hurdles [170,173]. In the scope of systemic delivery, the high immunogenicity of the adenovirus capsid causes rapid inactivation and clearance of the virus from blood circulation, and the hepatic tropism of the virion leads to poor tumor-targeted delivery and hepatotoxicity [174,175]. Furthermore, wide prevalence of patients with pre-existing adaptive immunity against adenovirus infections is also problematic, as neutralizing antibodies further contribute to rapid inactivation of the virus in patients which minimize the therapeutic benefit of oncolytic adenoviruses [176]. To address these challenges, diverse nanomaterial-based delivery systems have been investigated to either enhance local or systemic delivery of oncolytic adenoviruses (Fig. 6). This section will briefly discuss and summarize how different nanomaterial-based delivery systems have addressed the above-mentioned challenges toward optimal delivery of oncolytic adenoviruses.

Tumors in patients are highly heterogenic and among others CAR expression levels can vary greatly between different patients and even within the same patient, and in some cases may be completely abrogated [177,178]. These variable CAR expression levels between different tumor cells severely hampers oncolytic adenovirus infection efficiency in tumor tissues, as the cellular uptake of oncolytic adenovirus is CAR-dependent. To address this challenge, a number of different cationic polymers, such as arginine-grafted bioreducible polymer (ABP), methoxy poly(ethylene glycol)-b-poly{N-[N-(2-aminoethyl)-2-aminoethyl]-L-glutamate (PNLG), PEI-bile acid complex (DA3), multi-degradable bioreducible core-cross-linked PEI (rPEI), mPEG-PEI-g-Arg-S-S-Arg-g-PEI-mPEG (PPSA), and paclitaxel-conjugated ABP (APP), have been successfully synthesize and utilized to generate nanocomplexes with oncolytic adenoviruses that are recognized and internalized by tumor cells in a CAR-independent manner [179–185]. These cationic polymers bind to the negatively charged capsid proteins of oncolytic adenoviruses to generate cationic nanocomplexes, which bind and are subsequently internalized by cells by electrostatically interacting with negatively charged cell membranes rather than the CAR receptor. Indeed, oncolytic adenovirus complexed with any of the abovementioned cationic polymers showed superior internalization into wide range of cancer cell types regardless of their cellular CAR expression level, resulting in greater therapeutic transgene expression level, tumor growth inhibition, and higher level of intratumoral virus accumulation than naked oncolytic adenovirus.

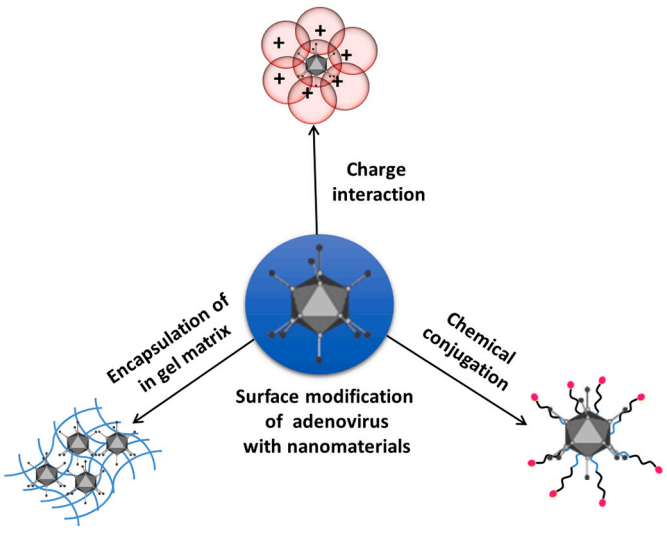


**Fig. 6.** Schematic presentation of overcoming barriers using adenovirus based nanomaterials.

Although cationic nanomaterials can exponentially boost oncolytic adenovirus internalization into cancer cells regardless of their CAR expression level, the positive zeta potential of the resulting nanocomplex results in poor systemic administrability due to nonspecific sequestration by normal cells and opsonization with serum proteins [186–188]. Despite these well-known hurdles to systemic application of cationic nanomedicines, ABP, PNLG, and APP were successfully utilized to generate nanocomplexes with oncolytic adenoviruses that elicited superior tumor growth inhibition than naked oncolytic adenoviruses after systemic administration [179,183,184]. One common characteristic for the investigated ABP-, PNLG-, and APP coated oncolytic adenovirus nanocomplexes is that they all exhibit sizes less than 200 nm in diameter, making them well-suited to benefit from enhanced permeability and retention (EPR) effect to passively target tumors after systemic administration [31,189]. Importantly, these adenovirus/polymer nanocomplexes achieved lower levels of hepatic sequestration and greater intratumoral accumulation of oncolytic adenoviruses than the corresponding naked virus, showing that careful optimization of these cationic nanocomplexes can improve tumor-specific accumulation of and confer liver detargeting as compared to systemically administered free adenovirus.

Alternative methods to overcome the CAR-dependence of oncolytic adenoviruses are based different nanomaterials containing active tumor targeting moieties, such as peptides (neurotensin, RGD), and antibodies (Herceptin), to generate tumor-targeted nanocomplexes with oncolytic adenovirus to redirect virion internalization to occur in a complementary target-specific and CAR-independent manner [190–194]. Similar to the application with nontargeted cationic polymers listed above, sufficient masking of the viral capsid with active tumor targeting ligands also nullifies viral capsid interactions with cell surface molecules, meaning that the cellular internalization of these complexes solely relies on the innate property of the tumor-targeted nanomaterial. Notably, several of these tumor-targeted nanomaterials have been successfully utilized for systemic delivery of oncolytic adenovirus to tumors overexpressing complementary receptors that can be targeted by the tumor targeting moieties to exert more potent antitumor effects and higher levels of intratumoral virus accumulation than naked oncolytic adenovirus or nontargeted control nanocomplexes [190–192,194]. Of note, both tumor-targeted nanocomplexes and nontargeted nanocomplexes exhibited similar pharmacokinetics, protection against adenovirus-specific neutralizing antibodies, and liver detargeting ability. Thus, the only notable difference between the two types of complexes after systemic administration is the tumor-specific accumulation of the ligand decorated complexes. Together, these findings demonstrate that inclusion of active tumor targeting moieties to nanocomplexes enhances intratumoral accumulation of the virus after systemic administration, while retaining the pharmacokinetic properties and stealth capacity to evade the host immune system as the nontargeted parental nanomaterial, to result in potent antitumor effects.

Aberrant physiological conditions, such as hypoxia-induced acidosis [195,196], of the tumor microenvironment can also be exploited to endow nanomaterials with improved tumor targeting ability. In the scope of oncolytic adenovirus delivery, a bioreducible and pH responsive polymer PPCBA (mPEG-piperazine-CBA) was successfully synthesized and utilized to generate nanocomplexes that could preferentially target the acidic conditions of the tumor microenvironment after systemic administration [197]. Coating the adenovirus surface with PPCBA enabled CAR-independent cellular internalization *via* micropinocytosis, which was superior to the naked virus. Importantly, the cell uptake of

the nanocomplexes and subsequent transgene expression by cancer cells were markedly improved under mildly acidic conditions (pH 6.0) that mimic the tumor microenvironment compared to normal physiological pH of 7.4, demonstrating the pH-dependent internalization of the complex. PPCBA-coated oncolytic adenovirus complexes exerted a stronger antitumor effect than naked oncolytic adenovirus after either intratumoral or intravenous injection. Further, systemic administration of PPCBA-coated adenovirus complex induced markedly lower levels of innate antiviral immune response and hepatotoxicity than naked oncolytic adenovirus, which demonstrates a good safety profile of the nanocomplex formulation for systemic delivery of oncolytic adenoviruses.

Most of the nontargeted and tumor-targeted nanomaterials mentioned above and administered either locally or systemically were shown to the protect virus against inactivation by adenovirus-specific neutralizing antibodies, thus lowering the chance of potential immunotoxicity. As rapid immune detection and clearance results in poor longevity of oncolytic viruses in the circulation and low accumulation in tumors [198], coating of the nanomaterials with stealth polymers like PEG may prolong circulation time and increase tumor disposition and finally in better antitumor effects. It is remarked that the immunological regulation of these nanocomplexes needs further evaluation in an immunocompetent animal model as the antitumor activity of the nanocomplex formulations discussed in this section have been conducted in human xenograft tumor models using immunocompromised hosts. Alternatively, utilization of controlled or sustained release systems, such as hydrogels, for the local delivery of oncolytic adenovirus has been shown to improve and prolong viral persistence in tumor tissues [199]. In detail, oncolytic adenovirus particles encapsulated in an injectable alginate gel was shown to retain its biological activity over longer periods under physiological conditions than naked virus. Importantly, time-dependent degradation of the gel in tumor tissues and sustained release of oncolytic adenoviruses led to prolonged tumor growth inhibition, as higher levels of virus was retained in tumor tissues up to 24 days after administration while a similar administered dose of naked oncolytic adenovirus rapidly decreased in time. Additionally, oncolytic adenovirus particles released from the alginate gel remaimed highly localized in tumor tissues, whereas naked oncolytic adenovirus nonspecifically shed to the liver from the injected tumor site to cause hepatotoxicity. Together, these findings demonstrated that sustained release of oncolytic adenovirus particles into the tumor tissues *via* local administration can prolong intratumoral viral persistence and prevent nonspecific shedding to normal tissues to result in both a better therapeutic efficacy and safety profile of the virus.

In summary, the studies discussed in this section demonstrate that cationic polymers together with targeting ligands can be utilized for surface decoration of oncolytic adenoviruses to introduce novel properties in the resulting complexes that cannot be achieved by genetic engineering of naked virus. These properties encompass tumor-targeted systemic delivery, evading detection by the host immune system. Moreover nonspecific shedding of virus from the injected site can be circumvented by loading the viruses into a injected hydrogel formulation. With the exponentially expanding and maturing field of nanomedicine, the future of nanomaterial-based delivery platforms for oncolytic adenovirus is very promising to maximize the therapeutic potential of oncolytic viruses for the treatment of patients.

## 8. Polymeric delivery systems for mRNA

In recent years there is an increased interest in the use of mRNA as therapeutic agents including vaccines. As opposed to pDNA, mRNA does not have to be delivered in the nucleus of a cell, which is major advantage. However, mRNA is more susceptible to nuclease degradation in physiological environments as compared to pDNA [200]. Nuclease-mediated degradation in the extracellular environment can be prevented by chemical modification of mRNA [201]. To mention, modification on 3′ and 5’ends of the mRNA protects against enzymatic degradation [44]. However, chemical modifications of mRNA is limited because the translational activity should be preserved after modification. Further, these modified mRNA’s are in their free form, and like other nucleic acid based drugs, hardly taken up by cells. Therefore, carriers with strong protective properties are required for the delivery of mRNA, alternatively or additionally to modification. Cationic polymers and/or lipids have been shown to be safe and versatile carriers for the intracellular delivery of mRNA with the aim to produce therapeutic proteins and antigens for vaccination purposes [202,203].

Polyplex micelles are frequently used as carriers of mRNA and prepared by mixing mRNA with poly(ethylene glycol) (PEG)-polycation block copolymers in aqueous solution, forming a structure consisting of a PEG shell and a core containing condensed mRNA. Micelle properties can be modulated by modification of the block copolymer design. The micelle can further be stabilized by crosslinking of the core with reversible disulfide bonds [204,205].

Although protected, nucleic acids in carriers can still be enzymatically degraded *via* two mechanisms; 1) carrier dissociation results in the release of nucleic acids and exposure to nucleases, or 2) nucleic acids encapsulated in the carrier are degraded by nucleases that penetrate the polyplex micelles. Carriers dissociate *via* polyion exchange reaction after exposure to anionic molecules (*i.e.* glycosaminoglycans [GAGs] expressed on the cell surfaces) [206]. PEG coating of polyplexes is a promising strategy to protect nucleic acid degradation by both mechanisms described. Studies using polyplex micelles of PEG-poly(lysine) (PLys) block copolymer and pDNA demonstrated high resistance to degradation by DNase and polyion exchange reaction by inhibiting the entry of DNase and polyanions into the micelle core [207]. However, nucleases are relatively small in size and can still penetrate into the micelle core after prolonged exposure. To prevent such penetration. For example, the construction of a hydrophobic physical barrier between the hydrophilic shell and core showed increased tolerability to both nucleases and polyanions and could prevent such penetration [208].

After the polyplex reaches the cytosol of target cells, dissociation has to take place to release the mRNA and to allow efficient transcription and translation. Such dissociation can be mediated by environment responsive crosslinkers that are cleaved only in intracellular environment, including disulfide crosslinks that are cleaved in response to the reductive environment of the cytosol [209,210], and ATP-responsive chemical bonding of phenylboronic acids (PBAs) and diol [211]. The introduction of hydrophobic compounds such as cholesterol provides another mechanism of polyplex stabilization through hydrophobic interactions [212]. Intravenous injection of micelles prepared from mRNA and poly(ethylene glycol) (PEG)-polycation block copolymers containing cholesterol, showed significantly enhanced blood retention of mRNA in comparison with control micelles without cholesterol [213]. Furthermore, *in vivo* tests demonstrated that cholesterol micelles generated efficient protein expression from the delivered mRNA in tumor tissue, inhibiting tumor growth, while micelles without cholesterol showed no therapeutic effect. Besides cholesterol, other compounds, such as tyrosine [214] and poly(ethylene glycol) methyl ether methacrylate (PEGMA) [215] have been used for polyplex stabilization.

The intrinsic immunogenic properties of pDNA and mRNA can induce adverse side effects *via* recognition by pattern recognition receptors (PRRs) that trigger innate immune responses. PRRs includes toll-like receptors (TLRs) that are mainly located in the endosome [216]. Immunogenicity can be reduced by avoiding TLR recognition in the endosome by chemical modification of mRNA molecules, and enhancing endosomal escape for minimizing exposure to TLRs. For example, partial replacement of mRNA nucleosides with chemically modified nucleosides (*i.e.* pseudouridine) was effective in inhibiting mRNA recognition by PRRs without causing a large decrease in mRNA translational activity [217,218]. In addition, immunogenicity can be controlled by carrier design. *In vivo* mRNA introduction using polyplex micelles demonstrated a reduced inflammatory response in the brain of mice compared to

naked mRNA [219].

## 9. Clinical status

Viral vectors are still dominating clinical gene therapy. Gendicine, a recombinant human p53 adenovirus, was approved in 2003 by the China Food and Drug Administration (CFDA) as the world's first oncolytic viral therapy for cancer [172,220]. Other important approved viral vectors are Glybera and Strimvelis. Glybera is used to treat patients suffering from lipoprotein lipase (LPL) deficiency, a genetic disorder caused by a defective gene (lipoprotein lipase) [2]. The adeno-associated virus serotype 1 (AAV1) viral vector introduces an intact copy of the human LPL gene into muscle cells which is subsequently translated into the therapeutic LPL enzyme. However, for reasons such as high costs and rarity of LPL deficiency, the world's first gene therapy product Glybera, was withdrawn from the market [221]. Strimvelis is an *ex vivo* gene therapy product to treat severe combined immunodeficiency caused by adenosine deaminase deficiency (ADA-SCID) [222]. The adenosine deaminase enzyme is essential for maintaining healthy lymphocytes. In clinical practice, lymphocyte producing CD34+ cells are extracted from the patient's bone marrow and a retroviral vector is used to insert a functional ADA gene into the CD34+ cells.

Viruses offer great efficiency for gene delivery, however, synthetic vectors are preferred due to safety concerns and relatively better possibilities for scaling up and GMP production. It is therefore expected that in the next decades synthetic gene delivery systems will dominate gene therapy clinical trials, whereas viral vectors may be more suited for *ex vivo* applications [19,223].

The CRIPSR/Cas9 technology provides a simple and efficient alternative for therapeutic gene editing and was rewarded with the Nobel Prize in Chemistry 2020. However, despite the great advantages, limitations remain which must be addressed for safe and clinical translation [224,225].

### 9.1. siRNA delivery

Cationic lipids and polymers are widely used as nonviral vectors both *in vitro* and *in vivo*. The first phase I siRNA clinical trial that utilizes a targeted nanoparticle delivery system (clinical registration number NCT00689065) started in 2008 and concerned a safety study of the CALAA-01 formulation to treat solid tumor cancers [154]. siRNAs were systemically administered to patients with solid tumors using a targeted nanoparticle delivery system consisting of a linear cyclodextrin-based polymer (CDP), a human transferrin (hTf) targeting ligand, PEG as hydrophilic an shielding polymer, and siRNA designed to reduce the expression of the M2 subunit of ribonucleotide reductase (RRM2) [154]. A dose-dependent accumulation of the targeted nanoparticles in tumors and a reduction in both RRM2 mRNA and protein by the RRM2-specific siRNA were observed. Moreover, it was demonstrated that the delivered siRNA engages in the RNAi machinery [111]. Taken together, these data demonstrate that systemic administration in humans results in specific gene inhibition *via* RNAi. However, dose-limiting toxicity was observed in several patients and the clinical trial was terminated [226].

In 2018, Onpattro was approved by the FDA after extensive clinical trials [227]. Onpattro is a LNP formulation, and a successful non-viral system for the delivery of siRNA to treat polyneuropathies resulting from the hereditary disease transthyretin-mediated amyloidosis (hATTR) and acts by inhibiting transthyretin (TTR) protein synthesis in the liver [220]. The clinical development and success of Onpattro paves the way for the development of a new class of non-viral nanomedicines based.

In November 2019, the second siRNA drug was approved for the treatment of adults with acute hepatic porphyria (AHP) [15]. Givosiran is a double-stranded small interfering ribonucleic acid (siRNA) covalently linked to a tri-antennary GalNAc ligand to enable specific delivery of the siRNA to hepatocytes [228]. The GalNAc ligand binds to the ASGPR on hepatocytes and triggers receptor-mediated endocytosis of the ligand-receptor complex, followed by release of the small interfering ribonucleic acid (siRNA) into the cytoplasm of the hepatocyte. This results in downregulation of aminolevulinate synthase 1 (ALAS1) mRNA through RNAinterference and prevents accumulation of neurotoxic aminolevulinic acid (ALA) and porphobilinogen (PBG), the key factors that are associated with APH attacks.

### 9.2. Delivery of mRNA encoding for therapeutic proteins

In 2017, Moderna launched a phase I clinical trial for intramural delivery of lipid nanoparticle-encapsulated OX40 ligand (OX40L) mRNA-2416 (ID: NCT03323398) (Table 2) [229]. OX40 and its binding partner OX40L are members of the tumor necrosis factor receptor (TNFR) and TNF superfamily and are crucial for many types of immune reactions mediated by T cells [44,230]. OX40 is expressed on activated CD4 and CD8 T cells, whereas OX40L is expressed on many antigen-presenting cells such as dendritic cells. OX40/OX40L interaction triggers the expansion, function and survival of memory and effector T lymphocytes which may enhance an immune response that subsequently results in the killing of nearby tumor cells. Safety and efficacy of mRNA encoding human OX40L was tested in patients with solid malignancies or lymphoma. Intramural injection of mRNA-2416 as LNP formulation was well tolerated, and showed increased OX40L protein expression and pro-inflammatory activity [231]. Taken together, the potential immunomodulatory and antitumor activities could be used to treat solid tumors and lymphoma.

Translate Bio is developing a LNP based formulation for the delivery of mRNA encoding fully functional cystic fibrosis transmembrane conductance regulator (CFTR) protein to the lung epithelial cells *via* inhalation (ID: NCT03375047) [232]. MRT5005 is the first clinical-stage phase I/II mRNA product candidate designed for the treatment of cystic fibrosis. In 2019, the interim results for safety and tolerability of MRT5005 were announced, summarizing the single ascending dose (SAD) portion (8, 16 and 24 mg) [233]. Moreover, MRT5005 was generally well tolerated at low and mid-doses, showed marked increases in lung function and no serious adverse effects were observed. The multiple ascending dose (MAD) study is still ongoing with data expected in 2020. This is the first time an mRNA therapeutic has been evaluated for its potential to treat a genetic disease, and the data indicate the potential of mRNA therapeutics for the treatment of lung diseases.

### 9.3. mRNA vaccine formulations

mRNA vaccine formulations represent a promising alternative to conventional vaccine formulations based on proteins or inactivated viruses because of their ability to encode a wide range of antigens and to possess intrinsic adjuvant effects. Further, potential mRNA vaccines allow rapid development and low-cost manufacturing, and even have possibilities for personalized vaccines [234]. Lipid nanoparticles with good biocompatibility are the most frequently studied mRNA vaccine delivery systems. Cationic liposomes composed of the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) or DOTMA, together with the helper lipid DOPE in combination with mRNA, have been developed as mRNA vaccines (Table 3) [44,235]. BioNTech has reported preferential expression of mRNA in dendritic cells after intravenous administration of RNA-lipoplexes containing DOTMA and DOPE [235]. The first melanoma patients treated with this formulation showed a positive immune response. Several other formulations are under investigation in clinical trials [44,236].

Development of prophylactic or therapeutic vaccines against infectious pathogens is the most efficient means to contain and prevent epidemics and a coronavirus (SARS-CoV-2) vaccine to prevent COVID-19 is under development [229]. Recently, ModernaTX Inc. has set-up a phase 3 clinical trial to assess the efficacy, safety, and immunogenicity of mRNA-1273 vaccine (ID: NCT04470427) [237]. The 1273 formulation

**Table 2**
Clinical trials involving non-viral mRNA formulations.

| Name | Therapeutic modality | Protein Target | Delivery Vehicle | Disease | ClinicalTrials.gov Identifier | Phase |
|---|---|---|---|---|---|---|
| mRNA-2416 | mRNA | OX40L | Lipid nanoparticle | Solid tumor or lymphoma | NCT03323398 | I |
| MRT5005 | mRNA | CFTR | Lipid nanoparticle | Cystic fibrosis | NCT03375047 | I/II |
| AZD-8601 | mRNA | VEGF-A | Naked mRNA (modified) | Heart failure | NCT03370887 | II |

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; OX40L, OX40 ligand; VEGF-A, vascular endothelial growth factor A. Adapted from [44].

**Table 3**
Clinical trials of mRNA vaccine formulations.

| Sponsor institution | Intervention/ treatment | API | Delivery Vehicle | Antigen | Disease | ClinicalTrials.gov Identifier | Phase |
|---|---|---|---|---|---|---|---|
| CureVac | CV7201 | mRNA | RNActive protamine | Rabies virus glycoprotein (RABV-G) | Rabies | NCT02241135 | I |
| | CV9201 | mRNA | RNActive protamine | TAAs: MAGEC1, MAGEC2, NY-ESO-1, survivin, 5 T4 | NSCLC | NCT00923312 | I/II |
| BioNTech | HPV vaccine | mRNA | Naked RNA | HPV antigen CD40 | HPV-driven squamous cell carcinoma | NCT03418480 | I/II |
| | Lipo-MERIT | mRNA | Lipo-MERIT, DOTMA (DOTAP)/DOPE lipoplex | TAAs: NYESO-1, MAGE-A3, tyrosinase, and TPTE | advanced melanoma | NCT02410733 | I |
| | IVAC | mRNA | Lipo-MERIT, DOTMA (DOTAP)/DOPE lipoplex | (1) 3 TAAs selected from a warehouse and p53 RNA; (2) Neo-Ag based on NGS screening | TNBC | NCT02316457 | I |
| Moderna | mRNA-1325 | mRNA | lipid nanoparticle-encapsulated mRNA | Zika virus antigen | Zika virus | NCT03014089 | I |
| | VAL-506440 | mRNA | lipid nanoparticle-encapsulated mRNA | H10N8 antigen | influenza | NCT03076385 | I |
| | mRNA-2416 | mRNA | lipid nanoparticle-encapsulated mRNA | human OX40L | solid tumor maliganancies or lymphoma | NCT03323398 | I/II |
| | mRNA-1273 | mRNA | Lipid nanoparticle-encapsulated mRNA | Human S protein | COVID-19 | NCT04470427 | III |

Abbreviations: API, active pharmaceutical ingredient; COVID, coronavirus disease; HPV, human papillomavirus; NGS, next generation sequencing; NSCLC, non-small lung cancer; TAA, tumor-associated antigen; TNBC, triple-negative breast cancer. Adapted from [44,237].

is a lipid nanoparticle-encapsulated mRNA-based vaccine that encodes for the transmembrane spike (S) glycoprotein of the coronavirus (SARS-CoV-2) which mediates the entrance of the virus into the host cells [238,239]. It is expected that antibodies targeting the S protein inhibit the viral entry into cells. Recently, Pfizer showed safety and efficacy of a COVID-19 vaccine that is based mRNA formulated in lipid nanoparticle [240]. The vaccine received approval by EMA and FDA and large vaccination campaigns will start in USA and Europe the beginning of 2021.

## 10. Conclusions

To date, a large variety of fascinating polyplexes has been rationally developed and investigated for nucleic acid therapy. Importantly, many issues revolve around efficient polyplex delivery, such as stability, targeted delivery and biodistribution. Improved understanding of specific biological barriers and novel therapies are needed to overcome these significant hurdles.

Polyplex delivery systems have been clinically evaluated but are still in the beginning. From the FDA database, there is only a limited number of completed and active human clinical trials. As discussed in Section 9, most of these trials are mRNA based therapeutics and vaccine therapies. The switch from classical pDNA gene transfer constructs to systems containing synthetic gene or protein-modulating nucleic acids (*i.e.*, mRNA, miRNA, antisense, and siRNA) has become the predominant focus for development, and has rapidly progressed to clinical trials. The ability of antisense and siRNA formulations to silence specific genes has applications to a wide variety of diseases.

## Dedication

This review is dedicated to Prof. Sung Wan Kim (1940-2020), a recognized Distinguished Professor of Pharmaceutics and Pharmaceutical Chemistry, and Bioengineering at the University of Utah. Two of the authors of this review (C.-O.Y. en W.E.H.) have collaborated with him and thank him for his great scientific insights, guidance and leadership.

## Authors

All authors contributed in writing of the review.

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## References

[1] M.F. Naso, B. Tomkowicz, W.L. Perry, W.R. Strohl, Adeno-associated virus (AAV) as a vector for gene therapy, BioDrugs 31 (2017) 317–334, https://doi.org/10.1007/s40259-017-0234-5.
[2] L.M. Bryant, D.M. Christopher, A.R. Giles, C. Hinderer, J.L. Rodriguez, J.B. Smith, E.A. Traxler, J. Tycko, A.P. Wojno, J.M. Wilson, Lessons learned from the clinical development and market authorization of Glybera, Hum. Gene Ther. Clin. Dev. 24 (2013) 55–64, https://doi.org/10.1089/humc.2013.087.
[3] M. Li, M. Zhao, Y. Fu, Y. Li, T. Gong, Z. Zhang, X. Sun, Enhanced intranasal delivery of mRNA vaccine by overcoming the nasal epithelial barrier via intra- a nd paracellular pathways, J. Control. Release 228 (2016) 9–19, https://doi.org/10.1016/j.jconrel.2016.02.043.
[4] Y. Rybakova, P.S. Kowalski, Y. Huang, J.T. Gonzalez, M.W. Heartlein, F. DeRosa, D. Delcassian, D.G. Anderson, mRNA delivery for therapeutic anti-HER2 antibody expression in vivo, Mol. Ther. 27 (2019) 1415–1423, https://doi.org/10.1016/j.ymthe.2019.05.012.
[5] Draft landscape of COVID-19 candidate vaccines. https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines, 2020 (accessed September 30, 2020).
[6] C. Martin, D. Lowery, mRNA vaccines: intellectual property landscape, Nat. Rev. Drug Discov. 19 (2020) 578, https://doi.org/10.1038/d41573-020-00119-8.

[7] V. Arechavala-Gomeza, K. Anthony, J. Morgan, F. Muntoni, Antisense oligonucleotide-mediated exon skipping for Duchenne muscular dystrophy: progress and challenges, Curr. Gene Ther. 12 (2012) 152–160, https://doi.org/10.2174/156652312800840621.

[8] M. Chiper, N. Tounsi, R. Kole, A. Kichler, G. Zuber, Self-aggregating 1.8 kDa polyethylenimines with dissolution switch at endosomal acidic pH are delivery carriers for plasmid DNA, mRNA, siRNA and exon-skipping oligonucleotides, J. Control. Release 246 (2017) 60–70, https://doi.org/10.1016/j.jconrel.2016.12.005.

[9] V.S. Patil, R. Zhou, T.M. Rana, Gene regulation by non-coding RNAs, Crit. Rev. Biochem. Mol. Biol. 49 (2014) 16–32, https://doi.org/10.3109/10409238.2013.844092.

[10] P. Moskwa, F.M. Buffa, Y. Pan, R. Panchakshari, P. Gottipati, R.J. Muschel, J. Beech, R. Kulshrestha, K. Abdelmohsen, D.M. Weinstock, M. Gorospe, A. L. Harris, T. Helleday, D. Chowdhury, MiR-182-mediated downregulation of BRCA1 impacts DNA repair and sensitivity to PARP inhibitors, Mol. Cell 41 (2011) 210–220, https://doi.org/10.1016/j.molcel.2010.12.005.

[11] Y. Fan, S. Yin, Y. Hao, J. Yang, H. Zhang, C. Sun, M. Ma, Q. Chang, J.J. Xi, MiR-19b promotes tumor growth and metastasis via targeting TP53, RNA 20 (2014) 765–772, https://doi.org/10.1261/rna.043026.113.

[12] B. Zhang, X. Pan, G.P. Cobb, T.A. Anderson, microRNAs as oncogenes and tumor suppressors, Dev. Biol. 302 (2007) 1–12, https://doi.org/10.1016/j.ydbio.2006.08.028.

[13] D. Guo, L. Barry, S.S.H. Lin, V. Huang, L.C. Li, RNAa in action: from the exception to the norm, RNA Biol. 11 (2014) 1221–1225, https://doi.org/10.4161/15476286.2014.972853.

[14] J.K. Nair, J.L.S. Willoughby, A. Chan, K. Charisse, M.R. Alam, Q. Wang, M. Hoekstra, P. Kandasamy, A.V. Kelin, S. Milstein, N. Taneja, J. Oshea, S. Shaikh, L. Zhang, R.J. Van Der Sluis, M.E. Jung, A. Akinc, R. Hutabarat, S. Kuchimanchi, K. Fitzgerald, T. Zimmermann, T.J.C. Van Berkel, M.A. Maier, K. G. Rajeev, M. Manoharan, Multivalent N -acetylgalactosamine-conjugated siRNA localizes in hepatocytes and elicits robust RNAi-mediated gene silencing, J. Am. Chem. Soc. 136 (2014) 16958–16961, https://doi.org/10.1021/ja505986a.

[15] L.J. Scott, Givosiran: first approval, Drugs 80 (2020) 335–339, https://doi.org/10.1007/s40265-020-01269-0.

[16] K. Kawabata, Y. Takakura, M. Hashida, The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake, Pharm. Res. An Off. J. Am. Assoc. Pharm. Sci. 12 (1995) 825–830, https://doi.org/10.1023/A:1016248701505.

[17] C. D'Andrea, D. Pezzoli, C. Malloggi, A. Candeo, G. Capelli, A. Bassi, A. Volonterio, P. Taroni, G. Candiani, The study of polyplex formation and stability by time-resolved fluorescence spectroscopy of SYBR green I-stained DNA, Photochem. Photobiol. Sci. 13 (2014) 1680–1689, https://doi.org/10.1039/c4pp00242c.

[18] S.C. De Smedt, J. Demeester, W.E. Hennink, Cationic polymer based gene delivery systems, Pharm. Res. 17 (2000) 113–126, https://doi.org/10.1023/A:1007548826495.

[19] L. Xu, T. Anchordoquy, Drug delivery trends in clinical trials and translational medicine: challenges and opportunities in the delivery of nucleic acid-based therapeutics, J. Pharm. Sci. 100 (2011) 38–52, https://doi.org/10.1002/jps.22243.

[20] H.K. de Wolf, C.J. Snel, F.J. Verbaan, R.M. Schiffelers, W.E. Hennink, G. Storm, Effect of cationic carriers on the pharmacokinetics and tumor localization of nucleic acids after intravenous administration, Int. J. Pharm. 331 (2007) 167–175, https://doi.org/10.1016/j.ijpharm.2006.10.029.

[21] F.J. Verbaan, C. Oussoren, I.M. van Dam, Y. Takakura, M. Hashida, D.J. A. Crommelin, W.E. Hennink, G. Storm, The fate of poly(2-dimethyl amino ethyl) methacrylate-based polyplexes after intravenous administration, Int. J. Pharm. 214 (2001) 99–101, https://doi.org/10.1016/S0378-5173(00)00642-6.

[22] S. Boeckle, K. von Gersdorff, S. van der Piepen, C. Culmsee, E. Wagner, M. Ogris, Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer, J. Gene Med. 6 (2004) 1102–1111, https://doi.org/10.1002/jgm.598.

[23] C. Arigita, N.J. Zuidam, D.J.A. Crommelin, W.E. Hennink, Association and dissociation characteristics of polymer/DNA complexes used for gene delivery, Pharm. Res. 16 (1999) 1534–1541, https://doi.org/10.1023/A:1015096302720.

[24] V.P. Torchilin, V.G. Omelyanenko, M.I. Papisov, A.A. Bogdanov, V.S. Trubetskoy, J.N. Herron, C.A. Gentry, Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity, BBA - Biomembr. 1195 (1994) 11–20, https://doi.org/10.1016/0005-2736(94)90003-5.

[25] P. Harvie, F.M.P. Wong, M.B. Bally, Use of poly(ethylene glycol)-lipid conjugates to regulate the surface attributes and transfection activity of lipid-DNA particles, in: J. Pharm. Sci, John Wiley and Sons Inc., 2000, pp. 652–663, https://doi.org/10.1002/(SICI)1520-6017(200005)89:5<652::AID-JPS11>3.0.CO;2-H.

[26] F.J. Verbaan, C. Oussoren, C.J. Snel, D.J.A. Crommelin, W.E. Hennink, G. Storm, Steric stabilization of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes mediates prolonged circulation and tumor targeting in mice, J. Gene Med. 6 (2004) 64–75, https://doi.org/10.1002/jgm.475.

[27] J. Fang, H. Nakamura, H. Maeda, The EPR effect: unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect, Adv. Drug Deliv. Rev. 63 (2011) 136–151, https://doi.org/10.1016/j.addr.2010.04.009.

[28] S.K. Cho, C. Dang, X. Wang, R. Ragan, Y.J. Kwon, Mixing-sequence-dependent nucleic acid complexation and gene transfer efficiency by polyethylenimine, Biomater. Sci. 3 (2015) 1124–1133, https://doi.org/10.1039/c5bm00041f.

[29] T. Blessing, J.S. Remy, J.P. Behr, Template oligomerization of DNA-bound cations produces calibrated nanometric particles [2], J. Am. Chem. Soc. 120 (1998) 8519–8520, https://doi.org/10.1021/ja980620o.

[30] M. Longmire, P.L. Choyke, H. Kobayashi, Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats, Nanomedicine 3 (2008) 703–717, https://doi.org/10.2217/17435889.3.5.703.

[31] Y. Nakamura, A. Mochida, P.L. Choyke, H. Kobayashi, Nanodrug delivery: is the enhanced permeability and retention effect sufficient for curing cancer? Bioconjug. Chem. 27 (2016) 2225–2238, https://doi.org/10.1021/acs.bioconjchem.6b00437.

[32] T. Lammers, F. Kiessling, W.E. Hennink, G. Storm, Drug targeting to tumors: principles, pitfalls and (pre-) clinical progress, J. Control. Release 161 (2012) 175–187, https://doi.org/10.1016/j.jconrel.2011.09.063.

[33] R. Gref, Y. Minamitake, M.T. Peracchia, V. Trubetskoy, V. Torchilin, R. Langer, Biodegradable long-circulating polymeric nanospheres, Science 80 (263) (1994) 1600–1603, https://doi.org/10.1126/science.8128245.

[34] M. Kursa, G.F. Walker, V. Roessler, M. Ogris, W. Roedl, R. Kircheis, E. Wagner, Novel shielded transferrin-polyethylene glycol-polyethylenimine/DNA complexes for systemic tumor-targeted gene transfer, Bioconjug. Chem. 14 (2003) 222–231, https://doi.org/10.1021/bc0256087.

[35] X. Yang, Q. Chen, J. Yang, S. Wu, J. Liu, Z. Li, D. Liu, X. Chen, Y. Qiu, Tumor-targeted accumulation of ligand-installed polymeric micelles influenced by surface PEGylation crowdedness, ACS Appl. Mater. Interfaces 9 (2017) 44045–44052, https://doi.org/10.1021/acsami.7b16764.

[36] D. Oupicky, M. Ogris, K.A. Howard, P.R. Dash, K. Ulbrich, L.W. Seymour, Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation, Mol. Ther. 5 (2002) 463–472, https://doi.org/10.1006/mthe.2002.0568.

[37] C.E. Nelson, J.R. Kintzing, A. Hanna, J.M. Shannon, M.K. Gupta, C.L. Duvall, Balancing cationic and hydrophobic content of PEGylated siRNA polyplexes enhances endosome escape, stability, blood circulation time, and bioactivity in vivo, ACS Nano 7 (2013) 8870–8880, https://doi.org/10.1021/nn403325f.

[38] J.S. Suk, Q. Xu, N. Kim, J. Hanes, L.M. Ensign, PEGylation as a strategy for improving nanoparticle-based drug and gene delivery, Adv. Drug Deliv. Rev. 99 (2016) 28–51, https://doi.org/10.1016/j.addr.2015.09.012.

[39] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Young, Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo, BBA - Biomembr. 1066 (1991) 29–36, https://doi.org/10.1016/0005-2736(91)90246-5.

[40] M.C. Woodle, Sterically stabilized liposome therapeutics, Adv. Drug Deliv. Rev. 16 (1995) 249–265, https://doi.org/10.1016/0169-409X(95)00028-6.

[41] G. Storm, S.O. Belliot, T. Daemen, D.D. Lasic, Surface modification of nanoparticles to oppose uptake by the mononuclear phagocyte system, Adv. Drug Deliv. Rev. 17 (1995) 31–48, https://doi.org/10.1016/0169-409X(95)00039-A.

[42] D.E. Owens Iii, N.A. Peppas, Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles, Int. J. Pharm. 307 (2006) 93–102, https://doi.org/10.1016/j.ijpharm.2005.10.010.

[43] U. Lächelt, E. Wagner, Nucleic acid therapeutics using Polyplexes: a journey of 50 years (and beyond), Chem. Rev. 115 (2015) 11043–11078, https://doi.org/10.1021/cr5006793.

[44] P.S. Kowalski, A. Rudra, L. Miao, D.G. Anderson, Delivering the messenger: advances in Technologies for Therapeutic mRNA delivery, Mol. Ther. 27 (2019) 710–728, https://doi.org/10.1016/j.ymthe.2019.02.012.

[45] K.G. Ford, B.E. Souberbielle, D. Darling, F. Farzaneh, Protein transduction: an alternative to genetic intervention? Gene Ther. 8 (2001) 1–4, https://doi.org/10.1038/sj.gt.3301383.

[46] R. Duncan, S.C.W. Richardson, Endocytosis and intracellular trafficking as gateways for nanomedicine delivery: opportunities and challenges, Mol. Pharm. 9 (2012) 2380–2402, https://doi.org/10.1021/mp300293n.

[47] E. Dauty, A.S. Verkman, Actin cytoskeleton as the principal determinant of size-dependent DNA mobility in cytoplasm: a new barrier for non-viral gene delivery, J. Biol. Chem. 280 (2005) 7823–7828, https://doi.org/10.1074/jbc.M412374200.

[48] G.L. Lukacs, P. Haggie, O. Seksek, D. Lechardeur, N. Freedman, A.S. Verkman, Size-dependent DNA mobility in cytoplasm and nucleus, J. Biol. Chem. 275 (2000) 1625–1629, https://doi.org/10.1074/jbc.275.3.1625.

[49] L. Pigeon, C. Gonçalves, D. Gosset, C. Pichon, P. Midoux, An E3-14.7K peptide that promotes microtubules-mediated transport of plasmid DNA increases polyplexes transfection efficiency, Small 9 (2013) 3845–3851, https://doi.org/10.1002/smll.201300217.

[50] K. Remaut, N. Symens, B. Lucas, J. Demeester, S.C. De Smedt, Cell division responsive peptides for optimized plasmid DNA delivery: the mitotic window of opportunity? J. Control. Release 179 (2014) 1–9, https://doi.org/10.1016/j.jconrel.2014.01.013.

[51] P. Zhang, E. Wagner, History of polymeric gene delivery systems, Top. Curr. Chem. 375 (2017), https://doi.org/10.1007/s41061-017-0112-0.

[52] F.E. Farber, J.L. Melnick, J.S. Butel, Optimal conditions for uptake of exogenous DNA by chinese hamster lung cells deficient in hypoxanthine-guanine phosphoribosyltransferase, Biochim. Biophys. Acta - Nucleic Acids Protein Synth. 390 (1975) 298–311, https://doi.org/10.1016/0005-2787(75)90350-0.

[53] E. Ramsay, J. Hadgraft, J. Birchall, M. Gumbleton, Examination of the biophysical interaction between plasmid DNA and the polycations, polylysine and polyornithine, as a basis for their differential gene transfection in-vitro, Int. J. Pharm. 210 (2000) 97–107, https://doi.org/10.1016/S0378-5173(00)00571-8.

[54] G.Y. W, C.H. W, Receptor-mediated in Vitro Gene Transformation by a Soluble DNA Carrier System*, 1987.

[55] S. Schreiber, E. Kämpgen, E. Wagner, D. Pirkhammer, J. Trcka, H. Korschan, A. Lindemann, R. Dorffner, H. Kittler, F. Kasteliz, Z. Küpcü, A. Sinski, K. Zatloukal, M. Buschle, W. Schmidt, M. Birnstiel, R.E. Kempe, T. Voigt, H. A. Weber, H. Pehamberger, R. Mertelsmann, E.B. Bröcker, K. Wolff, G. Stingl, Immunotherapy of metastatic malignant melanoma by a vaccine consisting of autologous interleukin 2-transfected cancer cells: outcome of a phase I study, Hum. Gene Ther. 10 (1999) 983–993, https://doi.org/10.1089/10430349950018382.
[56] L. Wightman, E. Patzelt, E. Wagner, R. Kircheis, Development of transferrin-polycation/DNA based vectors for gene delivery to melanoma cells, J. Drug Target. 7 (1999) 293–303, https://doi.org/10.3109/10611869909085512.
[57] E. Wagner, K. Zatloukal, M. Cotten, H. Kirlappos, K. Mechtler, D.T. Curiel, M. L. Birnstiel, Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 6099–6103, https://doi.org/10.1073/pnas.89.13.6099.
[58] H. Luthman, G. Magnusson, High efficiency polyoma DNA transfection of chloroquine treated cells, Nucleic Acids Res. 11 (1983) 1295–1308, https://doi.org/10.1093/nar/11.5.1295.
[59] M. Cotten, F. Langle-Rouault, H. Kirlappos, E. Wagner, K. Mechtler, M. Zenke, H. Beug, M.L. Birnstiel, Transferrin-polycation-mediated introduction of DNA into human leukemic cells: stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 4033–4037, https://doi.org/10.1073/pnas.87.11.4033.
[60] P. Erbacher, A.C. Roche, M. Monsigny, P. Midoux, Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA/lactosylated polylysine complexes, Exp. Cell Res. 225 (1996) 186–194, https://doi.org/10.1006/excr.1996.0169.
[61] J. Cheng, R. Zeidan, S. Mishra, A. Liu, S.H. Pun, R.P. Kulkarni, G.S. Jensen, N. C. Bellocq, M.E. Davis, Structure-function correlation of chloroquine and analogues as transgene expression enhancers in nonviral gene delivery, J. Med. Chem. 49 (2006) 6522–6531, https://doi.org/10.1021/jm060736s.
[62] C. Plank, B. Oberhauser, K. Mechtler, C. Koch, E. Wagner, The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems, J. Biol. Chem. 269 (1994) 12918–12924. https://europepmc.org/article/med/8175709 (accessed August 18, 2020).
[63] T.B. Wyman, F. Nicol, O. Zelphati, P.V. Scaria, C. Plank, F.C. Szoka, Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers, Biochemistry 36 (1997) 3008–3017, https://doi.org/10.1021/bi9618474.
[64] W. Li, F. Nicol, F.C. Szoka, GALA: a designed synthetic pH-responsive amphipathic peptide with applications in drug and gene delivery, Adv. Drug Deliv. Rev. 56 (2004) 967–985, https://doi.org/10.1016/j.addr.2003.10.041.
[65] O. Boussif, F. LezoualC'H, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 7297–7301, https://doi.org/10.1073/pnas.92.16.7297.
[66] L.M.P. Vermeulen, S.C. De Smedt, K. Remaut, K. Braeckmans, The proton sponge hypothesis: fable or fact? Eur. J. Pharm. Biopharm. 129 (2018) 184–190, https://doi.org/10.1016/j.ejpb.2018.05.034.
[67] I.M.S. Degors, C. Wang, Z.U. Rehman, I.S. Zuhorn, Carriers break barriers in drug delivery: endocytosis and endosomal escape of gene delivery vectors, Acc. Chem. Res. 52 (2019) 1750–1760, https://doi.org/10.1021/acs.accounts.9b00177.
[68] V. Kafil, Y. Omidi, Cytotoxic impacts of linear and branched polyethylenimine nanostructures in A431 cells, BioImpacts 1 (2011) 23–30, https://doi.org/10.5681/bi.2011.004.
[69] D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, T. Kissel, In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis, Biomaterials 24 (2003) 1121–1131, https://doi.org/10.1016/S0142-9612(02)00445-3.
[70] D. Fischer, T. Bieber, Y. Li, H.P. Elsässer, T. Kissel, A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity, Pharm. Res. 16 (1999) 1273–1279, https://doi.org/10.1023/A:1014861900478.
[71] D.Y. Furgeson, W.S. Chan, J.W. Yockman, S.W. Kim, Modified linear polyethylenimine-cholesterol conjugates for DNA complexation, Bioconjug. Chem. 14 (2003) 840–847, https://doi.org/10.1021/bc0340565.
[72] Z. Zhong, J. Feijen, M.C. Lok, W.E. Hennink, L.V. Christensen, J.W. Yockman, Y. H. Kim, S.W. Kim, Low molecular weight linear polyethylenimine-b-poly (ethylene glycol)-b-polyethylenimine triblock copolymers: synthesis, characterization, and in vitro gene transfer properties, Biomacromolecules 6 (2005) 3440–3448, https://doi.org/10.1021/bm050505n.
[73] Y.H. Choi, F. Liu, J.S. Kim, Y.K. Choi, J.S. Park, S.W. Kim, Polyethylene glycol-grafted poly-L-lysine as polymeric gene carder, J. Control. Release 54 (1998) 39–48, https://doi.org/10.1016/S0168-3659(97)00174-0.
[74] L. Wightman, R. Kircheis, V. Rössler, S. Garotta, R. Ruzicka, M. Kursa, E. Wagner, Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo, J. Gene Med. 3 (2001) 362–372, https://doi.org/10.1002/jgm.187.
[75] K. Itaka, A. Harada, Y. Yamasaki, K. Nakamura, H. Kawaguchi, K. Kataoka, In situ single cell observation by fluorescence resonance energy transfer reveals fast intra-cytoplasmic delivery and easy release of plasmid DNA complexed with linear polyethylenimine, J. Gene Med. 6 (2004) 76–84, https://doi.org/10.1002/jgm.470.
[76] C. Plank, K. Mechtler, F.C. Szoka, E. Wagner, Activation of the complement system by synthetic DNA complexes: a potential barrier for intravenous gene delivery, Hum. Gene Ther. 7 (1996) 1437–1446, https://doi.org/10.1089/hum.1996.7.12-1437.
[77] J. Senior, C. Delgado, D. Fisher, C. Tilcock, G. Gregoriadis, Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles, BBA - Biomembr. 1062 (1991) 77–82, https://doi.org/10.1016/0005-2736(91)90337-8.
[78] A.L. Klibanov, K. Maruyama, V.P. Torchilin, L. Huang, Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes, FEBS Lett. 268 (1990) 235–237, https://doi.org/10.1016/0014-5793(90)81016-H.
[79] R. Laga, R. Carlisle, M. Tangney, K. Ulbrich, L.W. Seymour, Polymer coatings for delivery of nucleic acid therapeutics, J. Control. Release 161 (2012) 537–553, https://doi.org/10.1016/j.jconrel.2012.02.013.
[80] T. Merdan, K. Kunath, H. Petersen, U. Bakowsky, K.H. Voigt, J. Kopecek, T. Kissel, PEGylation of poly(ethylene imine) affects stability of complexes with plasmid DNA under in vivo conditions in a dose-dependent manner after intravenous injection into mice, Bioconjug. Chem. 16 (2005) 785–792, https://doi.org/10.1021/bc049743q.
[81] G.F. Walker, C. Fella, J. Pelisek, J. Fahrmeir, S. Boeckle, M. Ogris, E. Wagner, Toward synthetic viruses: endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer in vitro and in vivo, Mol. Ther. 11 (2005) 418–425, https://doi.org/10.1016/j.ymthe.2004.11.006.
[82] K. Knop, R. Hoogenboom, D. Fischer, U.S. Schubert, Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives, Angew. Chem. Int. Ed. 49 (2010) 6288–6308, https://doi.org/10.1002/anie.200902672.
[83] A.S. Abu Lila, H. Kiwada, T. Ishida, The accelerated blood clearance (ABC) phenomenon: clinical challenge and approaches to manage, J. Control. Release 172 (2013) 38–47, https://doi.org/10.1016/j.jconrel.2013.07.026.
[84] M. Barz, R. Luxenhofer, R. Zentel, M.J. Vicent, Overcoming the PEG-addiction: well-defined alternatives to PEG, from structure-property relationships to better defined therapeutics, Polym. Chem. 2 (2011) 1900–1918, https://doi.org/10.1039/c0py00406e.
[85] T.T. Hoang Thi, E.H. Pilkington, D.H. Nguyen, J.S. Lee, K.D. Park, N.P. Truong, The importance of poly(ethylene glycol) alternatives for overcoming PEG immunogenicity in drug delivery and bioconjugation, Polymers (Basel) 12 (2020) 298, https://doi.org/10.3390/polym12020298.
[86] L. Beckert, L. Kostka, E. Kessel, A. Krhac Levacic, H. Kostkova, T. Etrych, U. Lächelt, E. Wagner, Acid-labile pHPMA modification of four-arm oligoaminoamide pDNA polyplexes balances shielding and gene transfer activity in vitro and in vivo, Eur. J. Pharm. Biopharm. 105 (2016) 85–96, https://doi.org/10.1016/j.ejpb.2016.05.019.
[87] E. Hara, M. Ueda, C.J. Kim, A. Makino, I. Hara, E. Ozeki, S. Kimura, Suppressive immune response of poly-(sarcosine) chains in peptide-nanosheets in contrast to polymeric micelles, in: J. Pept. Sci, John Wiley and Sons Ltd, 2014, pp. 570–577, https://doi.org/10.1002/psc.2655.
[88] A. Birke, D. Huesmann, A. Kelsch, M. Weilbächer, J. Xie, M. Bros, T. Bopp, C. Becker, K. Landfester, M. Barz, Polypeptoid-block-polypeptide copolymers: synthesis, characterization, and application of amphiphilic block copolypept(o)ides in drug formulations and miniemulsion techniques, Biomacromolecules 15 (2014) 548–557, https://doi.org/10.1021/bm401542z.
[89] P.M. Klein, K. Klinker, W. Zhang, S. Kern, E. Kessel, E. Wagner, M. Barz, Efficient shielding of polyplexes using heterotelechelic polysarcosines, Polymers (Basel) 10 (2018), https://doi.org/10.3390/polym10060689.
[90] T.A. Tockary, K. Osada, Q. Chen, K. MacHitani, A. Dirisala, S. Uchida, T. Nomoto, K. Toh, Y. Matsumoto, K. Itaka, K. Nitta, K. Nagayama, K. Kataoka, Tethered PEG crowdedness determining shape and blood circulation profile of polyplex micelle gene carriers, Macromolecules 46 (2013) 6585–6592, https://doi.org/10.1021/ma401093z.
[91] K. Osada, H. Oshima, D. Kobayashi, M. Doi, M. Enoki, Y. Yamasaki, K. Kataoka, Quantized folding of plasmid DNA condensed with block catiomer into characteristic rod structures promoting transgene efficacy, J. Am. Chem. Soc. 132 (2010) 12343–12348, https://doi.org/10.1021/ja102739b.
[92] S. Venkataraman, W.L. Ong, Z.Y. Ong, S.C. Joachim Loo, P.L. Rachel Ee, Y. Y. Yang, The role of PEG architecture and molecular weight in the gene transfection performance of PEGylated poly(dimethylaminoethyl methacrylate) based cationic polymers, Biomaterials 32 (2011) 2369–2378, https://doi.org/10.1016/j.biomaterials.2010.11.070.
[93] M.A. Wolfert, E.H. Schacht, V. Toncheva, K. Ulbrich, O. Nazarova, L.W. Seymour, Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers, Hum. Gene Ther. 7 (1996) 2123–2133, https://doi.org/10.1089/hum.1996.7.17-2123.
[94] M. Oba, K. Miyata, K. Osada, R.J. Christie, M. Sanjoh, W. Li, S. Fukushima, T. Ishii, M.R. Kano, N. Nishiyama, H. Koyama, K. Kataoka, Polyplex micelles prepared from ω-cholesteryl PEG-polycation block copolymers for systemic gene delivery, Biomaterials 32 (2011) 652–663, https://doi.org/10.1016/j.biomaterials.2010.09.022.
[95] L.Y. Chan, Y.L. Khung, C.Y. Lin, Preparation of messenger RNA nanomicelles via non-cytotoxic PEG-polyamine nanocomplex for intracerebroventicular delivery: a proof-of-concept study in mouse models, Nanomaterials 9 (2019) 67, https://doi.org/10.3390/nano9010067.
[96] K.M. Takeda, K. Osada, T.A. Tockary, A. Dirisala, Q. Chen, K. Kataoka, Poly (ethylene glycol) crowding as critical factor to determine pDNA packaging scheme into polyplex micelles for enhanced gene expression, Biomacromolecules 18 (2017) 36–43, https://doi.org/10.1021/acs.biomac.6b01247.
[97] K. Miyata, N. Nishiyama, K. Kataoka, Rational design of smart supramolecular assemblies for gene delivery: chemical challenges in the creation of artificial

viruses, Chem. Soc. Rev. 41 (2012) 2562–2574, https://doi.org/10.1039/c1cs15258k.
[98] C. Fella, G.F. Walker, M. Ogris, E. Wagner, Amine-reactive pyridylhydrazone-based PEG reagents for pH-reversible PEI polyplex shielding, Eur. J. Pharm. Sci. 34 (2008) 309–320, https://doi.org/10.1016/j.ejps.2008.05.004.
[99] A.M. Funhoff, S. Monge, R. Teeuwen, G.A. Koning, N.M.E. Schuurmans-Nieuwenbroek, D.J.A. Crommelin, D.M. Haddleton, W.E. Hennink, C.F. Van Nostrum, PEG shielded polymeric double-layered micelles for gene delivery, J. Control. Release 102 (2005) 711–724, https://doi.org/10.1016/j.jconrel.2004.11.005.
[100] B. Vroman, I. Ferreira, C. Jérôme, R. Jérôme, V. Préat, PEGylated quaternized copolymer/DNA complexes for gene delivery, Int. J. Pharm. 344 (2007) 88–95, https://doi.org/10.1016/j.ijpharm.2007.06.044.
[101] D.G. Abebe, R. Kandil, T. Kraus, M. Elsayed, O.M. Merkel, T. Fujiwara, Three-layered biodegradable micelles prepared by two-step self-assembly of PLA-PEI-PLA and PLA-PEG-PLA triblock copolymers as efficient gene delivery system, Macromol. Biosci. 15 (2015) 698–711, https://doi.org/10.1002/mabi.201400488.
[102] J.G. Piao, J.J. Yan, M.Z. Wang, D.C. Wu, Y.Z. You, A new method to cross-link a polyplex for enhancing in vivo stability and transfection efficiency, Biomater. Sci. 2 (2014) 390–398, https://doi.org/10.1039/c3bm60204d.
[103] M.R. Elzes, N. Akeroyd, J.F.J. Engbersen, J.M.J. Paulusse, Disulfide-functional poly(amido amine)s with tunable degradability for gene delivery, J. Control. Release 244 (2016) 357–365, https://doi.org/10.1016/j.jconrel.2016.08.021.
[104] L. Novo, L.Y. Rizzo, S.K. Golombek, G.R. Dakwar, B. Lou, K. Remaut, E. Mastrobattista, C.F. Van Nostrum, W. Jahnen-Dechent, F. Kiessling, K. Braeckmans, T. Lammers, W.E. Hennink, Decationized polyplexes as stable and safe carrier systems for improved biodistribution in systemic gene therapy, J. Control. Release 195 (2014) 162–175, https://doi.org/10.1016/j.jconrel.2014.08.028.
[105] Y. Fang, J. Xue, S. Gao, A. Lu, D. Yang, H. Jiang, Y. He, K. Shi, Cleavable PEGylation: a strategy for overcoming the "PEG dilemma" in efficient drug delivery, Drug Deliv. 24 (2017) 22–32, https://doi.org/10.1080/10717544.2017.1388451.
[106] H. Hatakeyama, H. Akita, H. Harashima, The polyethyleneglycol dilemma: advantage and disadvantage of PEGylation of liposomes for systemic genes and nucleic acids delivery to tumors, Biol. Pharm. Bull. 36 (2013) 892–899, https://doi.org/10.1248/bpb.b13-00059.
[107] I.A. Khalil, K. Kogure, H. Akita, H. Harashima, Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery, Pharmacol. Rev. 58 (2006) 32–45, https://doi.org/10.1124/pr.58.1.8.
[108] R.H. Prabhu, V.B. Patravale, M.D. Joshi, Polymeric nanoparticles for targeted treatment in oncology: current insights, Int. J. Nanomedicine 10 (2015) 1001–1018, https://doi.org/10.2147/IJN.S56932.
[109] J. Park, K. Singha, S. Son, J. Kim, R. Namgung, C.O. Yun, W.J. Kim, A review of RGD-functionalized nonviral gene delivery vectors for cancer therapy, Cancer Gene Ther. 19 (2012) 741–748, https://doi.org/10.1038/cgt.2012.64.
[110] R.M. Schiffelers, A. Ansari, J. Xu, Q. Zhou, Q. Tang, G. Storm, G. Molema, P.Y. Lu, P.V. Scaria, M.C. Woodle, Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle, Nucleic Acids Res. 32 (2004), e149, https://doi.org/10.1093/nar/gnh140.
[111] M.E. Davis, J.E. Zuckerman, C.H.J. Choi, D. Seligson, A. Tolcher, C.A. Alabi, Y. Yen, J.D. Heidel, A. Ribas, Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles, Nature 464 (2010) 1067–1070, https://doi.org/10.1038/nature08956.
[112] A.R. Hilgenbrink, P.S. Low, Folate receptor-mediated drug targeting: from therapeutics to diagnostics, J. Pharm. Sci. 94 (2005) 2135–2146, https://doi.org/10.1002/jps.20457.
[113] H. Mok, T.G. Park, Functional polymers for targeted delivery of nucleic acid drugs, Macromol. Biosci. 9 (2009) 731–743, https://doi.org/10.1002/mabi.200900044.
[114] M. Fernández, F. Javaid, V. Chudasama, Advances in targeting the folate receptor in the treatment/imaging of cancers, Chem. Sci. 9 (2018) 790–810, https://doi.org/10.1039/c7sc04004k.
[115] C. Dohmen, D. Edinger, T. Fröhlich, L. Schreiner, U. Lächelt, C. Troiber, J. Rädler, P. Hadwiger, H.P. Vornlocher, E. Wagner, Nanosized multifunctional polyplexes for receptor-mediated SiRNA delivery, ACS Nano 6 (2012) 5198–5208, https://doi.org/10.1021/nn300960m.
[116] E. Nogueira, A.C. Gomes, A. Preto, A. Cavaco-Paulo, Folate-targeted nanoparticles for rheumatoid arthritis therapy, Nanomed. Nanotechnol. Biol. Med. 12 (2016) 1113–1126, https://doi.org/10.1016/j.nano.2015.12.365.
[117] D.E. Large, J.R. Soucy, J. Hebert, D.T. Auguste, Advances in receptor-mediated, tumor-targeted drug delivery, Adv. Ther. 2 (2019) 1800091, https://doi.org/10.1002/adtp.201800091.
[118] T. Bus, A. Traeger, U.S. Schubert, The great escape: how cationic polyplexes overcome the endosomal barrier, J. Mater. Chem. B 6 (2018) 6904–6918, https://doi.org/10.1039/c8tb00967h.
[119] M.A. Mintzer, E.E. Simanek, Nonviral vectors for gene delivery, Chem. Rev. 109 (2009) 259–302, https://doi.org/10.1021/cr800409e.
[120] J. Behr, The proton sponge: a trick to enter cells the viruses did not exploit, Chimia (Aarau) 51 (1997) 34–36.
[121] J.M. Benns, J.S. Choi, R.I. Mahato, J.S. Park, Sung Wan Kim, pH-sensitive cationic polymer gene delivery vehicle: N-Ac-poly(L-histidine)-graft-poly(L-lysine) comb shaped polymer, Bioconjug. Chem. 11 (2000) 637–645, https://doi.org/10.1021/bc0000177.
[122] D.T. Curiel, S. Agarwal, E. Wagner, M. Cotten, Adenovirus enhancement of transferrin-polylysine-mediated gene delivery, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 8850–8854, https://doi.org/10.1073/pnas.88.19.8850.
[123] W. Zauner, D. Blaas, E. Kuechler, E. Wagner, Rhinovirus-mediated endosomal release of transfection complexes, J. Virol. 69 (1995) 1085–1092, https://doi.org/10.1128/jvi.69.2.1085-1092.1995.
[124] A.K. Varkouhi, M. Scholte, G. Storm, H.J. Haisma, Endosomal escape pathways for delivery of biologicals, J. Control. Release 151 (2011) 220–228, https://doi.org/10.1016/j.jconrel.2010.11.004.
[125] R. Ni, J. Zhou, N. Hossain, Y. Chau, Virus-inspired nucleic acid delivery system: linking virus and viral mimicry, Adv. Drug Deliv. Rev. 106 (2016) 3–26, https://doi.org/10.1016/j.addr.2016.07.005.
[126] K.G. de Bruin, C. Fella, M. Ogris, E. Wagner, N. Ruthardt, C. Bräuchle, Dynamics of photoinduced endosomal release of polyplexes, J. Control. Release 130 (2008) 175–182, https://doi.org/10.1016/j.jconrel.2008.06.001.
[127] N. Murthy, J. Campbell, N. Fausto, A.S. Hoffman, P.S. Stayton, Design and synthesis of pH-responsive polymeric carriers that target uptake and enhance the intracellular delivery of oligonucleotides, J. Control. Release 89 (2003) 365–374, https://doi.org/10.1016/S0168-3659(03)00099-3.
[128] Y. Matsumoto, K. Itaka, T. Yamasoba, K. Kataoka, Intranuclear fluorescence resonance energy transfer analysis of plasmid DNA decondensation from nonviral gene carriers, J. Gene Med. 11 (2009) 615–623, https://doi.org/10.1002/jgm.1338.
[129] J. Shi, B. Chou, J.L. Choi, A.L. Ta, S.H. Pun, Investigation of polyethylenimine/DNA polyplex transfection to cultured cells using radiolabeling and subcellular fractionation methods, Mol. Pharm. 10 (2013) 2145–2156, https://doi.org/10.1021/mp300651q.
[130] R.N. Cohen, M.A.E.M. van der Aa, N. Macaraeg, A.P. Lee, F.C. Szoka, Quantification of plasmid DNA copies in the nucleus after lipoplex and polyplex transfection, J. Control. Release 135 (2009) 166–174, https://doi.org/10.1016/j.jconrel.2008.12.016.
[131] M.J. Reilly, J.D. Larsen, M.O. Sullivan, Histone H3 tail peptides and poly (ethylenimine) have synergistic effects for gene delivery, Mol. Pharm. 9 (2012) 1031–1040, https://doi.org/10.1021/mp200372s.
[132] J.D. Larsen, N.L. Ross, M.O. Sullivan, Requirements for the nuclear entry of polyplexes and nanoparticles during mitosis, J. Gene Med. 14 (2012) 580–589, https://doi.org/10.1002/jgm.2669.
[133] M.A. Zanta, P. Belguise-Valladier, J.P. Behr, Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 91–96, https://doi.org/10.1073/pnas.96.1.91.
[134] C.W. Pouton, K.M. Wagstaff, D.M. Roth, G.W. Moseley, D.A. Jans, Targeted delivery to the nucleus, Adv. Drug Deliv. Rev. 59 (2007) 698–717, https://doi.org/10.1016/j.addr.2007.06.010.
[135] M.A.E.M. Van Der Aa, E. Mastrobattista, R.S. Oosting, W.E. Hennink, G.A. Koning, D.J.A. Crommelin, The nuclear pore complex: the gateway to successful nonviral gene delivery, Pharm. Res. 23 (2006) 447–459, https://doi.org/10.1007/s11095-005-9445-4.
[136] M.A.E.M. van der Aa, G.A. Koning, C. d'Oliveira, R.S. Oosting, K.J. Wilschut, W. E. Hennink, D.J.A. Crommelin, An NLS peptide covalently linked to linear DNA does not enhance transfection efficiency of cationic polymer based gene delivery systems, J. Gene Med. 7 (2005) 208–217, https://doi.org/10.1002/jgm.643.
[137] D.S. Manickam, D. Oupický, Multiblock reducible copolypeptides containing histidine-rich and nuclear localization sequences for gene delivery, Bioconjug. Chem. 17 (2006) 1395–1403, https://doi.org/10.1021/bc060104k.
[138] O. Madkhali, G. Mekhail, S.D. Wettig, Modified gelatin nanoparticles for gene delivery, Int. J. Pharm. 554 (2019) 224–234, https://doi.org/10.1016/j.ijpharm.2018.11.001.
[139] J.M. Dang, K.W. Leong, Natural polymers for gene delivery and tissue engineering, Adv. Drug Deliv. Rev. 58 (2006) 487–499, https://doi.org/10.1016/j.addr.2006.03.001.
[140] S.V. Zaitsev, A. Haberland, A. Otto, V.I. Vorob'ev, H. Haller, M. Böttger, H1 and HMG 17 extracted from calf thymus nuclei are efficient DNA carriers in gene transfer, Gene Ther. 4 (1997) 586–592, https://doi.org/10.1038/sj.gt.3300433.
[141] M. Böttger, F. Vogel, M. Platzer, U. Kiessling, K. Grade, M. Strauss, Condensation of vector DNA by the chromosomal protein HMG1 results in efficient transfection, BBA - Gene Struct. Expr. 950 (1988) 221–228, https://doi.org/10.1016/0167-4781(88)90014-0.
[142] J. Chen, R.J. Stickles, K.A. Daichendt, Galactosylated histone-mediated gene transfer and expression, Hum. Gene Ther. 5 (1994) 429–435, https://doi.org/10.1089/hum.1994.5.4-429.
[143] T. Kushibiki, N. Nagata-Nakajima, M. Sugai, A. Shimizu, Y. Tabata, Delivery of plasmid DNA expressing small interference RNA for TGF-β type II receptor by cationized gelatin to prevent interstitial renal fibrosis, J. Control. Release 105 (2005) 318–331, https://doi.org/10.1016/j.jconrel.2005.02.030.
[144] T. Kushibiki, N. Nagata-Nakajima, M. Sugai, A. Shimizu, Y. Tabata, Targeting of plasmid DNA to renal interstitial fibroblasts by cationized gelatin, Biol. Pharm. Bull. 28 (2005) 2007–2010, https://doi.org/10.1248/bpb.28.2007.
[145] F. Takeshita, Y. Minakuchi, S. Nagahara, K. Honma, H. Sasaki, K. Hirai, T. Teratani, N. Namatame, Y. Yamamoto, K. Hanai, T. Kato, A. Sano, T. Ochiya, Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen in vivo, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 12177–12182, https://doi.org/10.1073/pnas.0501753102.
[146] S. Mao, W. Sun, T. Kissel, Chitosan-based formulations for delivery of DNA and siRNA, Adv. Drug Deliv. Rev. 62 (2010) 12–27, https://doi.org/10.1016/j.addr.2009.08.004.

[147] K.L. Chang, Y. Higuchi, S. Kawakami, F. Yamashita, M. Hashida, Efficient gene transfection by histidine-modified chitosan through enhancement of endosomal escape, Bioconjug. Chem. 21 (2010) 1087–1095, https://doi.org/10.1021/bc1000609.
[148] I.K. Park, T.H. Kim, Y.H. Park, B.A. Shin, E.S. Choi, E.H. Chowdhury, T. Akaike, C. S. Cho, Galactosylated chitosan-graft-poly(ethylene glycol) as hepatocyte-targeting DNA carrier, J. Control. Release 76 (2001) 349–362, https://doi.org/10.1016/S0168-3659(01)00448-5.
[149] D. Lee, W. Zhang, S.A. Shirley, X. Kong, G.R. Hellermann, R.F. Lockey, S. S. Mohapatra, Thiolated chitosan/DNA nanocomplexes exhibit enhanced and sustained gene delivery, Pharm. Res. 24 (2007) 157–167, https://doi.org/10.1007/s11095-006-9136-9.
[150] F. Van De Manakker, T. Vermonden, C.F. Van Nostrum, W.E. Hennink, Cyclodextrin-based polymeric materials: synthesis, properties, and pharmaceutical/biomedical applications, Biomacromolecules 10 (2009) 3157–3175, https://doi.org/10.1021/bm901065f.
[151] S.H. Pun, N.C. Bellocq, A. Liu, G. Jensen, T. Machemer, E. Quijano, T. Schluep, S. Wen, H. Engler, J. Heidel, M.E. Davis, Cyclodextrin-modified polyethylenimine polymers for gene delivery, Bioconjug. Chem. 15 (2004) 831–840, https://doi.org/10.1021/bc049891g.
[152] H.P. Suzie, M.E. Davis, Development of a nonviral gene delivery vehicle for systemic application, Bioconjug. Chem. 13 (2002) 630–639, https://doi.org/10.1021/bc0155768.
[153] N.C. Bellocq, S.H. Pun, G.S. Jensen, M.E. Davis, Transferrin-containing, cyclodextrin polymer-based particles for tumor-targeted gene delivery, Bioconjug. Chem. 14 (2003) 1122–1132, https://doi.org/10.1021/bc034125f.
[154] M.E. Davis, The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic, Mol. Pharm. 6 (2009) 659–668, https://doi.org/10.1021/mp900015y.
[155] Tooru Ooya, Hak Soo Choi, Atsushi Yamashita, Nobuhiko Yui, Yuko Sugaya, Arihiro Kano, Atsushi Maruyama, Hidetaka Akita, Rie Ito, Kentaro Kogure, Hideyoshi Harashima, Biocleavable polyrotaxane−plasmid DNA polyplex for enhanced gene delivery, J. Am. Chem. Soc. 128 (2006) 3852–3853, https://doi.org/10.1021/JA055868+.
[156] J. Luten, C.F. van Nostrum, S.C. De Smedt, W.E. Hennink, Biodegradable polymers as non-viral carriers for plasmid DNA delivery, J. Control. Release 126 (2008) 97–110, https://doi.org/10.1016/j.jconrel.2007.10.028.
[157] C.K. Chen, P.K. Huang, W.C. Law, C.H. Chu, N.T. Chen, L.W. Lo, Biodegradable polymers for gene-delivery applications, Int. J. Nanomedicine 15 (2020) 2131–2150, https://doi.org/10.2147/IJN.S222419.
[158] Y.B. Lim, C.H. Kim, K. Kim, S.W. Kim, J.S. Park, Development of a safe gene delivery system using biodegradable polymer, poly[α-(4-aminobutyl)-L-glycolic acid] [19], J. Am. Chem. Soc. 122 (2000) 6524–6525, https://doi.org/10.1021/ja001033h.
[159] A. Akinc, A. Zumbuehl, M. Goldberg, E.S. Leshchiner, V. Busini, N. Hossain, S. A. Bacallado, D.N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. De Fougerolles, J.R. Dorkin, K. Narayanannair Jayaprakash, M. Jayaraman, M. John, V. Koteliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K.G. Rajeev, D.W.Y. Sah, J. Soutschek, I. Toudjarska, H. P. Vornlocher, T.S. Zimmermann, R. Langer, D.G. Anderson, A combinatorial library of lipid-like materials for delivery of RNAi therapeutics, Nat. Biotechnol. 26 (2008) 561–569, https://doi.org/10.1038/nbt1402.
[160] D.G. Anderson, D.M. Lynn, R. Langer, Semi-automated synthesis and screening of a large library of degradable cationic polymers for gene delivery, Angew. Chem. Int. Ed. 42 (2003) 3153–3158, https://doi.org/10.1002/anie.200351244.
[161] C. Lin, Z. Zhong, M.C. Lok, X. Jiang, W.E. Hennink, J. Feijen, J.F.J. Engbersen, Novel bioreducible poly(amido amine)s for highly efficient gene delivery, Bioconjug. Chem. 18 (2007) 138–145, https://doi.org/10.1021/bc060200l.
[162] J. Hoon Jeong, L.V. Christensen, J.W. Yockman, Z. Zhong, J.F.J. Engbersen, W. Jong Kim, J. Feijen, S. Wan Kim, Reducible poly(amido ethylenimine) directed to enhance RNA interference, Biomaterials 28 (2007) 1912–1917, https://doi.org/10.1016/j.biomaterials.2006.12.019.
[163] C. Lin, Z. Zhong, M.C. Lok, X. Jiang, W.E. Hennink, J. Feijen, J.F.J. Engbersen, Linear poly(amido amine)s with secondary and tertiary amino groups and variable amounts of disulfide linkages: synthesis and in vitro gene transfer properties, J. Control. Release 116 (2006) 130–137, https://doi.org/10.1016/j.jconrel.2006.09.009.
[164] P. Vader, L.J. Van Der Aa, J.F.J. Engbersen, G. Storm, R.M. Schiffelers, Disulfide-based poly(amido amine)s for siRNA delivery: effects of structure on siRNA complexation, cellular uptake, gene silencing and toxicity, Pharm. Res. 28 (2011) 1013–1022, https://doi.org/10.1007/s11095-010-0344-y.
[165] L. Novo, E. Mastrobattista, C.F. van Nostrum, T. Lammers, W.E. Hennink, Decationized polyplexes for gene delivery, Expert Opin. Drug Deliv. 12 (2015) 507–512, https://doi.org/10.1517/17425247.2015.988136.
[166] L. Novo, E.V.B. Van Gaal, E. Mastrobattista, C.F. Van Nostrum, W.E. Hennink, Decationized crosslinked polyplexes for redox-triggered gene delivery, J. Control. Release 169 (2013) 246–256, https://doi.org/10.1016/j.jconrel.2013.03.035.
[167] L. Novo, E. Mastrobattista, C.F. van Nostrum, W.E. Hennink, Targeted decationized polyplexes for cell specific gene delivery, Bioconjug. Chem. 25 (2014) 802–812, https://doi.org/10.1021/bc500074a.
[168] L.Y. Rizzo, S.K. Golombek, M.E. Mertens, Y. Pan, D. Laaf, J. Broda, J. Jayapaul, D. Möckel, V. Subr, W.E. Hennink, G. Storm, U. Simon, W. Jahnen-Dechent, F. Kiessling, T. Lammers, In vivo nanotoxicity testing using the zebrafish embryo assay, J. Mater. Chem. B 1 (2013) 3918–3925, https://doi.org/10.1039/c3tb20528b.
[169] S. Kunjachan, F. Gremse, B. Theek, P. Koczera, R. Pola, M. Pechar, T. Etrych, K. Ulbrich, G. Storm, F. Kiessling, T. Lammers, Noninvasive optical imaging of nanomedicine biodistribution, ACS Nano 7 (2013) 252–262, https://doi.org/10.1021/nn303955n.
[170] J. Hong, C.-O. Yun, Overcoming the limitations of locally administered oncolytic virotherapy, BMC Biomed. Eng. 1 (2019), https://doi.org/10.1186/s42490-019-0016-x.
[171] D. Kirn, Clinical research results with dl1520 (Onyx-015), a replication-selective adenovirus for the treatment of cancer: what have we learned? Gene Ther. 8 (2001) 89–98, https://doi.org/10.1038/sj.gt.3301377.
[172] K. Garber, China approves world's first oncolytic virus therapy for cancer treatment, J. Natl. Cancer Inst. 98 (2006) 298–300, https://doi.org/10.1093/jnci/djj111.
[173] J.W. Choi, Y.S. Lee, C.O. Yun, S.W. Kim, Polymeric oncolytic adenovirus for cancer gene therapy, J. Control. Release 219 (2015) 181–191, https://doi.org/10.1016/j.jconrel.2015.10.009.
[174] D. Kasala, A.R. Yoon, J. Hong, S.W. Kim, C.O. Yun, Evolving lessons on nanomaterial-coated viral vectors for local and systemic gene therapy, Nanomedicine 11 (2016) 1689–1713, https://doi.org/10.2217/nnm-2016-0060.
[175] A.R. Yoon, J. Hong, S.W. Kim, C.O. Yun, Redirecting adenovirus tropism by genetic, chemical, and mechanical modification of the adenovirus surface for cancer gene therapy, Expert Opin. Drug Deliv. 13 (2016) 843–858, https://doi.org/10.1517/17425247.2016.1158707.
[176] H. Fausther-Bovendo, G.P. Kobinger, Pre-existing immunity against ad vectors: humoral, cellular, and innate response, what's important? Hum. Vaccines Immunother. 10 (2014) 2875–2884, https://doi.org/10.4161/hv.29594.
[177] C.T. Giaginis, A.C. Zarros, M.A. Papaefthymiou, A.E. Papadopouli, I. K. Sfiniadakis, S.E. Theocharis, Coxsackievirus and adenovirus receptor expression in human endometrial adenocarcinoma: possible clinical implications, World J. Surg. Oncol. 6 (2008) 1–8, https://doi.org/10.1186/1477-7819-6-59.
[178] Y.Y. Ma, X.J. Wang, Y. Han, G. Li, H.J. Wang, S.B. Wang, X.Y. Chen, F.L. Liu, X. L. He, X.M. Tong, X.Z. Mou, Loss of coxsackie and adenovirus receptor expression in human colorectal cancer: a potential impact on the efficacy of adenovirus-mediated gene therapy in Chinese Han population, Mol. Med. Rep. 14 (2016) 2541–2547, https://doi.org/10.3892/mmr.2016.5536.
[179] D. Kasala, S.H. Lee, J.W. Hong, J.W. Choi, K. Nam, Y.H. Chung, S.W. Kim, C. O. Yun, Synergistic antitumor effect mediated by a paclitaxel-conjugated polymeric micelle-coated oncolytic adenovirus, Biomaterials 145 (2017) 207–222, https://doi.org/10.1016/j.biomaterials.2017.08.035.
[180] J.W. Choi, J. Kim, Q.N. Bui, Y. Li, C.O. Yun, D.S. Lee, S.W. Kim, Tuning surface charge and PEGylation of biocompatible polymers for efficient delivery of nucleic acid or adenoviral vector, Bioconjug. Chem. 26 (2015) 1818–1829, https://doi.org/10.1021/acs.bioconjchem.5b00357.
[181] J.W. Choi, J.P. Nam, K. Nam, Y.S. Lee, C.O. Yun, S.W. Kim, Oncolytic adenovirus coated with multidegradable bioreducible core-cross-linked polyethylenimine for cancer gene therapy, Biomacromolecules 16 (2015) 2132–2143, https://doi.org/10.1021/acs.biomac.5b00538.
[182] C.H. Lee, D. Kasala, Y. Na, M.S. Lee, S.W. Kim, J.H. Jeong, C.O. Yun, Enhanced therapeutic efficacy of an adenovirus-PEI-bile-acid complex in tumors with low coxsackie and adenovirus receptor expression, Biomaterials 35 (2014) 5505–5516, https://doi.org/10.1016/j.biomaterials.2014.03.060.
[183] J. Kim, Y. Li, S.W. Kim, D.S. Lee, C.O. Yun, Therapeutic efficacy of a systemically delivered oncolytic adenovirus - biodegradable polymer complex, Biomaterials 34 (2013) 4622–4631, https://doi.org/10.1016/j.biomaterials.2013.03.004.
[184] P.H. Kim, J. Kim, T. Il Kim, H.Y. Nam, J.W. Yockman, M. Kim, S.W. Kim, C. O. Yun, Bioreducible polymer-conjugated oncolytic adenovirus for hepatoma-specific therapy via systemic administration, Biomaterials 32 (2011) 9328–9342, https://doi.org/10.1016/j.biomaterials.2011.08.066.
[185] P.H. Kim, T. Il Kim, J.W. Yockman, S.W. Kim, C.O. Yun, The effect of surface modification of adenovirus with an arginine-grafted bioreducible polymer on transduction efficiency and immunogenicity in cancer gene therapy, Biomaterials 31 (2010) 1865–1874, https://doi.org/10.1016/j.biomaterials.2009.11.043.
[186] M. Ogris, S. Brunner, S. Schüller, R. Kircheis, E. Wagner, PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery, Gene Ther. 6 (1999) 595–605, https://doi.org/10.1038/sj.gt.3300900.
[187] C.F. Jones, R.A. Campbell, A.E. Brooks, S. Assemi, S. Tadjiki, G. Thiagarajan, C. Mulcock, A.S. Weyrich, B.D. Brooks, H. Ghandehari, D.W. Grainger, Cationic PAMAM dendrimers aggressively initiate blood clot formation, ACS Nano 6 (2012) 9900–9910, https://doi.org/10.1021/nn303472r.
[188] P. Chollet, M.C. Favrot, A. Hurbin, J.L. Coll, Side-effects of a systemic injection of linear polyethylenimine-DNA complexes, J. Gene Med. 4 (2002) 84–91, https://doi.org/10.1002/jgm.237.
[189] A.D. Wong, M. Ye, M.B. Ulmschneider, P.C. Searson, Quantitative analysis of the enhanced permeation and retention (EPR) effect, PLoS One 10 (2015), e0123461, https://doi.org/10.1371/journal.pone.0123461.
[190] Y. Na, J.W. Choi, D. Kasala, J.W. Hong, E. Oh, Y. Li, S.J. Jung, S.W. Kim, C. O. Yun, Potent antitumor effect of neurotensin receptor-targeted oncolytic adenovirus co-expressing decorin and Wnt antagonist in an orthotopic pancreatic tumor model, J. Control. Release 220 (2015) 766–782, https://doi.org/10.1016/j.jconrel.2015.10.015.
[191] J. Kim, H.Y. Nam, J.W. Choi, C.O. Yun, S.W. Kim, Efficient lung orthotopic tumor-growth suppression of oncolytic adenovirus complexed with RGD-targeted bioreducible polymer, Gene Ther. 21 (2014) 476–483, https://doi.org/10.1038/gt.2014.18.

[192] O.J. Kwon, E. Kang, J.W. Choi, S.W. Kim, C.O. Yun, Therapeutic targeting of chitosan-PEG-folate-complexed oncolytic adenovirus for active and systemic cancer gene therapy, J. Control. Release 169 (2013) 257–265, https://doi.org/10.1016/j.jconrel.2013.03.030.
[193] J. Kim, H.Y. Nam, T. Il Kim, P.H. Kim, J. Ryu, C.O. Yun, S.W. Kim, Active targeting of RGD-conjugated bioreducible polymer for delivery of oncolytic adenovirus expressing shRNA against IL-8 mRNA, Biomaterials 32 (2011) 5158–5166, https://doi.org/10.1016/j.biomaterials.2011.03.084.
[194] P.H. Kim, J.H. Sohn, J.W. Choi, Y. Jung, S.W. Kim, S. Haam, C.O. Yun, Active targeting and safety profile of PEG-modified adenovirus conjugated with herceptin, Biomaterials 32 (2011) 2314–2326, https://doi.org/10.1016/j.biomaterials.2010.10.031.
[195] A.R. Yoon, J.W. Hong, M. Kim, C.O. Yun, Hepatocellular carcinoma-targeting oncolytic adenovirus overcomes hypoxic tumor microenvironment and effectively disperses through both central and peripheral tumor regions, Sci. Rep. 8 (2018), https://doi.org/10.1038/s41598-018-20268-6.
[196] E. Oh, J. Hong, O.J. Kwon, C.O. Yun, A hypoxia- and telomerase-responsive oncolytic adenovirus expressing secretable trimeric TRAIL triggers tumour-specific apoptosis and promotes viral dispersion in TRAIL-resistant glioblastoma, Sci. Rep. 8 (2018), https://doi.org/10.1038/s41598-018-19300-6.
[197] C.Y. Moon, J.W. Choi, D. Kasala, S.J. Jung, S.W. Kim, C.O. Yun, Dual tumor targeting with pH-sensitive and bioreducible polymer-complexed oncolytic adenovirus, Biomaterials 41 (2015) 53–68, https://doi.org/10.1016/j.biomaterials.2014.11.021.
[198] M. Vähä-Koskela, A. Hinkkanen, Tumor restrictions to oncolytic virus, Biomedicines 2 (2014) 163–194, https://doi.org/10.3390/biomedicines2020163.
[199] J.W. Choi, E. Kang, O.J. Kwon, T.J. Yun, H.K. Park, P.H. Kim, S.W. Kim, J.H. Kim, C.O. Yun, Local sustained delivery of oncolytic adenovirus with injectable alginate gel for cancer virotherapy, Gene Ther. 20 (2013) 880–892, https://doi.org/10.1038/gt.2013.10.
[200] A. Yen, Y. Cheng, M. Sylvestre, H.H. Gustafson, S. Puri, S.H. Pun, Serum nuclease susceptibility of mRNA cargo in condensed Polyplexes, Mol. Pharm. 15 (2018) 2268–2276, https://doi.org/10.1021/acs.molpharmaceut.8b00134.
[201] X. Shen, D.R. Corey, Chemistry, mechanism and clinical status of antisense oligonucleotides and duplex RNAs, Nucleic Acids Res. 46 (2018) 1584–1600, https://doi.org/10.1093/nar/gkx1239.
[202] C. Meng, Z. Chen, G. Li, T. Welte, H. Shen, Nanoplatforms for mRNA therapeutics, Adv. Ther. 2000099 (2020), https://doi.org/10.1002/adtp.202000099.
[203] C. Zhang, G. Maruggi, H. Shan, J. Li, Advances in mRNA vaccines for infectious diseases, Front. Immunol. 10 (2019) 594, https://doi.org/10.3389/fimmu.2019.00594.
[204] A. Dirisala, S. Uchida, T.A. Tockary, N. Yoshinaga, J. Li, S. Osawa, L. Gorantla, S. Fukushima, K. Osada, K. Kataoka, Precise tuning of disulphide crosslinking in mRNA polyplex micelles for optimising extracellular and intracellular nuclease tolerability, J. Drug Target. 27 (2019) 670–680, https://doi.org/10.1080/1061186X.2018.1550646.
[205] B. Lou, S. De Koker, C.Y.J. Lau, W.E. Hennink, E. Mastrobattista, MRNA Polyplexes with post-conjugated GALA peptides efficiently target, transfect, and activate antigen presenting cells, Bioconjug. Chem. 30 (2019) 461–475, https://doi.org/10.1021/acs.bioconjchem.8b00524.
[206] M. Ruponen, S. Ylä-Herttuala, A. Urtti, Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies, Biochim. Biophys. Acta Biomembr. 1415 (1999) 331–341, https://doi.org/10.1016/S0005-2736(98)00199-0.
[207] S. Katayose, K. Kataoka, Remarkable increase in nuclease resistance of plasmid DNA through supramolecular assembly with poly(ethylene glycol)-poly(L-lysine) block copolymer, J. Pharm. Sci. 87 (1998) 160–163, https://doi.org/10.1021/js970304s.
[208] S. Osawa, K. Osada, S. Hiki, A. Dirisala, T. Ishii, K. Kataoka, Polyplex micelles with double-protective compartments of hydrophilic shell and thermoswitchable palisade of poly(oxazoline)-based block copolymers for promoted gene transfection, Biomacromolecules 17 (2016) 354–361, https://doi.org/10.1021/acs.biomac.5b01456.
[209] F. Meng, W.E. Hennink, Z. Zhong, Reduction-sensitive polymers and bioconjugates for biomedical applications, Biomaterials 30 (2009) 2180–2198, https://doi.org/10.1016/j.biomaterials.2009.01.026.
[210] L. Brülisauer, M.A. Gauthier, J.C. Leroux, Disulfide-containing parenteral delivery systems and their redox-biological fate, J. Control. Release 195 (2014) 147–154, https://doi.org/10.1016/j.jconrel.2014.06.012.
[211] J. Kim, Y.M. Lee, H. Kim, D. Park, J. Kim, W.J. Kim, Phenylboronic acid-sugar grafted polymer architecture as a dual stimuli-responsive gene carrier for targeted anti-angiogenic tumor therapy, Biomaterials 75 (2016) 102–111, https://doi.org/10.1016/j.biomaterials.2015.10.022.
[212] X.D. Guo, F. Tandiono, N. Wiradharma, D. Khor, C.G. Tan, M. Khan, Y. Qian, Y.-Y. Yang, Cationic micelles self-assembled from cholesterol-conjugated oligopeptides as an efficient gene delivery vector, Biomaterials 29 (2008) 4838–4846, https://doi.org/10.1016/j.biomaterials.2008.07.053.
[213] S. Uchida, H. Kinoh, T. Ishii, A. Matsui, T.A. Tockary, K.M. Takeda, H. Uchida, K. Osada, K. Itaka, K. Kataoka, Systemic delivery of messenger RNA for the treatment of pancreatic cancer using polyplex nanomicelles with a cholesterol moiety, Biomaterials 82 (2016) 221–228, https://doi.org/10.1016/j.biomaterials.2015.12.031.
[214] C. Troiber, D. Edinger, P. Kos, L. Schreiner, R. Kläger, A. Herrmann, E. Wagner, Stabilizing effect of tyrosine trimers on pDNA and siRNA polyplexes, Biomaterials 34 (2013) 1624–1633, https://doi.org/10.1016/j.biomaterials.2012.11.021.
[215] C. Cheng, A.J. Convertine, P.S. Stayton, J.D. Bryers, Multifunctional triblock copolymers for intracellular messenger RNA delivery, Biomaterials 33 (2012) 6868–6876, https://doi.org/10.1016/j.biomaterials.2012.06.020.
[216] R. Barbalat, S.E. Ewald, M.L. Mouchess, G.M. Barton, Nucleic acid recognition by the innate immune system, Annu. Rev. Immunol. 29 (2011) 185–214, https://doi.org/10.1146/annurev-immunol-031210-101340.
[217] K. Karikó, M. Buckstein, H. Ni, D. Weissman, Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA, Immunity 23 (2005) 165–175, https://doi.org/10.1016/j.immuni.2005.06.008.
[218] S. Uchida, K. Kataoka, K. Itaka, Screening of mRNA chemical modification to maximize protein expression with reduced immunogenicity, Pharmaceutics 7 (2015) 137–151, https://doi.org/10.3390/pharmaceutics7030137.
[219] S. Uchida, K. Itaka, H. Uchida, K. Hayakawa, T. Ogata, T. Ishii, S. Fukushima, K. Osada, K. Kataoka, In vivo messenger RNA introduction into the central nervous system using polyplex nanomicelle, PLoS One 8 (2013), e56220, https://doi.org/10.1371/journal.pone.0056220.
[220] W.W. Zhang, L. Li, D. Li, J. Liu, X. Li, W. Li, X. Xu, M.J. Zhang, L.A. Chandler, H. Lin, A. Hu, W. Xu, D.M.K. Lam, The first approved gene therapy product for cancer Ad-p53 (Gendicine): 12 years in the clinic, Hum. Gene Ther. 29 (2018) 160–179, https://doi.org/10.1089/hum.2017.218.
[221] M. Senior, After Glybera's withdrawal, what's next for gene therapy? Nat. Biotechnol. 35 (2017) 491–492, https://doi.org/10.1038/nbt0617-491.
[222] E. South, E. Cox, N. Meader, N. Woolacott, S. Griffin, Strimvelis® for treating severe combined immunodeficiency caused by adenosine deaminase deficiency: an evidence review group perspective of a NICE highly specialised technology evaluation, PharmacoEconomics - Open 3 (2019) 151–161, https://doi.org/10.1007/s41669-018-0102-3.
[223] M.P. Stewart, A. Sharei, X. Ding, G. Sahay, R. Langer, K.F. Jensen, In vitro and ex vivo strategies for intracellular delivery, Nature 538 (2016) 183–192, https://doi.org/10.1038/nature19764.
[224] F. Uddin, C.M. Rudin, T. Sen, CRISPR gene therapy: applications, limitations, and implications for the future, Front. Oncol. 10 (2020), https://doi.org/10.3389/fonc.2020.01387.
[225] D. Wilbie, J. Walther, E. Mastrobattista, Delivery aspects of CRISPR/Cas for in vivo genome editing, Acc. Chem. Res. 52 (2019) 1555–1564, https://doi.org/10.1021/acs.accounts.9b00106.
[226] J.E. Zuckerman, I. Gritli, A. Tolcher, J.D. Heidel, D. Lim, R. Morgan, B. Chmielowski, A. Ribas, M.E. Davis, Y. Yen, Correlating animal and human phase Ia/Ib clinical data with CALAA-01, a targeted, polymer-based nanoparticle containing siRNA, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 11449–11454, https://doi.org/10.1073/pnas.1411393111.
[227] A. Akinc, M.A. Maier, M. Manoharan, K. Fitzgerald, M. Jayaraman, S. Barros, S. Ansell, X. Du, M.J. Hope, T.D. Madden, B.L. Mui, S.C. Semple, Y.K. Tam, M. Ciufolini, D. Witzigmann, J.A. Kulkarni, R. van der Meel, P.R. Cullis, The Onpattro story and the clinical translation of nanomedicines containing nucleic acid-based drugs, Nat. Nanotechnol. 14 (2019) 1084–1087, https://doi.org/10.1038/s41565-019-0591-y.
[228] P.R. de Paula Brandão, S.S. Titze-de-Almeida, R. Titze-de-Almeida, Leading RNA interference therapeutics part 2: silencing delta-aminolevulinic acid synthase 1, with a focus on Givosiran, Mol. Diagnosis Ther. 24 (2020) 61–68, https://doi.org/10.1007/s40291-019-00438-6.
[229] Research Pipeline, MMA, CMV, Zika & Rare Diseases - Moderna. https://www.modernatx.com/pipeline, 2020 (accessed August 31, 2020).
[230] M. Croft, S. Salek-Ardakani, J. Song, T. So, P. Bansal-Pakala, Regulation of T Cell Immunity by OX40 and OX40L. https://www.ncbi.nlm.nih.gov/books/NBK5990/, 2013 (accessed October 4, 2020).
[231] A. Jimeno, S. Gupta, R. Sullivan, K.T. Do, W.L. Akerley, D. Wang, D. Teoh, K. Schalper, S.J. Zacharek, J. Sun, A.S. Laino, J. Frederick, H. Zhou, W. Randolph, S. Pascarella, L. Johansen, P.S. Cohen, R.S. Meehan, T.M. Bauer, Abstract CT032: A phase 1/2, open-label, multicenter, dose escalation and efficacy study of mRNA-2416, a lipid nanoparticle encapsulated mRNA encoding human OX40L, for intratumoral injection alone or in combination with durvalumab for patients with advanced malignancies, in: Cancer Res., American Association for Cancer Research (AACR), 2020, p. CT032, https://doi.org/10.1158/1538-7445.am2020-ct032.
[232] mRNA Therapeutics and Vaccines, Translate Bio|Pipeline, 2020 https://translate.bio/pipeline/ (accessed August 31, 2020).
[233] Translate Bio Announces Interim Results from Phase 1/2 Clinical Trial of MRT5005 in Patients with Cystic Fibrosis. https://investors.translate.bio/investors/news-and-events, 2019 (accessed October 4, 2020).
[234] N. Pardi, M.J. Hogan, F.W. Porter, D. Weissman, mRNA vaccines-a new era in vaccinology, Nat. Rev. Drug Discov. 17 (2018) 261–279, https://doi.org/10.1038/nrd.2017.243.
[235] L.M. Kranz, M. Diken, H. Haas, S. Kreiter, C. Loquai, K.C. Reuter, M. Meng, D. Fritz, F. Vascotto, H. Hefesha, C. Grunwitz, M. Vormehr, Y. Hüsemann, A. Selmi, A.N. Kuhn, J. Buck, E. Derhovanessian, R. Rae, S. Attig, J. Diekmann, R. A. Jabulowsky, S. Heesch, J. Hassel, P. Langguth, S. Grabbe, C. Huber, Ö. Türeci, U. Sahin, Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy, Nature 534 (2016) 396–401, https://doi.org/10.1038/nature18300.
[236] R.A. Jabulowsky, C. Loquai, M. Diken, L.M. Kranz, H. Haas, S. Attig, N. Bidmon, J. Buck, E. Derhovanessian, J. Diekmann, D. Fritz, V. Jahndel, A. Kemmer-Brueck, K. Kuehlcke, A.N. Kuhn, P. Langguth, U. Luxemburger, M. Meng, F. Mueller, R. Rae, F. Sari, D. Schwarck-Kokarakis, C. Seck, K. Spieß, M. Witt, J.C. Hassel, J. Utikal, R. Kaufmann, S. Kreiter, C. Huber, O. Tuereci, U. Sahin, Abstract CT032:

A first-in-human phase I/II clinical trial assessing novel mRNA-lipoplex nanoparticles for potent cancer immunotherapy in patients with malignant melanoma, in: Cancer Res., American Association for Cancer Research (AACR), 2016, p. CT032, https://doi.org/10.1158/1538-7445.am2016-ct032.

[237] A Study to Evaluate Efficacy, Safety, and Immunogenicity of mRNA-1273 Vaccine in Adults Aged 18 Years and Older to Prevent COVID-19 - Full Text View - ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT04470427, 2020 (accessed September 1, 2020).

[238] A.C. Walls, Y.J. Park, M.A. Tortorici, A. Wall, A.T. McGuire, D. Veesler, Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein, Cell 181 (2020) 281–292, e6, https://doi.org/10.1016/j.cell.2020.02.058.

[239] L.A. Jackson, E.J. Anderson, N.G. Rouphael, P.C. Roberts, M. Makhene, R. N. Coler, M.P. McCullough, J.D. Chappell, M.R. Denison, L.J. Stevens, A. J. Pruijssers, A. McDermott, B. Flach, N.A. Doria-Rose, K.S. Corbett, K. M. Morabito, S. O'Dell, S.D. Schmidt, P.A. Swanson, M. Padilla, J.R. Mascola, K. M. Neuzil, H. Bennett, W. Sun, E. Peters, M. Makowski, J. Albert, K. Cross, W. Buchanan, R. Pikaart-Tautges, J.E. Ledgerwood, B.S. Graham, J.H. Beigel, An mRNA vaccine against SARS-CoV-2 — preliminary report, N. Engl. J. Med. (2020), https://doi.org/10.1056/nejmoa2022483.

[240] F.P. Polack, S.J. Thomas, N. Kitchin, J. Absalon, A. Gurtman, S. Lockhart, J. L. Perez, G. Pérez Marc, E.D. Moreira, C. Zerbini, R. Bailey, K.A. Swanson, S. Roychoudhury, K. Koury, P. Li, W.V. Kalina, D. Cooper, R.W. Frenck, L. L. Hammitt, Ö. Türeci, H. Nell, A. Schaefer, S. Ünal, D.B. Tresnan, S. Mather, P. R. Dormitzer, U. Şahin, K.U. Jansen, W.C. Gruber, Safety and efficacy of the BNT162b2 mRNA covid-19 vaccine, N. Engl. J. Med. (2020), https://doi.org/10.1056/NEJMoa2034577. NEJMoa2034577.