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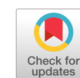
Optimizing synthetic nucleic acid and protein nanocarriers: The chemical evolution approach



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ABSTRACT

Optimizing synthetic nanocarriers is like searching for a needle in a haystack. How to find the most suitable carrier for intracellular delivery of a specified macromolecular nanoagent for a given disease target location? Here, we review different synthetic 'chemical evolution' strategies that have been pursued. Libraries of nanocarriers have been generated either by unbiased combinatorial chemistry or by variation and novel combination of known functional delivery elements. As in natural evolution, definition of nanocarriers as sequences, as barcode or design principle, may fuel chemical evolution. Screening in appropriate test system may not only provide delivery candidates, but also a refined understanding of cellular delivery including novel, unpredictable mechanisms. Combined with rational design and computational algorithms, candidates can be further optimized in subsequent evolution cycles into nanocarriers with improved safety and efficacy. Optimization of nanocarriers differs for various cargos, as illustrated for plasmid DNA, siRNA, mRNA, proteins, or genome-editing nucleases.
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Contents

1. Introduction . . . 31
2. Chemical evolution of delivery nanoagents . . . 31
   2.1. Molecular conjugates . . . 31
   2.2. Poly amino acid diblock polymers . . . 32
   2.3. Multi-block copolymer libraries . . . 32
   2.4. Combinatorial chemistry libraries . . . 34
   2.5. Sequence-defined macromolecules . . . 35
   2.6. Barcoded evolution of nanoagents *in vivo* . . . 38
3. Optimizing for various macromolecular cargos . . . 39
   3.1. pDNA delivery . . . 40
   3.2. siRNA delivery . . . 42
   3.3. mRNA delivery . . . 43
   3.4. Intracellular protein delivery . . . 45
   3.5. Delivery of genome-editing nucleases . . . 46

Abbreviations: ASGPR, asialoglycoprotein receptor; CART, charge-altering releasable transporters; CPP, cell-penetrating peptide; DET, diethylene triamine; eGFP, enhanced green fluorescent protein; mRNA, messenger RNA; OAA, oligoaminoamide; PACE, poly(amine-co-ester); PBAE, poly (β-amino ester); pCL, polycaprolactone; pDIPAMA, poly(2-diisopropylaminoethylmethacrylate); pDMAEMA, poly[(2-dimethylamino)ethyl methacrylate]; pDNA, plasmid DNA; PEG, polyethylene glycol; PEI, polyethylenimine; pHEMA, poly(2-hydroxyethyl methacrylate); pHPMA, poly[N-(2-hydroxypropyl) methacrylamide]; pLys, poly(L)lysine; PMO, phosphorodiamidate morpholino oligomer; pOEGMA, poly[oligo(ethylene glycol) monomethyl ether methacrylate]; pTMAEMA, poly[(2-trimethylamino)ethyl methacrylate]; siRNA, small interfering RNA; SPS, solid phase synthesis; Stp, succinoyl tetraethylene pentamine; TEP = TEPA, tetraethylene pentamine.

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4. Conclusions . . . 47
Acknowledgements . . . 48
References . . . 48

1. Introduction

Nucleic acid and/or protein macromolecule based nanomedicines present an exciting avenue in medical care. Within the last three decades almost three-thousand gene therapy clinical trials have been performed, and at least nine gene therapy products reached medical market authorization, see [1] and http://www.abedia.com/wiley. Furthermore, at least eight oligonucleotide [2] and one siRNA [3,4] drug products have been approved by the major medical agencies, and numerous clinical trials have reached advanced stages. All these macromolecular products depend on their successful intracellular delivery, which still appears as a critical bottleneck. It is not surprising that the current gene therapy products all are containing viral vectors or genetically modified cells. After a continuous struggle and refinement over thirty years, the development of effective retro/lentiviral vectors, adenovirus-associated viral (AAV) vectors or other viral vectors has paved the way towards approved medical drugs. In distinction, the marketed antisense agents (oligonucleotides) and RNA interference agents (siRNAs) all are synthetic nucleic acids. Here as well, advances in delivery chemistry have triggered the medical breakthrough [2,5]. The design of lipid nanoparticles (LNPs) for delivery into hepatocytes [6] was the basis for the first marketed siRNA drug, Patisiran [4]. Recently, the design of asialoglycoprotein receptor (ASGPR) targeted, chemically completely modified siRNA conjugated with tri-(N-acetylgalactosamine) ligand opened another way for liver-specific RNA interference therapy [7,8]. Both LNPs and ASGPR-targeted siRNA conjugates are tested in advanced clinical studies where the target gene is located in liver hepatocytes. The medical efficacy of such nanomedicines is remarkable, considering that often only a small fraction (~1%) of active agent reaches the molecular target location [9], which usually is the cytosol, sometimes the nucleus of target cells. A further refinement of delivery carriers, especially for target tissues outside the liver, will have a tremendous impact on future nanomedicines.

Reasons for the slow progress in delivery of macromolecules are manifold. First of all, apart from recombinant proteins, macromolecular drugs present a new development challenge for pharmaceutical industry; synthetic and analytical tools had to be steadily developed to meet the requirement for macromolecule design at pharmaceutical grade and scale. Secondly, the different phases of extracellular and intracellular delivery demand bioresponsive actions of carriers, to alter their biophysical properties in a dynamic (pH-, redox- or enzyme-sensitive) mode [10–17]. Thirdly, recent experience tells that different macromolecular cargos (proteins, siRNA, mRNA, pDNA), often with different intracellular target sites, may require different carriers [18–23]. And fourth, the various different target organs and cells, and the different therapeutic modes (transient or permanent action) dictate different requirements to nanocarriers; optimization for *ex vivo* delivery into cultured cell most likely will not correlate with optimal *in vivo* delivery [24]. Reflecting all these hurdles, how can we optimize macromolecular drug delivery? Here we report on recent strategies to overcome the mentioned challenges, with the main focus on chemical evolution approaches toward optimized delivery of intracellularly active nucleic acid or protein therapeutics.

2. Chemical evolution of delivery nanoagents

Nature designed viruses as highly potent intracellular delivery agents, based on only four different nucleotides, twenty amino acids, in many cases also lipids and carbohydrates. With such a restricted chemical space of building blocks, and the requirement to assemble viruses under aqueous physiological *in vivo* conditions and disassemble them again under *in vivo* conditions ('assembly – disassembly paradox'), one might predict that synthetic nanoparticles generated under optimized chemical conditions utilizing numerous chemical building blocks might be far more effective. The impediment to design such optimized nanoparticles simply resides in the lack of knowledge about the perfect combination of building blocks. Random testing even of only a fraction of possible combinations of building blocks and their fast recycling would not result in optimized nanoagents within reasonable time. How did nature develop viruses and even more sophisticated, living structures? To our knowledge, the ingredients have been: *i)* defining structures in form of precise sequences, *ii)* storage (and reproduction) of this sequence information (via the genetic code), *iii)* subtle stochastic changes of this information with reproduction and time, and *iv)* a lot of time. It took natural evolution on earth several hundred million years to develop the first living organisms [25].

Artificial evolution strategies on an accelerated time scale are expected to be key measures for optimizing synthetic nanoagents. This may involve rational design of building blocks based on a better understanding of the delivery process, combined with empirical screening of diverse chemical compound libraries, computational prediction by machine learning algorithms, virtual screening and, last but not least, storage of information on the precise molecular structure of the evolved nanoagents. In the following, examples of rational designs and evolution-based designs of different carrier types are presented, for illustrating the current stage of chemical evolution of delivery nanoagents (Fig. 1).

2.1. Molecular conjugates

Initial efforts in intracellular delivery of RNA and DNA focused on transfection reagents such as poly(L)lysine (pLys), polyarginine, polyornithine, or DEAE-dextran [26–28]. These polycationic carriers can electrostatically bind, compact and protect the nucleic acid in form of nanoscaled polyplexes [29,30]. Complexation of negatively charged nucleic acid with a small excess of carrier results in supercharged nanoparticles with positive surface charge, most convenient for enhanced cell interaction and nonspecific intracellular uptake [31,32]. Intracellular release from endocytic vesicles such as endolysosomes was identified as a critical delivery hurdle for the majority of carriers including polylysine [33], but less critical for endosomal cationizable carriers such as polyethylenimine (PEI) [34]. Several polycations were found to be toxic due to unspecific interaction with biomolecules [35,36] and lack of degradability. These kinds of observations stimulated the search for biocompatible cationic backbones, which are more effective in intracellular release and biodegradability (see below).

A different direction in optimization has been the modification of basic carriers such as pLys with other delivery modules [37]. Modules can be receptor-binding targeting ligands, nanoparticle surface shielding domains, or lipid membrane-disturbing agents. To enhance endosomal escape into the cytosol, molecular conjugates with membrane-destabilizing peptides [38–40], or defective viral particles [41–43] were designed. To increase cellular receptor-specific uptake, molecular conjugates with targeting ligands were synthesized [44,45]. Despite encouraging proof-of-concept studies, incorporation of ligands neither guarantees *in vivo* target specificity nor target receptor binding due to protein corona formation on the nanoparticle [46].

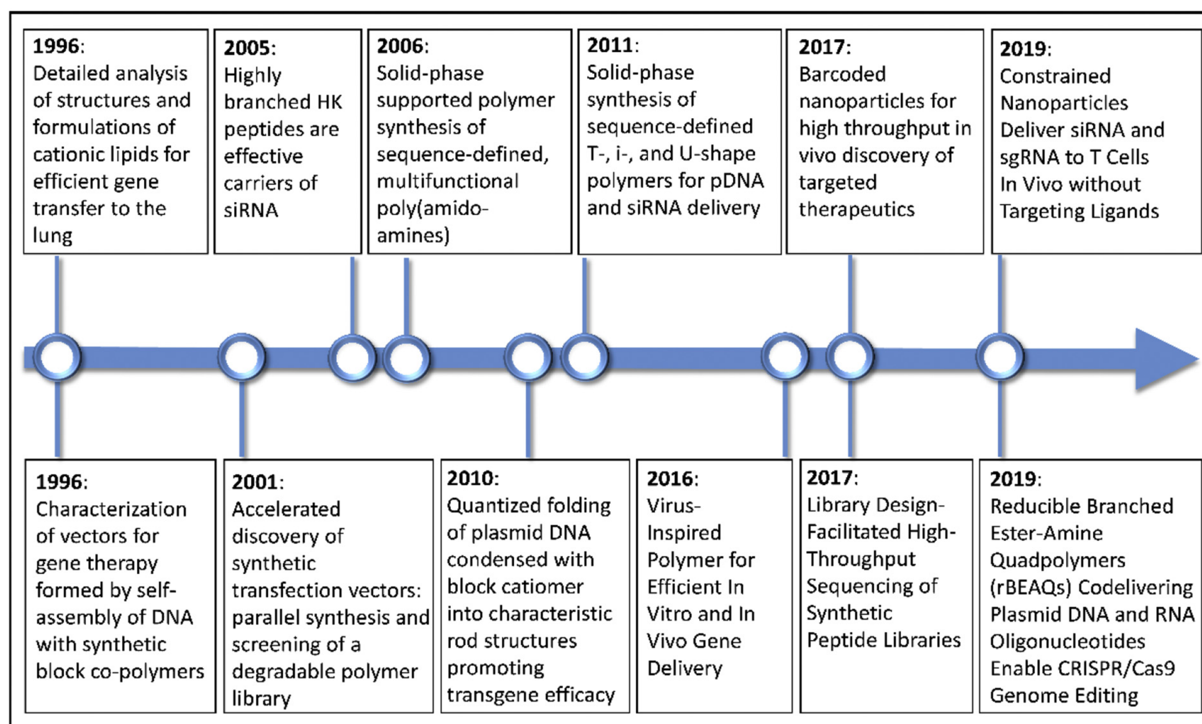


Fig. 1. Schematic timeline of synthetic evolution strategies. Titles of representative reports which are discussed in the following sections and also can be found in the list of references.

Thus, incorporation of hydrophilic surface shielding modules [47], including polyethylene glycol (PEG) [48–56], poly[N-(2-hydroxypropyl) methacrylamide] (pHPMA) [57–59], hydroxyethyl starch [60], hyaluronic acid [61–63], or polysarcosine [64–66] often enhanced *in vivo* bioavailibility and also reduced toxic unspecific interactions. The mentioned delivery modules (compaction, shielding, targeting, endosomal escape) do not necessarily synergize [67]; for example, shielding may antagonize endolysosomal membrane destabilization. For this purpose, dynamic molecular conjugates and polyplexes were generated, which disassemble in specific (acidic, enzymatic or bioreducing) microenvironments [13,14,68–72]. In a nutshell, such carrier conjugates imitate virus-like cell entry processes [73,74]. Encouraging therapeutic efficacies were demonstrated in several preclinical tumor models, such as described in [75–81]. Despite their high complexity, several of these molecular conjugate polyplexes reached the stage of application in human clinical studies [53,82–84]. Nevertheless, conjugation of two or more macromolecules (polymers, proteins, peptides) usually presents a chemical challenging process with polydispersity in backbones and ill-defined conjugation sites or topologies; technical alternatives include the design of high-precision polymers or defined lower molecular weight chemical entities.

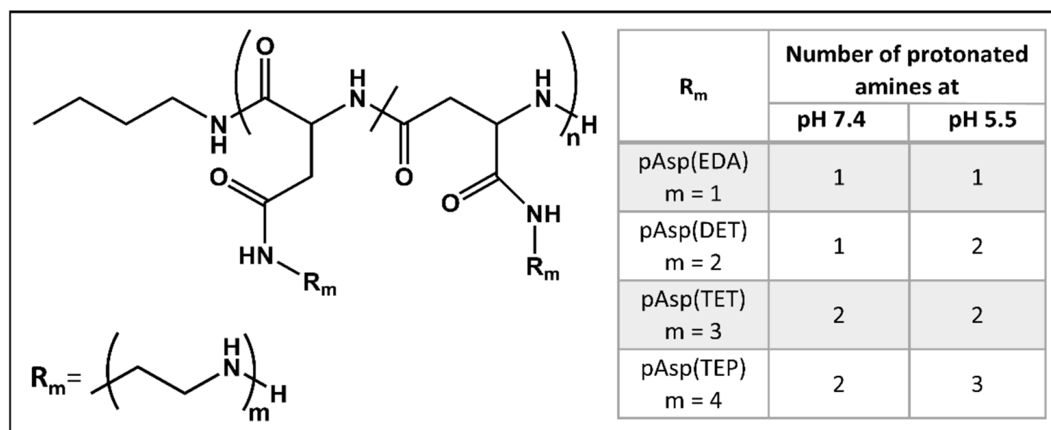
2.2. Poly amino acid diblock polymers

In thoughtful and systematic studies, Kataoka and collaborators designed PEG-polycation block copolymers for chemical evolution of pDNA, siRNA and mRNA polyplexes. PEG blocks were found to significantly affect nanoparticle shape, condensation, and transcriptional availability of pDNA [85–88]. PEG-pLys packages the pDNA into rod structures, with a quantized length of pDNA folding n times [85]. PEG shielding increased blood circulation time, but reduced transfection efficacy because of hampered cellular uptake. Systemic treatment with cRGD-targeted polyplexes with a rod length below 200 nm displayed antitumor efficacy against pancreatic cancer, whereas polyplexes with higher rod length, despite superior blood circulation, had negligible antitumor efficacy [86]. PEG-pLys with acid-labile linkage was applied to generate rod-shaped pDNA polyplexes, which under acidic conditions experience a removal of PEG from the polyplex and a dynamic change to compacted globules [87]. Further chemical evolution involved a series of PEG-pLys block copolymers with differing molecular weights of both pLys and PEG segments, for obtaining a refined understanding of pDNA packaging and gene transfer activity (see also Section 3.1).

In order to replace pLys with more effective cationic delivery carriers, Kazunori Kataoka and colleagues amidated PEG-poly(aspartic acid) (pAsp) with various oligoethylenimines [89–92]. Diethylene triamine (DET) modified PEG-pAsp(DET) polyplexes showed a high biocompatibility and good diffusion into tissue as demonstrated in multi-cell spheroids [90]. For siRNA delivery, steroyl groups were introduced into pAsp(DET) [92]. Comparison of the various oligoamine side chains, an 'even-odd rule' (Fig. 2) was established based on the differing pK values affecting endosomal buffering and escape. Polymers containing DET or tetraethylene pentamine (TEP) providing (after amidation) even units of protonatable aminoethanes showed best efficiency in pDNA transfections [93]. For mRNA delivery, this rule was unclear and was valid only for transfection levels at 12 h (DET and TEP best); after 48 h, the odd repeat triethylene tetramine (TET) displayed best transfection efficacy, followed by TEP [94]. For intravenous delivery of messenger RNA (mRNA), the ω-terminus of the PEG-pAsp(TEP) block copolymer was provided with a cholesterol moiety to increase the polyplex stability by hydrophobic interaction. Upon intravenous injection, mRNA polyplexes showed enhanced blood circulation in comparison to Chol-free polyplexes. PEG-pAsp(TEP)-Chol based delivery of mRNA coding for the anti-angiogenic protein sFlt-1 inhibited growth of subcutaneous implanted pancreatic cancer in mice [95].

2.3. Multi-block copolymer libraries

Based on the efficiency of reversible addition-fragmentation chain transfer (RAFT) polymerization [96,97] multifunctional transfection block copolymers can be synthesized with good yields and low polydispersity. In this field, initial nucleic acid delivery studies were made with polymethacrylates [98–104] such as cationic poly[(2-dimethylamino)



R_m	Number of protonated amines at	
	pH 7.4	pH 5.5
pAsp(EDA) m = 1	1	1
pAsp(DET) m = 2	1	2
pAsp(TET) m = 3	2	2
pAsp(TEP) m = 4	2	3

Fig. 2. 'Even-odd rule'. Polyplexes from polymers with an even number of aminoethylene units in the side chains lead to higher endosomal buffer capacity (due to change of protonation between pH 7.4 and pH 5.5) and higher transfection efficiency than odd numbered ones [93].

ethyl methacrylate] (pDMAEMA), or poly[(2-trimethylamino)ethyl methacrylate] (pTMAEMA), often prepared as co-blocks with hydrophilic pHPMA or PEG for improved biocompatibility and improved polyplex circulation in blood. A library of defined methacrylate polymers bearing pendant primary, secondary, and tertiary amino groups was synthesized using monomers (2-aminoethyl)-methacrylate, N-methyl-(2-aminoethyl)-methacrylate, or N,N-dimethyl-(2-aminoethyl)-methacrylate, respectively, in order to determine the effect of the amino substitution on pDNA transfection [105]. Studies on cellular uptake and intracellular release suggested endosomal release effective via pore formation in lipid-membranes, where methacrylate-based polyplexes with a high primary amino group content mediated the best pDNA transfection, and polyplexes with tertiary amino groups the lowest pDNA transfection.

The group of Suzie Pun designed stabilized dual responsive nanoparticles for pDNA delivery *in vivo* [106]. The polyplexes were based on a ternary amphiphilic block copolymer containing a disulfide-linked hydrophobic polycaprolactone (pCL) block, a pH-sensitive tetraethylene pentamine (TEPA)-modified poly(glycidyl methacrylate) block, and a hydrophilic oligo(ethylene glycol) monomethyl ether methacrylate (OEGMA) block (Fig. 3A). The pOEGMA block provides polyplex shielding in the blood circulation, the pCL block provides hydrophobic polyplex destabilization until intracellular disulfide cleavage, and the TEPA block endosomal cationization and endosomal escape. Subsequent work of the same lab optimized endosomal release of pDNA polyplexes by synthesizing VIPER (Fig. 3B) [107]. This 'virus-inspired polymer for endosomal release' presents a methacrylate copolymer containing polycationic pDMAEMA for binding and compaction of DNA, a pOEGMA block for shielding, a pH-sensitive poly(2-diisopropylaminoethylmethacrylate) pDIPAMA block and a neighboring poly(pyridyl disulfide ethyl methacrylate) pPDSEMA block. The latter activated disulfide block was coupled with lytic melittin peptides. Upon endosomal acidification the pDIPAMA block undergoes a transition from a hydrophobic to a hydrophilic phase, thus exposing the melittin peptides of the neighboring block in the endolysosomal compartment; this triggers endosomal membrane disruption and polyplex escape into the cell cytosol. Upon intratumoral administration VIPER facilitated pDNA delivery into KB tumors in mice. The carrier was also found effective for siRNA delivery and gene silencing in the lung [108]. In further evolution, the same research lab successfully replaced melittin with other lytic peptides [109]. The endosomal responsiveness of pDIPAMA derivatives was utilized for nucleic acid delivery also by other investigators, for example in the design of siRNA micelleplexes for cancer immunotherapy by Yaping Li, Haijun Yu and collaborators [110,111].

pDNA polyplexes may face the dilemma of insufficient stability in the blood circulation on the one hand, but at the same time limited release of pDNA from polycationic carriers inside the target cell. Therefore, in addition to chemically stable polymethacrylate polycations, also dynamic degradable analogues were synthesized. For example, Kwon and colleagues applied acid-degradable cationic ketal-linked methacrylamides, which were photo-polymerized in the presence of pDNA. Under acidifying endosomal conditions, polyplexes lose the cationic groups of the polymethacrylate carrier, which enhances cargo pDNA release and pulmonary gene transfer after intratracheal delivery in mouse lung [112]. To address the 'polyplex dilemma' in a slightly different manner, Cheng et al [113] designed the pH-sensitive triblock copolymer p(PMA-PMBA)-b-p(OEGMA-DMAEMA), consisting of poly

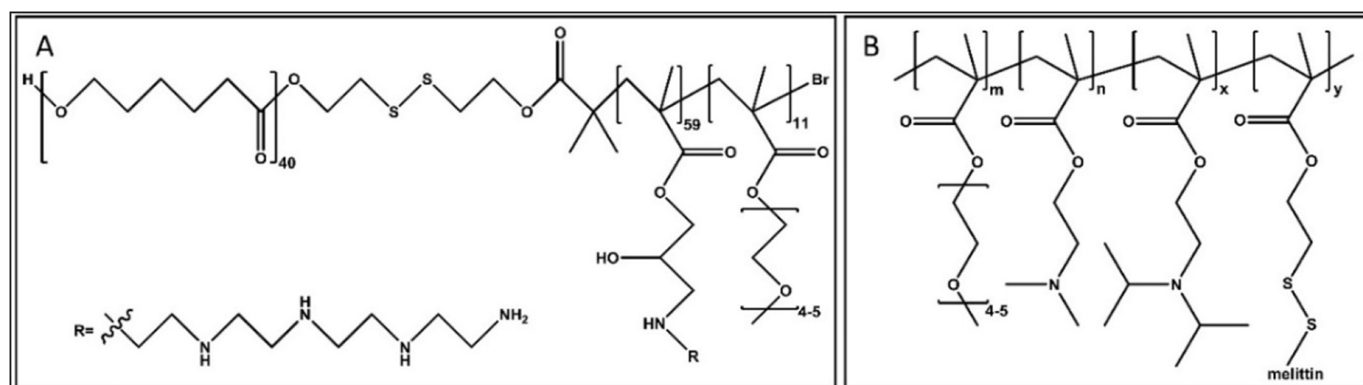


Fig. 3. Block co-polymers based on methacrylate backbones. (A) OEGMA terminal triblock copolymer (PCL_{40}-SS-P(GMA-TEPA)$_{59}$-P(OEGMA)$_{11}$ [106]. (B) VIPER [107].

(propargyl methacrylate-graft-propyl-(4-methoxy-benzylidene)-amine)-block-poly(oligo(ethylene glycol) monomethyl ether methacrylate)-co-poly(2-(dimethylamino)ethyl methacrylate). The hydrophobic p(PMA-PMBA) domain enhances polyplex stability at physiological neutral pH. Upon endosomal acid-catalyzed cleavage of benzaldimines the domain undergoes a hydrophilic transition, thus enabling the release of pDNA. In comparison to the pH-stable analogue, *in vitro* gene transfer and also *in vivo* intraventricular gene transfer into brain was improved, although the *in vivo* efficiency remained moderate [113].

As a pioneer in methacrylate polyplexes [101–103], Wim Hennink and his team expanded the area and generated 'decationized polyplexes' as a completely novel class of methacrylate polyplexes [114–116]. These polyplexes were based on the terpolymer p(HPMA-DMAE-co-PDTEMA)-b-PEG, consisting of a PEG block and the cationic block HPMA-DMAE (with pHPMA modified with dimethylaminoethyl carbonate at the hydroxyl group) containing 15% copolymerized N-(pyridyldithioethyl) methacrylamide (PDTMA). After pDNA polyplex formation with this polymer, the PDTMA units can be used for dithiol mediated crosslinkage into disulfide-stabilized polyplexes. By subsequent hydrolysis of the carbonate esters at pH 8.5, the cationic DMAE groups are removed. The resulting decationized pDNA polyplexes are stabilized by the formed disulfide crosslinks in the absence of polyelectrolyte interaction; they showed excellent biocompatibility, enhanced residence time in blood circulation and increased accumulation in tumor. Analogous decationized polyplexes were also generated for siRNA delivery [117].

Evolution of nucleic acid carriers requires a focus on the specific selected target. The clinical breakthrough of cancer immunotherapy by chimeric antigen receptor (CAR) T cells raised interest in efficient *ex vivo* transfection of primary human T cells. With this focus, Pun and coworkers optimized methacrylate copolymers for pDNA and mRNA delivery into the Jurkat human T cell line and primary human T cells [118]. VIPER and several other linear, comb and sunflower pDMAEMA block copolymer architectures based on poly(2-hydroxyethyl methacrylate) (pHEMA) cores were evaluated. A subset of comb- and sunflower-shaped pHEMA$_{25}$-g-pDMAEMA$_{16}$ polymers were identified which can mediate transfection of Jurkat cells with efficiencies up to 50% with minimal toxicity. Transfection of both CD4+ and CD8+ primary human T cells with mRNA and pDNA were demonstrated with high viability and at efficiencies up to 25% and 18%, respectively.

2.4. Combinatorial chemistry libraries

More than two decades ago, Seng Cheng and colleagues published the result of the first large library screen of lipoplexes for gene transfer to the lung [119]. The study included several remarkable aspects: screening a huge number of cationic lipids; screening for the best formulation using the most authentic small animal model, which is the mouse lung *in vivo*; and discovery of the optimized formulation (containing lipid *#67*, a spermine – cholesterol derivative linked in T-shape configuration). This formulation was >100-fold more potent than previously tested lipoplexes. For development of novel cationic polymers with improved delivery and biocompatibility, the initial screen focused on replacement of PLL with linear and hyperbranched poly (amino esters) [120,121]. Short oligoethylenimines were converted by reaction with 1,4-butanediol diacrylate or 1,6-hexanediol diacrylate into degradable PEIs which are applied as transfection carriers *in vitro* and also *in vivo* [122–124]. Also, linear beta-amino ester polymers were produced via the same Michael addition starting from 4-aminobutanol [125]. The degradable PEI mimics showed favorable transfection activities. The team of Robert Langer developed a breakthrough concept by generating large combinatorial libraries of biodegradable poly(β-amino esters) (PBAE) based on Michael addition of a series of primary or secondary amine monomers to several different diacrylates (Fig. 4A) [126–128]. Such a large, 2350-member library was screened by a semi-automated high-throughput process for pDNA delivery, and 46 polymers were identified with higher transfection activity than the gold standard PEI [128]. A second generation library was generated containing 486 PBAEs, which were characterized for biophysical and pDNA transfection properties [129]. Interestingly, the top nine polymers all contained amino alcohols as building blocks, and the three top polymers differed in structure by only one carbon. The two best polymers condensed pDNA to the smallest particle sizes of 71 and 79 nm.

For siRNA delivery, Robert Langer, Daniel Anderson and colleagues designed an analogous Michael addition-based combinatorial library of over 1,200 structural diverse structures termed lipidoids [132]. These lipid-like structures were obtained by combinatorial Michael addition of lipidic acrylate esters or amides to various mono- to oligoamines. Lipidoid 98-N12-5, based on addition of five dodecylacrylamides to triethylentetramine (Fig. 4B), performed best *in vitro*, and upon formulation with cholesterol and PEG-lipid, also mediated excellent *in vivo* gene silencing in liver hepatocytes of treated mice [130,132]. A novel combinatorial library based on epoxide-addition chemistry (Fig. 4C) yielded the highly potent lipidoid C12-200 (Fig. 4D), which was able to mediate *in vivo* gene silencing in mouse liver at doses below 0.01 mg/kg [131,133]. A subsequent high-throughput study of 1,536 structurally distinct nanoparticles with cationic cores and variable hydrophilic shells was performed by Daniel Siegwart and Anderson based on epoxide-functionalized hydrophilic

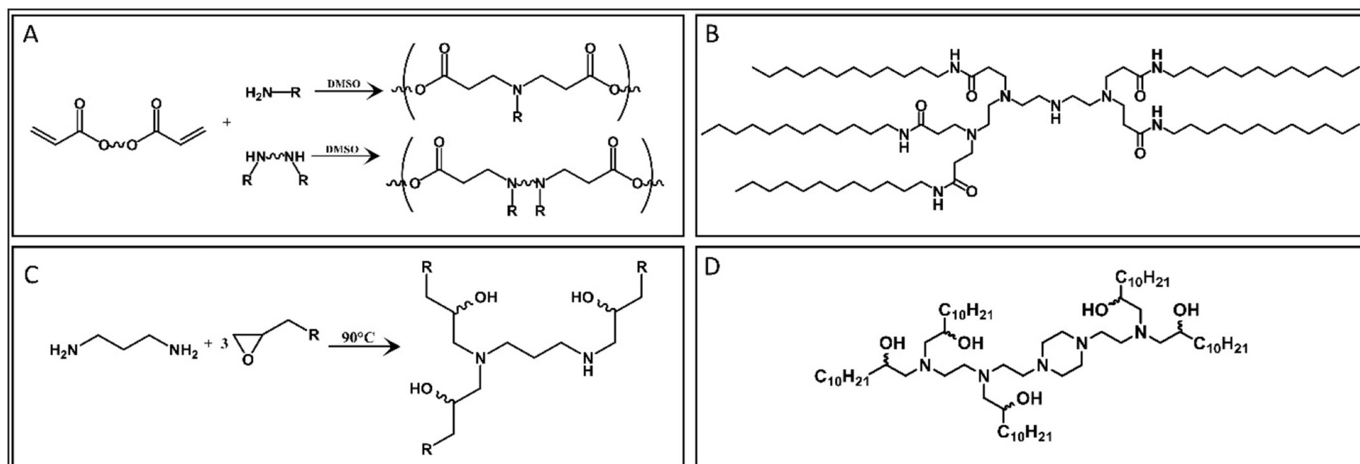


Fig. 4. Lipidoids for siRNA delivery. (A) Combinatorial libraries of biodegradable poly(β-amino esters) via Michael addition of a series of primary or secondary amine monomers to several different diacrylate monomers [127]. (B) Lipidoid 98-N12-5 [130]. (C) Epoxide addition based libraries [131]. (D) Lipidoid C12-200 [131].

block polymers that were combinatorially cross-linked with a diverse library of amines [134]. Cross-linkers with tertiary dimethylamine or piperazine groups and potential buffering capacity, as well as thin hydrophilic shells were favorable. Covalent cholesterol attachment to the polymeric carrier allowed siRNA transfection in hepatocytes from mouse liver *in vivo*.

The mentioned reports make clear that the cargo (pDNA or siRNA or other nucleic acids) as well as the specific application dictate the optimum nanocarrier, which needs to be identified by individual library screening. Consistently, using a library of low charge density poly (amine-co-ester) terpolymers generated by enzyme catalyzed polymerization, the Mark Saltzman lab demonstrated that different PACE polymers are required for optimized transfection of either pDNA, mRNA, or siRNA [22].

2.5. Sequence-defined macromolecules

Multifunctional molecular conjugates present a real challenge for precise chemical production. Nevertheless, their structure is by far exceeded in complexity, functionality, and high precision by natural macromolecules. The secret of natural proteins is their definition and information storage as (linear) sequences. Such sequences present virtual information, in nature stored on DNA or RNA templates, with this information translated into functional real structure by reading via the genetic code. For biomimetic evolution of artificial macromolecular carriers, their definition as sequences is a key requirement. In short, sequences *per se* present perfectly defined information of an intangible construction plan in biomacromolecules, which can be stored on material templates (as in nature) or in digital virtual form (in artificial synthetic evolution). Execution of the construction plan may proceed in alternative ways; either by direct use of the digital code for artificial assembly by a chemical synthesizer into sequence-defined macromolecules; or via reading an artificial code from a real template by recognition with building block-loaded adapter molecules.

Chemical synthesis of peptide and oligonucleotide sequences is well established, and the generation of precise sequence-defined artificial macromolecules is a recently emerging field [135–137]. We and others aimed at development of peptide-like chemical evolution strategies for intracellular macromolecule delivery (Fig. 5), where artificial amino acids with superior carrier properties would be integrated. Initial evolution of sequence-defined transfection agents was based on standard peptides. First studies explored the requirements for nucleic acid binding by introducing the cationic amino acids lysine, arginines or ornithines [138,139], disulfide-forming nanoparticle-stabilizing cysteines [140,141], endosomal-buffering histidines [141–147] or membrane-destabilizing peptides [148,149]. In their pioneering work, Christian Plank et al. [139] synthesized a series of branched oligocationic peptides and found that minimally six to eight cationic amino acids (arginine > lysine ~ ornithine) are required to condense pDNA into active gene delivery particles. The team of James Mixson systematically evaluated linear and branched peptide libraries containing lysines (for nucleic acid binding) and histidines (for endosomal buffering) in various ratios (e.g. HK, H2K, H3K) and numbers of branches (e.g. linear, four or eight branches). Addition of a histidine-rich tail, for example in peptide H2K4bT, significantly improved pDNA gene transfer activity, most likely by enhanced buffering capacity of the carrier [143]. Efficiencies were found to also depend on the target cells. In one study in combination with cationic liposomes, a linear HK peptide was found to transfect primary cells more effectively than the branched analogue [144]. Initial cellular uptake or size of the complexes could not explain the differences. Interestingly, a strong relation was found between the optimal type of HK carrier and the differing endocytic vesicle pH of different cell types. By altering the fraction of cationizable histidines, the endosomal pH of a cell may determine the amount of DNA released from the linear or branched HK polymer. In the primary cells in which the linear HK liposomes were the best carriers, the endolysosomes were highly acidic (pH of <5.0). Conversely, in cell lines where the branched HK liposomes were optimal, the pH of endolysosomes was above 6.0. These results with the HK liposome carriers were predictive of which cells could be transfected efficiently by the branched HK polymers alone. Whereas the linear HK polymers without liposomes were ineffective pDNA carriers in all tested cell cultures, the branched HK polymers without liposomes effectively transfected cells with a higher endosomal pH. For siRNA complexes, the H3K8b peptide was highly effective in gene silencing, whereas the effective pDNA carrier H2K4b was ineffective for siRNA [145].

In addition to natural amino acids, artificial building blocks were also introduced, such as triethylene tetramine or analogues developed by

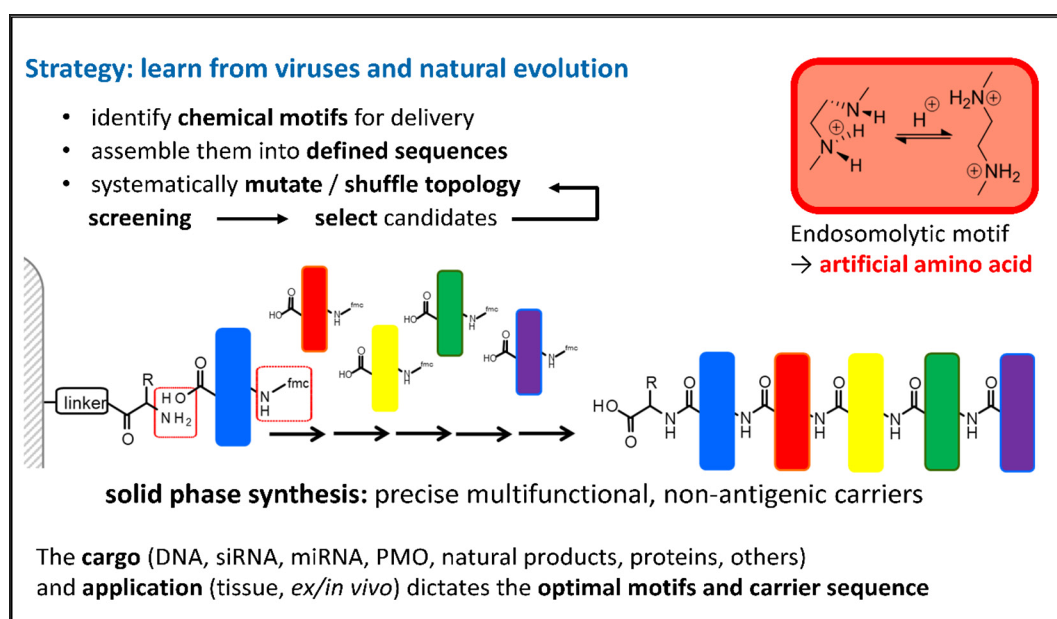


Fig. 5. Chemical evolution strategy for optimizing sequence-defined nanocarriers. The endosomally cationizable diamino-ethane motif is displayed as example of a delivery motif which can be converted into an artificial amino acid and subsequently applied in the SPS sequence assembly.

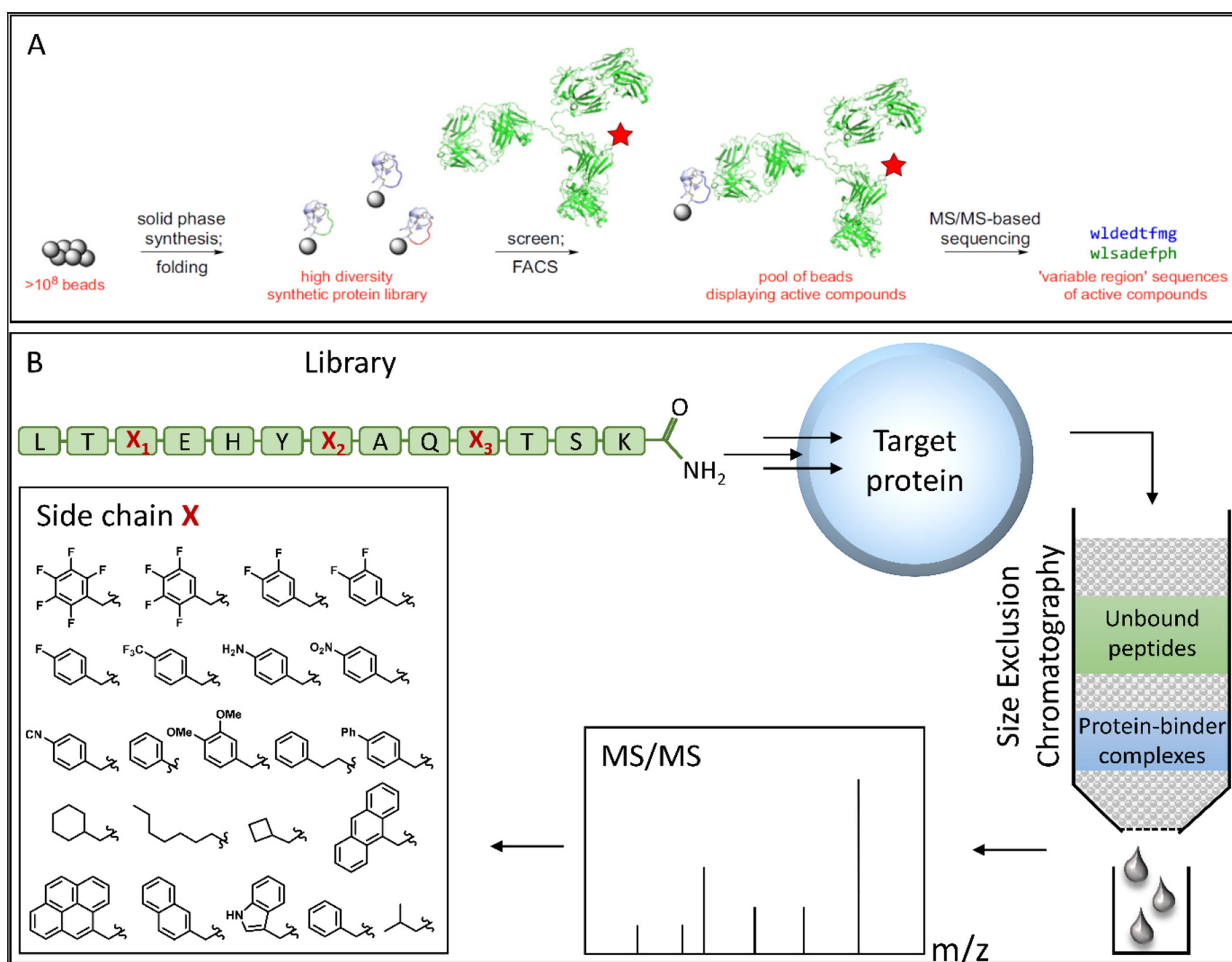


Fig. 6. Synthetic artificial peptide libraries (A) for xenoprotein engineering, reproduced from [155] with permissions of *Proc. Natl. Acad. Sci. U. S. A.*; *D*-amino acids are in lowercase; (B) for identifying protein-protein interaction inhibitors, compare [156]; the example displays MDM2 binders identified from a third evolution library containing non-canonical amino acids.

Zheng-Rong Lu and collaborators [150–152]. Fatty acids were also incorporated as lipophilic domains [151], such as in the evolution of the PepFect cell-penetrating peptide series of Ulo Langel and colleagues [149,153,154]. Bradley Pentelute and coworkers introduced innovative technologies for peptide library synthesis including artificial 'non-canonical' amino acids for generation of xenopeptides and xenoproteins, and nano shotgun LC-MS/MS sequencing for high-throughput library screening [155–158]. The potency of their novel chemical evolution methodology was demonstrated by identification of xenoprotein interactors (Fig. 6A) and or protein - protein interaction (PPI) inhibitors (Fig. 6B) with far higher potency compared to previous conventional peptides. For innovative drug delivery, the Pentelute lab introduced several artificial macrocyclization technologies to improve peptide-based drugs, such as providing an enhanced transfer of a transportan-10 analogue or a BIM BH3 domain across the blood-brain-barrier [159]. To improve the intracellular delivery of splice-switching phosphorodiamidate morpholino oligonucleotides (PMOs), they conjugated PMOs with arginine-rich cell-penetrating peptides (CPPs) that had been first optimized by perfluoroaryl macrocyclization and bicyclization [160]. In addition, based on results with 64 PMO-CPP conjugates, machine learning algorithms were developed for computational prediction of suitable CPPs, and validated with seven novel CPP sequences that all proved to be effective [161]. Thus, empirical library screen and machine learning might synergize in a chemical evolution process.

Laura Hartmann and Hans Börner [162–167] adopted solid phase synthesis (SPS) for the sequence-defined alignment of completely unnatural chemical units. Instead of incorporating α-amino acids with standard protective groups, they performed alternating coupling of diamines (3,3'diamino-N-methyl-dipropylamine, or a bis (tBoc)-protected spermine, or a disulfide-containing unit) followed by succinic acid anhydride as dicarboxylic acid unit in each round of coupling. The resulting oligo(amidoamines) were applied for pDNA polyplex formation and thus, to our knowledge, present the first examples of completely artificial sequence-defined nanocarriers. Subsequently, to combine the known efficient transfection properties of PEI with the methodology of automated Fmoc peptide synthesis, David Schaffert and colleagues designed artificial oligo(aminoethylene) amino acids with amino-terminal Fmoc and internal tBoc protective groups [168–170]. These artificial amino acids contain three to five repeats of the protonatable aminoethylenimine motif. The novel building blocks were assembled in combination with standard Fmoc and side chain protected α-amino acids by SPS into more than 1000 defined oligo (ethanamino)amide (OAA) sequences. Oligomer architectures included linear [169,171], two-arm [169], three-arm [169,172], four-arm [169,170,173] and comb architectures [174]. Branching points were provided by the two (α, ε) amines of lysine introduced as either via bis(Fmoc) lysine or Fmoc/Dde lysine, which after simultaneous or subsequent deprotection during synthesis can result in pseudo-symmetric

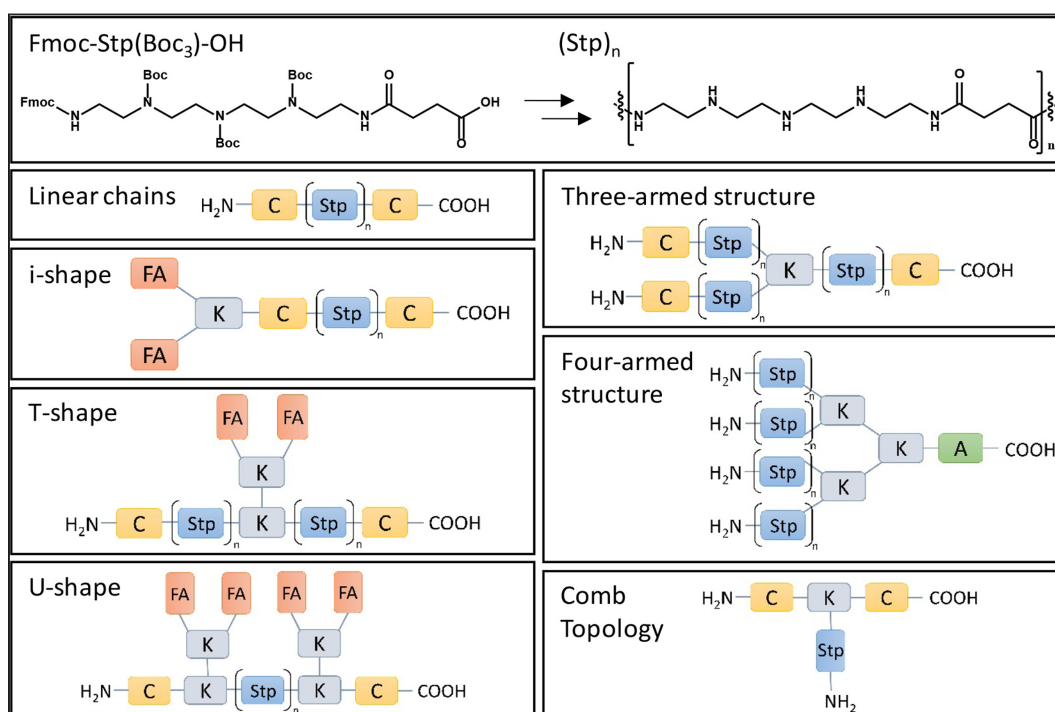


Fig. 7. Protected artificial amino acid Fmoc-Stp(Boc_3)-OH and sequential assembly into Stp-based oligo(ethanamino)amide (OAA) sequences with different structural topologies [169–174]. Stp, succinoyl tetraethylene pentamine; C, cysteine; K, lysine; A, alanine; FA, fatty acid.

or asymmetric branches. Optionally, lipo-oligoaminoamides were designed with i-shape, T-shape, or U-shape topology [169,172,175] by introducing bis (acyl)-modified lysines as hydrophobic polyplex stabilizating domains (Fig. 7). In several different topologies, terminal cysteines served for the stabilization of polyplexes by formation of bioreducible disulfide bridges. Due to the precision of the chemical design, simple open questions on structure –activity relationships were addressable. Testing the length of a linear sequence based on the building block Stp (succinyl TEP, containing three protonatable nitrogens per unit when integrated into the oligomer backbone) as requirement for pDNA compaction and gene transfer, an optimum length of 20 Stp units was identified, representing 61 protonable nitrogens and in total 100 nitrogens. pDNA transfection efficiency was about fivefold higher and cytotoxicity about tenfold lower as compared with the 'gold standard' linear PEI 22kDa, which contains around 500 (± 200) protonable nitrogens [171].

For further optimization of pDNA carriers, the design of four-arms based on two branching lysines strongly reduced the number of coupling steps, and terminal cysteines increased polyplex stability and transfection efficiency. With regard to the total number of nitrogens, the type of selected polyamino acid played a more critical role than extending the length of arms beyond three building blocks. Units containing pentaethylene hexamine (Sph) were more effective than tetraethylene pentamine (Stp), which were more effective than triethylene tetramine (Gtt) building blocks [170]. A closer inspection of the total protonation capacity as well as the cationization pH confirmed independently the 'even-odd' rule which had been developed by Kataoka and colleagues (see Section 2.2). Gtt and Sph with even numbered protonatable amino groups displayed higher total endolysosomal buffer capacity than the odd number analogue Stp. Within the relevant endolysosomal pH range, Gtt has a maximum buffer capacity at pH 5, and Stp a maximum around pH 7. Alternating incorporation of histidines increased the total buffer capacity with a more continuous cationization pH profile and, especially for the 'odd' Stp building block, strongly enhanced gene transfer both in the *in vitro* and *in vivo* setting [173]. The even-odd rule was well applicable when comparing linear and comb topologies and their differing properties in pDNA complex formation, endosomal buffer capacity, cellular binding and intracellular uptake, and gene expression. In linear topologies, the two terminal amines of building blocks are consumed by amidation, resulting in three (odd) or four (even) amines for Stp and Sph oligomers, respectively. Branched topology (with only one amine amidated) reverses properties, with four (even) and five (odd) protonatable amines for Stp and Sph. Consistently, the comb topology increased endosomal pH buffering for the Stp comb structures, but reduced endosomal buffering for the Sph comb structures. For both carriers, the combs mediated a higher overall cellular uptake in comparison to the linear topologies. In sum, Stp combs displayed a combined advantage in both, buffering and cellular uptake, and a strong (up to >100-fold) increase in pDNA transfection, whereas Sph combs mediated only a moderately (up to fourfold) enhanced transfection over the linear topology [174]. Incorporated Gtt building blocks enhance endosomal buffering, but (due to lowest number of nitrogens) display less pDNA binding; this could be compensated by incorporating cysteines, which form polyplex-stabilizing disulfide cross-links [173].

Beyond pDNA compaction and core nanoassembly, incorporation of surface shielding and targeting domains was considered. Sequence-defined solid-phase-assisted assembly enables all-in-one synthesis of peptide or small-molecule targeting ligands attached to precise monodisperse PEG molecules and the nucleic-acid binding and endosomal buffering core OAA backbone. For example, monodisperse PEGylated two-arm oligomers with targeting ligands such as cRGD or B6 [176], folate [173,177], c-Met binding peptide [178,179], EGFR-binding peptide GE11 [180], or an IL-6 receptor binding peptide [181] were synthesized with defined uniform molecular weight, and receptor-mediated cellular uptake of polyplexes was confirmed resulting in enhanced gene transfer. By variation of the molecular weight of the PEG domain, its influence on biophysical and biological properties was elucidated. PEGylation was found to interfere with the endosomal escape function of the PEI-like OAA polyplexes, which is a limitation that also was previously detected for targeted, PEG-shielded PEI polyplexes [182,183]. Importantly,

enhancement of endosomal buffer capacity by incorporation of histidines into the cationic OAA backbone compensated for this bottleneck [173,177,178].

PEGylation and incorporation of related hydrophilic domains affected also pDNA compaction; screening PEG domains from 12 to 72 ethylenoxide (EO) units demonstrated optimum compaction without PEG or 12 EO units, moderate compaction with 24 EO and lack of compaction with 48 or 72 EO repeat units [184]. In other studies similar low-molecular-weight PEG was found as favorable with ligand-PEG-shielded nanoparticles [185] or liposomes [186]. pDNA compaction was found as important requirement for intravenous systemic administration *in vivo* in mice in a HUH7 hepatocellular carcinoma xenograft model. pDNA gene transfer via a cMet binding peptide – $(EO)_{24}$ – (Stp/His)-Cys two-arm OAA was highly successful, but only if 1/3 of oligomers was substituted by an analogous PEG-free three-arm compacting oligomer. The formulation without or with compacting oligomer did not significantly differ in cell culture transfection [178].

Incorporation of tyrosine tripeptides at both ends of ligand-PEG-two arm oligoaminoamides was considered for nanoparticle stabilization by aromatic π-π stacking [177], which also improved pDNA polyplex compaction. In a recent study, pDNA was compared with far smaller minicircle (MC) DNA in OAA formulations [187]. While the DNA type (number of base pairs) controlled the nanoparticle size, the carriers dominated the shape of polyplexes. c-Met-targeted, tyrosine tripeptide-containing OAA polyplexes presented compact structures with a rod size of 65–100nm for pDNA and 35–40nm for MC. As compared to their tyrosine-free pDNA analogues, the optimized MC nanostructure facilitated an ~200-fold enhanced gene transfer in c-Met-positive DU145 prostate carcinoma cells.

An alternative strategy for designing well-compacted surface-shielded polyplexes is the pre-formation of compacted pDNA core nanoparticles followed by post-modification with a shielding/targeting shell [188]. One recent example was the design of pDNA polyplexes with four-arm OAAs post-modified with acid-labile pHPMA. This enabled 'deshielding' in the endosomal environment, resulting in favorable gene transfer into tumor cells *in vitro* and *in vivo* [189]. Another example presents the disulfide-based modification of pDNA lipopolyplexes with a bidentate PEG–GE11 agent for EGF receptor-mediated gene transfer [190].

Optimization for other cargos such as small molecule drugs [191,192], phosphorodiamidate morpholino oligomer (PMO), siRNA, mRNA, or proteins including Cas9/sgRNA ribonucleoprotein (RNP) particles revealed significantly different requirements for nanocarriers as outlined in other sections. For example, in the evolution of siRNA carriers, polyplex stabilization by hydrophobic fatty acid domains was highly important (see Section 3.2). PMOs present a chemically different class of small, uncharged nucleic acid analogues that has already resulted in the exon-skipping drug Exondys, which is FDA-approved for treatment of Muscular Dystrophy. After screening a fatty acid lipo-OAA mini-library for small PMO molecules, Ulrich Lächelt and colleagues identified linolenic acid-containing oligomer PMO conjugates as most potent RNA slice-switching agents [193]. Far superior endosomal escape properties of the linolenic acid (containing three double bonds) over the analogous saturated stearic acid derivative appear as the most likely explanation for this non-predictable finding. Screening an analogous T-shaped lipo-OAA library for delivery of Cas9/sgRNA RNPs revealed a hydroxy-stearic acid (OHSteA) containing lipo-OAA as superior over analogues with unsaturated or saturated fatty acids without hydroxylation, displaying improved cellular uptake and endosomal release, an increased nuclear association and the highest CRISPR/Cas9 mediated gene knock-out [194].

Another example illustrating the advantage of sequence-defined artificial peptide-based evolution is the recent work by Sören Reinhard and colleagues [17]. He realized that the artificial Stp-based OAAs are not degradable by the lysosomal enzyme cathepsin B. A degradability of OAA nanocarriers in lysosomes however might be highly advantageous, blocking undesired cytotoxic lysosomal lytic activity of the nanocarrier. Designing a mini-library by inserting single natural *(L)* amino acids or dipeptides into a Stp backbone, followed by a library screen exposing to cathepsin B under endolysosomal conditions, resulted in the discovery of simple dipeptide cathepsin cleavage sites. Inserting for example *(L)*-RR into lipo-OAAs for intended lysosomal degradation resulted in siRNA nanocarriers with strongly reduced cytotoxicity as compared to their *(D)*-rr analogues.

A different assembly mode of sequence-defined macromolecular structures is the direct supramolecular assembly on a real template, as it happens in the natural evolutionary process. Such a DNA template-assisted synthesis was reported by David Liu and colleagues, where monomers were pre-arranged at a DNA template and translated in an enzyme-free process into sequence-defined synthetic polymers [195]. In a recent study, the same group designed highly functionalized nucleic acid polymers (HFNAPs) assembled by ligase-mediated DNA-templated polymerization, starting with 32 building blocks that contain diverse side chains on a DNA backbone [196]. Repeated evolution cycles of polymer translation, selection and reverse translation, led to HFNAPs, which can bind PCSK9 with high nM affinity.

2.6. Barcoded evolution of nanoagents in vivo

For chemical evolution of nanocarriers and their nucleic acid nanoparticles, analytical methods for high-throughput screening of compound libraries are of utmost importance, both *in vitro*, but even more *in vivo*. As reviewed above (Section 2.5), Pentelute and coworkers applied nano shotgun LC-MS/MS sequencing as high-throughput method for screening compounds with defined unique molecular mass such as peptide analogues. Phage display library screening presents a commonly used molecular biology approach for fishing target-binding, phage-displayed peptides by panning the library to the desired target, followed by identifying the selected peptide via the phage-encoded nucleic acid sequence. This potent evolution strategy has been transferred into the chemistry field. For example, DNA-encoded chemical libraries [197,198] have been generated by coupling chemical molecules to unique nucleic acid sequences (DNA barcodes). James Dahlman and colleagues [199] introduced DNA barcoding for high throughput screening and identification of suitable nanocarriers for *in vivo* delivery (Fig. 8). Single-stranded DNA barcodes were approximately 60 nucleotides long, terminally phosphorothioate–stabilized oligonucleotides, containing eight to ten central nucleotides as barcode and the 3' and 5' ends as adapter sequences for subsequent Illumina deep sequencing. Individual barcodes can be incorporated into the interior of different nanoparticles without affecting their activity. Upon simultaneous injection in a single mouse, the biodistribution of thirty different nanoparticles to eight tissue was monitored by deep sequencing [199,200]. A follow-up paper analyzed differences of nucleic acid nanoparticle *in vivo* delivery between wild-type and Cav1 knockout mice; for this purpose, the authors optimized the barcode secondary structure, thereby making the DNA amplification much easier, and enabling concurrent ddPCR readouts [201]. In addition, the researchers made the following important observation; by comparing delivery of 281 barcoded lipid nanoparticles (LNPs) to endothelial cells and macrophages *in vitro* and *in vivo*, the researcher found that *in vitro* delivery to immortalized mouse macrophages (RAWs) did not predict *in vivo* delivery to macrophages, and in vitro delivery to mouse aortic endothelial cells (iMAECs) did not predict *in vivo* delivery to endothelial cells of heart, lung, or bone marrow [24]. In many cases LNPs that performed well in vivo did not rank highly *in vitro* and thus would have been lost by an *in vitro* pre-screen; and LNPs top ranking *in vitro* did not perform well *in vivo.*

Beyond monitoring biodistribution only, in the next step the barcode-based system was further developed to measure functional delivery of hundreds barcoded LNPs within a single mouse. This novel strategy for *in vivo* evolution was termed 'Fast identification of nanoparticle delivery (FIND)' [202–204]. Functional delivery of RNA, such as Cre

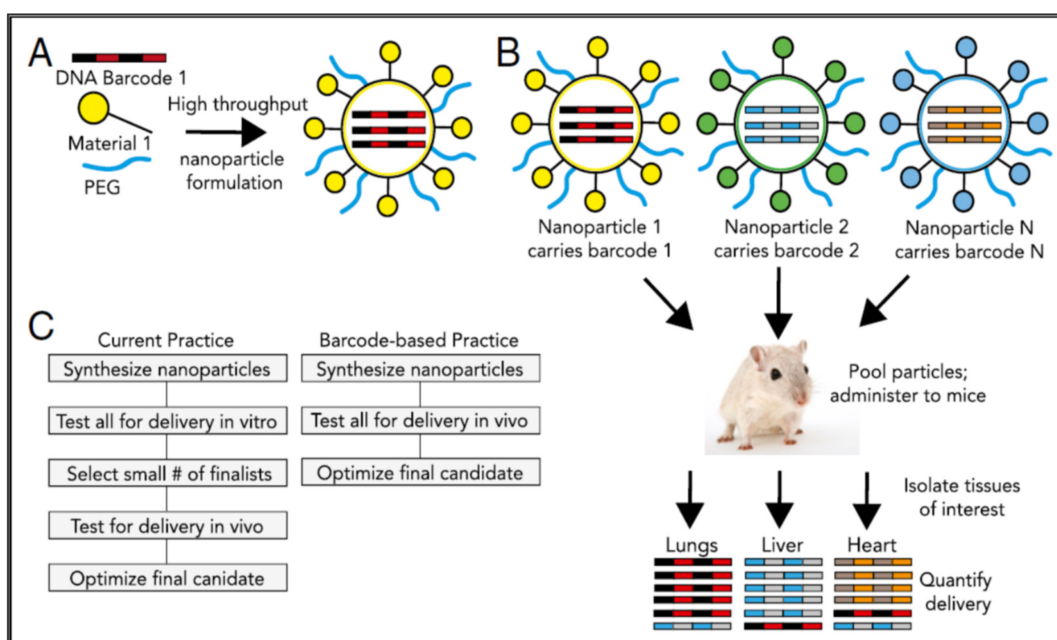


Fig. 8. High throughput nanocarrier *in vivo* screening by DNA barcode sequences. DNA barcoded nanoparticles for high throughput *in vivo* nanoparticle delivery. (A) Nanoparticles carry individual DNA barcodes. (B) Each nanoparticle carries a distinct barcode. Particles are then pooled and administered simultaneously to mice. Tissues are then isolated, and delivery is quantified by sequencing the barcodes. In this example, nanoparticle 1 delivers to the lungs, nanoparticle 2 delivers to the liver, and nanoparticle N delivers to the heart. (C) The barcode-based practice enables multiplexed nanoparticle-distribution studies *in vivo*. Reproduced from Dahlman et al. [199] with permission of *Proc. Natl. Acad. Sci. U. S. A.*

mRNA or specific siRNA, in the appropriate reporter mouse models (Lox-Stop-Lox-tdTomato mice or GFP mice, respectively) results in gene editing or gene silencing, which triggers induction/reduction of a reporter signal in the successfully transfected cells/organs. Successfully transfected cells can be isolated by cell sorting and analyzed for the barcoded bioactive LNPs. LNPs had been previously optimized to deliver Cas9 mRNA and sgRNA to hepatocytes. In their novel work, Sago et al [202] measured the functional mRNA delivery of more than 250 LNPs *in vivo* to multiple cell types. They identified two LNPs (7C2 and 7C3) with an altered tropism that efficiently deliver siRNA, mRNA, and single-guide RNA (sgRNA) to endothelial cells.

By combining FIND with bioinformatics, *in vivo* RNA delivery was optimized in a directed evolution process. For example, Dahlman and coworkers identified LNP ‘BM1’ that delivers siRNA or sgRNA to bone marrow endothelial cells (BMECs). BMEC tropism was not related to the size of LNP, but changed with cholesterol content and PEG structure in the formulation [203]. Apparently, significant changes to vascular targeting can be made by simple changes in chemical composition instead of using active targeting ligands.

The same research group applied FIND to quantify *in vivo* delivery of 75 distinct mRNA LNPs to 28 cell types. They discovered that an LNP containing oxidized cholesterol delivers Cre mRNA for DNA editing into hepatic endothelial cells and Kupffer cells at low dose of 0.05 mg/kg, fivefold more potently than into hepatocytes. Cholesterol oxidation on the hydrocarbon tail linked with sterol ring D outperformed cholesterol modifications on sterol ring B [204]. Most recently Dahlman’s team applied 168 DNA-barcoded siGFP nanoparticles for identifying functional siRNA *in vivo* delivery to nine cell types in transgenic eGFP mice. Constrained lipid nanoparticles (cLNPs), containing an adamantly lipid, deliver siRNA and sgRNA to T lymphocytes at low doses and (in contrast to previously reported LNPs) do not preferentially target hepatocytes [205]. In sum, within a short period the barcode-based design has already proven to be a powerful strategy of chemical evolution towards optimized nanocarriers [206], providing solutions which would not have been predictable by rational design and classical screening. Provided that barcodes can be integrated within the interior of stable nanoparticles, and those would not expose or exchange nucleic acid barcodes with each other, the strategy could also be applied to polyplexes or other class of nanomaterials.

3. Optimizing for various macromolecular cargos

Delivery requirements of various macromolecular cargos are different (Table 1), based on their different sizes, charges, chemical properties and different intracellular sites of action [18–23]. Conversely, different classes of nanocarriers offer characteristics, which are more or less suitable for a given cargo. For standard cationic polymers [23,30], polyplex formation is an entropy-driven process based on the electrostatic interaction between the multivalent polycationic polymer with the polyanionic nucleic acid cargo. A larger number of charges per

Table 1
Differences in physicochemical properties of the different cargos and their requirements for delivery

Cargo	Physicochemical properties	Requirements for delivery
pDNA MC DNA	Large (5–15 kbp) ds DNA, circular, bacterial vector backbone; minicircle (3–4 kbp) without backbone	Compaction into nanoparticle dimension, Protection against nucleases, Intranuclear delivery
siRNA	Small (21–23 bp) ds RNA, can be partly/completely chemically modified to reduce innate immune responses and enhance biostability lower polyplex stability	Stable incorporation into nanoparticle or chemical conjugation to targeting unit, Cytosolic delivery, incorporation of guide strand into the RNA-induced silencing complex
mRNA	Medium sized ss RNA, instable, can be partly modified to reduce immunogenicity and enhance stability	Synthetically modifications of the mRNA, binding to the translational machinery
Proteins	Variable in (medium) size, charge/isoelectric point, hydrophilicity, stability, bioactivity	Extra/intracellular, various organelles, release in bioactive form; carriers need to be adjusted for every protein cargo

bp, base pairs; ds, double stranded; kbp, kilo base pairs; mRNA, messenger RNA; ss, single stranded [19,23,209,210].

polycationic carrier and also the polyanionic nucleic acid is favorable for stability of these polyelectrolyte complexes (PECs). Clear differences between large (pDNA, higher polyplex stability) and small (siRNA, lower polyplex stability) cargos can be seen. Covalent conjugates between siRNA and cationic polymers have been investigated to overcome the stability problem [207,208].

In case of LNPs [6], hydrophobic lipid-lipid interactions, co-assembly of (mono/di)-cationic lipids into polycationic lipid micelles and liposomal structure dominate, resulting in incorporation and encapsulation of the nucleic acid cargo. Obviously electrostatic interactions are only partly responsible for LNP formation, therefore the size of nucleic acid cargo is less influential, and formulations are also suitable for small cargos such as siRNA.

Lipopolyplexes present an intermediate class of nanomaterials based on lipid-conjugated polycations, which self-assemble into larger polycationic micelles and electrostatically interact with negatively charged nucleic acid cargos. For all classes of carriers, optimization for a specific cargo type requires variation of the cationic domain (size, number of charges) and lipid domain of the carrier, optionally mixed lipid compositions (cationic lipid, cholesterol, phospholipid, PEG-lipid), N/P charge ratio and the formulation process.

The main challenges for successful delivery of all types of cargo are the formation of stable nanoparticles with suitable size, protection of the cargo against destabilization by serum proteins and degradation, a delayed elimination from the blood circulation (renal clearance, liver and spleen uptake) and lack of inflammatory and immune responses. Nanoparticle shielding and targeting ligands were introduced, particle size and colloidal stability of nanoparticles variegated to obtain homing to target tissues, such as tumors via passive targeting and the enhanced permeation and retention (EPR effect) due to vascular leakage, or active transendothelial transport [211,212]. Within target cells, successful intracellular transport, including endosomal escape, cargo release, cytosolic migration and optionally nuclear import have to be achieved [19,209,213]. In the following, chemical evolution- based optimization of nanocarriers is illustrated for the cargos pDNA, siRNA, mRNA, proteins and genome-editing nucleases.

3.1. pDNA delivery

A long-term experience with antitumoral pDNA polyplexes is largely based on targeted and surface shielded PEI conjugates. Tumoral delivery of therapeutic agents was enhanced by targeting ligands such as transferrin or EGF and included pDNA encoding TNF-α [75,80,214] or sodium iodide symporter (NIS) [81,215], and tumor regressions were observed in mouse tumor models. In both therapeutic modality, gene transfer to a subpopulation of tumor target cells is sufficient, because the secreted TNF-α may react at neighboring cells (including endothelium), and also NIS enables targeted radionucleotide therapy with a strong by-stander effect on neighbouring untransfected tissue.

PEI is an effective pDNA transfection agent but displays significant, molecular-weight dependent cytotoxicity and low degradability. Kazunori Kataoka and colleagues had optimized biodegradable PEG-polyamino acid diblocks (see Section 2.2) for pDNA compaction. They observed a higher *in vitro* gene expression with more extended rod-shaped nanoparticles than with compact globular shaped polyplexes [88]. However, a small polyplex size was important for *in vivo* efficiency [86]. The researchers applied a series of PEG-pLys block copolymers with various molecular weights of pLys and PEG segments for pDNA complexation. These studies showed that rod-shaped polyplexes formed when the tethered PEG chains covering pDNA in a pre-condensed state were dense enough to overlap each other (reduced tethering density (RTD) > 1) (see Fig. 9), whereas globular polyplexes were obtained when PEG segments did not overlap (RTD < 1) [88]. The observations are consistent with related studies using other PEG-polycation polyplexes [216,217]. Importantly, in a cell-free system the rod-shaped more extended nanoparticles mediated higher gene expression than the globular polyplexes [88].

The lab advanced the pDNA binding carrier structures by designing amidated PEG-poly(aspartic acid) (pAsp) derivatives with good buffering capacity in the endolysosomal range, yielding PEG-pAsp(DET) and PEG-pAsp(TEP) as most effective carriers [93]. For further optimization of *in vivo* delivery, the cyclic RGD peptide was installed as tumor targeting ligand, and cholesterol was attached to the other end of the nanocarrier for improved packaging by hydrophobic stabilization. The latter step was critical for efficacy and enabled the use of high-molecular weight (20 kDa) PEG, which on the other hand improved polyplex blood circulation. The created cRGD-PEG-pAsp(DET)-cholesteryl polyplexes were tested in a subcutaneous BxPC3 pancreatic cancer xenograft mouse model, achieving improved accumulation at the tumor site. Potent tumor growth suppression was obtained by antiangiogenic sFlt-1 gene transfer [218]. The expression of soluble sFlt-1 exerts antiangiogenesis by trapping angiogenic molecules (VEGF); such a paracrine strategy by-passes the need of transfecting the majority of tumor target cells.

Jianjun Cheng and colleagues developed poly(glutamic acid vinylbenzylester) pGlu(OBzV) into a library of cationic endosomolytic α-helical pDNA nanocarriers pGlu(OBz-X) [219]. For this purpose, the vinylgroups were oxidized to the benzaldehyde derivatives, which were reductively aminated with a mini-library of 31 amines and oligoamines (see Fig. 10). The nanocarriers were evaluated for pDNA delivery, with the top candidate outperforming the gold standard PEI by 12-fold. Subsequently the delivery system was also applied for Cas9 pDNA/sgRNA co-delivery [220].

Jordan Green and coworkers extended the combinatorial design of poly(β-amino esters) (PBAEs, see Section 2.4) for synthesis of

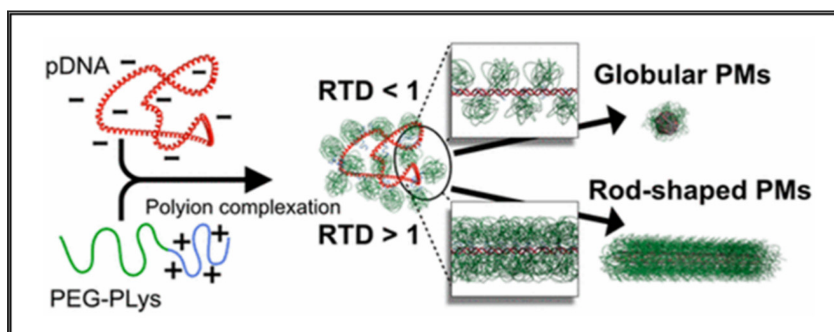


Fig. 9. PEG crowding as decisive factor in pDNA packaging into polyplexes. PM, polymer micelles; RTD, reduced tethering density. Reproduced from Takeda et al. [88] with permissions of American Chemical Society. Copyright 2017.

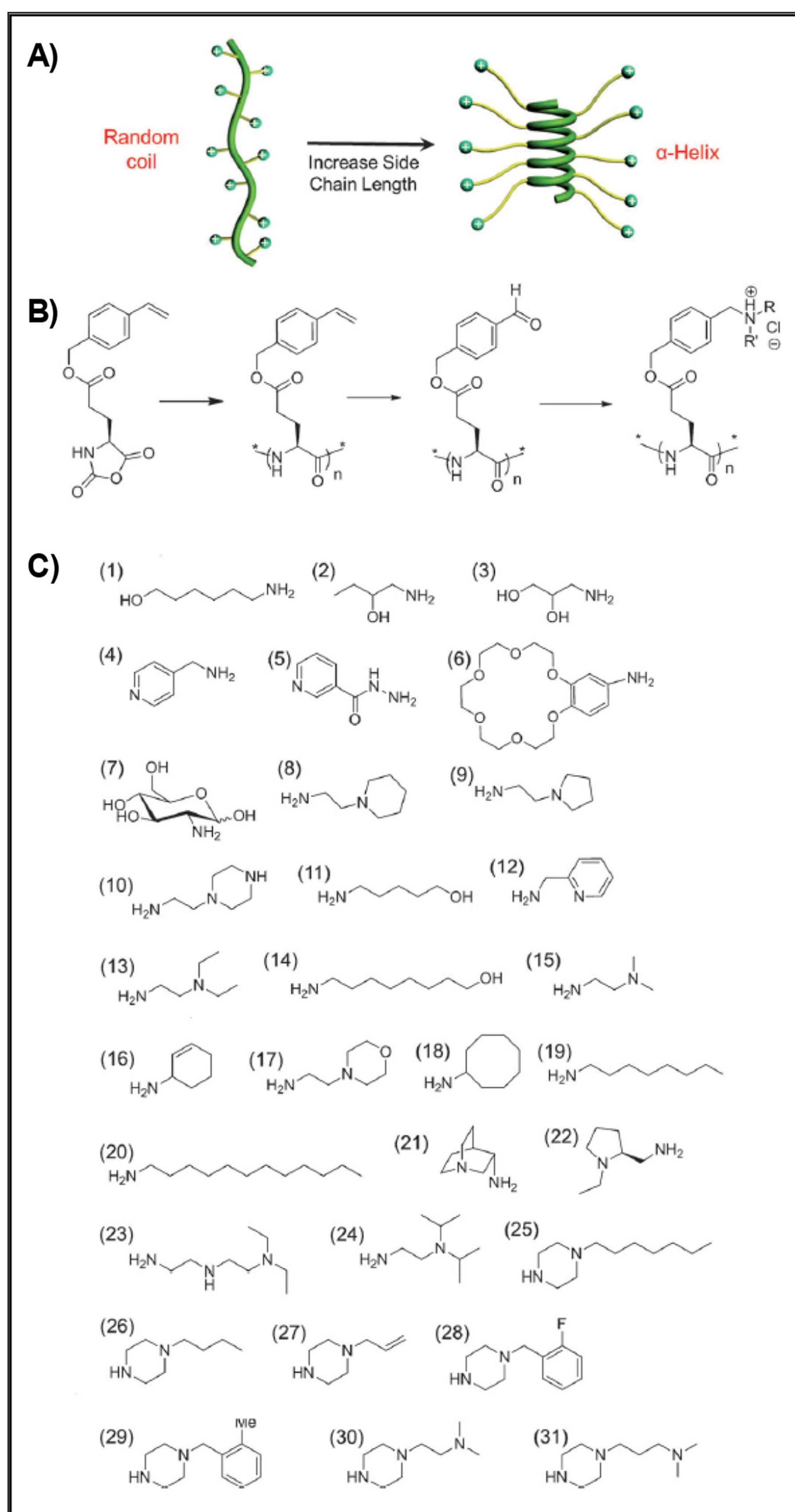


Fig. 10. (A) Polypeptide with charged side chains and the random coil to helix transformation in response to elongated side chains. (B) Synthesis of polypeptides. (C) Amines used to synthesize pGlu(OBz-X). Reproduced from Gabrielson et al. [219] with permission. Copyright 2012 WILEY WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

multifunctional branched poly(ester amine) quadpolymers (BEAQs). Synthesis occurred via A2 + B2/B3 + C1 Michael addition, starting from small acrylate and amine monomers, followed by subsequent capping with amine-containing small molecules [221]. These BEAQs present advantages over previously reported linear PBAEs, particularly for volume-limited applications. A moderate degree of branching was favorable for effective pDNA transfer to retinal pigment epithelial cells. Structural properties, including good endosomal buffering capacity and sufficient tertiary amine content, correlated with gene transfer efficacy.

In optimizing sequence-defined OAAs (see Section 2.5) for pDNA delivery, Petra Kos et al faced the dilemma between polyplex shielding and pDNA compaction. In their case, co-formulation of ligand-PEG-two-arm OAAs with PEG-free three-arms was critically required for polyplex stabilization and functional *in vivo* delivery into tumors [178]. Furthermore, the presence of a tumor–targeting ligand (such as c-Met binding peptide) as well as the presence of protonatable histidines in the carrier backbone were strictly necessary for the *in vivo* gene transfer. Based on this concept, c-Met targeted pDNA polyplexes were able to systemically deliver NIS pDNA into a subcutaneous HUH7 hepatocellular carcinoma xenograft in mice [179]. Functional NIS expression was detected using the diagnostic radioisotope iodide ^{123}I by scintigraphy. Three cycles of intravenous polyplex and therapeutic radioiodide administration retarded the tumor growth and prolonged the survival of mice.

By introducing the antitumoral cascade targeting ligand peptide I_6P_7, Rongqin Huang and collaborators generated glioma-targeted pDNA polyplexes [181]. The interleukin-6 peptide fragment I_6P_7 provides multiple functions, including blood-brain-barrier crossing, glioma-targeting, and direct tumor-inhibition by binding the IL-6 receptor without cellular activation. Intravenously applied I_6P_7-Stp-His/pDNA polyplexes were delivered into orthotopic U87 glioma in the mouse xenograft model; delivered pDNA encoding inhibitor of growth 4 (pING4) was successfully expressed in the glioma, which significantly prolonged the survival time of treated mice.

Nonviral pDNA carriers have also been developed for indications different from cancer. Zheng-Rong Lu and collaborators had optimized the cationizable lipopeptide like carrier ECO as pDNA and siRNA carrier (see also next Section 3.2) in several chemical evolution cycles by SPS. They applied ECO/pDNA nanoparticle for gene therapy of inherited retinal disorders by subretinal injection in genetic mouse models of Lebers' congenital amaurosis (LCA) [222] and Stargardt disease (STGD) [223], demonstrating therapeutic effects in these forms of blindness.

3.2. siRNA delivery

Small interfering RNA (siRNA, 2 nm helix diameter, 7 nm length, 21–23 base pairs) presents a far smaller double-stranded nucleic acid than pDNA. This opens a far broader range of formulation options, with small sizes of chemically modified small conjugates of free siRNA, unimolecular siRNA nanoplexes (for example, with a coat only 1 nm thicks), to various liposomal or polymer micelle formulations, which usually are in the size range of around 100 nm. Obviously, a greater flexibility provides an opportunity [5,224,225], but also a challenge for chemical evolution processes: should siRNA be chemically further minimized into drug-like molecules (to better diffuse into tissues)? Or should multiple siRNAs be formulated into larger virus-like delivery nanoparticles (to provide disease-focused localization) [226]? Quo vadis? Which direction to go? Irrespective of these challenging alternative choices, the siRNA drug concept has already proven to be a success story. Within two decades since discovery of the RNAi mechanism, the first siRNA drug product, Patisiran (Onpattro) [4], reached the medical market in 2018, for therapy of hereditary transthyretin (TTR)-mediated amyloidosis (ATTR amyloidosis). Mutations in the TTR gene (producing a tetrameric protein, also called pre-albumin) result in tetramer destabilization, amyloid fibrils and plaques, and rapidly progressive, debilitating morbidity (polyneuropathy, cardiomyopathy) with high mortality. Patisiran contains siRNA directed against TTR mRNA formulated into 60 – 100 nm PEGylated LNPs. The design of LNPs for delivery is based on a long-term development by Cullis and collaborators [6,227]. The formulation contains four lipidic excipients: cholesterol, distearyl phosphatidyl choline; DLin-MC3-DMA as special cationic lipid, consisting of a dimethylamino-propionic ester of a carba-analogue of di-linoleic acid glyceride; and the sheddable PEG derivative PEG2k-C-DMG. The cationic lipid DLin-MC3-DMA supports nucleic acid incorporation and endosomal membrane destabilization. The dimyristylated PEG agent stabilized the formulation but is gradually shed off in blood circulation; this triggers incorporation of an apolipoprotein E corona [228], which results in targeted delivery to the LDL receptor and subsequent gene silencing in liver hepatocytes.

Alternative nanoformulations include siRNA conjugates with cholesterol [229], which enhanced the biodistribution to a series of tissues. More recently, conjugates with tri-(N-acetyl-galactosamine)-PEG ligand were designed for targeting the hepatocyte-specific asialoglycoprotein receptor (ASGPR); combined with complete chemical modification of siRNA backbone, upon administration via the subcutaneous route this opened another very potent way for liver-specific RNA interference therapy [7,8]. ASGPR-targeted siRNA-PEG-GalNAc conjugates are currently evaluated in several advanced clinical studies with target genes being located in liver hepatocytes. The first subcutaneous siRNA-PEG-GalNAc conjugate product Givosiran, targeting aminolevulinic acid synthase 1 (ALAS1) for the treatment of acute hepatic porphyria, received market approval in November 2019. Gene silencing of ALAS1 reduces build-up of neurotoxic aminolevulinic acid (ALA) and porphobilinogen (PBG) metabolites [230].

Further chemical evolution of LNP formulation focused on higher potency at low doses, thus enhancing the biocompatibility at therapeutic dosage. By screening combinatorial libraries Daniel Anderson, Robert Langer and colleagues had identified cationic lipidoids such as 98-N12-5 [130,132] or C12-200 [131,133], which as part of siRNA LNP formulations were able to mediate *in vivo* gene silencing in mouse liver; in case of C12-200 efficient gene silencing was seen at doses below 10 μg/kg. Hideyoshi Harashima and coworkers invested efforts in optimizing pH-responsive cationic lipids as well as active targeting strategies beyond the liver [231,232]. In recent work they systematically derivatized the hydrophilic head group and hydrophobic tails of YSK12-C4, a pH-sensitive cationic lipid that was previously developed in their laboratory. Studies revealed that hydrophilic head significantly affected the apparent pKa of the final LNP product, which is a key factor in both intrahepatic distribution and endosomal escape. In contrast, the hydrophobic tail strongly affected intrahepatic distribution without depending on apparent pKa. A structure-activity relationship study enabled the selection of a potent LNP composed of a pH-sensitive cationic lipid CL4H6 that showed efficient gene silencing at very low dose (50% silencing at 2.5 μg/kg), biodegradability and good biocompatibility. Compared to the previously developed LNPs, a superior efficiency for endosomal escape, cytosolic release and *in vivo* gene silencing was observed [233]. Furthermore, the same research group developed tumor-targeted siRNA LNP formulations. They compared RGD peptide-modified lipid nanoparticles (RGD-LNPs) with analogous PEG-LNPs in a lung tumor metastasis model. Accumulation of PEG-LNPs in the tumor-bearing lung was lower than that for the RGD-LNPs. Intravenous administration of siRNA RGD-LNP induced gene silencing in the metastasized but not the normal lung. RGD-LNP of antiangiogenic siRNA against DLL4 greatly prolonged the survival of tumor mice [234].

Existing LNP and GalNAc conjugate technologies appear to be suitable for medical developments where the target cells are liver hepatocytes. For systemic targeting other tissues and organs, delivery technology still needs to be explored and optimized. As a significant step towards therapy, tumor-targeted siRNA polyplexes have already been evaluated in first clinical studies in cancer patients. The nanoparticles were based on transferrin-coated cyclodextrin-

oligocation for active targeting the transferrin receptor. Complexes of siRNA against the M2 subunit of ribonucleotide reductase demonstrated gene silencing (and target protein reduction) in distant patient tumor tissue [84].

Zheng-Rong Lu and coworkers designed lipo-peptide like cationizable siRNA carriers with endosomal pH-dependent membrane disruptive capabilities, for the intracellular endosomal release of the internalized siRNA. These carriers consist of three domains: cationizable head, amino acid-based linkers including cysteines, histidines or lysines, and two terminal hydrophobic oleic acids. Initial studies (published between 2007 and 2009) identified (1-aminoethyl) iminobis [N-(oleoyl cysteinyl histinyl-1-aminoethyl) propionamide] (EHCO) as potent siRNA carrier, which upon attachment of bombesin or a RGD peptide via PEG spacers triggered receptor-mediated uptake and gene silencing in tumors *in vitro* and *in vivo* [151,235,236]. As published in 2013 [152], a new mini-library screen of SPS-prepared lipopeptides was performed to optimize the number of protonable amines and pKa of the cationic domain, the degree of unsaturation of the lipid tail, and the presence of histidines. The histidine-free carriers ECO (bis-oleic acid) and ECLn (bis-linolic acid) mediated the best siRNA silencing activity. Subsequently, ECO was successfully applied in RGD-PEG siRNA nanoformulations for therapeutic tumor-targeting. Intravenous injections of RGD-targeted ECO/sibeta3 nanoparticles reduced MDA-MB-231 breast tumor growth and metastasis [237]. Most recently, the system was used for therapeutic gene silencing of the onco-lncRNA DACR, resulting in reduced tumor growth [238].

In our own optimization of tumor receptor-targeted sequence-defined OAAs (compare Section 2.5) for siRNA delivery, two different strategies were pursued. The first strategy aimed at generation of small unimolecular siRNA nanoplexes with a thin coat of ligand-PEG-OAA two arm molecules; nanoplex sizes increased only by 2 nm or 4 nm beyond free siRNA, depending on the length of PEG of 24 or 48 EO units, respectively [239]. Nanoplexes required stabilization by cysteine-based disulfide bridges within the OAA coat; furthermore, siRNA conjugation with an endosomal escape peptide (INF7) was strictly required for gene silencing efficacy. Folic acid (FolA) [239] or glutaminylated methotrexate [240] were used as targeting ligands. With the latter ligand, which also may act as intracellular dihydrofolate reductase inhibitor, antitumoral activity (50% cures of mice) was obtained after intratumoral administration of siRNA nanoplexes against spindle motor protein EG5 [240]. Systemic administration, however, was ineffective, as the nanoplexes were rapidly cleared via the kidney and could be recovered from the urine of treated mice.

As second strategy, for systemic administration, siRNA polyplexes of sizes around 100 nm were formulated by including non-PEGylated OAAs. For siRNA delivery, polyplex stabilization was required, either by defined cysteine-based disulfide cross-links and/or the incorporation of hydrophobic lipid domains [172]. Including terminal tyrosine tripeptide sequences for stabilization via π-π interaction resulted in further improvement [241]. A mini-library screen of lipo-OAAs for the effect of incorporated fatty acids on efficacy and biocompatibility revealed an impact on polyplex stability, endosomolytic activity and also cytotoxicity. The cytotoxicity issue was resolved by incorporation of designer cleavage sites into the lipo-OAA carrier, such as an SSBB disulfide building block between cationic and lipid domain, which are cleavable in the cytosol [242], or lysosomal cathepsin B cleavage sites for intended lysosomal degradation [17].

For incorporation of active targeting function, targeted combination polyplexes (TCPs) were generated [243]. In the siRNA polyplex formation, a targeted FolA-PEG two-arm or four-arm OAA was reacted with a non-PEGylated three-arm OAA. The reaction was performed by fast directed coupling of thionitrobenzoic acid (TNB) cysteine-modified OAA with the free terminal cysteine thiol groups of the other OAA via disulfide formation. In a variation of the strategies, Dian-Jang Lee et al designed targeted lipopolyplexes (TLPs) by combination of the targeted FolA-PEG two-arm with a T-shaped lipo-OAA [244]. For both, TCP and TLP, optimized combinations were tested *in vivo* and demonstrated gene silencing in a subcutaneous L1210 mouse leukemia model after intravenous administration.

Well-compacted surface-shielded siRNA lipopolyplexes were also formulated in an alternative mode, by pre-formation of compacted siRNA core complexes followed by post-modification with a PEG-targeting ligand shell. FolA, transferrin, or an EGFR binding peptide was immobilized via a cysteine-based linkage [245–247]. More recently, the strategy was expanded to the use of orthogonal, copper-free click chemistry. Lipo-OAAs were synthesized with a terminal azido function, and formulated siRNA lipoplexes were treated with DBCO-PEG-ligands. By these means, FolA-targeted siRNA nanoparticles were generated, which upon intravenous application presented tumor-targeted EG5 gene silencing and antitumoral action in combination with the antitumoral natural product pretubulysin [248]. Analogously, EGF receptor-targeted lipopolyplexes were formulated for EG5 siRNA/methotrexate co-delivery [249] or EG5 siRNA/pretubulysin co-delivery [250], demonstrating clear combination effects as tested in EGFR positive tumor cell cultures. As a novel type of combination therapy, a dual antitumoral conjugate of EG5 siRNA with the pro-apoptotic peptide KLK was designed [251]. For tumoral delivery, this bioreducible siRNA conjugate was formulated in a T-shaped azido-lipo-OAA and click-coated with DBCO-PEG-AP1 as ligand construct for targeting the IL4 receptor [251]. Both mitochondrial destabilization by cytosolic released KLK peptide and EG5 silencing triggered mitotic block was demonstrated.

An extension of this 'chemical evolution' screen for siRNA delivery carriers [252] addressed the question about a possible beneficial role of histidines. As reported above, histidines as endosomal buffering components had been found beneficial for pDNA delivery. However, histidines (which carry no charge at physiological pH), may also reduce polyplex stability. For pDNA polyplexes which (due to the far larger number of base pairs) are more stable than siRNA polyplexes, this usually does not present a problem. For example, pDNA/PEI polyplexes require >1M salt for dissociation, whereas PEI polyplexes of siRNA dissociate already with 0.5M sodium chloride [253]. For siRNA polyplex performance, incorporation of histidines can be a balancing act. Extra measures have to be taken for polyplex stabilization to counteract an unfavorable histidine effect. In case of T-shaped lipo-OAAs with a short cationizable Stp backbone without and with incorporated histidines, the histidine-free carrier displayed a better gene silencing performance [251]. Analogous siRNA lipo-polyplexes formed with lipo-OAAs containing an extended Stp backbone (40 aminoethylene nitrogens) were found to be more stable, and incorporation of alternating histidines further improved gene silencing efficiency [254]. The observations are well consistent with published data. For siRNA carriers like ECO that contain only short cationic domains but lytic fatty acid domains, histidines might be dispensable, as reported by Lu and collaborators [152]. Histidines integrated into lipid-free larger cationic oligolysine peptides, such as the highly effective gene silencing H3K8b peptide, are required for endosomal escape activity [145].

3.3. mRNA delivery

Therapeutic mRNA delivery has become increasingly attractive in recent years. It has a great potential for addressing medical applications like immunotherapy, gene editing, regenerative medicine and vaccines [255,256]. To improve the stability of the nucleic acid under physiological conditions, and to decrease the immune response, synthetically modifications of the rather unstable mRNA are necessary. In addition, the development of efficient delivery carriers for mRNA is required to protect the cargo as well as for effective cellular uptake and cytosolic release [256]. The new therapeutic procedure offers new challenges and opportunities for the delivery of polymers. Because of the poor pharmacokinetic properties of mRNA, Rudolf Zentel and colleagues developed decationizable block copolymers containing disulfide-linked primary

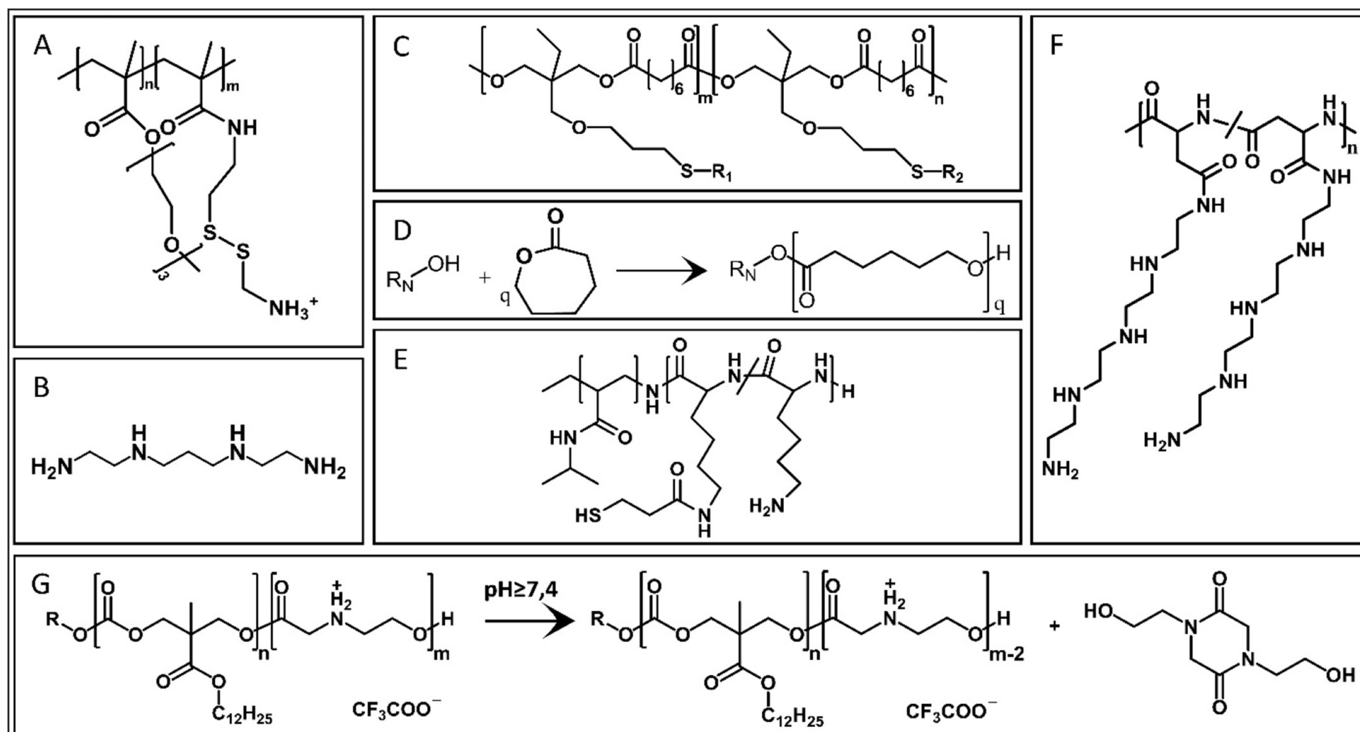


Fig. 11. mRNA delivery carriers. (A) Reductive decationizable block copolymers [257]. (B) Ethylenimine-propylenimine-ethylenimine (EPE) sequence motif as superior building block [258]. (C) Functional and degradable poly(trimethylolpropane allyl ether-co-suberoyl chloride) library [255]. (D) Ring opening polymerization of lactones initiated by a tertiary amino-alcohol [260]. (E) PNIPAM pLys(SH) [264]. (F) PEG-pAsp(TEP) block copolymers [95]. (G) Biodegradation of a CART copolymer [265].

amines (Fig. 11A) that form polyplexes with negatively charged mRNA. Different translation functionalities and polyplex structures were found depending on the composition of the block copolymers. They noted mRNA release of the nano-sized polyplexes in the reducing environment of the cytosol [257]. Christian Dohmen and collaborators evaluated cationic lipids and polymers formed with different defined oligoamines. They found that a modified PEI-like carrier with an (aminoethylene-aminopropylene-aminoethylene) sequence (Fig. 11B) is more efficient than aminoethylene or aminopropylene based carriers, and leads to enhanced mRNA transfection [258].

Several researchers developed different degradable polymer-lipid nanoparticles for systemic delivery of mRNA to the lung [255,256,259]. James Kaczmarek et al formulated mRNA nanoparticles with degradable poly(β-amino ester)-lipid (PBAE-lipid) for functional delivery to the lungs. Through coformulation with lipid-PEG they obtained serum-stable nanoparticles with increased *in vitro* potency and most potent *in vivo* mRNA delivery to the lungs [259]. By means of polymer synthesis and nanoparticle formulation the potency of the degradable, polymeric nanoparticles can be increased. They also demonstrated the ability of particles to functionally deliver mRNA to pulmonary endothelial and immune cells by using Cre reporter gene mice [256]. Moreover, a combinatorial polyester library (Fig. 11C) containing 480 distinct chemical structures was generated by Daniel Siegwart and coworkers with the aim to identify mRNA carriers. Surface shielding with 5% pluronic F127 improved the stability of mRNA polyplexes, and enabled predominant lung gene transfer upon intravenous application in mice [255].

To enable tissue-selective mRNA delivery, novel ionizable amino-polyesters were designed by Piotr Kowalski et al (Fig. 11D). Carriers were synthesized by ring opening polymerization of lactones with tertiary amino-alcohols and applied for mRNA formulation into LNPs. Effective mRNA expression in lung endothelium, liver hepatocytes and splenic antigen presenting cells was found [260]. Standard LNPs as developed for the siRNA therapeutic Patisiran (see Section 3.2) show effective hepatocyte delivery by LDL receptor mediated uptake; the small fraction (~1%) of cargo released into the cytosol is an early event (5–15 min after endocytosis [261,262]). The efficiency on other tissues and cell types is highly variable. Recently, Arwyn Jones, Marianne Ashford and colleagues evaluated intracellular trafficking of mRNA LNPs in 30 different tumor cell lines, comparing highly transfecting cell lines with moderately or poorly transfecting cell lines [263]. High-transfecting cells showed rapid LNP uptake and trafficking through an organized endocytic pathway to lysosomes or rapid exocytosis was observed in well transfectable cells. Low-transfecting cells displayed slower endosomal trafficking of LNPs to lysosomes and defective endocytic organization and acidification. In line with [261,262], efficient LNP transfection relies on an early and narrow endosomal escape window.

Wenfei Dong and collaborators generated stable polymeric nanoformulations by complexing mRNA with poly(N-isopropylacrylamide) (PNIPAM)-pLys–thiol (Fig. 11E) and cRGD-PEG-pLys-thiol for systemic delivery to tumors. Thermo-responsive PNIPAM was incorporated for stabilization and mRNA protection during systemic circulation, redox-responsive disulfide linkages for extracellular stabilization and intracellular mRNA release. Furthermore, the PEG-conjugated cRGD ligand on the surface of the nanoformulation improved tumor accumulation, and achieved potent gene expression of the loaded mRNA via integrin-facilitated cellular uptake [264].

Hydrophobic polyplex stabilization was also essential in the work of Kataoka and coworkers for optimizing systemic mRNA delivery by PEG-pAsp(TEP) block copolymers (Fig. 11F). A cholesterol moiety had to be attached to the ω-terminus of the copolymer for enhanced blood retention upon intravenous administration. The i.v. administration of PEG-pAsp(TEP)-Chol polyplexes for the delivery of mRNA encoding the anti-angiogenic protein sFlt-1 resulted in a remarkable growth inhibition of pancreatic cancer in a subcutaneous inoculation mouse model [95].

Amphiphilic charge-altering releasable transporters (CARTs) are degradable materials for mRNA transfer and have been developed by Robert Waymouth and coworkers (Fig. 11G). The step-economical

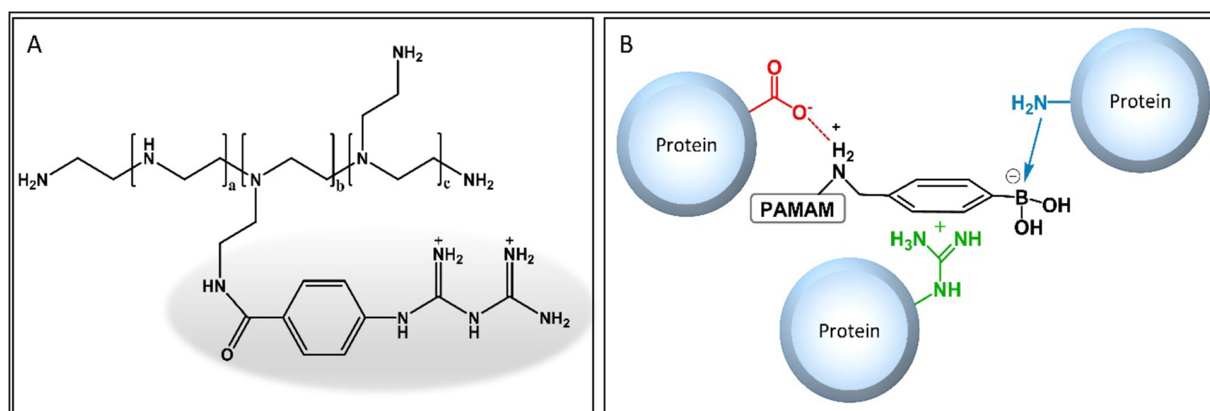


Fig. 12. Polycationic protein carriers. (A) Phenyl-biguanidine-benzoic acid (PBGBA) modified branched PEI (25 kDa) containing in average 60 PBGBA per bPEI [276]. (B) Protein interactions with phenyl-boronic acid modified cationic G5 PAMAM dendrimer via nitrogen-boronate complexation, cation-π and ionic interactions enables dendrimer binding with either negatively or positively charged proteins [277].

synthesis of CARTs was achieved by an organocatalytic oligomerization. These transporters are capable of first building CART/mRNA complexes, protecting and delivering mRNA into cells. Subsequently CARTs undergo a degradative intramolecular rearrangement, degrading to neutral small molecules by controlled self-immolation, resulting in the release of mRNA into the cytoplasm and translation into protein [265]. Evolutionary optimization was possible using CART libraries incorporating different lipidic side groups. CART/mRNA complexes were demonstrated to successfully transfect lymphocytes *in vitro* and in mice *in vivo* [266]. Upon intratumoral injection, they can effectively trigger antitumor immune responses [267].

3.4. Intracellular protein delivery

Extracellularly active recombinant therapeutic proteins such as growth factors and antibodies have already captured a major fraction of the pharmaceutical market [268]. Delivery of proteins to intracellular target locations is a recent emerging task, fueled by developments of nanobodies with intracellular molecular targets or high-precision DNA endonucleases such as Cre recombinase, zinc finger nuclease, TALENs, or Cas9 protein for genome editing (see also Section 3.5). In this novel research area, numerous different delivery approaches have been recently reported [269,270]. These include physical incorporation into degradable hydrogels [271–275] or lipidic carriers, conjugation or complexation with polymers such as phenyl-biguanide modified [276] or phenyl-boronic acid modified [277] polycations (Fig. 12), targeting ligands or other functional transduction domains [278–282], or covalent reversible recharging followed by electrostatic polycation complex formation [283–285]. In contrast to the previously discussed cargos DNA, siRNA, and mRNA, which are physicochemically uniform within their compound class, protein cargos may very much differ in size, charge/isoelectric point, hydrophilicity and stability. When screening libraries of sequence-defined OAA oligomers for delivery of different protein cargos (such as enhanced green fluorescent protein (eGFP), LacZ, RNase, or nanobodies), our own learning experience has been that different carriers or carrier concepts were best for the different protein cargos. In the nutshell, for every protein cargo an independent "chemical evolution search" for the most suitable nanocarrier has to be performed.

A main delivery effort is the specific attachment of the protein formulation to the target cell, in order to trigger cellular uptake by endocytosis or related mechanisms. Subsequent release to the cytosol is the next challenge, followed by intracellular release at the target location (cytosol, nucleus) in bioactive form. Thus, in our work, proteins were bioreversibly conjugated with sequence-defined carriers. Optionally, linkers were used that are removed traceless under endosomal acidic conditions [286,287]. Alternatively, redox-sensitive disulfide linkages were applied for bioreduction in the cytosol [288,289].

Screening a sequence-defined oligoaminoamide (OAA) library for eGFP protein delivery presented a tetra oleoyl-modified protonatable OAA containing a folate-PEG domain for receptor targeting and nanoparticle shielding, and a protein disulfide linkage, as the best intracellular eGFP delivery agent into folate receptor-expressing tumor cells [289] (Fig. 13A). The formulation consisted of small worm-like nanomicelle

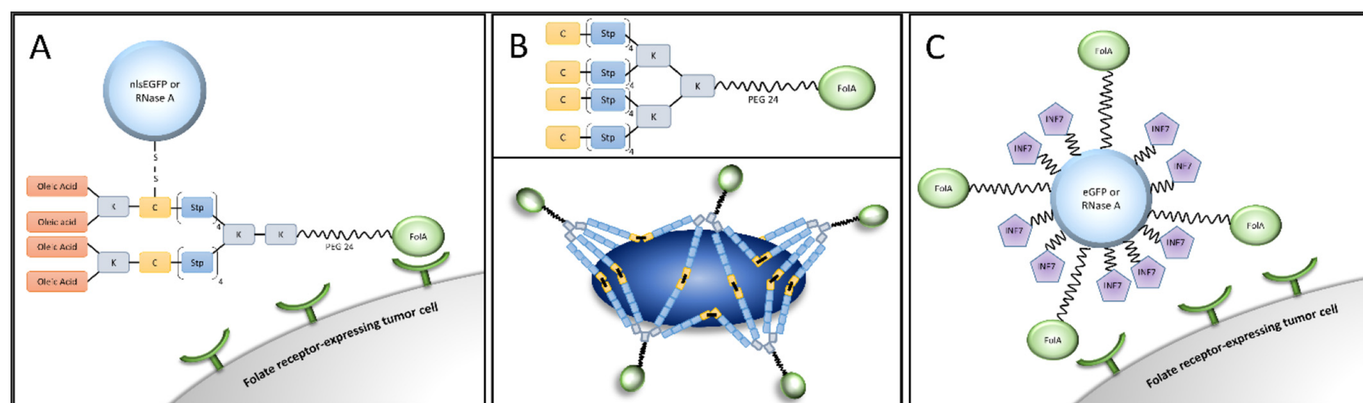


Fig. 13. Protein delivery strategies by solid-phase synthesized nanocarriers. (A) Bioreversible protein disulfide linked lipo-OAA [289,290]. (B) Folate-PEG linked four-arm OAAs for bioreversibly caging nanobody cargos [291]. (C) Traceless reversible protein conjugation with a targeting ligand (folate-PEG) and an endosomal escape peptide (INF7) [292].

rods with approximately 10 nm in diameter and 30 nm hydrodynamic diameter. Formulation of the bioreversible carrier conjugates with helper lipid resulted in formation of 50 nm proteoliposomes with similar protein delivery efficiency [290]. Replacing eGFP by antitumoral RNase A resulted in highly effective cytosolic delivery and the desired tumor cell killing.

Interestingly, when screening the library for intracellular delivery of very small (2 nm) nanobodies, the disulfide-linked lipo-OAA was not the most suitable delivery solution. Instead, a folate-PEG linked four-arm OAA containing a terminal cysteine on each arm (Fig. 13B) was found as most effective, surprisingly without covalent linkage to the nanobody protein [291]. The very small size of the nanoplexes and the strict requirement of the cysteines on the carrier with disulfide forming capacity is consistent with bioreversible caging of the nanobodies within a disulfide cross-linked OAA net coating the nanobody. Nanoplexes were internalized via receptor-mediated endocytosis and partly released into the cytosol. Bioreductive cytosolic release of nanobodies was verified via intracellular staining of nanobody target proteins in living cells. Fig. 13C illustrates the third protein delivery strategy as evaluated in our group. In contrast to the other approaches, carrier-free delivery formulations of eGFP or RNase A were developed by direct, traceless reversible protein conjugation with folate-PEG targeting ligands and influenza-derived endosomal escape peptide (INF7) functionalization [292]. Folate ligand incorporation was required and sufficient for cellular uptake. For the smaller and basic RNase A protein, additional INF7 functionalization was required and sufficient for cytosolic delivery and potent cell killing; for the larger non-basic eGFP protein, cytosolic delivery was limited despite INF7 incorporation. In another complementary approach, Ulrich Lächelt and colleagues generated multifunctional hybrid nanosystems by coordinative self-assembly of His-tagged proteins (such as H_6-eGFP or H_6-transferrin), apoptotic peptides, or mitochondrial cytochrome c onto metal—organic frameworks (MOFs) [293]. Intracellular delivery and cell killing by the apoptotic cargos was observed.

In summary, on the one hand, the biochemical difference of proteins enables the exploration of many different delivery strategies; on the other hand, generic protein delivery solutions are futile, and individual protein cargos request individual optimizations by chemical evolution.

3.5. Delivery of genome-editing nucleases

Over the past several years great advances in precise genome modifying nucleases such as zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), meganucleases (MN), or CRISPR Cas9/sgRNA [294,295] and other have opened a new era for genetic manipulation of model organisms [296] as well as the development of gene therapeutics [297–299]. For this purpose, nonviral vectors offer a broad and safer alternative to viral vectors, to overcome viral vector problems such as high occurrence of preexisting or induced immune response, or unintended sustained expression of programmable nucleases, which might lead to off-target cleavage. Transient expression profiles of nonviral vectors, which usually present a disadvantage for treatment of most genetic diseases, can be advantageously applied for transient expression of genome–modifying nucleases. Different intracellular delivery modalities for the genome-editing agents can be pursued: as gene expression constructs based on pDNA [300] or mRNA [299]; as recombinant protein, or as Cas9 protein/sgRNA ribonucleoprotein formulation [194,298]. Especially, the easy design of the CRISPR-associated protein 9 (Cas9)/ single guide RNA (sgRNA) system has boosted genome editing strategies. CRISPR/Cas9 is considered to be an innovative tool for highly efficient genome engineering [298,300,301]. Therefore development of efficient and safe delivery technologies, as also reviewed in this ADDR issue [302–304], is of utmost importance.

Kam Leong, Jianjun Cheng and collaborators applied the cationic α-helical polypeptide poly(γ-4-((2-(piperidin-1-yl)ethyl)aminomethyl) benzyl-L-glutamate), which was developed out of a library screen (see Fig. 10) for the co-delivery of sgRNA and pDNA expressing Cas9. PEGylated nanoparticles were obtained by including PEG-polythymidine$_{40}$ in the polyplex formulation. Single or multiplex gene editing was performed *in vitro* with an up to 47% efficiency. *In vivo* targeting gene deletion of polo-like kinase 1 (Plk1) suppressed the growth of HeLa tumors in mice and prolonged the animal survival rate [220].

Junjui Huang and colleagues developed a new strategy for the delivery of large pDNA coding for Cas9 and sgRNA for editing of two different genes (β-globin, rhomboid 5 homolog 1) by using PEI-β-cyclodextrin as cationic polymer to form nanocomplexes [300]. Zheng-Rong Lu and coworker optimized their bis (oleoyl-cysteine) oligoaminopeptide system ECO (see Sections 3.1 and 3.2) by introducing triamino-triethylamine as a novel cationizable head group and optionally additional linker lysine or histidines. ECO and iECO (without any further linker amino acids) presented the most effective agents for intracellular delivery of pDNA expressing CRISPR/Cas9, resulting in genome editing [305].

Gleb Sukhorukov and colleagues reported on pDNA-based delivery of CRISPR Cas9 components, improved by polymeric and hybrid microcarriers made of degradable polymers coated by a biocompatible shell of silica [306]. Using a combinatorial design of poly(β-amino esters) (see Section 2.4), Jordan Green and colleagues had developed branched ester-amine quadpolymers (BEAQs) for pDNA transfer (see Section 3.1). To co-encapsulate and deliver Cas9 pDNA and sgRNA in the same biodegradable nanoparticle system, Green and collaborators extended the chemical evolution to the design of reducible BEAQs (rBEAQs) with a library varying the polymer branching, reducibility and hydrophobicity. These variations resulted in 40% Cas9-facilitated gene knockout in human embryonic kidney 293T cells, which also confirms the presence as well as the bioactivity of the two components in the same cell [307].

mRNA delivery enables high and transient expression of genome-modifying nucleases. Anthony Conway et al co-formulated LNPs with a novel ionizable lipid and mRNA encoding ZFNs. They achieved highly effective *in vivo* genome editing of several therapeutic gene targets such as the TTR or PCSK9 gene in hepatocytes [308]. Standard LNPs (see Sections 2.4 and 3.2) preferably deliver nucleic acids (such as siRNA or mRNA) into liver hepatocytes. James Dahlman and coworkers applied their high-throughput barcode-based technology FIND (see Section 2.6) to measure efficient LNP-based *in vivo* delivery mRNA and sgRNA into multiple organs. In this directed evolution process, they found more than 250 LNPs delivering mRNA to multiple cell types. They also identified two LNPs, 7C2 and 7C3, that efficiently deliver sgRNA and mRNA to endothelial cells. The LNP 7C3 was found to deliver Cas9 mRNA and sgRNA to splenic endothelial cells as efficiently as to hepatocytes, opening the opportunity for endothelial cell gene editing [202]. The team of Daniel Siegwart designed zwitterionic amino lipids nanoparticles for delivery of Cas9 mRNA plus sgRNAs. Co-delivery of Cas9 mRNA/LoxP sgRNA via an intravenous route resulted in rescued tdTomato fluorescent protein expression in the liver, lung and kidneys of Lox-Stop-Lox tdTomato transgenic mice [309].

As the CRISPR-Cas9 protein activity is required only for the short period of the genome editing process, direct intracellular protein delivery into the cell nucleus is a further attractive option, preferable in form of an active Cas9 protein/sgRNA ribonucleoprotein complex. Delivery of protein cargos across cell membranes as well as endosome escape is a persistent challenge, which has to be met by strategies as outlined in Section 3.4. Jennifer Doudna and colleagues designed CRISPR-Cas9

protein conjugates, harboring N-acetylgalactosamine (GalNAc) trimers as targeting ligands to achieve effective ASGPR-mediated endocytosis into hepatocytes for liver-specific genome editing [310]. They could demonstrate gene editing in HepG2 cell culture when co-applied together with an endosomolytic peptide ppTG21.

David Liu and collaborators developed protein delivery for Cre recombinase, TALE, or Cas9 nuclease by conversion into negatively supercharged proteins followed by complexation with standard cationic liposomes. Supercharging was achieved by recombinant fusion with other negatively charged proteins or, in case of Cas9, by loading with the negatively charged sgRNA [311]. Subsequently the same research group applied bioreducible lipid carriers for improved intracellular release of the ribonucleoprotein agent [312]. Yoo Kyung Kang et al followed a different approach for CRISPR Cas9 protein delivery. They conjugated Cas9 endonuclease covalently with cationic branched PEI, and subsequently used the cationic conjugate for complexation with sgRNA. The formed nanoparticles were applied for genome editing of methicillin-resistant Staphylococcus aureus (MRSA) [298].

Yuan Ping, Yijun Cheng and coworkers had generated effective carriers for intracellular protein delivery by modifying cationic amine-terminated polyamidoamine (PAMAM) generation 5 dendrimers with phenylboronic acid (PBA) residues (see also Section 3.4). This modification has made it possible to bind with either negatively or positively charged proteins, assembling them into nanoparticles with potent cytosolic delivery, and maintaining the protein bioactivity after intracellular release. Amongst several protein cargos, they demonstrated efficient Cas9 protein/sgRNA co-delivery and gene editing [277]. Wenfu Zheng, Xingyu Jiang and colleagues reported on genome editing hybrid nanoparticles assembled from a core of gold nanoclusters modified with HIV tat peptide, Cas9 protein and sgRNA expressing pDNA, coated with a PEG-lipid shell. Using sgRNA targeting Polo-like kinase-1 (Plk1), a more than 70% down-regulation of Plk1 was observed in melanoma cells *in vitro* and a 75% suppression of melanoma growth in mice *in vivo* [313].

4. Conclusions

Extracellular and intracellular delivery of therapeutic nucleic acids or proteins has been the focus of numerous activities now already over 58 years, since first successful enhancement of poliovirus RNA delivery with basic proteins [314]. About three thousand gene therapy clinical trials (see http://www.abedia.com/wiley [1]) have been performed, yielding at current stage nine gene therapy products. Numerous chemistry efforts resulted in one siRNA and at least eight synthetic oligonucleotides that were approved as drug products. Further nucleic acid products are in advanced clinical evaluation and will reach the market soon. In all these nanomedicine developments, efficient and safe transfer of the therapeutic nanoagent into the desired target tissues and cells of patients are still the most critical limitation and bottleneck.

Nature designed viruses as highly potent intracellular delivery agents, using a very limited tool-box of chemical building blocks assembled under physiological conditions. We may learn from the elaborative mechanisms of viruses for infecting cells and might try to imitate them by generating 'artificial viruses'. However, numerous different classes of viruses with widely different entry mechanisms exist. To paraphrase the quote from Richard Feynman 'What I cannot create I do not understand': being fascinated about viruses, does not

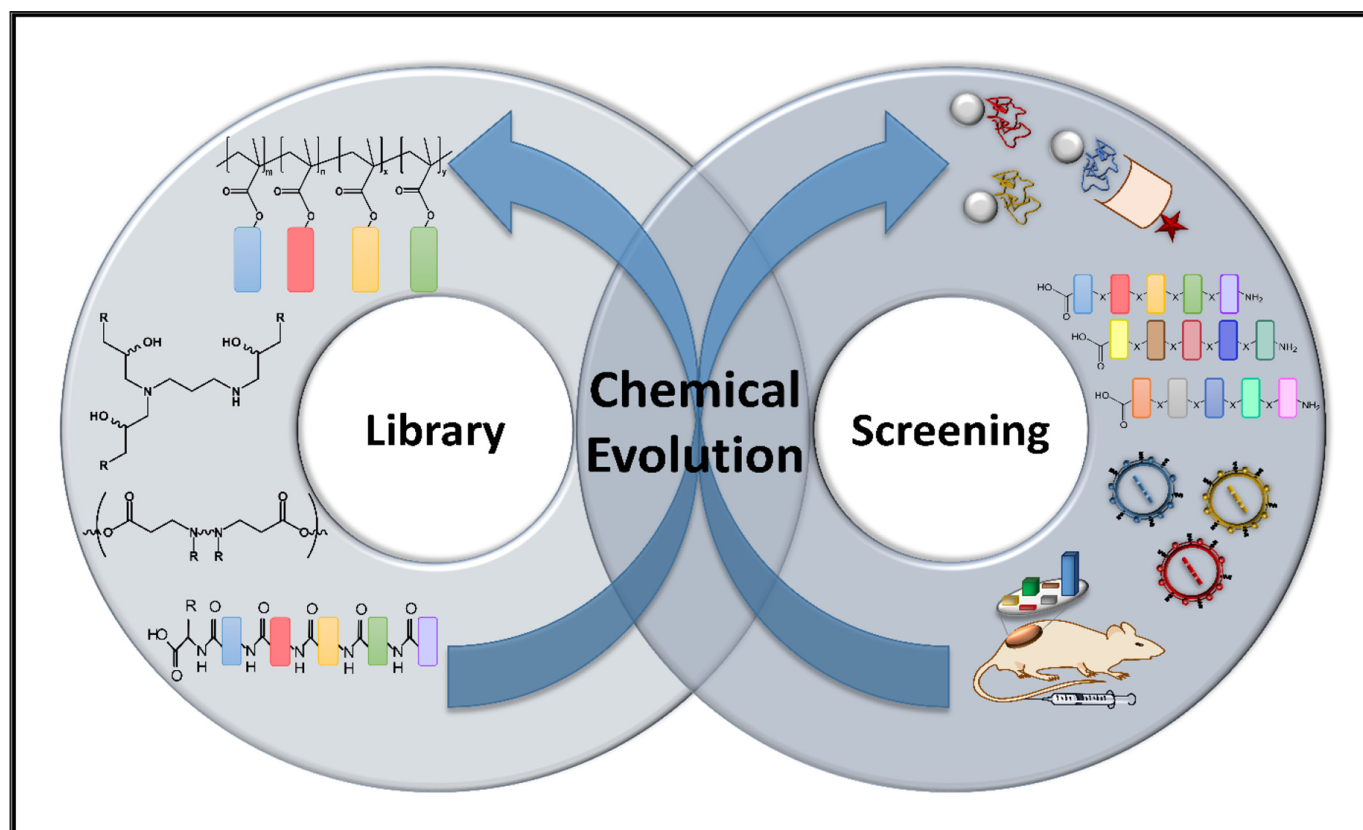


Fig. 14. Chemical evolution of nanocarriers. Chemical libraries can be combinatorial or sequence-defined. Screening in appropriate *in vitro* or *in vivo* test models is supposed to (physically or functionally) enrich nanocarriers with favorable properties. Nanocarriers can be identified either directly (by sequence) or indirectly (by barcoding), providing input for the next evolution cycle.

mean to understand them and being able to create them. Viruses may be regarded as snapshots of natural evolution, and their diverse optimized processes may not be general applicable in an artificial setting. It might be more useful to learn from the general principles of natural evolution and adopt them to artificial synthetic designs and selection processes termed here as "chemical evolution". In theory, refined synthetic nanocarriers designed from a far broader space of chemical building blocks and synthesized using a whole range of optimized chemical conditions might be more effective in delivery than natural viruses. We 'simply' lack the knowledge about their perfect construction plan.

General design principles for synthetic chemical evolution of nanocarriers (Fig. 14) could be as follows:

i. Generation of libraries of chemical structures defined as retrievable 'Sequences', assembled from a set of chemical building blocks.
ii. Definition as 'Sequences' can be based on the actual sequence of subunits or on integrated (DNA or peptide) barcodes.
iii. A 'Sequence' can be linear (as in natural nucleic acids and peptides) or might contain branching (such as in dendrons).
iv. 'Sequences' need to be retrievable in terms of structure identification (high-end analytics from genomics/proteomics) and regeneration of the structure (directed synthesis). The sequence information should be storable *in silico* or on a template (compare with the genetic code in natural evolution).
v. Libraries may be the result of random or designed small changes in sequence of building blocks ('mutations'), or rearrangement of previously found useful domains ('shuffling'). Analogous gene shuffling has been applied in natural and artificial evolution (just for example, note the abundant use of DNA binding zinc fingers in ~2800 human proteins [315]).
vi. Screening of the library has to be performed in appropriate test models, for identification and selection of most suitable nanocarrier candidates as starting point for the next refined library and next evolution cycle.
vii. Different macromolecular cargos (illustrated here for pDNA siRNA, mRNA, proteins and genome–editing agents) may require different nanocarriers due to their different physicochemical properties and their different intracellular target sites and modes of action.
viii. The different therapeutic modes (transient or permanent action; low or high level of intervention required for therapeutic effects) as well as the various different target organs and cell types may dictate different requirements to the nanocarrier. Optimization for *ex vivo* delivery into cultured cell most likely will not correlate with optimal *in vivo* delivery. Therefore, applying the most relevant screening model is of utmost importance. Keep in mind: even in the best case, you only get what you actually asked/screened for.

Chemical evolution may involve rational design of improved building blocks based on a better understanding of the delivery processes. It may be fueled by empirical screening of diverse chemical compound libraries, computational prediction by machine learning algorithms, virtual screening. Chemical evolution can also be regarded as an evolution of knowledge. To illustrate this with one example of endosomal escape: in the early nineties, when this step was discovered to be ineffective in nonviral gene transfer, viral endosomolytic mechanisms were introduced to overcome this bottleneck [38,316]. It also triggered the search for endosomolytic agents and the discovery that a simple cationizable polymer, PEI, can overcome the endosomal barrier via "The proton sponge: A trick to enter cells the viruses did not exploit" [317,318]. A refined molecular understanding of the aminoethylene protonation characteristics was developed and described as the "even-odd" effect [93], which pointed out that not every proton sponge might be applicable. Evolution continues with the notion that different intracellular endolysosomal sorting mechanisms in different cell types and tissues may strongly influence the selection of the most productive mechanism of cationizable nanocarriers [144,263].

In conclusion, different chemical evolution strategies have already been pursued, as reported herein. Starting from rational design of multifunctional molecular conjugates and block copolymer libraries, screening of combinatorial chemistry libraries or sequence-defined peptide-like macromolecules has been performed, largely *ex vivo* in cell culture, but in part also in more relevant *in vivo* mouse models. DNA- or peptide- barcoded nanoagents, combined with high-end genomics/MS analytics for identification of nanocarriers, present encouraging options for future *in vivo* chemical evolution.

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References

[1] S.L. Ginn, A.K. Amaya, I.E. Alexander, M. Edelstein, M.R. Abedi, Gene therapy clinical trials worldwide to 2017 – an update, J. Gene Med. 20 (2018)e3015.
[2] C.A. Stein, D. Castanotto, FDA-approved oligonucleotide therapies in 2017, Mol. Ther. 25 (2017) 1069–1075.
[3] R. Titze-de-Almeida, C. David, S.S. Titze-de-Almeida, The race of 10 synthetic RNAi-based drugs to the pharmaceutical market, Pharm. Res. 34 (2017) 1339–1363.
[4] D. Adams, A. Gonzalez-Duarte, W.D. O'Riordan, C.C. Yang, M. Ueda, A.V. Kristen, I. Tournev, H.H. Schmidt, T. Coelho, J.L. Berk, K.P. Lin, G. Vita, S. Attarian, V. Plante-Bordeneuve, M.M. Mezei, J.M. Campistol, J. Buades, T.H. Brannagan 3rd, B.J. Kim, J. Oh, Y. Parman, Y. Sekijima, P.N. Hawkins, S.D. Solomon, M. Polydefkis, P.J. Dyck, P.J. Gandhi, S. Goyal, J. Chen, A.L. Strahs, S.V. Nochur, M.T. Sweetser, P.P. Garg, A.K. Vaishnaw, J.A. Gollob, O.B. Suhr, Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis, N. Engl. J. Med. 379 (2018) 11–21.
[5] M. Egli, M. Manoharan, Re-engineering RNA molecules into therapeutic agents, Acc. Chem. Res. 52 (2019) 1036–1047.
[6] P.R. Cullis, M.J. Hope, Lipid nanoparticle systems for enabling gene therapies, Mol. Ther. 25 (2017) 1467–1475.
[7] J.K. Nair, H. Attarwala, A. Sehgal, Q. Wang, K. Aluri, X. Zhang, M. Gao, J. Liu, R. Indrakanti, S. Schofield, P. Kretschmer, C.R. Brown, S. Gupta, J.L.S. Willoughby, J.A. Boshar, V. Jadhav, K. Charisse, T. Zimmermann, K. Fitzgerald, M. Manoharan, K.G. Rajeev, A. Akinc, R. Hutabarat, M.A. Maier, Impact of enhanced metabolic stability on pharmacokinetics and pharmacodynamics of GalNAc-siRNA conjugates, Nucleic Acids Res. 45 (2017) 10969–10977.
[8] D.J. Foster, C.R. Brown, S. Shaikh, C. Trapp, M.K. Schlegel, K. Qian, A. Sehgal, K.G. Rajeev, V. Jadhav, M. Manoharan, S. Kuchimanchi, M.A. Maier, S. Milstein, Advanced siRNA designs further improve in vivo performance of GalNAc-siRNA conjugates, Mol. Ther. 26 (2018) 708–717.
[9] A. Wittrup, J. Lieberman, Knocking down disease: a progress report on siRNA therapeutics, Nat. Rev. Genet. 16 (2015) 543–552.
[10] D. Oupicky, V. Diwadkar, Stimuli-responsive gene delivery vectors, Curr. Opin. Mol. Ther. 5 (2003) 345–350.
[11] E. Wagner, Programmed drug delivery: nanosystems for tumor targeting, Exp. Op. Biol. Ther. 7 (2007) 587–593.
[12] M. Sun, K. Wang, D. Oupicky, Advances in stimulus-responsive polymeric materials for systemic delivery of nucleic acids, Adv. Healthcare Mater. 7 (2018).
[13] S. Hager, E. Wagner, Bioresponsive polyplexes – chemically programmed for nucleic acid delivery, Exp. Op. Drug Del. 15 (2018) 1067–1083.
[14] D.J. Peeler, D.L. Sellers, S.H. Pun, pH-sensitive polymers as dynamic mediators of barriers to nucleic acid delivery, Bioconjug. Chem. 30 (2019) 350–365.
[15] P.M. Klein, E. Wagner, Bioreducible polycations as shuttles for therapeutic nucleic acid and protein transfection, Antioxid. Redox Signal. 21 (5) (2014) 804–817.
[16] H. Hatakeyama, H. Akita, K. Kogure, M. Oishi, Y. Nagasaki, Y. Kihira, M. Ueno, H. Kobayashi, H. Kikuchi, H. Harashima, Development of a novel systemic gene delivery system for cancer therapy with a tumor-specific cleavable PEG-lipid, Gene Ther. 14 (2007) 68–77.
[17] S. Reinhard, Y. Wang, S. Dengler, E. Wagner, Precise enzymatic cleavage sites for improved bioactivity of siRNA lipo-polyplexes, Bioconjug. Chem. 29 (2018) 3649–3657.

[18] A. Kwok, S.L. Hart, Comparative structural and functional studies of nanoparticle formulations for DNA and siRNA delivery, Nanomedicine 7 (2011) 210–219.

[19] C. Scholz, E. Wagner, Therapeutic plasmid DNA versus siRNA delivery: common and different tasks for synthetic carriers, J. Control. Release 161 (2012) 554–565.

[20] C. Goncalves, S. Akhter, C. Pichon, P. Midoux, Intracellular availability of pDNA and mRNA after transfection: a comparative study among polyplexes, lipoplexes, and lipopolyplexes, Mol. Pharm. 13 (2016) 3153–3163.

[21] A.K. Blakney, G. Yilmaz, P.F. McKay, C.R. Becer, R.J. Shattock, One size does not fit all: the effect of chain length and charge density of poly(ethylene imine) based copolymers on delivery of pDNA, mRNA, and RepRNA polyplexes, Biomacromolecules 19 (2018) 2870–2879.

[22] A.C. Kauffman, A.S. Piotrowski-Daspit, K.H. Nakazawa, Y. Jiang, A. Datye, W.M. Saltzman, Tunability of biodegradable poly(amine- co-ester) polymers for customized nucleic acid delivery and other biomedical applications, Biomacromolecules 19 (2018) 3861–3873.

[23] L. Peng, E. Wagner, Polymeric carriers for nucleic acid delivery: current designs and future directions, Biomacromolecules 20 (2019) 3613–3626.

[24] K. Paunovska, C.D. Sago, C.M. Monaco, W.H. Hudson, M.G. Castro, T.G. Rudoltz, S. Kalathoor, D.A. Vanover, P.J. Santangelo, R. Ahmed, A.V. Bryksin, J.E. Dahlman, A direct comparison of in vitro and in vivo nucleic acid delivery mediated by hundreds of nanoparticles reveals a weak correlation, Nano Lett. 18 (2018) 2148–2157.

[25] A.C. Allwood, M.R. Walter, B.S. Kamber, C.P. Marshall, I.W. Burch, Stromatolite reef from the Early Archaean era of Australia, Nature 441 (2006) 714–718.

[26] A. Vaheri, J.S. Pagano, Infectious poliovirus RNA: a sensitive method of assay, Virology 27 (1965) 434–436.

[27] J.H. McCutchan, J.S. Pagano, Enchancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyl-dextran, J. Natl. Cancer Inst. 41 (1968) 351–357.

[28] 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 390 (1975) 298–311.

[29] P.L. Felgner, Y. Barenholz, J.P. Behr, S.H. Cheng, P. Cullis, L. Huang, J.A. Jessee, L. Seymour, F. Szoka, A.R. Thierry, E. Wagner, G. Wu, Nomenclature for synthetic gene delivery systems, Hum. Gene Ther. 8 (1997) 511–512.

[30] U. Lächelt, E. Wagner, Nucleic acid therapeutics using polyplexes: a journey of 50 years (and beyond), Chem. Rev. 115 (2015) 11043–11078.

[31] K.A. Mislick, J.D. Baldeschwieler, Evidence for the role of proteoglycans in cation-mediated gene transfer, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 12349–12354.

[32] I. Kopatz, J.S. Remy, J.P. Behr, A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin, J. Gene Med. 6 (2004) 769–776.

[33] 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.

[34] S.M. Zou, P. Erbacher, J.S. Remy, J.P. Behr, Systemic linear polyethylenimine (L-PEI)-mediated gene delivery in the mouse, J. Gene Med. 2 (2000) 128–134.

[35] O.M. Merkel, R. Urbanics, P. Bedocs, Z. Rozsnyay, L. Rosivall, M. Toth, T. Kissel, J. Szebeni, In vitro and in vivo complement activation and related anaphylactic effects associated with polyethylenimine and polyethylenimine-graft-poly(ethylene glycol) block copolymers, Biomaterials 32 (2011) 4936–4942.

[36] C. Plank, K. Mechtler, F.C. Szoka Jr., 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.

[37] E. Wagner, M. Ogris, W. Zauner, Polylysine-based transfection systems utilizing receptor-mediated delivery, Adv. Drug Deliv. Rev. 30 (1998) 97–113.

[38] E. Wagner, C. Plank, K. Zatloukal, M. Cotten, M.L. Birnstiel, Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 7934–7938.

[39] M. Meyer, A. Philipp, R. Oskuee, C. Schmidt, E. Wagner, Breathing life into polycations: functionalization with pH-responsive endosomolytic peptides and polyethylene glycol enables siRNA delivery, J. Am. Chem. Soc. 130 (2008) 3272–3273.

[40] J.G. Schellinger, J.A. Pahang, R.N. Johnson, D.S. Chu, D.L. Sellers, D.O. Maris, A.J. Convertine, P.S. Stayton, P.J. Horner, S.H. Pun, Melittin-grafted HPMA-oligolysine based copolymers for gene delivery, Biomaterials 34 (2013) 2318–2326.

[41] 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.

[42] R.J. Cristiano, L.C. Smith, M.A. Kay, B.R. Brinkley, S.L. Woo, Hepatic gene therapy: efficient gene delivery and expression in primary hepatocytes utilizing a conjugated adenovirus-DNA complex, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 11548–11552.

[43] W. Zauner, D. Blaas, E. Kuechler, E. Wagner, Rhinovirus-mediated endosomal release of transfection complexes, J. Virol. 69 (1995) 1085–1092.

[44] G.Y. Wu, C.H. Wu, Receptor-mediated gene delivery and expression in vivo, J. Biol. Chem. 262 (1988) 14621–14624.

[45] E. Wagner, M. Zenke, M. Cotten, H. Beug, M.L. Birnstiel, Transferrin-polycation conjugates as carriers for DNA uptake into cells, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 3410–3414.

[46] M. Tonigold, J. Simon, D. Estupinan, M. Kokkinopoulou, J. Reinholz, U. Kintzel, A. Kaltbeitzel, P. Renz, M.P. Domogalla, K. Steinbrink, I. Lieberwirth, D. Crespy, K. Landfester, V. Mailander, Pre-adsorption of antibodies enables targeting of nanocarriers despite a biomolecular corona, Nat. Nanotechnol. 13 (2018) 862–869.

[47] 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.

[48] R. Kircheis, S. Schuller, S. Brunner, M. Ogris, K.H. Heider, W. Zauner, E. Wagner, Polycation-based DNA complexes for tumor-targeted gene delivery in vivo, J. Gene Med. 1 (1999) 111–120.

[49] M. Ogris, S. Brunner, S. Schuller, 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.

[50] 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.

[51] T. Blessing, M. Kursa, R. Holzhauser, R. Kircheis, E. Wagner, Different strategies for formation of pegylated EGF-conjugated PEI/DNA complexes for targeted gene delivery, Bioconjug. Chem. 12 (2001) 529–537.

[52] 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.

[53] M.W. Konstan, P.B. Davis, J.S. Wagener, K.A. Hilliard, R.C. Stern, L.J. Milgram, T.H. Kowalczyk, S.L. Hyatt, T.L. Fink, C.R. Gedeon, S.M. Oette, J.M. Payne, O. Muhammad, A.G. Ziady, R.C. Moen, M.J. Cooper, Compacted DNA nanoparticles administered to the nasal mucosa of cystic fibrosis subjects are safe and demonstrate partial to complete cystic fibrosis transmembrane regulator reconstitution, Hum. Gene Ther. 15 (2004) 1255–1269.

[54] 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.

[55] O.M. Merkel, D. Librizzi, A. Pfestroff, T. Schurrat, K. Buyens, N.N. Sanders, S.C. De Smedt, M. Behe, T. Kissel, Stability of siRNA polyplexes from poly(ethylenimine) and poly(ethylenimine)-g-poly(ethylene glycol) under in vivo conditions: effects on pharmacokinetics and biodistribution measured by Fluorescence Fluctuation Spectroscopy and Single Photon Emission Computed Tomography (SPECT) imaging, J. Control. Release 138 (2009) 148–159.

[56] A. Taschauer, W. Polzer, F. Alioglu, M. Billerhart, S. Decker, T. Kittelmann, E. Geppl, S. Elmenofi, M. Zehl, E. Urban, H. Sami, M. Ogris, Peptide-targeted polyplexes for aerosol-mediated gene delivery to CD49f-overexpressing tumor lesions in lung, Mol. Ther. Nucleic Acids 18 (2019) 774–786.

[57] K.D. Fisher, K. Ulbrich, V. Subr, C.M. Ward, V. Mautner, D. Blakey, L.W. Seymour, A versatile system for receptor-mediated gene delivery permits increased entry of DNA into target cells, enhanced delivery to the nucleus and elevated rates of transgene expression, Gene Ther. 7 (2000) 1337–1343.

[58] R.C. Carlisle, T. Etrych, S.S. Briggs, J.A. Preece, K. Ulbrich, L.W. Seymour, Polymer-coated polyethylenimine/DNA complexes designed for triggered activation by intracellular reduction, J. Gene Med. 6 (2004) 337–344.

[59] R.N. Johnson, D.S. Chu, J. Shi, J.G. Schellinger, P.M. Carlson, S.H. Pun, HPMA-oligolysine copolymers for gene delivery: optimization of peptide length and polymer molecular weight, J. Control. Release 155 (2011) 303–311.

[60] M. Noga, D. Edinger, R. Klager, S.V. Wegner, J.P. Spatz, E. Wagner, G. Winter, A. Besheer, The effect of molar mass and degree of hydroxyethylation on the controlled shielding and deshielding of hydroxyethyl starch-coated polyplexes, Biomaterials 34 (2013) 2530–2538.

[61] M. Hornof, M. de la Fuente, M. Hallikainen, R.H. Tammi, A. Urtti, Low molecular weight hyaluronan shielding of DNA/PEI polyplexes facilitates CD44 receptor mediated uptake in human corneal epithelial cells, J. Gene Med. 10 (2008) 70–80.

[62] T. Ito, C. Yoshihara, K. Hamada, Y. Koyama, DNA/polyethyleneimine/hyaluronic acid small complex particles and tumor suppression in mice, Biomaterials 31 (2010) 2912–2918.

[63] T. Sato, M. Nakata, Z. Yang, Y. Torizuka, S. Kishimoto, M. Ishihara, In vitro and in vivo gene delivery using chitosan/hyaluronic acid nanoparticles: Influences of molecular mass of hyaluronic acid and lyophilization on transfection efficiency, J. Gene Med. 19 (2017).

[64] P. Heller, A. Birke, D. Huesmann, B. Weber, K. Fischer, A. Reske-Kunz, M. Bros, M. Barz, Introducing peptoplexes: polylysine-block-polysarcosine based polyplexes for transfection of HEK 293T cells, Macromol. Biosci. 14 (2014) 1380–1395.

[65] P. Heller, D. Hobernik, U. Lächelt, M. Schinnerer, B. Weber, M. Schmidt, E. Wagner, M. Bros, M. Barz, Combining reactive triblock copolymers with functional crosslinkers: a versatile pathway to disulfide stabilized-polyplex libraries and their application as pDNA vaccines, J. Control. Release 258 (2017) 146–160.

[66] P.M. Klein, K. Klinker, W. Zhang, S. Kern, E. Kessel, E. Wagner, M. Barz, Efficient shielding of polyplexes using heterotelechelic polysarcosines, Polymers 10 (2018).

[67] J. Chen, K. Wang, J. Wu, H. Tian, X. Chen, Polycations for gene delivery: dilemmas and solutions, Bioconjug. Chem. 30 (2019) 338–349.

[68] G.F. Walker, V. Knorr, L. Allmendinger, F.F. Paintner, E. Wagner, An acetal-based PEGylation reagent for pH-sensitive shielding of DNA polyplexes. Bioconjug. Chem. 18 (2007) 1218–1225.

[69] 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.

[70] V. Knorr, M. Ogris, E. Wagner, An acid sensitive ketal-based polyethylene glycol-oligoethylenimine copolymer mediates improved transfection efficiency at reduced toxicity, Pharm. Res. 25 (2008) 2937–2945.

[71] J.A. Wolff, D.B. Rozema, Breaking the bonds: non-viral vectors become chemically dynamic, Mol. Ther. 16 (2008) 8–15.

[72] D.B. Rozema, A.V. Blokhin, D.H. Wakefield, J.D. Benson, J.C. Carlson, J.J. Klein, L.J. Almeida, A.L. Nicholas, H.L. Hamilton, Q. Chu, J.O. Hegge, S.C. Wong, V.S.

Trubetskoy, C.M. Hagen, E. Kitas, J.A. Wolff, D.L. Lewis, Protease-triggered siRNA delivery vehicles, J. Control. Release 209 (2015) 57–66.
[73] E. Wagner, Strategies to improve DNA polyplexes for in vivo gene transfer: will "artificial viruses" be the answer? Pharm. Res. 21 (2004) 8–14.
[74] T. Luo, H. Liang, R. Jin, Y. Nie, Virus inspired and mimetic designs in nonviral gene delivery, J. Gene Med. 21 (2019)e3090.
[75] R. Kircheis, E. Ostermann, M.F. Wolschek, C. Lichtenberger, C. Magin-Lachmann, L. Wightman, M. Kursa, E. Wagner, Tumor-targeted gene delivery of tumor necrosis factor-alpha induces tumor necrosis and tumor regression without systemic toxicity, Cancer Gene Ther. 9 (2002) 673–680.
[76] 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.
[77] A. Ndoye, G. Dolivet, A. Hogset, A. Leroux, A. Fifre, P. Erbacher, K. Berg, J.P. Behr, F. Guillemin, J.L. Merlin, Eradication of p53-mutated head and neck squamous cell carcinoma xenografts using nonviral p53 gene therapy and photochemical internalization, Mol. Ther. 13 (2006) 1156–1162.
[78] A. Shir, M. Ogris, W. Roedl, E. Wagner, A. Levitzki, EGFR-homing dsRNA activates cancer-targeted immune response and eliminates disseminated EGFR-overexpressing tumors in mice, Clin. Cancer Res. 17 (2011) 1033–1043.
[79] G. Abourbeh, A. Shir, E. Mishani, M. Ogris, W. Rodl, E. Wagner, A. Levitzki, PolyIC GE11 polyplex inhibits EGFR-overexpressing tumors, IUBMB Life 64 (2012) 324–330.
[80] B. Su, A. Cengizeroglu, K. Farkasova, J.R. Viola, M. Anton, J.W. Ellwart, R. Haase, E. Wagner, M. Ogris, Systemic TNFalpha gene therapy synergizes with liposomal doxorubicine in the treatment of metastatic cancer, Mol. Ther. 21 (2013) 300–308.
[81] K.A. Schmohl, A. Gupta, G.K. Grunwald, M. Trajkovic-Arsic, K. Klutz, R. Braren, M. Schwaiger, P.J. Nelson, M. Ogris, E. Wagner, J.T. Siveke, C. Spitzweg, Imaging and targeted therapy of pancreatic ductal adenocarcinoma using the theranostic sodium iodide symporter (NIS) gene, Oncotarget 8 (2017) 33393–33404.
[82] S. Schreiber, E. Kampgen, E. Wagner, D. Pirkhammer, J. Trcka, H. Korschan, A. Lindemann, R. Dorffner, H. Kittler, F. Kasteliz, Z. Kupcu, A. Sinski, K. Zatloukal, M. Buschle, W. Schmidt, M. Birnstiel, R.E. Kempe, T. Voigt, H.A. Weber, H. Pehamberger, R. Mertelsmann, E.B. Brocker, 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.
[83] K. Anwer, M.N. Barnes, J. Fewell, D.H. Lewis, R.D. Alvarez, Phase-I clinical trial of IL-12 plasmid/lipopolymer complexes for the treatment of recurrent ovarian cancer, Gene Ther. 17 (2010) 360–369.
[84] M.E. Davis, J.E. Zuckerman, C.H. 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.
[85] 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.
[86] A. Dirisala, K. Osada, Q. Chen, T.A. Tockary, K. Machitani, S. Osawa, X. Liu, T. Ishii, K. Miyata, M. Oba, S. Uchida, K. Itaka, K. Kataoka, Optimized rod length of polyplex micelles for maximizing transfection efficiency and their performance in systemic gene therapy against stroma-rich pancreatic tumors, Biomaterials 35 (2014) 5359–5368.
[87] T.A. Tockary, K. Osada, Y. Motoda, S. Hiki, Q. Chen, K.M. Takeda, A. Dirisala, S. Osawa, K. Kataoka, Rod-to-globule transition of pDNA/PEG-Poly(l-Lysine) polyplex micelles induced by a collapsed balance between DNA rigidity and PEG crowdedness, Small 12 (2016) 1193–1200.
[88] 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.
[89] 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.
[90] M. Han, Y. Bae, N. Nishiyama, K. Miyata, M. Oba, K. Kataoka, Transfection study using multicellular tumor spheroids for screening non-viral polymeric gene vectors with low cytotoxicity and high transfection efficiencies, J. Control. Release 121 (2007) 38–48.
[91] K. Miyata, M. Oba, M. Nakanishi, S. Fukushima, Y. Yamasaki, H. Koyama, N. Nishiyama, K. Kataoka, Polyplexes from poly(aspartamide) bearing 1,2-diaminoethane side chains induce pH-selective, endosomal membrane destabilization with amplified transfection and negligible cytotoxicity, J. Am. Chem. Soc. 130 (2008) 16287–16294.
[92] H.J. Kim, A. Ishii, K. Miyata, Y. Lee, S. Wu, M. Oba, N. Nishiyama, K. Kataoka, Introduction of stearoyl moieties into a biocompatible cationic polyaspartamide derivative, PAsp(DET), with endosomal escaping function for enhanced siRNA-mediated gene knockdown, J. Control. Release 145 (2010) 141–148.
[93] H. Uchida, K. Miyata, M. Oba, T. Ishii, T. Suma, K. Itaka, N. Nishiyama, K. Kataoka, Odd-even effect of repeating aminoethylene units in the side chain of N-substituted polyaspartamides on gene transfection profiles, J. Am. Chem. Soc. 133 (2011) 15524–15532.
[94] H. Uchida, K. Itaka, T. Nomoto, T. Ishii, T. Suma, M. Ikegami, K. Miyata, M. Oba, N. Nishiyama, K. Kataoka, Modulated protonation of side chain aminoethylene repeats in N-substituted polyaspartamides promotes mRNA transfection, J. Am. Chem. Soc. 136 (2014) 12396–12405.
[95] 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.
[96] C. Boyer, V. Bulmus, T.P. Davis, V. Ladmiral, J. Liu, S. Perrier, Bioapplications of RAFT polymerization, Chem. Rev. 109 (2009) 5402–5436.
[97] M.H. Stenzel, RAFT polymerization: an avenue to functional polymeric micelles for drug delivery, Chem. Commun. (2008) 3486–3503.
[98] M.A. Wolfert, E.H. Schacht, V. Toncheva, K. Ulbrich, O. Nazarova, L.W. Seymour, P.R. Dash, D. Oupicky, Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers, Hum. Gene Ther. 7 (1996) 2123–2133.
[99] N. Murthy, J. Campbell, N. Fausto, A.S. Hoffman, P.S. Stayton, Bioinspired pH-responsive polymers for the intracellular delivery of biomolecular drugs, Bioconjug. Chem. 14 (2003) 412–419.
[100] K.A. Howard, P.R. Dash, M.L. Read, K. Ward, L.M. Tomkins, O. Nazarova, K. Ulbrich, L.W. Seymour, Influence of hydrophilicity of cationic polymers on the biophysical properties of polyelectrolyte complexes formed by self-assembly with DNA, Biochim. Biophys. Acta 1475 (2000) 245–255.
[101] J.Y. Cherng, P. van de Wetering, H. Talsma, D.J. Crommelin, W.E. Hennink, Effect of size and serum proteins on transfection efficiency of poly ((2-dimethylamino) ethyl methacrylate)-plasmid nanoparticles, Pharm. Res. 13 (1996) 1038–1042.
[102] P. van de Wetering, J.Y. Cherng, H. Talsma, D.J. Crommelin, W.E. Hennink, 2-(Dimethylamino)ethyl methacrylate based (co)polymers as gene transfer agents, J. Control. Release 53 (1998) 145–153.
[103] F.J. Verbaan, C. Oussoren, C.J. Snel, D.J. 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.
[104] M. Barz, M. Tarantola, K. Fischer, M. Schmidt, R. Luxenhofer, A. Janshoff, P. Theato, R. Zentel, From defined reactive diblock copolymers to functional HPMA-based self-assembled nanoaggregates, Biomacromolecules 9 (2008) 3114–3118.
[105] A.K. Trutzschler, T. Bus, M. Reifarth, J.C. Brendel, S. Hoeppener, A. Traeger, U.S. Schubert, Beyond gene transfection with methacrylate-based polyplexes-the influence of the amino substitution pattern, Bioconjug. Chem. 29 (2018) 2181–2194.
[106] H. Wei, L.R. Volpatti, D.L. Sellers, D.O. Maris, I.W. Andrews, A.S. Hemphill, L.W. Chan, D.S. Chu, P.J. Horner, S.H. Pun, Dual responsive, stabilized nanoparticles for efficient in vivo plasmid delivery, Angew. Chem. Int. Ed. 52 (2013) 5377–5381.
[107] Y. Cheng, R.C. Yumul, S.H. Pun, Virus-inspired polymer for efficient in vitro and in vivo gene delivery, Angew. Chem. Int. Ed. 55 (2016) 12013–12017.
[108] D.P. Feldmann, Y. Cheng, R. Kandil, Y. Xie, M. Mohammadi, H. Harz, A. Sharma, D.J. Peeler, A. Moszczynska, H. Leonhardt, S.H. Pun, O.M. Merkel, In vitro and in vivo delivery of siRNA via VIPER polymer system to lung cells, J. Control. Release 276 (2018) 50–58.
[109] D.J. Peeler, S.N. Thai, Y. Cheng, P.J. Horner, D.L. Sellers, S.H. Pun, pH-sensitive polymer micelles provide selective and potentiated lytic capacity to venom peptides for effective intracellular delivery, Biomaterials 192 (2019) 235–244.
[110] T. Wang, D. Wang, H. Yu, M. Wang, J. Liu, B. Feng, F. Zhou, Q. Yin, Z. Zhang, Y. Huang, Y. Li, Intracellularly acid-switchable multifunctional micelles for combinational photo/chemotherapy of the drug-resistant tumor, ACS Nano 10 (2016) 3496–3508.
[111] W. Wang, M. Saeed, Y. Zhou, L. Yang, D. Wang, H. Yu, Non-viral gene delivery for cancer immunotherapy, J. Gene Med. 21 (2019), e3092.
[112] I.K. Ko, A. Ziady, S. Lu, Y.J. Kwon, Acid-degradable cationic methacrylamide polymerized in the presence of plasmid DNA as tunable non-viral gene carrier, Biomaterials 29 (2008) 3872–3881.
[113] Y. Cheng, D.L. Sellers, J.Y. Tan, D.J. Peeler, P.J. Horner, S.H. Pun, Development of switchable polymers to address the dilemma of stability and cargo release in polycationic nucleic acid carriers, Biomaterials 127 (2017) 89–96.
[114] L. Novo, E.V. 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.
[115] 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.
[116] 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.
[117] L. Novo, K.M. Takeda, T. Petteta, G.R. Dakwar, J.B. van den Dikkenberg, K. Remaut, K. Braeckmans, C.F. van Nostrum, E. Mastrobattista, W.E. Hennink, Targeted decationized polyplexes for siRNA delivery, Mol. Pharm. 12 (2015) 150–161.
[118] B.R. Olden, Y. Cheng, J.L. Yu, S.H. Pun, Cationic polymers for non-viral gene delivery to human T cells, J. Control. Release 282 (2018) 140–147.
[119] E.R. Lee, J. Marshall, C.S. Siegel, C. Jiang, N.S. Yew, M.R. Nichols, J.B. Nietupski, R.J. Ziegler, M.B. Lane, K.X. Wang, N.C. Wan, R.K. Scheule, D.J. Harris, A.E. Smith, S.H. Cheng, Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung, Hum. Gene Ther. 7 (1996) 1701–1717.
[120] Y.B. Lim, S.O. Han, H.U. Kong, Y. Lee, J.S. Park, B. Jeong, S.W. Kim, Biodegradable polyester, poly[alpha-(4-aminobutyl)-L-glycolic acid], as a non-toxic gene carrier, Pharm. Res. 17 (2000) 811–816.
[121] Y.B. Lim, S.M. Kim, H. Suh, J.S. Park, Biodegradable, endosome disruptive, and cationic network-type polymer as a highly efficient and nontoxic gene delivery carrier, Bioconjug. Chem. 13 (2002) 952–957.

[122] M.L. Forrest, J.T. Koerber, D.W. Pack, A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery, Bioconjug. Chem. 14 (2003) 934–940.
[123] J. Kloeckner, S. Bruzzano, M. Ogris, E. Wagner, Gene carriers based on hexanediol diacrylate linked oligoethylenimine: effect of chemical structure of polymer on biological properties, Bioconjug. Chem. 17 (2006) 1339–1345.
[124] V. Russ, H. Elfberg, C. Thoma, J. Kloeckner, M. Ogris, E. Wagner, Novel degradable oligoethylenimine acrylate ester-based pseudodendrimers for in vitro and in vivo gene transfer, Gene Ther. 15 (2008) 18–29.
[125] A. Akinc, D.G. Anderson, D.M. Lynn, R. Langer, Synthesis of poly(beta-amino ester)s optimized for highly effective gene delivery, Bioconjug. Chem. 14 (2003) 979–988.
[126] D.M. Lynn, D.G. Anderson, D. Putnam, R. Langer, Accelerated discovery of synthetic transfection vectors: parallel synthesis and screening of a degradable polymer library, J. Am. Chem. Soc. 123 (2001) 8155–8156.
[127] A. Akinc, D.M. Lynn, D.G. Anderson, R. Langer, Parallel synthesis and biophysical characterization of a degradable polymer library for gene delivery, J. Am. Chem. Soc. 125 (2003) 5316–5323.
[128] 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.
[129] D.G. Anderson, A. Akinc, N. Hossain, R. Langer, Structure/property studies of polymeric gene delivery using a library of poly(beta-amino esters), Mol. Ther. 11 (2005) 426–434.
[130] A. Akinc, M. Goldberg, J. Qin, J.R. Dorkin, C. Gamba-Vitalo, M. Maier, K.N. Jayaprakash, M. Jayaraman, K.G. Rajeev, M. Manoharan, V. Koteliansky, I. Rohl, E.S. Leshchiner, R. Langer, D.G. Anderson, Development of lipidoid-siRNA formulations for systemic delivery to the liver, Mol. Ther. 17 (2009) 872–879.
[131] K.T. Love, K.P. Mahon, C.G. Levins, K.A. Whitehead, W. Querbes, J.R. Dorkin, J. Qin, W. Cantley, L.L. Qin, T. Racie, M. Frank-Kamenetsky, K.N. Yip, R. Alvarez, D.W. Sah, A. de Fougerolles, K. Fitzgerald, V. Koteliansky, A. Akinc, R. Langer, D.G. Anderson, Lipid-like materials for low-dose, in vivo gene silencing, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 1864–1869.
[132] 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, J.K. Narayanannair, M. Jayaraman, M. John, V. Koteliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K.G. Rajeev, D.W. 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.
[133] K.P. Mahon, K.T. Love, K.A. Whitehead, J. Qin, A. Akinc, E. Leshchiner, I. Leshchiner, R. Langer, D.G. Anderson, Combinatorial approach to determine functional group effects on lipidoid-mediated siRNA delivery, Bioconjug. Chem. 21 (2010) 1448–1454.
[134] D.J. Siegwart, K.A. Whitehead, L. Nuhn, G. Sahay, H. Cheng, S. Jiang, M. Ma, A. Lytton-Jean, A. Vegas, P. Fenton, C.G. Levins, K.T. Love, H. Lee, C. Cortez, S.P. Collins, Y.F. Li, J. Jang, W. Querbes, C. Zurenko, T. Novobrantseva, R. Langer, D.G. Anderson, Combinatorial synthesis of chemically diverse core-shell nanoparticles for intracellular delivery, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 12996–13001.
[135] J.F. Lutz, M. Ouchi, D.R. Liu, M. Sawamoto, Sequence-controlled polymers, Science 341 (2013) 1238149.
[136] S.C. Solleder, R.V. Schneider, K.S. Wetzel, A.C. Boukis, M.A.R. Meier, Recent progress in the design of monodisperse, sequence-defined macromolecules, macromol, Rapid Commun. 38 (2017).
[137] S. Celasun, D. Remmler, T. Schwaar, M.G. Weller, F. Du Prez, H.G. Borner, Digging into the sequential space of thiolactone precision polymers: a combinatorial strategy to identify functional domains, Angew. Chem. Int. Ed. 58 (2019) 1960–1964.
[138] M.S. Wadhwa, D.L. Knoell, A.P. Young, K.G. Rice, Targeted gene delivery with a low molecular weight glycopeptide carrier, Bioconjug. Chem. 6 (1995) 283–291.
[139] C. Plank, M.X. Tang, A.R. Wolfe, F.C. Szoka Jr., Branched cationic peptides for gene delivery: role of type and number of cationic residues in formation and in vitro activity of DNA polyplexes, Hum. Gene Ther. 10 (1999) 319–332.
[140] D.L. McKenzie, K.Y. Kwok, K.G. Rice, A potent new class of reductively activated peptide gene delivery agents, J. Biol. Chem. 275 (2000) 9970–9977.
[141] M. Stevenson, V. Ramos-Perez, S. Singh, M. Soliman, J.A. Preece, S.S. Briggs, M.L. Read, L.W. Seymour, Delivery of siRNA mediated by histidine-containing reducible polycations, J. Control. Release 130 (2008) 46–56.
[142] Q.R. Chen, L. Zhang, S.A. Stass, A.J. Mixson, Branched co-polymers of histidine and lysine are efficient carriers of plasmids, Nucleic Acids Res. 29 (2001) 1334–1340.
[143] Q. Leng, A.J. Mixson, Modified branched peptides with a histidine-rich tail enhance in vitro gene transfection, Nucleic Acids Res. 33 (2005), e40.
[144] Q.R. Chen, L. Zhang, P.W. Luther, A.J. Mixson, Optimal transfection with the HK polymer depends on its degree of branching and the pH of endocytic vesicles, Nucleic Acids Res. 30 (2002) 1338–1345.
[145] Q. Leng, P. Scaria, J. Zhu, N. Ambulos, P. Campbell, A.J. Mixson, Highly branched HK peptides are effective carriers of siRNA, J. Gene Med. 7 (2005) 977–986.
[146] Q. Leng, S.T. Chou, P.V. Scaria, M.C. Woodle, A.J. Mixson, Increased tumor distribution and expression of histidine-rich plasmid polyplexes, J. Gene Med. 16 (2014) 317–328.
[147] Q. Leng, A.J. Mixson, The neuropilin-1 receptor mediates enhanced tumor delivery of H2K polyplexes, J. Gene Med. 18 (2016) 134–144.
[148] T.B. Wyman, F. Nicol, O. Zelphati, P.V. Scaria, C. Plank, F.C. Szoka Jr., Design, synthesis, and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers, Biochemistry 36 (1997) 3008–3017.
[149] K. Ezzat, S.E. Andaloussi, E.M. Zaghloul, T. Lehto, S. Lindberg, P.M. Moreno, J.R. Viola, T. Magdy, R. Abdo, P. Guterstam, R. Sillard, S.M. Hammond, M.J. Wood, A.A. Arzumanov, M.J. Gait, C.I. Smith, M. Hallbrink, U. Langel, PepFect 14, a novel cell-penetrating peptide for oligonucleotide delivery in solution and as solid formulation, Nucleic Acids Res. 39 (2011) 5284–5298.
[150] X.L. Wang, R. Jensen, Z.R. Lu, A novel environment-sensitive biodegradable polydisulfide with protonatable pendants for nucleic acid delivery, J. Control. Release 120 (2007) 250–258.
[151] X.L. Wang, S. Ramusovic, T. Nguyen, Z.R. Lu, Novel polymerizable surfactants with pH-sensitive amphiphilicity and cell membrane disruption for efficient siRNA delivery, Bioconjug. Chem. 18 (2007) 2169–2177.
[152] A.S. Malamas, M. Gujrati, C.M. Kummitha, R. Xu, Z.R. Lu, Design and evaluation of new pH-sensitive amphiphilic cationic lipids for siRNA delivery, J. Control. Release 171 (2013) 296–307.
[153] T. Lehto, R. Abes, N. Oskolkov, J. Suhorutsenko, D.M. Copolovici, I. Mager, J.R. Viola, O.E. Simonson, K. Ezzat, P. Guterstam, E. Eriste, C.I. Smith, B. Lebleu, A. El Samir, U. Langel, Delivery of nucleic acids with a stearylated (RxR)4 peptide using a non-covalent co-incubation strategy, J. Control. Release 141 (2010) 42–51.
[154] S.E. Andaloussi, T. Lehto, I. Mager, K. Rosenthal-Aizman, I.I. Oprea, O.E. Simonson, H. Sork, K. Ezzat, D.M. Copolovici, K. Kurrikoff, J.R. Viola, E.M. Zaghloul, R. Sillard, H.J. Johansson, F. Said Hassane, P. Guterstam, J. Suhorutsenko, P.M. Moreno, N. Oskolkov, J. Halldin, U. Tedebark, A. Metspalu, B. Lebleu, J. Lehtio, C.I. Smith, U. Langel, Design of a peptide-based vector, PepFect6, for efficient delivery of siRNA in cell culture and systemically in vivo, Nucleic Acids Res. 39 (2011) 3972–3987.
[155] Z.P. Gates, A.A. Vinogradov, A.J. Quartararo, A. Bandyopadhyay, Z.N. Choo, E.D. Evans, K.H. Halloran, A.J. Mijalis, S.K. Mong, M.D. Simon, E.A. Standley, E.D. Styduhar, S.Z. Tasker, F. Touti, J.M. Weber, J.L. Wilson, T.F. Jamison, B.L. Pentelute, Xenoprotein engineering via synthetic libraries, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) E5298–e5306.
[156] F. Touti, Z.P. Gates, A. Bandyopadhyay, G. Lautrette, B.L. Pentelute, In-solution enrichment identifies peptide inhibitors of protein-protein interactions, Nat. Chem. Biol. 15 (2019) 410–418.
[157] B.L. Pentelute, L. Wang, Editorial overview: chemistry for biopolymers to investigate and even move beyond nature, Curr. Opin. Chem. Biol. 34 (2016) v–vi.
[158] A.A. Vinogradov, Z.P. Gates, C. Zhang, A.J. Quartararo, K.H. Halloran, B.L. Pentelute, Library design-facilitated high-throughput sequencing of synthetic peptide libraries, ACS Comb. Sci. 19 (2017) 694–701.
[159] C.M. Fadzen, J.M. Wolfe, C.F. Cho, E.A. Chiocca, S.E. Lawler, B.L. Pentelute, Perfluoroarene-based peptide macrocycles to enhance penetration across the blood-brain barrier, J. Am. Chem. Soc. 139 (2017) 15628–15631.
[160] J.M. Wolfe, C.M. Fadzen, R.L. Holden, M. Yao, G.J. Hanson, B.L. Pentelute, Perfluoroaryl bicyclic cell-penetrating peptides for delivery of antisense oligonucleotides, Angew. Chem. Int. Ed. 57 (2018) 4756–4759.
[161] J.M. Wolfe, C.M. Fadzen, Z.N. Choo, R.L. Holden, M. Yao, G.J. Hanson, B.L. Pentelute, Machine learning to predict cell-penetrating peptides for antisense delivery, ACS Cent. Sci. 4 (2018) 512–520.
[162] L. Hartmann, E. Krause, M. Antonietti, H.G. Borner, Solid-phase supported polymer synthesis of sequence-defined, multifunctional poly(amidoamines), Biomacromolecules 7 (2006) 1239–1244.
[163] L. Hartmann, S. Häfele, R. Peschka-Süss, M. Antonietti, H.G. Börner, Sequence positioning of disulfide linkages to program the degradation of monodisperse poly (amidoamines), Macromolecules 40 (2007) 7771–7776.
[164] L. Hartmann, S. Hafele, R. Peschka-Suss, M. Antonietti, H.G. Borner, Tailor-made poly(amidoamine)s for controlled complexation and condensation of DNA, Chem. 14 (2008) 2025–2033.
[165] L. Hartmann, H.G. Börner, Precision polymers: monodisperse, monomer-sequence-defined segments to target future demands of polymers in medicine, Adv. Mater. 21 (2009) 3425–3431.
[166] L. Hartmann, Polymers for control freaks: sequence-defined poly(amidoamine)s and their biomedical applications, Macromol. Chem. Phys. 212 (2011) 8–13.
[167] S.A. Hill, C. Gerke, L. Hartmann, Recent developments in solid-phase strategies towards synthetic, sequence-defined macromolecules, Chem. Asian J. 13 (2018) 3611–3622.
[168] D. Schaffert, N. Badgujar, E. Wagner, Novel Fmoc-polyamino acids for solid-phase synthesis of defined polyamidoamines, Org. Lett. 13 (2011) 1586–1589.
[169] D. Schaffert, C. Troiber, E.E. Salcher, T. Fröhlich, I. Martin, N. Badgujar, C. Dohmen, D. Edinger, R. Kläger, G. Maiwald, K. Farkasova, S. Seeber, K. Jahn-Hofmann, P. Hadwiger, E. Wagner, Solid-phase synthesis of sequence-defined T-, i-, and U-shape polymers for pDNA and siRNA delivery, Angew. Chem. Int. Ed. 50 (2011) 8986–8989.
[170] E.E. Salcher, P. Kos, T. Fröhlich, N. Badgujar, M. Scheible, E. Wagner, Sequence-defined four-arm oligo(ethanamino)amides for pDNA and siRNA delivery: Impact of building blocks on efficacy, J. Control. Release 164 (2012) 380–386.
[171] C. Scholz, P. Kos, L. Leclercq, X. Jin, H. Cottet, E. Wagner, Correlation of length of linear oligo(ethanamino) amides with gene transfer and cytotoxicity, ChemMedChem 9 (2014) 2104–2110.
[172] T. Fröhlich, D. Edinger, R. Klager, C. Troiber, E. Salcher, N. Badgujar, I. Martin, D. Schaffert, A. Cengizeroglu, P. Hadwiger, H.P. Vornlocher, E. Wagner, Structure-activity relationships of siRNA carriers based on sequence-defined oligo (ethane amino) amides, J. Control. Release 160 (2012) 532–541.
[173] U. Lächelt, P. Kos, F.M. Mickler, A. Herrmann, E.E. Salcher, W. Rodl, N. Badgujar, C. Brauchle, E. Wagner, Fine-tuning of proton sponges by precise diaminoethanes and histidines in pDNA polyplexes, Nanomedicine 10 (2014) 35–44.
[174] C. Scholz, P. Kos, E. Wagner, Comb-like oligoaminoethane carriers: change in topology improves pDNA delivery, Bioconjug. Chem. 25 (2014) 251–261.
[175] D. Schaffert, C. Troiber, E. Wagner, New sequence-defined polyaminoamides with tailored endosomolytic properties for plasmid DNA delivery, Bioconjug. Chem. 23 (2012) 1157–1165.

[176] I. Martin, C. Dohmen, C. Mas-Moruno, C. Troiber, P. Kos, D. Schaffert, U. Lächelt, M. Teixido, M. Günther, H. Kessler, E. Giralt, E. Wagner, Solid-phase-assisted synthesis of targeting peptide-PEG-oligo(ethane amino)amides for receptor-mediated gene delivery, Org. Biomol. Chem. 10 (2012) 3258–3268.
[177] D. He, K. Müller, A. Krhac Levacic, P. Kos, U. Lächelt, E. Wagner, Combinatorial optimization of sequence-defined oligo(ethanamino)amides for folate receptor-targeted pDNA and siRNA delivery, Bioconjug. Chem. 27 (2016) 647–659.
[178] P. Kos, U. Lächelt, A. Herrmann, F.M. Mickler, M. Döblinger, D. He, A. Krhac Levacic, S. Morys, C. Bräuchle, E. Wagner, Histidine-rich stabilized polyplexes for cMet-directed tumor-targeted gene transfer, Nanoscale 7 (2015) 5350–5362.
[179] S. Urnauer, S. Morys, A. Krhac Levacic, A.M. Müller, C. Schug, K.A. Schmohl, N. Schwenk, C. Zach, J. Carlsen, P. Bartenstein, E. Wagner, C. Spitzweg, Sequence-defined cMET/HGFR-targeted polymers as gene delivery vehicles for the theranostic sodium iodide symporter (NIS) gene, Mol. Ther. 24 (2016) 1395–1404.
[180] P. Kos, U. Lächelt, D. He, Y. Nie, Z. Gu, E. Wagner, Dual-targeted polyplexes based on sequence-defined peptide-PEG-oligoamino amides, J. Pharm. Sci. 104 (2015) 464–475.
[181] S. Wang, S. Reinhard, C. Li, M. Qian, H. Jiang, Y. Du, U. Lächelt, W. Lu, E. Wagner, R. Huang, Antitumoral cascade-targeting ligand for IL-6 receptor-mediated gene delivery to glioma, Mol. Ther. 25 (2017) 1556–1566.
[182] 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.
[183] D. Schaffert, M. Kiss, W. Rodl, A. Shir, A. Levitzki, M. Ogris, E. Wagner, Poly(I:C)-mediated tumor growth suppression in EGF-receptor overexpressing tumors using EGF-polyethylene glycol-linear polyethylenimine as carrier, Pharm. Res. 28 (2011) 731–741.
[184] S. Morys, A. Krhac Levacic, S. Urnauer, S. Kempter, S. Kern, J.O. Radler, C. Spitzweg, U. Lächelt, E. Wagner, Influence of defined hydrophilic blocks within oligoaminoamide copolymers: compaction versus shielding of pDNA nanoparticles, Polymers 9 (2017).
[185] J.M. Williford, M.M. Archang, I. Minn, Y. Ren, M. Wo, J. Vandermark, P.B. Fisher, M.G. Pomper, H.Q. Mao, Critical length of PEG grafts on lPEI/DNA nanoparticles for efficient in vivo delivery, ACS Biomater. Sci. Eng. 2 (2016) 567–578.
[186] J.F. Stefanick, J.D. Ashley, T. Kiziltepe, B. Bilgicer, A systematic analysis of peptide linker length and liposomal polyethylene glycol coating on cellular uptake of peptide-targeted liposomes, ACS Nano 7 (2013) 2935–2947.
[187] A.K. Levacic, S. Morys, S. Kempter, U. Lächelt, E. Wagner, Minicircle versus plasmid DNA delivery by receptor-targeted polyplexes, Hum. Gene Ther. 28 (2017) 862–874.
[188] M. Ogris, G. Walker, T. Blessing, R. Kircheis, M. Wolschek, E. Wagner, Tumor-targeted gene therapy: strategies for the preparation of ligand-polyethylene glycol-polyethylenimine/DNA complexes, J. Control. Release 91 (2003) 173–181.
[189] 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.
[190] S. Morys, S. Urnauer, C. Spitzweg, E. Wagner, EGFR targeting and shielding of pDNA lipopolyplexes via bivalent attachment of a sequence-defined PEG agent, Macromol. Biosci. 18 (2018) 1700203.
[191] I. Truebenbach, J. Gorges, J. Kuhn, S. Kern, E. Baratti, U. Kazmaier, E. Wagner, U. Lächelt, Sequence-defined oligoamide drug conjugates of pretubulysin and methotrexate for folate receptor targeted cancer therapy, Macromol. Biosci. 17 (2017) 1600520.
[192] I. Truebenbach, S. Kern, D.M. Loy, M. Höhn, J. Gorges, U. Kazmaier, E. Wagner, Combination chemotherapy of L1210 tumors in mice with pretubulysin and methotrexate lipo-oligomer nanoparticles, Mol. Pharm. 16 (2019) 2405–2417.
[193] J. Kuhn, P.M. Klein, N. Al Danaf, J.Z. Nordin, S. Reinhard, D.M. Loy, M. Höhn, S. El Andaloussi, D.C. Lamb, E. Wagner, Y. Aoki, T. Lehto, U. Lächelt, Supramolecular assembly of aminoethylene-lipopeptide PMO conjugates into RNA splice-switching nanomicelles, Adv. Funct. Mater. 29 (2019) 1906432.
[194] J. Kuhn, Y. Lin, A. Krhac Levacic, N. Al Danaf, L. Peng, M. Hohn, D.C. Lamb, E. Wagner, U. Lächelt, Delivery of Cas9/sgRNA ribonucleoprotein complexes via hydroxystearyl oligoamino amides, Bioconjug. Chem. 31 (2020) 729–742.
[195] J. Niu, R. Hili, D.R. Liu, Enzyme-free translation of DNA into sequence-defined synthetic polymers structurally unrelated to nucleic acids, Nat. Chem. 5 (2013) 282–292.
[196] Z. Chen, P.A. Lichtor, A.P. Berliner, J.C. Chen, D.R. Liu, Evolution of sequence-defined highly functionalized nucleic acid polymers, Nat. Chem. 10 (2018) 420–427.
[197] Y. Li, R. De Luca, S. Cazzamalli, F. Pretto, D. Bajic, J. Scheuermann, D. Neri, Versatile protein recognition by the encoded display of multiple chemical elements on a constant macrocyclic scaffold, Nat. Chem. 10 (2018) 441–448.
[198] D. Neri, R.A. Lerner, DNA-encoded chemical libraries: a selection system based on endowing organic compounds with amplifiable information, Annu. Rev. Biochem. 87 (2018) 479–502.
[199] J.E. Dahlman, K.J. Kauffman, Y. Xing, T.E. Shaw, F.F. Mir, C.C. Dlott, R. Langer, D.G. Anderson, E.T. Wang, Barcoded nanoparticles for high throughput in vivo discovery of targeted therapeutics, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 2060–2065.
[200] M.P. Lokugamage, C.D. Sago, J.E. Dahlman, Testing thousands of nanoparticles in vivo using DNA barcodes, Curr. Opin. Biomed. Eng. 7 (2018) 1–8.
[201] C.D. Sago, M.P. Lokugamage, G.N. Lando, N. Djeddar, N.N. Shah, C. Syed, A.V. Bryksin, J.E. Dahlman, Modifying a commonly expressed endocytic receptor retargets nanoparticles in vivo, Nano Lett. 18 (2018) 7590–7600.
[202] C.D. Sago, M.P. Lokugamage, K. Paunovska, D.A. Vanover, C.M. Monaco, N.N. Shah, M. Gamboa Castro, S.E. Anderson, T.G. Rudoltz, G.N. Lando, P. Munnilal Tiwari, J.L. Kirschman, N. Willett, Y.C. Jang, P.J. Santangelo, A.V. Bryksin, J.E. Dahlman, High-throughput in vivo screen of functional mRNA delivery identifies nanoparticles for endothelial cell gene editing, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) E9944–e9952.
[203] C.D. Sago, M.P. Lokugamage, F.Z. Islam, B.R. Krupczak, M. Sato, J.E. Dahlman, Nanoparticles that deliver RNA to bone marrow identified by in vivo directed evolution, J. Am. Chem. Soc. 140 (2018) 17095–17105.
[204] K. Paunovska, A.J. Da Silva Sanchez, C.D. Sago, Z. Gan, M.P. Lokugamage, F.Z. Islam, S. Kalathoor, B.R. Krupczak, J.E. Dahlman, Nanoparticles containing oxidized cholesterol deliver mRNA to the liver microenvironment at clinically relevant doses, Adv. Mater. 31 (2019), e1807748.
[205] M.P. Lokugamage, C.D. Sago, Z. Gan, B.R. Krupczak, J.E. Dahlman, Constrained nanoparticles deliver siRNA and sgRNA to T cells in vivo without targeting ligands, Adv. Mater. 31 (2019), e1902251.
[206] K. Paunovska, D. Loughrey, C.D. Sago, R. Langer, J.E. Dahlman, Using large datasets to understand nanotechnology, Adv. Mater. 31 (2019), e1902798.
[207] M. Meyer, C. Dohmen, A. Philipp, D. Kiener, G. Maiwald, C. Scheu, M. Ogris, E. Wagner, Synthesis and biological evaluation of a bioresponsive and endosomolytic siRNA−polymer conjugate, Mol. Pharm. 6 (2009) 752–762.
[208] D.B. Rozema, D.L. Lewis, D.H. Wakefield, S.C. Wong, J.J. Klein, P.L. Roesch, S.L. Bertin, T.W. Reppen, Q. Chu, A.V. Blokhin, J.E. Hagstrom, J.A. Wolff, Dynamic polyconjugates for targeted in vivo delivery of siRNA to hepatocytes, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 12982–12987.
[209] R.K. Thapa, M.O. Sullivan, Gene delivery by peptide-assisted transport, Curr. Opin. Biomed. Eng. 7 (2018) 71–82.
[210] M.S. Draz, B.A. Fang, P. Zhang, Z. Hu, S. Gu, K.C. Weng, J.W. Gray, F.F. Chen, Nanoparticle-mediated systemic delivery of siRNA for treatment of cancers and viral infections, Theranostics 4 (2014) 872–892.
[211] H. Maeda, The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting, Adv. Enzym. Regul. 41 (2001) 189–207.
[212] S. Sindhwani, A.M. Syed, J. Ngai, B.R. Kingston, L. Maiorino, J. Rothschild, P. MacMillan, Y. Zhang, N.U. Rajesh, T. Hoang, J.L.Y. Wu, S. Wilhelm, A. Zilman, S. Gadde, A. Sulaiman, B. Ouyang, Z. Lin, L. Wang, M. Egeblad, W.C.W. Chan, The entry of nanoparticles into solid tumours, Nat. Mater. (2020) https://doi.org/10.1038/s41563-019-0566-2.
[213] S. Brunner, E. Furtbauer, T. Sauer, M. Kursa, E. Wagner, Overcoming the nuclear barrier: cell cycle independent nonviral gene transfer with linear polyethylenimine or electroporation, Mol. Ther. 5 (2002) 80–86.
[214] E. Wagner, R. Kircheis, G.F. Walker, Targeted nucleic acid delivery into tumors: new avenues for cancer therapy, Biomed. Pharmacother. 58 (2004) 152–161.
[215] K. Klutz, D. Schaffert, M.J. Willhauck, G.K. Grunwald, R. Haase, N. Wunderlich, C. Zach, F.J. Gildehaus, R. Senekowitsch-Schmidtke, B. Goke, E. Wagner, M. Ogris, C. Spitzweg, Epidermal growth factor receptor-targeted (131)I-therapy of liver cancer following systemic delivery of the sodium iodide symporter gene, Mol. Ther. 19 (2011) 676–685.
[216] J. DeRouchey, G.F. Walker, E. Wagner, J.O. Radler, Decorated rods: a "bottom-up" self-assembly of monomolecular DNA complexes, J. Phys. Chem. B 110 (2006) 4548–4554.
[217] J. DeRouchey, C. Schmidt, G.F. Walker, C. Koch, C. Plank, E. Wagner, J.O. Radler, Monomolecular assembly of siRNA and poly(ethylene glycol)-peptide copolymers, Biomacromolecules 9 (2008) 724–732.
[218] Z. Ge, Q. Chen, K. Osada, X. Liu, T.A. Tockary, S. Uchida, A. Dirisala, T. Ishii, T. Nomoto, K. Toh, Y. Matsumoto, M. Oba, M.R. Kano, K. Itaka, K. Kataoka, Targeted gene delivery by polyplex micelles with crowded PEG palisade and cRGD moiety for systemic treatment of pancreatic tumors, Biomaterials 35 (2014) 3416–3426.
[219] N.P. Gabrielson, H. Lu, L. Yin, D. Li, F. Wang, J. Cheng, Reactive and bioactive cationic alpha-helical polypeptide template for nonviral gene delivery, Angew. Chem. Int. Ed. 51 (2012) 1143–1147.
[220] H.X. Wang, Z. Song, Y.H. Lao, X. Xu, J. Gong, D. Cheng, S. Chakraborty, J.S. Park, M. Li, D. Huang, L. Yin, J. Cheng, K.W. Leong, Nonviral gene editing via CRISPR/Cas9 delivery by membrane-disruptive and endosomolytic helical polypeptide, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) 4903–4908.
[221] D.R. Wilson, Y. Rui, K. Siddiq, D. Routkevitch, J.J. Green, Differentially branched ester amine quadpolymers with amphiphilic and PH-sensitive properties for efficient plasmid DNA delivery, Mol. Pharm. 16 (2019) 655–668.
[222] D. Sun, B. Sahu, S. Gao, R.M. Schur, A.M. Vaidya, A. Maeda, K. Palczewski, Z.R. Lu, Targeted multifunctional lipid ECO plasmid DNA nanoparticles as efficient non-viral gene therapy for leber's congenital amaurosis, Mol. Ther. Nucleic Acids 7 (2017) 42–52.
[223] D. Sun, R.M. Schur, A.E. Sears, S.Q. Gao, A. Vaidya, W. Sun, A. Maeda, T. Kern, K. Palczewski, Z.R. Lu, Non-viral gene therapy for stargardt disease with ECO/pRHO-ABCA4 self-assembled nanoparticles, Mol. Ther. 28 (2020) 293–303.
[224] Y. Dong, D.J. Siegwart, D.G. Anderson, Strategies, design, and chemistry in siRNA delivery systems, Adv. Drug Deliv. Rev. 144 (2019) 133–147.
[225] E. Wagner, Polymers for siRNA delivery: inspired by viruses to be targeted, dynamic, and precise, Acc. Chem. Res. 45 (2012) 1005–1013.
[226] E. Wagner, Biomaterials in RNAi therapeutics: quo vadis? Biomater. Sci. 1 (2013) 804–809.
[227] S.C. Semple, A. Akinc, J. Chen, A.P. Sandhu, B.L. Mui, C.K. Cho, D.W.Y. Sah, D. Stebbing, E.J. Crosley, E. Yaworski, I.M. Hafez, J.R. Dorkin, J. Qin, K. Lam, K.G. Rajeev, K.F. Wong, L.B. Jeffs, L. Nechev, M.L. Eisenhardt, M. Jayaraman, M. Kazem, M.A. Maier, M. Srinivasulu, M.J. Weinstein, Q. Chen, R. Alvarez, S.A. Barros, S. De, S.K. Klimuk, T. Borland, V. Kosovrasti, W.L. Cantley, Y.K. Tam, M. Manoharan, M.A. Ciufolini, M.A. Tracy, A. de Fougerolles, I. MacLachlan, P.R. Cullis, T.D. Madden, M.J. Hope, Rational design of cationic lipids for siRNA delivery, Nat. Biotechnol. 28 (2010) 172–176.

[228] A. Akinc, W. Querbes, S. De, J. Qin, M. Frank-Kamenetsky, K.N. Jayaprakash, M. Jayaraman, K.G. Rajeev, W.L. Cantley, J.R. Dorkin, J.S. Butler, L. Qin, T. Racie, A. Sprague, E. Fava, A. Zeigerer, M.J. Hope, M. Zerial, D.W. Sah, K. Fitzgerald, M.A. Tracy, M. Manoharan, V. Koteliansky, A. Fougerolles, M.A. Maier, Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms, Mol. Ther. 18 (2010) 1357–1364.

[229] J. Soutschek, A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R.K. Pandey, T. Racie, K.G. Rajeev, I. Röhl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan, H.P. Vornlocher, Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs, Nature 432 (2004) 173–178.

[230] P.R. de Paula Brandao, 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. Diagn. Ther. 24 (2020) 61–68.

[231] I.A. Khalil, Y. Yamada, H. Harashima, Optimization of siRNA delivery to target sites: issues and future directions, Expert Opin. Drug Deliv. 15 (2018) 1053–1065.

[232] Y. Sato, H. Matsui, R. Sato, H. Harashima, Neutralization of negative charges of siRNA results in improved safety and efficient gene silencing activity of lipid nanoparticles loaded with high levels of siRNA, J. Control. Release 284 (2018) 179–187.

[233] Y. Sato, K. Hashiba, K. Sasaki, M. Maeki, M. Tokeshi, H. Harashima, Understanding structure-activity relationships of pH-sensitive cationic lipids facilitates the rational identification of promising lipid nanoparticles for delivering siRNAs in vivo, J. Control. Release 295 (2019) 140–152.

[234] Y. Sakurai, T. Hada, A. Kato, Y. Hagino, W. Mizumura, H. Harashima, Effective therapy using a liposomal siRNA that targets the tumor vasculature in a model murine breast cancer with lung metastasis, Mol. Ther. Oncolytics 11 (2018) 102–108.

[235] X.L. Wang, R. Xu, X. Wu, D. Gillespie, R. Jensen, Z.R. Lu, Targeted systemic delivery of a therapeutic siRNA with a multifunctional carrier controls tumor proliferation in mice, Mol. Pharm. 6 (2009) 738–746.

[236] X.-L. Wang, R. Xu, Z.-R. Lu, A peptide-targeted delivery system with pH-sensitive amphiphilic cell membrane disruption for efficient receptor-mediated siRNA delivery, J. Control. Release 134 (2009) 207–213.

[237] J.G. Parvani, M.D. Gujrati, M.A. Mack, W.P. Schiemann, Z.R. Lu, Silencing beta3 integrin by targeted ECO/siRNA nanoparticles inhibits EMT and metastasis of triple-negative breast cancer, Cancer Res. 75 (2015) 2316–2325.

[238] A.M. Vaidya, Z. Sun, N. Ayat, A. Schilb, X. Liu, H. Jiang, D. Sun, J. Scheidt, V. Qian, S. He, H. Gilmore, W.P. Schiemann, Z.R. Lu, Systemic delivery of tumor-targeting siRNA nanoparticles against an oncogenic LncRNA facilitates effective triple-negative breast cancer therapy, Bioconjug. Chem. 30 (2019) 907–919.

[239] 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.

[240] D.J. Lee, E. Kessel, D. Edinger, D. He, P.M. Klein, L. Voith von Voithenberg, D.C. Lamb, U. Lächelt, T. Lehto, E. Wagner, Dual antitumoral potency of EG5 siRNA nanoplexes armed with cytotoxic bifunctional glutamyl-methotrexate targeting ligand, Biomaterials 77 (2016) 98–110.

[241] 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.

[242] P.M. Klein, S. Reinhard, D.J. Lee, K. Müller, D. Ponader, L. Hartmann, E. Wagner, Precise redox-sensitive cleavage sites for improved bioactivity of siRNA lipopolyplexes, Nanoscale 8 (2016) 18098–18104.

[243] D.J. Lee, D. He, E. Kessel, K. Padari, S. Kempter, U. Lächelt, J.O. Radler, M. Pooga, E. Wagner, Tumoral gene silencing by receptor-targeted combinatorial siRNA polyplexes, J. Control. Release 244 (2016) 280–291.

[244] D.J. Lee, E. Kessel, T. Lehto, X. Liu, N. Yoshinaga, K. Padari, Y.C. Chen, S. Kempter, S. Uchida, J.O. Radler, M. Pooga, M.T. Sheu, K. Kataoka, E. Wagner, Systemic delivery of folate-PEG siRNA lipopolyplexes with enhanced intracellular stability for in vivo gene silencing in leukemia, Bioconjug. Chem. 28 (2017) 2393–2409.

[245] K. Müller, P.M. Klein, P. Heissig, A. Roidl, E. Wagner, EGF receptor targeted lipo-oligocation polyplexes for antitumoral siRNA and miRNA delivery, Nanotechnology 27 (2016) 464001.

[246] K. Müller, E. Kessel, P.M. Klein, M. Hohn, E. Wagner, Post-PEGylation of siRNA lipo-oligoamino amide polyplexes using tetra-glutamylated folic acid as ligand for receptor-targeted delivery, Mol. Pharm. 13 (2016) 2332–2345.

[247] W. Zhang, K. Müller, E. Kessel, S. Reinhard, D. He, P.M. Klein, M. Höhn, W. Rödl, S. Kempter, E. Wagner, Targeted siRNA delivery using a lipo-oligoaminoamide nanocore with an influenza peptide and transferrin shell, Adv. Healthcare Mater. 5 (2016) 1493–1504.

[248] P.M. Klein, S. Kern, D.J. Lee, J. Schmaus, M. Höhn, J. Gorges, U. Kazmaier, E. Wagner, Folate receptor-directed orthogonal click-functionalization of siRNA lipopolyplexes for tumor cell killing in vivo, Biomaterials 178 (2018) 630–642.

[249] B. Steinborn, I. Truebenbach, S. Morys, U. Lächelt, E. Wagner, W. Zhang, Epidermal growth factor receptor targeted methotrexate and small interfering RNA co-delivery, J. Gene Med. 20 (2018), e3041.

[250] I. Truebenbach, W. Zhang, Y. Wang, S. Kern, M. Höhn, S. Reinhard, J. Gorges, U. Kazmaier, E. Wagner, Co-delivery of pretubulysin and siEG5 to EGFR overexpressing carcinoma cells, Int. J. Pharm. 569 (2019) 118570.

[251] J. Luo, M. Höhn, S. Reinhard, D.M. Loy, P.M. Klein, E. Wagner, IL4-receptor-targeted dual antitumoral apoptotic peptide—siRNA conjugate lipoplexes, Adv. Funct. Mater. 29 (2019) 1900697.

[252] J. Luo, E. Wagner, Y. Wang, Artificial peptides for antitumoral siRNA delivery, J. Mater. Chem. B 8 (2020) 2020–2031.

[253] A. Zintchenko, A. Philipp, A. Dehshahri, E. Wagner, Simple modifications of branched PEI lead to highly efficient siRNA carriers with low toxicity, Bioconjug. Chem. 19 (2008) 1448–1455.

[254] Y. Wang, J. Luo, I. Truebenbach, S. Reinhard, P.M. Klein, M. Höhn, S. Kern, S. Morys, D.M. Loy, E. Wagner, W. Zhang, Double click-functionalized siRNA polyplexes for gene silencing in epidermal growth factor receptor-positive tumor cells, ACS Biomater. Sci. Eng. 6 (2) (2020) 1074–1089.

[255] Y. Yan, H. Xiong, X. Zhang, Q. Cheng, D.J. Siegwart, Systemic mRNA delivery to the lungs by functional polyester-based carriers, Biomacromolecules 18 (2017) 4307–4315.

[256] J.C. Kaczmarek, K.J. Kauffman, O.S. Fenton, K. Sadtler, A.K. Patel, M.W. Heartlein, F. DeRosa, D.G. Anderson, Optimization of a degradable polymer-lipid nanoparticle for potent systemic delivery of mRNA to the lung endothelium and immune cells, Nano Lett. 18 (2018) 6449–6454.

[257] L. Nuhn, L. Kaps, M. Diken, D. Schuppan, R. Zentel, Reductive decationizable block copolymers for stimuli-responsive mRNA delivery, Macromol. Rapid Commun. 37 (2016) 924–933.

[258] A. Jarzebinska, T. Pasewald, J. Lambrecht, O. Mykhaylyk, L. Kummerling, P. Beck, G. Hasenpusch, C. Rudolph, C. Plank, C. Dohmen, A single methylene group in oligoalkylamine-based cationic polymers and lipids promotes enhanced mRNA delivery, Angew. Chem. Int. Ed. 55 (2016) 9591–9595.

[259] J.C. Kaczmarek, A.K. Patel, K.J. Kauffman, O.S. Fenton, M.J. Webber, M.W. Heartlein, F. DeRosa, D.G. Anderson, Polymer-lipid nanoparticles for systemic delivery of mRNA to the lungs, Angew. Chem. Int. Ed. 55 (2016) 13808–13812.

[260] P.S. Kowalski, U. Capasso Palmiero, Y. Huang, A. Rudra, R. Langer, D.G. Anderson, Ionizable amino-polyesters synthesized via ring opening polymerization of tertiary amino-alcohols for tissue selective mRNA delivery, Adv. Mater. 5 (2018)e1801151.

[261] J. Gilleron, W. Querbes, A. Zeigerer, A. Borodovsky, G. Marsico, U. Schubert, K. Manygoats, S. Seifert, C. Andree, M. Stoter, H. Epstein-Barash, L. Zhang, V. Koteliansky, K. Fitzgerald, E. Fava, M. Bickle, Y. Kalaidzidis, A. Akinc, M. Maier, M. Zerial, Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape, Nat. Biotechnol. 31 (2013) 638–646.

[262] A. Wittrup, A. Ai, X. Liu, P. Hamar, R. Trifonova, K. Charisse, M. Manoharan, T. Kirchhausen, J. Lieberman, Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown, Nat. Biotechnol. 33 (2015) 870–876.

[263] E.J. Sayers, S.E. Peel, A. Schantz, R.M. England, M. Beano, S.M. Bates, A.S. Desai, S. Puri, M.B. Ashford, A.T. Jones, Endocytic profiling of cancer cell models reveals critical factors influencing LNP-mediated mRNA delivery and protein expression, Mol. Ther. 27 (2019) 1950–1962.

[264] Q. Chen, R. Qi, X. Chen, X. Yang, S. Wu, H. Xiao, W. Dong, A targeted and stable polymeric nanoformulation enhances systemic delivery of mRNA to tumors, Mol. Ther. 25 (2017) 92–101.

[265] C.J. McKinlay, J.R. Vargas, T.R. Blake, J.W. Hardy, M. Kanada, C.H. Contag, P.A. Wender, R.M. Waymouth, Charge-altering releasable transporters (CARTs) for the delivery and release of mRNA in living animals, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) E448–E456.

[266] C.J. McKinlay, N.L. Benner, O.A. Haabeth, R.M. Waymouth, P.A. Wender, Enhanced mRNA delivery into lymphocytes enabled by lipid-varied libraries of charge-altering releasable transporters, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) E5859–e5866.

[267] O.A.W. Haabeth, T.R. Blake, C.J. McKinlay, A.A. Tveita, A. Sallets, R.M. Waymouth, P.A. Wender, R. Levy, Local delivery of Ox40l, Cd80, and Cd86 mRNA kindles global anticancer immunity, Cancer Res. 79 (2019) 1624–1634.

[268] H.A. Lagasse, A. Alexaki, V.L. Simhadri, N.H. Katagiri, W. Jankowski, Z.E. Sauna, C. Kimchi-Sarfaty, Recent advances in (therapeutic protein) drug development, F1000Research 6 (2017)113.

[269] X. Liu, F. Wu, Y. Ji, L. Yin, Recent advances in anti-cancer protein/peptide delivery, Bioconjug. Chem. 30 (2019) 305–324.

[270] Y.W. Lee, D.C. Luther, J.A. Kretzmann, A. Burden, T. Jeon, S. Zhai, V.M. Rotello, Protein delivery into the cell cytosol using non-viral nanocarriers, Theranostics 9 (2019) 3280–3292.

[271] K.H. Bae, M. Kurisawa, Emerging hydrogel designs for controlled protein delivery, Biomater. Sci. 4 (2016) 1184–1192.

[272] J. Dai, W. Long, Z. Liang, L. Wen, F. Yang, G. Chen, A novel vehicle for local protein delivery to the inner ear: injectable and biodegradable thermosensitive hydrogel loaded with PLGA nanoparticles, Drug Dev. Ind. Pharm. 44 (2018) 89–98.

[273] T. Jiang, S. Shen, T. Wang, M. Li, B. He, R. Mo, A substrate-selective enzyme-catalysis assembly strategy for oligopeptide hydrogel-assisted combinatorial protein delivery, Nano Lett. 17 (2017) 7447–7454.

[274] M.C. Koetting, J.F. Guido, M. Gupta, A. Zhang, N.A. Peppas, pH-responsive and enzymatically-responsive hydrogel microparticles for the oral delivery of therapeutic proteins: effects of protein size, crosslinking density, and hydrogel degradation on protein delivery, J. Control. Release 221 (2016) 18–25.

[275] A. Matsumoto, M. Tanaka, H. Matsumoto, K. Ochi, Y. Moro-Oka, H. Kuwata, H. Yamada, I. Shirakawa, T. Miyazawa, H. Ishii, K. Kataoka, Y. Ogawa, Y. Miyahara, T. Suganami, Synthetic "smart gel" provides glucose-responsive insulin delivery in diabetic mice, Sci. Adv. 3 (2017)eaaq0723.

[276] C. Yu, E. Tan, Y. Xu, J. Lv, Y. Cheng, A guanidinium-rich polymer for efficient cytosolic delivery of native proteins, Bioconjug. Chem. 30 (2019) 413–417.

[277] C. Liu, T. Wan, H. Wang, S. Zhang, Y. Ping, Y. Cheng, A boronic acid-rich dendrimer with robust and unprecedented efficiency for cytosolic protein delivery and CRISPR-Cas9 gene editing, Sci. Adv. 5 (2019)eaaw8922.

[278] S.R. Schwarze, A. Ho, A. Vocero-Akbani, S.F. Dowdy, In vivo protein transduction: delivery of a biologically active protein into the mouse, Science 285 (1999) 1569–1572.

[279] R.K. June, K. Gogoi, A. Eguchi, X.S. Cui, S.F. Dowdy, Synthesis of a pH-sensitive nitrilotriacetic linker to peptide transduction domains to enable intracellular delivery of histidine imidazole ring-containing macromolecules, J. Am. Chem. Soc. 132 (2010) 10680–10682.
[280] N. Nischan, H.D. Herce, F. Natale, N. Bohlke, N. Budisa, M.C. Cardoso, C.P. Hackenberger, Covalent attachment of cyclic TAT peptides to GFP results in protein delivery into live cells with immediate bioavailability, Angew. Chem. Int. Ed. 54 (2015) 1950–1953.
[281] A.F.L. Schneider, A.L.D. Wallabregue, L. Franz, C.P.R. Hackenberger, Targeted subcellular protein delivery using cleavable cyclic cell-penetrating peptides, Bioconjug. Chem. 30 (2019) 400–404.
[282] Y. Kawaguchi, S. Ise, Y. Azuma, T. Takeuchi, K. Kawano, T.K. Le, J. Ohkanda, S. Futaki, Dipicolylamine/metal complexes that promote direct cell-membrane penetration of octaarginine, Bioconjug. Chem. 30 (2019) 454–460.
[283] Y. Lee, S. Fukushima, Y. Bae, S. Hiki, T. Ishii, K. Kataoka, A protein nanocarrier from charge-conversion polymer in response to endosomal pH, J. Am. Chem. Soc. 129 (2007) 5362–5363.
[284] Y. Lee, T. Ishii, H. Cabral, H.J. Kim, J.H. Seo, N. Nishiyama, H. Oshima, K. Osada, K. Kataoka, Charge-conversional polyionic complex micelles-efficient nanocarriers for protein delivery into cytoplasm, Angew. Chem. Int. Ed. 48 (2009) 5309–5312.
[285] A. Tao, G.L. Huang, K. Igarashi, T. Hong, S. Liao, F. Stellacci, Y. Matsumoto, T. Yamasoba, K. Kataoka, H. Cabral, Polymeric micelles loading proteins through concurrent ion complexation and pH-cleavable covalent bonding for in vivo delivery, Macromol. Biosci. 20 (2019)e1900161.
[286] K. Maier, E. Wagner, Acid-labile traceless click linker for protein transduction, J. Am. Chem. Soc. 134 (2012) 10169–10173.
[287] X. Liu, P. Zhang, D. He, W. Rödl, T. Preiss, J.O. Rädler, E. Wagner, U. Lächelt, pH-reversible cationic RNase A conjugates for enhanced cellular delivery and tumor cell killing, Biomacromolecules 17 (2016) 173–182.
[288] K. Maier, I. Martin, E. Wagner, Sequence defined disulfide-linked shuttle for strongly enhanced intracellular protein delivery, Mol. Pharm. 9 (2012) 3560–3568.
[289] P. Zhang, D. He, P.M. Klein, X. Liu, R. Röder, M. Döblinger, E. Wagner, Enhanced intracellular protein transduction by sequence defined tetra-oleoyl oligoaminoamides targeted for cancer therapy, Adv. Funct. Mater. 25 (2015) 6627–6636.
[290] P. Zhang, B. Steinborn, U. Lächelt, S. Zahler, E. Wagner, Lipo-oligomer nanoformulations for targeted intracellular protein delivery, Biomacromolecules 18 (2017) 2509–2520.
[291] R. Röder, J. Helma, T. Preiss, J.O. Rädler, H. Leonhardt, E. Wagner, Intracellular delivery of nanobodies for imaging of target proteins in live cells, Pharm. Res. 34 (2017) 161–174.
[292] X. Liu, P. Zhang, W. Rödl, K. Maier, U. Lächelt, E. Wagner, Toward artificial immunotoxins: traceless reversible conjugation of RNase A with receptor targeting and endosomal escape domains, Mol. Pharm. 14 (2017) 1439–1449.
[293] R. Röder, T. Preiss, P. Hirschle, B. Steinborn, A. Zimpel, M. Höhn, J.O. Rädler, T. Bein, E. Wagner, S. Wuttke, U. Lächelt, Multifunctional nanoparticles by coordinative self-assembly of His-tagged units with metal-organic frameworks, J. Am. Chem. Soc. 139 (2017) 2359–2368.
[294] J.A. Doudna, E. Charpentier, Genome editing. The new frontier of genome engineering with CRISPR-Cas9, Science 346 (2014)1258096.
[295] M. Nishiga, L.S. Qi, J.C. Wu, Therapeutic genome editing in cardiovascular diseases, Adv Drug Deliv Rev (2020 Feb 21) https://doi.org/10.1016/j.addr.2020.02.003.
[296] A. Zarei, V. Razban, S.E. Hosseini, S.M.B. Tabei, Creating cell and animal models of human disease by genome editing using CRISPR/Cas9, J. Gene Med. 21 (2019), e3082.
[297] X. Xu, T. Wan, H. Xin, J. Wu, Y. Ping, Delivery of CRISPR/Cas9 for therapeutic genome editing, J. Gene Med. 21 (2019)e3107.
[298] Y.K. Kang, K. Kwon, J.S. Ryu, H.N. Lee, C. Park, H.J. Chung, Nonviral genome editing based on a polymer-derivatized CRISPR nanocomplex for targeting bacterial pathogens and antibiotic resistance, Bioconjug. Chem. 28 (2017) 957–967.
[299] H.X. Zhang, Y. Zhang, H. Yin, Genome editing with mRNA encoding ZFN, TALEN, and Cas9, Mol. Ther. 27 (2019) 735–746.
[300] Z. Zhang, T. Wan, Y. Chen, Y. Chen, H. Sun, T. Cao, Z. Songyang, G. Tang, C. Wu, Y. Ping, F.J. Xu, J. Huang, Cationic polymer-mediated CRISPR/Cas9 plasmid delivery for genome editing, Macromol. Rapid Commun. 40 (2019), e1800068.
[301] X. Chen, Y. Chen, H. Xin, T. Wan, Y. Ping, Near-Infrared Engineering of Photothermal NanoCRISPR for Programmable Genome Editing, Proc. Natl. Acad. Sci. U.S.A. 117 (2020) 2395–2405.
[302] H. Tang, X. Zhao, X. Jiang, Synthetic multi-layer nanoparticles for CRISPR-Cas9 genome editing, Adv. Drug Deliv. Rev. (2020) https://doi.org/10.1016/j.addr.2020.03.001In this issue.
[303] C.F. Xu, G.J. Chen, Y.L. Luo, Y. Zhang, G. Zhao, Z.D. Lu, A. Czarna, Z. Gu, J. Wang, Rational designs of in vivo CRISPR-Cas delivery systems. Adv. Drug Deliv. Rev. (2019) https://doi.org/10.1016/j.addr.2019.11.005 In this issue.
[304] T. Wan, Y. Ping, Delivery of genome-editing biomacromolecules for treatment of lung genetic disorders, Adv. Drug Del. Rev. (2020)In this issue.
[305] D. Sun, Z. Sun, H. Jiang, A.M. Vaidya, R. Xin, N.R. Ayat, A.L. Schilb, P.L. Qiao, Z. Han, A. Naderi, Z.R. Lu, Synthesis and evaluation of pH-sensitive multifunctional lipids for efficient delivery of CRISPR/Cas9 in gene editing, Bioconjug. Chem. 30 (2019) 667–678.
[306] A.S. Timin, A.R. Muslimov, K.V. Lepik, O.S. Epifanovskaya, A.I. Shakirova, U. Mock, K. Riecken, M.V. Okilova, V.S. Sergeev, B.V. Afanasyev, B. Fehse, G.B. Sukhorukov, Efficient gene editing via non-viral delivery of CRISPR-Cas9 system using polymeric and hybrid microcarriers, Nanomedicine 14 (2018) 97–108.
[307] Y. Rui, D.R. Wilson, K. Sanders, J.J. Green, Reducible branched ester-amine quadpolymers (rBEAQs) codelivering plasmid DNA and RNA oligonucleotides enable CRISPR/Cas9 genome editing, ACS Appl. Mater. Interfaces 11 (2019) 10472–10480.
[308] A. Conway, M. Mendel, K. Kim, K. McGovern, A. Boyko, L. Zhang, J.C. Miller, R.C. DeKelver, D.E. Paschon, B.L. Mui, P.J.C. Lin, Y.K. Tam, C. Barbosa, T. Redelmeier, M.C. Holmes, G. Lee, Non-viral delivery of zinc finger nuclease mRNA enables highly efficient in vivo genome editing of multiple therapeutic gene targets, Mol. Ther. 27 (2019) 866–877.
[309] J.B. Miller, S. Zhang, P. Kos, H. Xiong, K. Zhou, S.S. Perelman, H. Zhu, D.J. Siegwart, Non-viral CRISPR/Cas gene editing in vitro and in vivo enabled by synthetic nanoparticle co-delivery of Cas9 mRNA and sgRNA, Angew. Chem. Int. Ed. 56 (2017) 1059–1063.
[310] R. Rouet, B.A. Thuma, M.D. Roy, N.G. Lintner, D.M. Rubitski, J.E. Finley, H.M. Wisniewska, R. Mendonsa, A. Hirsh, L. de Onate, J. Compte Barron, T.J. McLellan, J. Bellenger, X. Feng, A. Varghese, B.A. Chrunyk, K. Borzilleri, K.D. Hesp, K. Zhou, N. Ma, M. Tu, R. Dullea, K.F. McClure, R.C. Wilson, S. Liras, V. Mascitti, J.A. Doudna, Receptor-mediated delivery of CRISPR-Cas9 endonuclease for cell-type-specific gene editing, J. Am. Chem. Soc. 140 (2018) 6596–6603.
[311] J.A. Zuris, D.B. Thompson, Y. Shu, J.P. Guilinger, J.L. Bessen, J.H. Hu, M.L. Maeder, J.K. Joung, Z.Y. Chen, D.R. Liu, Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo, Nat. Biotechnol. 33 (2015) 73–80.
[312] M. Wang, J.A. Zuris, F. Meng, H. Rees, S. Sun, P. Deng, Y. Han, X. Gao, D. Pouli, Q. Wu, I. Georgakoudi, D.R. Liu, Q. Xu, Efficient delivery of genome-editing proteins using bioreducible lipid nanoparticles, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 2868–2873.
[313] P. Wang, L. Zhang, Y. Xie, N. Wang, R. Tang, W. Zheng, X. Jiang, Genome editing for cancer therapy: delivery of Cas9 Protein/sgRNA plasmid via a gold nanocluster/lipid core-shell nanocarrier, Adv. Sci. 4 (2017) 1700175.
[314] C.E. Smull, E.H. Ludwig, Enhancement of the plaque forming capacity of poliovirus ribonucleic acid with basic proteins, J. Bacteriol. 84 (1962) 1035–1040.
[315] C. Andreini, L. Banci, I. Bertini, A. Rosato, Counting the zinc-proteins encoded in the human genome, J. Proteome Res. 5 (2006) 196–201.
[316] 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.
[317] 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.
[318] J.P. Behr, The proton sponge: a trick to enter cells the viruses did not exploit, Chimia 51 (1997) 34–36.