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REVIEW ARTICLE

Targeting the Inside of Cells with Biologicals: Chemicals as a Delivery Strategy

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Abstract
Delivering macromolecules into the cytosol or nucleus is possible in vitro for DNA, RNA and proteins, but translation for clinical use has been limited. Therapeutic delivery of macromolecules into cells requires overcoming substantially higher barriers compared to the use of small molecule drugs or proteins in the extracellular space. Breakthroughs like DNA delivery for approved gene therapies and RNA delivery for silencing of genes (patisiran, ONPATTRO®, Alnylam Pharmaceuticals, Cambridge, MA, USA) or for vaccination such as the RNA-based coronavirus disease 2019 (COVID-19) vaccines demonstrated the feasibility of using macromolecules inside cells for therapy. Chemical carriers are part of the reason why these novel RNA-based therapeutics possess sufficient efficacy for their clinical application. A clear advantage of synthetic chemicals as carriers for macromolecule delivery is their favourable properties with respect to production and storage compared to more bioinspired vehicles like viral vectors or more complex drugs like cellular therapies. If biologicals can be applied to intracellular targets, the druggable space is substantially broadened by circumventing the limited utility of small molecules for blocking protein–protein interactions and the limitation of protein-based drugs to the extracellular space. An in depth understanding of the macromolecular cargo types, carrier types and the cell biology of delivery is crucial for optimal application and further development of biologicals inside cells. Basic mechanistic principles of the molecular and cell biological aspects of cytosolic/nuclear delivery of macromolecules, with particular consideration of protein delivery, are reviewed here. The efficiency of macromolecule delivery and applications in research and therapy are highlighted.

Key Points

Classic drugs often only reach a small portion of all disease-relevant molecules.

Delivering DNA, RNA or proteins as drugs could substantially increase the possibilities for therapeutic intervention.

Delivery by chemical carriers allowed ground-breaking new therapies, including coronavirus disease 2019 (COVID-19) vaccines, but a thorough mechanistic understanding of the delivery methods is critical for successful application to biological molecules with different properties.

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1 Introduction

Carriers for intracellular delivery can be categorized into compartments or molecules including lipids, polymers and inorganic carriers. Bioinspired compartments are for instance exosomes, viral vectors, bacterial ghosts or red blood cell ghosts, and bioinspired molecules as carriers include peptides or proteins [1, 2]. Employing a bacterial type 3 secretion system for delivery is another example of a bioinspired delivery strategy [3].

Different from bioinspired carriers, chemicals have certain advantages as drug carriers, including low production costs, potential for large scale production and often favourable properties for storage like high stability [4–6]. Especially if cargoes are delivered to many cells like whole tissues or organs, the ability to produce high quantities of carrier and cargo material for delivery can be decisive (for gene delivery based on adeno-associated viruses, doses in orders of magnitude up to 10^{14} vector genomes per kilogram body weight have been used [7]). Chemical carriers may reach higher loading capacities than viral vectors for

cargoes like DNA [4, 6] and impose little or no size limits to nucleic acid cargoes. They are less immunogenic and considered safer than viral vectors [4, 8]. Accessibility to rational design allows engineering carriers with specific properties required for individual steps in the delivery process.

Drugs based on non-viral nucleic acid delivery have been approved [9–14]. Cytosolic delivery of proteins, although practised in vitro [15–20], is not as advanced in its clinical utility, although targeting proteins directly allows interference in ways that are not possible with other approaches [21]. Besides circumventing the risk for insertional mutagenesis that is associated with DNA delivery [4], delivering proteins has the potential to individually target functions that are encoded in post-translational modifications, protein conformations, splice variants or different functional epitopes of a protein among other advantages of protein–protein interference [21]. Interfering with protein–protein interactions is a challenge for small molecules, and they are most suitable for targets with hydrophobic pockets. Protein-based drugs are most suitable for extracellular drugs. Considering targets with hydrophobic pockets or extracellular targets as “druggable”, a majority of 80% of targets remain “undruggable” [22].

The possibility of drugging the intracellular space with macromolecules could substantially contribute to unlocking the many targets that are still considered undruggable. Chemical carriers based on lipids, polymers and inorganic carriers for macromolecule delivery will be reviewed here, with an emphasis on protein delivery.

2 Cargoes

2.1 Comparison of DNA, RNA and Protein Delivery by Chemicals

2.1.1 Proteins Compared to Nucleic Acids for Cytosolic Delivery

Cargo delivery can be divided into several distinct steps, including the association of cargo with carriers, contact of the complexes with the cell surface and uptake into cells by endocytosis, and endosomal release is usually required [23–26]. Although similar for some cargoes and carriers, there are distinct differences between the transfection of proteins and nucleic acids. In contrast to the negatively charged, comparably uniform physicochemical properties of nucleic acids, proteins are highly diverse in size, structure and charge distribution. A lipid-based carrier, for example, had to be optimized to allow delivery of an antibody [27]. A way to address the diversity of protein properties that complicates finding a carrier that fits all proteins is linking an entity to the protein that mediates association with the carrier. For example, fusion of a negatively charged entity to proteins has been shown to allow repurposing nucleic acid-transfection reagents for protein delivery [18, 28]. Although proteins can be “anionized”, this does not guarantee delivery by a cationic lipid-based carrier. Vice versa, a protein being cationic may not always be an obstacle to delivery [24]. For protein delivery with cationic lipid-based carriers, the ratios of surface areas and hydrophobic interactions of the cargo protein with the carrier were more important than charge ratios [24, 25]. To enhance attachment of proteins to carriers, natural polyphenols have been proposed as potential bridging ligands to a particular polymer-based carrier type [29, 30]. In contrast to nucleic acids, the integrity of the protein’s tertiary structure is essential for functionality. A critical difference between nucleic acid delivery and protein delivery is the presence or absence of amplification. While many nucleic acids benefit from inherent amplification effects, for most proteins, the number of molecules delivered is the actual amount that can be effective. An exception are enzymes, for which delivery of a single protein can result in many substrate molecules to be converted, i.e. an amplified effect from a low dose of delivered cargo.

2.1.2 RNA Compared to DNA for Cytosolic/Nuclear Delivery

The physicochemically more uniform nature of nucleic acids substantially simplifies the search for universally applicable carriers, but there are still some distinct differences between RNA and DNA with relevance to delivery [31]. Efficacies might be different depending on whether the cargo is plasmid DNA (pDNA) or short interfering RNA (siRNA), even with the same carrier type [32]. One of the reasons for this is the difference in barriers that need to be overcome by RNA or DNA. Cytosolic delivery is sufficient for RNA and many proteins, but the requirement for nuclear access is an additional barrier for DNA delivery [32, 33]. Further, complex stability differs depending on whether carriers associate with pDNA or RNA [32, 34]. The size of nucleic acids affects complex stability; if the electrostatic interaction with cationic carriers is limited because nucleic acids are small like siRNA, the stability of complexes is lower and complexes are more sensitive to dissociation by salts compared to larger nucleic acids like DNA [32, 34]. The release of counter ions upon electrostatic interaction between carrier and cargo increases entropy. The more extensive electrostatic interactions of the larger DNA molecule can confer more stability to DNA-carrier complexes compared to RNA-carrier complexes [32]. High complex stability is advantageous before delivery to the final site of action, because it can have a

protective effect on nucleic acids against degradation [35]. High stability with a resulting difficulty with regard to releasing the cargo from the carrier is often a disadvantage after the final site of action is reached [36] if release from the carrier is required for cargo function. The difference in size between DNA plasmids and short RNA oligonucleotides can also be relevant in the context of endocytic uptake. Large DNA molecules have to be condensed by polycations to smaller particles [32, 37, 38], which are in a size range of particles that are taken up by endocytosis [39]. For comparison, DNA can be sized in the range of micrometres [32, 37, 38], while the length of siRNA is in the range of a few nanometres [32, 40, 41].

2.1.3 RNA and Proteins Compared as Potential Drugs

An advantage that both proteins and RNA have in common is the lower risk they pose because they do not integrate into the genome [21, 42]. An advantage of the special case of delivering antibody encoding messenger RNA (mRNA) instead of delivering antibodies as recombinant proteins is the circumvention of aberrant post-translational modifications [43]. An advantage of mRNA-encoded versus recombinant antibodies as proteins is also their cost-effectiveness, ease of production and no requirement to optimize each sequence individually [43]. The therapeutic space could furthermore broaden due to the increased number of accessible targets if mRNA-encoded antibodies are applied to bind intracellular targets, which extracellularly applied antibodies usually cannot reach, including knockdown at the protein level by cytosolic intracellular antibodies (intrabodies) or endoplasmic reticulum (ER) retained intrabodies (ER intrabodies) [21, 44].

2.1.4 Macromolecules in the Absence of Carriers or Delivery Systems

It is noteworthy that "naked" RNA, i.e. RNA in the absence of a carrier, is internalized and expressed in dendritic cells (DCs). Already in 1990, Wolff et al. had found naked RNA to be expressed in mice [45], and naked RNA is taken up by many cell types [43, 46], although it is in most cell types entrapped in endosomes and degraded in the lysosome, reaching the cytosol only inefficiently [42, 46]. DCs are an exception, taking up naked RNA with some efficiency [42, 47]. Uptake of naked RNA into DCs was found to involve receptor-mediated endocytosis and macropinocytosis. To allow expression of the RNA-encoded protein, endosomal escape is required, but the exact mechanism of escape is still insufficiently understood [47]. "Naked" DNA has also been reported to be expressed (Wolff et al. in 1990) [45], and a "naked" protein has been reported to be delivered to cells showing activity in the cell nucleus [48, 49].

2.1.5 Temporal Control of Cargo Efficacy

The duration of activity for RNA and proteins is transient and limited by dilution due to cell division within days or, in non-dividing cells, by degradation within weeks [32, 50]. The transient nature of RNA or protein cargoes can be of therapeutic value [42]. If a protein is too long-lived, and if this is not desired for the application, its half-life could be modulated by one of the strategies to influence degradation of a protein of interest [51]. Antibodies that have been delivered as proteins were detectable for up to at least 4 days after delivery [16]. The silencing effect induced by siRNA may last between a few days and more than 3 weeks, depending on how often cells divide and dilute the delivered siRNA [50]. If DNA does not express episomally (extrachromosomally) but integrates into the host genome, the genetic modification can become permanent [32, 52]. For example, a single intramuscular injection of a viral vector that delivered an antibody gene led to lifelong expression of the antibody in mice [53]. Besides the duration of activity, the time until activity starts after administration differs between cargo types. Protein activity can start immediately upon successful delivery to the site of action, without delay. After successful delivery of RNA or DNA, there is a delay until the desired activity takes effect. This delay ranges from a few hours to several days. For example, expression from RNA was detectable already 2 h after administration [54, 55]; expression of DNA from plasmids or delivered by viral vectors was detectable in the range of 1 to several days post inoculation [55–57]. Expression of mRNA-encoded antibodies peaked at approximately 24 h after administration and lasted for up to several days or weeks [58–60].

2.1.6 Comparison of Quantities of Cargo Required for Efficacy

The dose required for an effect can substantially vary among proteins, from high amounts to only a single protein such as a toxin molecule [61, 62]. For DNA, one to a few transcribed pDNA molecules in the nucleus were claimed to be sufficient for detectable protein expression, due to amplification via transcription and translation [32, 63]. Although gene silencing involves recycling of siRNA, which represents an inherent amplification effect, still more than only a single or a few siRNA molecules are required in the cytosol for successful silencing [32]. Different from the catalytic process of silencing by siRNA, there is no amplification effect for antisense oligonucleotides, but they require equimolar annealing [64]. The expression levels of mRNA-encoded antibodies required for neutralization of targets are expected to be higher than those required for the purpose of vaccination. Naked mRNA was described to be sufficient to induce immune responses, but is potentially not sufficient for applications that require

Table 1 Comparison of delivery requirements for DNA, RNA and proteins

	DNA	mRNA	siRNA	Proteins
Amplification of effect	++ [32]	+ [42]	+ [32]	–
Predictable association with carrier via ionic interaction between negatively charged cargo and positively charged carrier	+	+	+	–
Functionality with little dependence on tertiary structure	+	+	+	–
Integrity of the genome ensured	–	+	+	+
Typically transient activity	–	+	+	+
Compartment	Nucleus	Cytosol	Cytosol	Any compartment including cytosol, nucleus, endosomes, lysosomes, ER, Golgi, mitochondria
Efficient delivery also to non-dividing cells	–	+	+	+

– indicates no, + indicates yes, ++ indicates yes and enhanced relative to "+"

ER endoplasmic reticulum, *mRNA* messenger RNA, *siRNA* short interfering RNA

higher amounts of protein and that target other cells than DCs [42].

A comparison of delivery requirements for DNA, RNA and proteins is provided in Table 1.

2.2 Protein Delivery Applied in Vitro According to Research Areas

In contrast to in vitro delivery of nucleic acids, which has been an established routine procedure for decades, the delivery of proteins by chemical carriers has remained an exception. Investigating in which research areas protein delivery with chemical carriers has already been applied and which application fields might be particularly promising for this approach may allow valuable conclusions in view of potential future applications. Examples are, therefore, reviewed in Table 2. An example that compared protein delivery of the same cargo with different lipid-based carriers used Fc-Cre as a cargo, a protein consisting of Cre recombinase fused to the constant region of an antibody [16]. Examples of protein delivery by chemical carriers include applications with potential as research-area-independent tools and with potential in the research areas of oncology, neurology, hereditary diseases or metabolic conditions (Table 2). An application of protein delivery that has been pursued by various researchers independently of each other is genome modification (Table 2).

3 Carriers

Chemicals used as carriers for biological cargoes typically belong to the group of lipid-based, polymer-based or inorganic nanoparticles [90]. Depending on composition and structural assembly, carriers differ in the mechanism by which they can be loaded with cargo. Premature cargo release is an unwanted effect, but cargo release is often necessary for function [36, 91]. To avoid the trade-off between requiring complex stability for delivering cargoes into cells and complex instability for release of cargoes at their site of action, stimuli-responsive carriers are an option to initiate cargo release only when desired [92–94].

A carrier ideally fulfils functions in a temporal sequence of tasks, exhibiting specific properties at defined time points [64]. Implementing all functions required for delivery in a time- and location-controlled way as "programmed delivery" [64, 95] is a challenge because carriers often need to reconcile opposed properties like being stable outside cells, but dissociating from cargo inside cells, being inert against surfaces, but attaching to the cell surface, and possessing the ability to destabilize membranes of intracellular vesicles but not the plasma membrane or others like the mitochondrial or nuclear membrane [64]. Furthermore, carriers need to fulfil functions that are specific to the cargo type; for example, they need to condense large nucleic acids like DNA [32, 37, 38].

3.1 Lipid-Based Carriers

Lipid-based carriers are typically amphiphiles, containing a non-polar fatty acid chain and a polar head or charged head group. Their amphipathic character allows them to self-assemble in different supramolecular structures like micellar structures, bilayers or vesicles [96, 97]. Lipid nanoparticles commonly employed for nucleic acid delivery are different from liposomes in their structure, for example, assemblies containing micellar structures are referred to as lipid nanoparticles [90].

Table 2 Protein cargoes and their research and potential application areas

Protein	Reagent/carrier type	Cell/organism	Aim/reported effect/research area	References
Cre	Polymer-based	Reporter cell line based on T cell line	Genome modification	[65]
Fc-Cre	Lipid-based (Pulsin, Bioporter, Ab-DeliverIN)	Reporter cell line based on SC1 cells	Genome modification	[16]
Cre Recombinase, TALE- and Cas9-based transcription factors, negatively super-charged proteins	Cationic lipid-based	Injection into cochlea of mice, human cultured cells	Genome modification	[28]
Cas9	Gold nanoparticle- polymer-based carrier	Local injection into mice	Genome modification Murine model of Duchenne muscular dystrophy	[66]
Cas9	Gold nanoparticle-based carrier	Intracranial injection into mice	Genome modification, treatment of symptoms in mice caused by fragile X syndrome in autism spectrum disorders	[67]
Cas9 ribonuclein complex	Polymer-based biodegradable nanocapsule	Murine RPE tissue and skeletal muscle, local administration	Genome modification	[68]
Cas9 with anionic tag	Gold functionalized to have a positive surface charge	HeLa, HEK293T	Genome modification	[69]
Nucleoside kinases	Lipid-based (Bioporter)	Human osteosarcoma and CHO cell lines	Potential application in oncology, increase of sensitivity to nucleoside analogues like ganciclovir	[70]
Caspase 3	Gold-based supramolecular assemblies [71, 72], gold with pH sensitive polymer [73], iron oxide nanogel [74]	HeLa [71, 72], gastric cancer cell line [73], murine colon cancer cell line CT26 [74]	Potential for inducing cell death	[71–74]
Fluorescent antibody, caspase 3, caspase 8, and granzyme B	Lipid-based (TFA-DODAPL: DOPE also called BioPorter)	Cell lines including HeLa-S3, NIH3T3, Jurkat and primary cells	Potential for inducing cell death	[24]
Anti-HPV16 E6 antibody	Lipid-based	CaSki, SiHa cells	Downregulation of oncogene activity	[75]
Antibody for Ki-67 inactivation	Lipid-based carrier and photosensitizer	HeLa	Inactivation of a biomarker for proliferating cancer cells	[76]
Anti-S100A4	Liposome-based	Breast cancer 4T1 cells, xenograft mouse model	Oncology, application for inhibition of metastasis	[77]
Anti-Gasdermin B (GSDMB) antibody	Polymer-based capsules	Her2 expressing breast carcinoma cell lines, xenograft mouse models	Oncology, anti-tumour effects in Her2 over-expressing breast cancer models	[78]
Anti-pPKCθ (Thr538)	Polymer-based Noncovalent complexation	Ex vivo delivery into human PBMCs	Modulation of protein kinase Cθ signal-ling, which is associated with activation, proliferation and differentiation of T cells	[79]
Anti-SMC2 antibodies	Polymeric micelles	HCT116	Oncology, inhibition of SMC2 as a poten-tial therapeutic strategy	[80]
Anti-MRP1 antibody, anti-RelA antibody (IgGs were linked to anionic polypep-tides)	Lipid-based (cationic lipids originally used for nucleic acid delivery)	HEK293T, HT1080, A549	Inhibition of drug efflux pump, inhibition of transcription factor NFkappaB	[81]
Anti-gamma Tubulin, anti-actin, anti-Golgi protein, anti-NFkappaB antibodies	Polymersome-based, pH sensitive	NIH3T3, bEnd3 cell lines and primary human dermal fibroblast cells	Labelling of proteins in living cells and interference with cellular processes	[82, 83]

Table 2 (continued)

Protein	Reagent/carrier type	Cell/organism	Aim/reported effect/research area	References
Anti-lamin antibody	PEI, noncovalent complexation	Human fibroblasts	Potential for tracking proteins and functional studies	[84]
Anti-nuclear pore complex as a transiently anionized IgG	Polymer-based pH-sensitive micelles, noncovalent complexation	Murine colon carcinoma cell line C26	Potential for tracking proteins and functional studies	[85]
Anti-nuclear pore complex antibody	Liposome-based carrier with a cationic peptide (R8) and a pH-sensitive fusogenic peptide	HeLa	Analysis of intracellular molecules	[86]
Anti-cytokeratin 8 (K8) antibody	Liposome-based	HeLa	Potential application in cystic fibrosis research	[27]
Anti-synuclein antibody, small GTPase rhoG	Polymer-based	Neurons and neuronal cell lines	Neuronal disorders	[87]
Anti-alpha synuclein co-delivered with recombinant(His)6-TRIM21	Polymers based on synthetic mimics of poly-arginine-based peptides	SH-SY5Y	Relevance in the context of Parkinson's disease, targeted proteolysis	[88]
Anti-GSH antibody and several further IgGs	Polymers based on synthetic mimics of poly-arginine-based peptides	HeLa	Potential relevance for oxidative stress research	[88]
Anti-PTP1B	Polymers based on synthetic mimics of poly-arginine-based peptides	HeLa, A431	Relevance in the context of diabetes and obesity	[88]
GFP, Sox2, p53	Hollow gold nanoshells with surface functionalization and light-sensitive elements	Prostate cancer cell line PPC-1	Proof of principle for delivery system, potential relevance of cargoes in oncology	[89]

Identifiers starting with "CVCL" are Expasy accessions

4T1 mouse cancer cell line (CVCL_0125), *A431* human cancer cell line (CVCL_0037), *A549* human cancer cell line (CVCL_0023), *bEnd3* transformed mouse cell line (CVCL_0170), *C26* murine colon carcinoma cell line, *Cas9* CRISPR associated 9, *CaSki* human cancer cell line (CVCL_1100), *CHO* Chinese hamster ovary cells (CVCL_0213), *Cre* Cre recombinase (Cre: "causes recombination"), *CRISPR* clustered regularly interspaced short palindromic repeats, *CT26* mouse colon cancer cell line (CVCL_7254), *DOPE* dioleoylphosphatidylethanolamine, *Fc-Cre* fusion protein of Cre recombinase and the constant region of an antibody (Fc: "fragment crystallizable"), *GDP* guanine diphosphate, *GFP* green fluorescent protein, *GSH* glutathione, *GTP* guanine triphosphate, *GTPase* enzyme that catalyzes the hydrolysis of GTP to GDP and phosphate, *HCT116* human cancer cell line (CVCL_0291), *HEK293T* human transformed cell line (CVCL_0063), *HeLa* human cancer cell line (CVCL_0030), *HeLa-S3* human cancer cell line (CVCL_0058), *Her2* receptor tyrosine-protein kinase erbB-2, $(His)_6$ peptide consisting of 6 histidines, *HPV16 E6* human papillomavirus type 16 protein E6, *HT1080* human cancer cell line (CVCL_0317), *IgG* immunoglobulin G, *Ki-67* proliferation marker protein Ki-67, *MRP1* multidrug resistance-associated protein 1, *NFkappaB* nuclear factor kappa B, *NIH3T3* spontaneously immortalized mouse cell line (CVCL_0594), *p53* cellular tumour antigen p53, *PBMCs* peripheral blood mononuclear cells, *PEI* polyethylenimine, *PPC-1* human prostate cancer cell line (CVCL_4778), *pPKCθ (Thr538)* phosphorylated protein kinase Cθ (threonine 538), *PTP1B* synonym to PTPN1 (i.e. tyrosine-protein phosphatase non-receptor type 1), *R8* a peptide composed of 8 arginines, *RelA* transcription factor p65, *RhoG* Rho-related GTP-binding protein RhoG, *RPE* retinal pigment epithelium, *S100A4* S100 calcium binding protein A4, *SC1* adherent mouse-derived cell line (ATCC no. CRL-1404), *SH-SY5Y* human cancer cell line (CVCL_0019), *SMC2* structural maintenance of chromosomes protein 2, *Sox2* transcription factor SOX-2, *TALE* transcription activator-like effector, *TFA-DODAPL* trifluoroacetylated lipopolyamine, *TRIM21* tripartite motif containing-21

The inner aqueous compartment of liposomes can carry cargoes or the surface of carriers can be loaded with cargoes [24, 25, 96, 97]. Liposomal inner compartments allow carriage of cargoes with various physicochemical properties [98]. Complex formation of carriers with cargoes typically involves ionic interactions [32]. The ratio of surface areas and potentially hydrophobic interactions were relevant for protein cargoes and a cationic lipid-based carrier [25]. Encapsulation of cargoes in liposomes has been described early. Szoka et al. [98] introduced cargoes in an aqueous buffer into a lipid mixture in an organic solvent. The organic solvent was subsequently removed by evaporation, but protein cargoes can be exposed to the organic solvent and denature as a consequence. Still, 41% of the protein alkaline phosphatase was found to remain active following this cargo-loading procedure [98]. Liposomes can also be prepared without exposing cargoes to organic solvents, for example, by film rehydration [97], by mixing lipid components in an organic solvent and drying the mixture on a solid support like a glass surface, where lipids form a thin layer. When subsequently adding an aqueous solution containing the cargo, vesicles are formed [97].

Typical components of lipid-based carriers are a mixture of four major components: cationic or ionisable lipids, phospholipids, a sterol like cholesterol and a lipid-anchored polyethylene glycol (PEG) [14, 43, 55, 90, 99]. Each component has individual functions, including vial and storage stability provided by lipid-anchored PEG, phospholipids make up the particle structure, cholesterol plays a role in stability and cationic or ionisable lipids promote ionic interactions with negatively charged cargoes as well as cellular uptake and endosomal escape [90, 100]. New-generation lipids are neutral at physiological pH and only become cationic at acidic pH, which reduced toxicity and enhanced efficiency [43, 101]. For example, a lipid formulation that is used as an approved drug for the delivery of an RNA vaccine consisted of ((4-hydroxybutyl)azanediyl)bis(hexane-6,1-diyl)bis(2-hexyldecanoate) as an ionisable lipid, 2-[(PEG)-2000]-*N*,*N*-ditetradecylacetamide as a PEGylated lipid and cholesterol and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) as structural lipids [14, 102].

The size of liposomes can be influenced by the choice of the pore size of a membrane through which liposomes can be passed after preparation (extrusion) [97, 103]. The size of lipoplexes depends on various factors, including those that affect complex formation and aggregation [31]. The stability of lipid-based carriers comprises physical and chemical aspects. The physical stability of liposomes includes, for example, their resistance against rupture upon stretching. The chemical stability of lipid-based carriers can be limited by proneness of unsaturated fatty acid chains to oxidation and also by hydrolysis of ester bonds [97].

In summary, lipid-based carriers for cargo delivery can have the advantage of being accessible to many researchers even without requiring chemical synthesis, if mixing of defined reagents in certain optimized ratios is sufficient for preparation of cargo-loaded carriers.

3.2 Polymer-Based Carriers

Polymer-based carriers are a very diverse group of carriers in their chemical composition, structure and the mechanism by which they carry cargoes [64, 90, 93]. In contrast to the exactly defined chemical properties of individual components of lipid-based carriers, polymers are often inhomogeneous mixtures with a size distribution. Cargo-loading procedures for polymer-based carriers include cargo association with the surface of carriers, cargo entrapment in the matrix of polymers and encapsulation *in* or conjugation *to* the polymer [18, 90].

Copolymers are amphiphilic if composed of a hydrophilic and a hydrophobic polymer block linked together, resulting in similar properties to those of amphiphilic lipids [97]. They can self-assemble and, according to the bilayer-to-vesicle model, assemble to micelles that become bilayer sheets, which eventually close to vesicles (polymersomes) driven by edge energy [94]. The critical packaging parameter [$P_c = \nu/(a \times l)$, with ν as the volume of the hydrophobic part, a as the area of the hydrophilic part and l as the length of the block copolymer] can help estimate which morphology is most likely formed [97]. For example, with a $P_c < 1/3$, spherical micelles form, with $½ < P_c < 1$, vesicles form, and at $P_c = 1$, planar bilayers form [97, 104]. Polymersomes tend to be more stable than liposomes, can be stiffer and more resistant against rupture upon stretching than liposomes [97] and often have substantially thicker bilayers (5–50 nm) compared to the thin bilayers (3–5 nm) and large inner compartments of liposomes [97]. The risk for disassembly of vesicles upon dilution can be overcome by cross-linking and keeps cargo release independent of dilution [93]. Covalent bonds between polymer blocks are usually more stable than the ester bond in phospholipids. Loading of polymersomes with cargoes is similar to loading liposomes, e.g. by film rehydration [97]. Because there is little control over vesicle size with film rehydration, extrusion, sonication or freeze-thaw cycles are often applied after preparation. Freeze-thaw cycles, especially, are harmful for proteins. A preparation method with better size control is microfluidics based, but has a low throughput compared to film rehydration. Further preparation methods for polymersomes are reviewed elsewhere [94, 97].

In situ cross-linking is used for preparing polymeric nanocontainers, forming around a template that either remains or

is removed, leaving a hollow shell [93]. Loading may be limited to the particle's surface, because the polymer shell is an obstacle to loading macromolecules into the cavity. A strategy circumventing this obstacle is in situ encapsulation of protein cargoes, which can be achieved by chemically functionalizing the protein with polymerizable groups, letting monomers adsorb to the protein and subsequently polymerizing the monomers as a shell around the protein [18, 68, 93, 105]. In situ encapsulated enzymes may not require release from the polymeric shell if substrates are small molecules that readily diffuse through the polymer meshwork [105]. The polymer shell's purpose can be protection of cargoes from the surrounding environment, like from proteases [105, 106]. If protein cargoes need to be released from the nanocapsule, degradable polymers can be used [68, 105]. The stimuli responsiveness of polymers can include stimuli like enzymes, temperature, light, electric stimuli, magnetic stimuli or ultrasound [92–94]. Particularly relevant in the physiological context are pH-, redox- or enzyme-responsive properties, allowing release upon acidification of endosomes or by reduction in the cytosol [68, 92–94, 107].

Nanoparticles from complex hyperbranched polymeric structures (dendrimers) can be covalently or non-covalently linked to biologicals, for example, via charge interactions [90, 108]. Polycations like polyethylenimine (PEI) or polyamidoamine (PAMAM) can exhibit molecular weight-dependent cytotoxicity. To address PEI's weight-dependent trade-off between cytotoxicity and efficacy, stimuli responsive linkers connecting low molecular weight polymers have been suggested and allowed high transfection efficiency at much reduced cytotoxicity [64, 109, 110].

In summary, polymer-based carriers are highly versatile and highly accessible to rational design when applying the relevant expertise. The assembly mechanism for cargo and carrier has similarities for amphiphilic lipid- and polymer-based carriers. The versatile material properties obtainable with polymeric carriers is an opportunity to strategically engineer parameters affecting delivery efficiency, which are governed by cell-biological and physiological aspects discussed in detail in the section on cells and clinical applications.

3.3 Inorganic Carriers

Inorganic carriers comprise materials like gold, silica or carbon nanotubes. Functionalizing the surface of inorganic nanoparticles can be used to promote loading cargoes [18, 105]. For example, the surface of silica particles was functionalized by covalently linking a hydrophobic surface coating via silanes, which facilitates loading proteins via hydrophobic interactions [111, 112]. A variety of proteins adsorb spontaneously on carbon nanotubes [113]. However, it is important to maintain the protein's function during any type of loading process. Surface functionalization can also promote loading cargoes via electrostatic interactions [105, 114]. Loading by formation of supramolecular assemblies from gold nanoparticle-based carriers and cargoes has been reported by Rotello et al. [71, 114, 115]. Carriers have been loaded by non-covalent affinity binding via His-tagged proteins on gold nanoparticles functionalized with an anti-His aptamer [116]. Covalent linkage of cargo molecules to carriers is an option if cargoes do not have to be released from carriers or if stimuli-responsive release is possible [117]. Release of cargoes that were covalently linked to the inorganic carrier can be stimuli responsive, for example, gold nanoparticles functionalized via thiol groups released the covalently linked molecules in the reducing cytosolic environment with glutathione [105, 118].

Inorganic nanoparticles are often of interest for applications requiring special material properties, like certain imaging applications [90], but may not require cytosolic delivery. Inorganic carriers that have been used for the purpose of intracellular delivery include mesoporous silica nanoparticles used as carriers for chromobodies. Delivery efficiency was assumed to originate from a proton sponge effect of the His_6 tag of the chromobodies, but was very limited in the absence of endosomolytic reagents [119]. Naked gold nanoparticles conjugated with siRNA did not result in silencing and thus did not deliver siRNA to the cytosol [99]. However, inorganic nanoparticles can be functionalized, for example, with polymers to add properties of the polymer. Inorganic nanoparticles that organized into supramolecular structures have been proposed to act via membrane fusion [71, 115]. A non-endocytosis-based entry path that involves membrane disruption has been proposed as a mechanism for delivery of carbon-based carriers, but was associated with toxicity depending on dose and exposure time [20, 120].

Eventually, inorganic carriers may allow particular applications that are not possible with organic carriers, but lipid- or polymer-based carriers have so far been used much more commonly for delivering biological macromolecules.

3.4 Combinations of Chemicals with Other Reagents or Physical Methods

Combination approaches have been described early, like two components consisting of the polymer poly-L-lysine (PLL) as a carrier to bind and condense DNA for internalization and the small molecule chloroquine to promote endosomal escape [33, 121]. Bioinspired molecules have been combined with chemicals, for example, a peptide derived from the cell-penetrating peptide TAT has been combined with the small molecule UNC7938 [122]. Further combination

approaches include peptides combined with lipids [86, 123], liposomes that have been provided with a polymer shell (capsosomes) [97, 124] or lipid/polymer hybrid vehicles, which were combined with a peptide as vehicles for ER targeting [125].

Chemicals have been combined with physical methods like photochemical internalization (PCI) [126–129]. PCI requires a reagent (photosensitizer) that needs to localize in endosomal membranes and forms reactive singlet oxygen, destroying the vesicle membranes upon light exposure [129, 130]. Combination of TAT with PCI was found to result in a certain cytotoxicity, which can be explained by efficient lysis of endosomes accompanied by release of calcium into the cytosol and cell death, but lytic effects were found to also depend on the exact chemical properties of the TAT conjugate [131, 132]. Photochemical disruption of endosomal membranes has been combined with bioinspired reagents like peptides and lipid-based, polymer-based and also inorganic carriers [126–129, 133–136] and has also been used to deliver proteins [130, 137]. The combination of inorganic glass beads with mechanical "hitting" to deliver photocaged antibodies into cells has also been described [138].

Combination approaches benefit from combined mechanisms acting towards successful delivery. Therefore, they are particularly promising in terms of delivery efficiency, but how well combination approaches can be translated to therapeutic application varies substantially between approaches.

4 Cells

4.1 Mechanisms of Cytosolic Access

The inherent properties of cells and cellular processes are important to understand when dealing with the delivery of cargoes to their site of action. An example that illustrates the relevance of considering the role of cells in the delivery process is given by von Gersdorff et al., who compared different cell lines and found evidence for cell specificity of the gene delivery process [139]. Particles for nucleic acid delivery typically possess a positive net charge, which allows carrier/cargo complexes to interact with anionic proteoglycans on the cell surface [23, 26]. Much of what is known about delivery with chemical carriers has been observed in the context of DNA delivery, which has a long history [64] of being applied (since the 1960s) [33, 140]. When assessing the cytosolic delivery of proteins, additional aspects need to be considered to ensure methods that avoid artefacts are chosen [16, 26, 141]. Since many carriers for cytosolic delivery of macromolecules are lipid based or polymer based, the following will mostly focus on these carrier types.

The nuclear membrane has to be crossed by some cargoes. Permeabilization of the nuclear membrane by the polymer PEI has been reported as one possible mechanism of nuclear entry, but is associated with toxicity [110]. Although nuclear delivery may occur without cell division [142, 143], it occurred to a lesser extent in cells that were non-dividing [144]. Nuclear access is possible upon nuclear breakdown during cell division, which is absent in quiescent and non-dividing cells. If cells were close to mitosis in the cell cycle, transfection was 30- to 500-fold higher with lipid- or polymer-based carriers [144–146]. Nuclear delivery also depends on circumventing degradation by cytosolic enzymes, for example, nucleic acids degrade rapidly within approximately 1–2 h after injection to the cytosol [8, 35, 147]. Complexation of DNA to PEI resulted in a tenfold increase in nuclear delivery compared to naked DNA that had been injected into the cytoplasm [142, 147, 148]. Complexation of cargoes with carriers can protect from degradation [8, 35, 149], but the functionality of cargoes inside cells in most cases also requires release from the carrier [8, 150]. For example, expression levels and the time point at which expression from cargo-DNA molecules starts were observed to depend on the release of cargo from the carrier [150–152]. Ideally, DNA remains complexed in the cytosol for protection against nucleases and dissociates from carriers to provide undisturbed access for the transcription machinery to the DNA [148].

An obstacle that needs to be overcome for DNA, RNA and proteins alike is the escape of cargoes from endocytic vesicles [16, 33]. Most carriers are taken up by endocytosis, endosomal entrapment has been described as common [19, 114, 153], and escape of siRNA is thought to be a rate limiting step for many carriers [99, 154]. Final readouts like transfection efficiency represent the result of a combined effect of all hurdles, which complicates identifying individual limiting barriers. However, endosomal escape is known as a major limiting factor and crucial determinant for delivering macromolecules to the cytosol [16, 26, 33, 128, 155, 156]. Even with the use of a lipid-based reagent for endosomal disruption, endosomal entrapment was still marked [19, 27], and the endosomal escape mechanism described by the proton sponge hypothesis is assumed to be generally low in efficiency [115].

Endosomal escape via a targeted disruption of endosomal membranes can be achieved by PCI, but most delivery strategies have to rely on other mechanisms for endosomal escape. Mechanisms for cytosolic entry that have been proposed include fusion with membranes, pore formation, transient disruption or lysis of endosomes [1, 33, 153].

Fusion has been discussed as a potential mechanism to use to enter the cytosol, and in vitro experiments have shown fusion of liposomes [157]. A minor entry path of

siRNA lipoplexes additional to endocytosis was assumed to potentially be based on fusion with the plasma membrane [158]. Continued delivery of cargo in the presence of various endocytosis inhibitors was suggested to indicate fusion as a major mechanism [159]. A certain structural arrangement has been associated with fusion [160]. Incorporating viral components into liposomes was proposed for generating "fusogenic liposomes" [161–164]. Carriers based on gold nanoparticle supramolecular assemblies have been proposed to act by fusion [71, 115, 165]. However, it can be difficult to determine whether fusion is the primary mode of delivery. Only part of the siRNA cargo of a lipid-based carrier was released from a vesicle to the cytosol [166], which was argued to be inconsistent with fusion, which was expected to result in complete cargo release [153]. Although lipoplexes were assumed to potentially fuse directly with the membrane by some [167–169], others reported the uptake to be energy dependent, and lipoplexes were concluded not to fuse with the plasma membrane, but to be endocytosed [169, 170].

Some bacterial peptides that associate with the rim of *pores* may stabilize open pores by reducing line tension [171]. From experiments with viral peptides, pore formation is known as one of several mechanisms for endosomal escape, but it can be highly size dependent, allowing only molecules of small size to pass. In the case of human rhinovirus serotype 2 (HRV2)-facilitated release of dextran from endosomes, ~ 27% of internalized 10-kDa-sized dextran molecules escaped from endosomes compared to only 2% of internalized 70-kDa dextran molecules [172]. The dimensions of macromolecules were argued by some to be too large for passing through pores potentially formed in the process of delivery by lipid- or polymer-based carriers [153, 156], making it an unlikely escape mechanism for lipoplexes or polyplexes [153]. Pore formation could also be conceived to influence the likelihood for endosomal lysis. Endosomal lysis was discussed as a potential escape mechanism [1]. Pore formation has been proposed to promote endosomal lysis [173], but also to counteract endosomal escape because leakiness of vesicles through pores would reduce osmotic swelling and potential bursting of endosomes [174, 175]. Alternatively, the fusion pore model and the transient pore model both suggest the vesicle remains intact [176].

The proton sponge hypothesis was described in the 90s as a possible explanation for how endosomal escape might occur [33, 108, 177], but has since been under discussion [1, 156, 175, 178, 179], because it cannot explain all observations sufficiently. It refers to carriers containing many nitrogen atoms that can be protonated and "soak up" protons like a sponge. Because acidification of endosomes via proton influx has to be balanced by Cl^- influx, buffering was expected to lead to unbalanced Cl^- ions and thereby cause osmotic pressure and endosome rupture. Protonation of polymeric carriers was additionally expected to result in charge repulsion causing expansion of the polymer network, which may further promote endosome rupture [177]. The proton sponge effect was, for example, assumed to be valid for polymers like PEI [108], PAMAM [180] and poly[2-(dimethylamino)ethyl methacrylate) (PDMAEMA) [33, 181].

Evidence arguing for and against the validity of the hallmarks of the proton sponge hypothesis, the buffering effect affecting acidification of endosomes, chloride accumulation and endosomal swelling, carrier expansion and further aspects have been analysed in detail in an excellent review by Vermeulen et al. [175]. Increased *buffering* capacity of carriers has been associated with increased transfection efficiency in some examples [175, 182–184], while the decrease of PEI's buffering capacity by chemical modification resulted in lowered transfection efficiency. If PEI was not protonated any more in endosomes, gene expression was reduced, consistent with reduced endosomal escape [175, 185, 186]. Evidence that does not seem to agree with the proton sponge hypothesis includes the observation that not all substances with buffering capacity induce endosomal escape and increasing the buffering capacity has not always increased transfection efficiency [175, 187]. For example, a polymer modified with an additional amine group per monomer to provide buffering capacity did not result in the expected effect, but decreased the transfection efficiency. The limiting factor for transfection efficiency was found to be endosomal entrapment of the polymer with the additional amine group, because transfection efficiency was restored by a membrane disruptive reagent. As a conclusion, it was doubted whether the proton sponge hypothesis has general validity considering the aspect of buffering capacity [187]. Also, results with PEI derivatives possessing a decreased buffer capacity but leading to markedly increased transfection efficiencies are not expected according to the proton sponge hypothesis [175, 188]. Therefore, buffering capacity and transfection efficiency might not always be correlated [33, 187, 189], so additional aspects may determine the final transfection efficiency and endosomal escape. *Acidification* towards pH 5 occurs quickly in endocytic vesicles, within only a few minutes [190]. Intracellular pH measurements seem to not always confirm the proton sponge hypothesis [178, 191]. Although polyplexes did not change the lysosomal pH and Benjaminsen et al., therefore, concluded the proton sponge effect not to be the dominant escape mechanism [178], acidification can be delayed even if the luminal pH of endosomes is not changed [33]. Consequently, buffering effects may not necessarily increase pH as a final result, but slowed down acidification of endosomes in the presence of buffering polymers was reported, consistent with the proton sponge hypothesis [175, 178, 182]. Evidence has been provided for proton influx being accompanied by *chloride ion influx*, and vesicles have been observed to

increase in size with PEI [182]. Although this is consistent with unbalanced chloride ions causing *osmotic swelling of vesicles*, it has been questioned whether vesicles are likely to reach the osmotic pressure required for rupture [175, 192]. If not entirely responsible for making endosomes rupture, osmotic pressure could still be imagined to aid in disrupting membranes [175, 192]. The size of endosomes is cell-type dependent, and smaller vesicles rupture more easily unless potential vesicle leakiness does not exceed a certain threshold, above which it will not rupture independently of its size [174, 175]. Even only a few bursts of vesicles might be sufficient for transfection, if they occur [175, 179]. While burst-like release from endosomes associated with polymer-based carriers was reported by some [179, 193], no bursting of endosomes was observed by others for a lipid-based carrier [99]. Even burst-like release of cargoes from endosomes delivered by polymer-based carriers did not lead to complete lysis of endosomes, but endosomes remained intact after a sudden local release event [179]. For a lipid-based carrier, cargo release was not altered by a proton pump inhibitor [99], and results were consistent with a mechanism based on lipid exchange [99, 194]. *Expansion of carriers* has been proposed to assist endosomal escape by causing mechanical pressure [156, 195]. According to the "umbrella hypothesis", electrostatic repulsion of groups that become charged upon protonation in a polymer causes the polymer to expand from a collapsed to an extended conformation, which might aid endosomal escape. An increase of volume by swelling of the polymer has indeed resulted in higher transfection efficiency, for example, fractured PAMAM dendrimers having an increased volume change upon protonation caused higher transfection efficiency compared to intact dendrimers [196, 197]. A pH-responsive polymer increased its diameter from ~ 200 to ~ 550 nm [198]. Transfection efficiency was higher for nanoparticles with higher swelling [199]. Carrier expansion might be a contributory factor to endosomal escape among further mechanisms [175, 191, 198].

4.2 Updated Model for the Mechanism of Endosomal Escape and Further Hypotheses

Successful escape from vesicles most likely occurs after multiple mechanisms have acted together [200]. Revised proton sponge hypothesis versions and further endosomal release theories have emerged and been discussed [33, 153]. Schubert et al. discusses the models of "polyplex hole formation" and "free polymer hole formation" [33]. These models differ from the proton sponge hypothesis in taking charge-driven interaction of carriers with the membrane into account, which might complement the proton sponge hypothesis in explaining endosomal escape, as also suggested by Vermeulen et al. [33, 175].

Buffering, swelling of vesicles, carrier expansion upon pH change, local membrane destabilization and potentially further aspects might influence endosomal release separately or combined, and the escape mechanism has to be considered individually for each carrier [33, 175, 200]. Based on the discussion by Vermeulen et al., an endosomal escape mechanism can potentially be imagined as follows: a polymeric carrier buffers protons, leading to both osmotic pressure causing swelling of endosomes and to charge repulsion causing expansion of the polymer. A local perturbance of the membrane may eventually trigger disruption of the already swollen and potentially mechanically, by expanded polymers, strained endosomal membrane [175]. According to Rehman et al., the release of endosomal contents originates from a particular region, which might point to the local disturbance [179]. However, if membrane destabilization does not lead to "pinching" of a vesicle that is close to bursting, it might instead lead to leakiness of the vesicle, which may counteract the build-up of osmotic pressure and render endosomal escape inefficient compared to bursting endosomes [174, 175].

Interaction of carriers with membranes may, therefore, be an important factor for endosomal escape. Simulations, studies with supported lipid bilayers (SLBs), in vitro experiments with the patch clamp method and atomic force microscopy have been used to analyse the effect of polymers on membranes [201–204]. Artificial lipid bilayers and cell membranes are damaged by polycationic organic nanoparticles, for example, membranes are damaged by PEI, PAMAM or PLL [201–203, 205–207]. Cationic polymers were found to cause membrane hole formation and thinning and disruption of membranes [201, 202, 205]. For example, PEI was found to interact with and disrupt lysosomal membranes by electron microscopy [208]. Studies with SLBs revealed thinning of membranes or hole formation induced by cationic polymers [201], and membrane defects were detected in living cells using patch clamp [203]. There is evidence that free PEI polymers, i.e. polymers that are not complexed with cargo, can play an important role in destabilizing membranes. Non-complexed PEI contributes to high transfection efficiencies, but also to toxicity [209, 210]. Free polymer was suggested to interact with membranes in a "carpet structure", causing defects or intercalating into the plasma membrane even before endocytosis [33, 210]. Lipid-based carriers were suggested to engage in destabilization of endosomal membranes [211] and were reported to cause perturbance of endosomal membranes by promoting the transition from bilayers to nonbilayer lipid structures [194]. An event that was suggested to initiate the process described as the "fusion pore model" is the occurrence of anionic lipids in the inner leaflet of the endosomal membrane, which have by "flip-flop" turned from the outer cytosolic to the inner

luminal leaflet of the endosomal membrane, promoting lipid mixture and cargo translocation [176]. The "flip-flop" mechanism was proposed to be induced by the carrier/cargo complex and assumed to have initiated endosomal escape of oligonucleotides delivered by a cationic lipid-based carrier [212]. Cationic lipid-based carriers may interact with anionic lipids that have, upon destabilization, rearranged by flip-flop to the inner monolayer of endosomal vesicles and promote lipid mixing and endosomal escape [176, 211]. The biomimetic reagent TAT, which is also cationic, has been found to induce lipid mixing in endosomes [213, 214].

Nanoscale hole formation or local destabilization of the membrane while the whole endosome remains intact was proposed as a possible consequence of polymer interaction with membranes and is assumed to be a charge-driven local destabilization leading to transient nanoholes [33, 179, 201]. This is consistent with rupture of entire vesicles being unlikely or rare and with the observation of cargo release by transient local bursts instead of complete vesicle lysis by live imaging [33, 99, 179].

Vesicle budding and collapse is a model proposed to explain macromolecular escape consistent with escape under maintained endosomal integrity [153]. It has been questioned whether the size of holes formed in the presence of carriers would be sufficient to let cargoes or their complexes with carriers pass [33, 153, 215]. According to this model, membrane curvature is induced in the membrane, followed by budding off of a small entity, which is assumed to subsequently disintegrate in the cytosol, releasing its contents while leaving the endosome behind intact [153]. The model of vesicle budding and collapse was described for cell-penetrating peptides [153, 216], but not for other carriers. Authors argue it might potentially apply to further carriers and explain escape even if membrane holes are too small for letting macromolecules pass.

Some carrier types may induce endosomal escape upon *disassembly*, which has been proposed to cause an osmotic shock due to the increased particle number, leading to vesicle rupture [156, 217]. The mechanism of escape for a type of hydrolysable polymersomes was furthermore suggested to involve action as a detergent upon degradation, thereby, destabilizing the endolysosome [200, 218].

Apart from the mainly carrier-focused models for endosomal escape mentioned before, *less carrier-focused* mechanisms have been proposed. Leakiness of vesicles has been suggested as a possible mechanism contributing to endosomal escape, has been attributed to macropinosomes including macropinosomal lysis [99, 219] and is cell-type dependent [174]. Also, naked RNA has been reported to be delivered, i.e. delivery of RNA without any carrier-induced silencing [45, 220], although the mechanism by which it escapes macropinosomes and reaches the cytosol is still incompletely understood [42, 47].

If the mechanism of endosomal escape is qualitatively correctly described, it might eventually be the efficiency with which this mechanism acts that governs the number of molecules able to reach the cytosol or nucleus.

4.3 Efficiency

High apparent endosomal escape efficiency has been attributed to some reagents like dimeric fluorescent TAT (dfTAT), although the percentage of molecules that entered cells was acknowledged to be small compared to the extracellularly administered amount [148]. PCI-mediated delivery has been reported to allow efficient endosomal escape [129, 130].

Detectability of endosomal escape by conventional fluorescence microscopy is unusual, because dilution of cargoes from burst endosomes in the cytosol often renders them undetectable [99]. Gilleron et al. used electron microscopy to detect escaped cargo, because only a few hundred cargo molecules might escape from endosomal compartments and release from bursts of vesicles was rare, as indicated by the stable numbers and contents of vesicles over time. Therefore, escaped cargo might not be detectable by standard fluorescence microscopy [99]. Often, endosomal escape is detected by indicators that are sensitive to even small amounts of escaped cargo. For example, gene knockdown was reported to require only 2000 cytosolic siRNAs or fewer [166]. A number of 500 siRNAs per cell allowed a 50% target reduction in vivo in rats [221], and an estimated number of 370 siRNA copies per mouse liver cell was required for a 50% knockdown, being within a similar range [222]. For DNA as a cargo molecule, a number of 75–3000 copies of plasmids in the nucleus was sufficient to induce measurable transgene expression [36]. Further readouts employed as indicators of endosomal escape include toxicity or enzymatic activity, like that of beta-lactamase or Cre recombinase. Toxicity as an indicator for endosomal escape can be highly sensitive, because single toxin molecules can be sufficient to kill a cell [61, 62, 223]. For beta-lactamase activity, 50 molecules in a single cell have been sufficient for detection [224]. For Cre recombinase, four Cre molecules are theoretically sufficient for recombination [148]. Therefore, proof of endosomal escape does not always originate from escape of large amounts of cargoes if detection methods are highly sensitive.

Only approximately 1–2% of siRNA applied with a lipid-based carrier was reported to escape into the cytosol from endosomes [99]. A similar range with about 3.5% of internalized siRNAs reaching the cytosol was reported for siRNA associated with a lipid-based carrier by another study [166]. Naked RNA was reported to be capable of entering the cytosol as well, but fewer than one in 10,000 molecules typically reach the cytosol [42].

For plasmids delivered by lipid-based or polymer-based carriers, only 1–5% of the total applied doses were reported

to reach the nuclear fraction, corresponding to between 75 and 50,000 plasmids per nucleus, depending on the applied DNA dose [36]. Only 10–20% of the total dose was detected in total cell lysates after application of lipid-based or polymer-based carriers [36]. Glover et al. analysed the endosomal escape efficiency and nuclear delivery of a polymer-based and a lipid-based carrier by real time polymerase chain reaction (PCR) and flow cytometric detection of protein expression. Depending on the carrier, per cell, 0.8% and 0.4% of the total amount of DNA cargo administered reached the nucleus. Approximately 350 plasmids/h were delivered within the first 8 h of exposure, and after 24 h of exposure, LipofectAMINE2000 (LF2K) resulted in the delivery of 8.3×10^3 plasmids and PEI in the delivery of 1.8×10^4 plasmids to the nucleus from a total of 4 µg of plasmid (2.2×10^6 plasmids per cell) to which cells were exposed [225]. According to Gilleron et al., approximately 200 siRNA-gold particles escaped endosomes per cell during a 6-h time period in HeLa in vitro or hepatocytes in vivo, which corresponds to a maximum of 2000–4000 siRNA molecules per cell [99]. In HeLa cells, endosomal escape occurred in only 10% of the endosomes containing polyplexes, although endosomes were small and, therefore, even pose favourable conditions for escape [174].

Biomimetic approaches are not the focus of this review, but for comparison the TAT peptide and Antennapedia peptide (pAntp) were reported to result in an endosomal escape efficiency of ~ 2% [148, 226, 227]. The pAntp was reported to result in ~ 2% of internalized peptides reaching the cytosol, which was only 0.015% of the total amount applied, resulting from an experiment in which 10^6 cells were incubated for 2 h with 1 µM pAntp (corresponding to ~ 1.2×10^8 molecules/cell; 9×10^5 molecules/cell pAntp were internalized and 1.8×10^4 molecules/cell reached the cytosol) [148, 227].

DNA delivery was found by Rehman et al. to originate from only one to two polyplexes per cell on average [179]. Complexation to a carrier can protect cargoes until the nuclear barrier has been overcome, as the comparison of microinjected free and complexed DNA has demonstrated. While less than 0.1% of naked DNA reached the nucleus after microinjection, 1% of DNA in a polyplex reached the nucleus after microinjection [142]. The same plasmid number in the nucleus may not always result in the same expression levels, but also depends on how accessible plasmids are for the cellular expression machinery, for example, a similar number of plasmids per nucleus resulted in a ten- to 100-fold higher luciferase expression if plasmids had been delivered by lipofectamine compared to those that had been delivered by PEI [36]. The efficacy of the cargo at its site of action may further be related to its individual mode of action. Knockdown has been observed to be non-proportional: only 370 copies of siRNA mediated a 50% knockdown, but to reach an 80% knockdown, 2200 copies of siRNA were required [197, 222]. Protein expression from DNA may reach saturation, even if the number of plasmids/nucleus continues to rise. For example, with a 3.5- and 17.8-fold increase in plasmids/nucleus, protein expression increased only 1.3- and 1.4-fold in a mouse melanoma cell line [36]. With 90% of CRISPR Cas9 (clustered regularly interspaced short palindromic repeats associated protein 9) co-delivered with guide RNA to HeLa cells, a gene editing efficiency of up to 30% was reached [69]. The speed of delivery has been observed to be rapid, with endosomal escape occurring within 5–15 min of endocytosis or as reported by Wittrup et al. [166].

In vivo, the most easily accessible tissue is liver tissue, but efficiency is low even in liver tissue, with only 0.1% of total siRNA delivered, while the remaining fraction is degraded or does not reach the cytosol [197, 222]. In vivo delivery by local injection into the cochlea of mice resulted in Cas9:single guide RNA (sgRNA) complex-mediated genome modification in 13–20% of outer hair cells near the injection site [28].

4.4 Endocytosis/Intracellular Trafficking and Its Relevance to Escape

The question might arise as to whether the barrier that carriers have to overcome for escaping endosomes is independent of the endocytic pathway, or whether the uptake route may influence delivery efficiency. Phagocytosis mainly allows special cell types (phagocytes) to internalize large particles like pathogens and target them to the lysosome for degradation. Pinocytosis includes the endocytosis subtypes clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis (CvME), clathrin- and caveolae-independent endocytosis or macropinocytosis [228, 229] and serves functions like nutrient uptake or cellular signalling [229, 230]. Macropinocytosis allows uptake of larger volumes of extracellular fluid, and the formation of membrane ruffles preceding macropinocytosis can be triggered by growth factors, viruses or bacteria [231].

Although the mechanism by which *intracellular trafficking might affect delivery efficiency* is not fully understood, there is evidence for an influence of the uptake route on final delivery efficiency [232–235]. Transfection with PEI was most efficient when uptake took place via CvME [234], which was not associated with lysosomal localization of polyplexes [233, 235]. Clathrin-mediated uptake resulted in lower transfection efficiency and lysosomal degradation [233]. Contrary to these findings, in a study with lipid- and polymer-based carriers, high transfection efficiency was found to be associated with CME, but not caveolae-mediated uptake and the endocytic route taken was more decisive than the amount of internalized

particles [236]. A difference in internalization efficiency and cytosolic delivery efficiency was also observed in an example of transferrin-receptor-mediated uptake of polyplexes. More than 95% of cells had taken up transferrin polyplexes, but cytosolic delivery was moderate, requiring chloroquine for enhancement [237]. CME followed by macropinocytosis was a delivery route for siRNA and a lipid-based carrier, with a major contribution of macropinocytosis to gene silencing [99]. A comparison of uptake into four different cell lines showed internalization to occur through multiple endocytic pathways, but mostly only one endocytic pathway resulted in cargo delivery. Which pathway was productive was cell-type dependent [169]. Besides depending on the carrier, uptake routes are generally highly cell-type dependent [33, 238]. In HEK293 cells, RNA interference (RNAi) was facilitated via Graf1-mediated endocytosis (GME) [169], a pathway via which also dextran is internalized [239]. Arf6-dependent endocytosis (ADE) initiated RNAi in HeLa and HepG2 cells [169]. Blocking "non-productive pathways", i.e. pathways that did not contribute to cytosolic delivery, could be a strategy to improve delivery efficiency by shifting cargo uptake to "productive pathways", as demonstrated by Vocelle et al. [169].

Autophagy was reported to lower delivery efficiency [240] and can be triggered by carriers [240, 241] by carrier-induced damage of endosomal membranes [166, 241]. Autophagy is considered to be a barrier to delivery by some [1, 240], but others argue it may have no influence on delivery efficiency, with endosomal escape occurring earlier than autophagy [1, 166].

Recycling may affect gene delivery efficiency [242, 243]. Recycling was reported to limit the cytosolic delivery of siRNA, and up to 70% of internalized siRNA lipid complexes were exocytosed. Lack of a regulator of recycling pathways increased retention of carriers in the cells and increased gene silencing [242].

Intracellular trafficking and how deeply cargoes are transported into the cell is relevant for DNA delivery [197]. PEI/DNA complexes were transported within minutes along microtubules to the perinuclear region [244]. Uptake of cargoes exclusively at the periphery, e.g. by fusion with the plasma membrane, would be disadvantageous for nuclear delivery because large complexes typically sized 80–500 nm diffuse slowly through the cytoplasm without active transport [197, 245]. Further, it is preferable if destabilization of membranes occurs in the endolysosomal system, because plasma membrane destabilization affects cell viability [191]. Exposure to the cytoplasmic environment for prolonged time periods increases the risk for cargo degradation [147].

The *size* of endosomal vesicles may influence escape efficiency, because it is relevant for a vesicle's propensity to rupture [174]. The internalization route can be influenced by particle size [39, 246]. DCs efficiently internalize particles sized below 500 nm, which comprises the typical size range of viruses (~ 20–450 nm) [247, 248]. Macropinocytosis allows uptake of large particles, macropinosomes having a size range of approximately > 0.2–5 µm [249–251]. CME can be an uptake route for particles with sizes of ~ 200 nm or less. Clathrin-independent endocytosis was suggested to internalize particles larger than 200 nm and up to at least 500 nm in size. This might be relevant for delivery efficiency, because 200-nm-sized particles, but not 500-nm-sized particles, trafficked to late endosomes and lysosomes in the murine melanoma cell line B16 [39]. However, it is cell-type and carrier-type dependent which endocytic pathway is productive in contributing to cytosolic cargo delivery [139]. For example, CME was a productive pathway for a lipid-based carrier in HeLa cells or for a polymer-based carrier in Cos-7 cells. For other cell line and carrier combinations, clathrin-independent endocytosis was the major productive uptake route, or more than one pathway was productive [139, 233]. Liposomes and polymersomes typically have sizes within a broad range of several tens of nanometres to up to more than 10 µm [94, 97]. Lipid- or polymer-based nanoparticles are typically in the range of several tens to several hundreds of nanometres [1]. Polymer nanocontainers are typically sized 10–500 nm [93]. Inorganic carriers vary much in size, depending on the individual carrier type, ranging from below 10 nm to several micrometres [1]. Besides their individual particle diameter, inorganic nanoparticles can form larger superstructures assembled from smaller particles [71, 115]. The most efficient delivery was observed for particles sized 130–215 nm in a study with different carriers and a myoblast cell line [236]. A size range of 10–60 nm has been suggested to be most suitable for internalization of most nanoparticles into non-phagocytic cells. Although smaller nanoparticles were found to internalize better, those that are below 30 nm were also reported to be insufficient activators of endocytosis, narrowing optimal size ranges further [252].

Possible unwanted fates of internalized material include trafficking to recycling endosomes followed by exocytosis or trafficking to lysosomes or uptake into autophagosomes followed by transfer to lysosomes before endosomal escape occurs [33]. Impaired integrity of lysosomes may cause cell death [253, 254], rupture of lysosomes can trigger apoptosis [156, 255, 256] and, therefore, might not be a suitable escape mechanism. There is a limited window of opportunity within which escape may occur, an *optimal time point for escape* likely being between early endosomes and fusion with lysosomes [33, 156]. Results from experiments with siRNAs and lipid-based carriers were consistent with escape from early endocytic structures that are hybrid compartments sharing characteristics of early and late endosomes [99]. In experiments with Niemann-Pick C1 protein (NPC1)-deficient cells, accumulation in enlarged late endosomes

was associated with greater gene silencing efficiency, suggesting late endosomes as sites of escape for lipid-delivered siRNA [242]. Late endosomes were also the preferential site of escape for oligonucleotides applied in the presence of small molecule compounds for enhancing endosomal release [257]. Endosomal release was observed in a narrow time window of only ~ 10 min for siRNA with a lipid-based carrier [166]. The window of opportunity for escape may broaden if trafficking is delayed. The formation of early/late endosome hybrid compartments with delayed trafficking to lysosomes could be imagined to allow cargoes to spend more time in a compartment from which escape may occur [99, 242, 258]. Impeding intracellular trafficking to lysosomal compartments was proposed to re-route cargoes into a novel compartment and increase the chance of cargo escape from vesicles [176, 259]. Also, the amount internalized in cells that is not exocytosed, i.e. the general retention of cargo in cells, may influence escape efficiency [242, 243]. The window of opportunity for cytosolic entry might, therefore, be broadened by escape from recycling pathways and influenced by the amount of cargo and residence time within the cell. Nevertheless, the efficiency of endosomal escape can still vary substantially, even if all treated cells have internalized carriers and cargoes [236, 260]. Analysing internalization routes of materials into cells requires a certain scrutiny when interpreting data, because small molecule inhibitors commonly used for endocytic pathways often lack specificity [261]. Furthermore, continued uptake of a substance under inhibition of a pathway is not always informative, because the pathway is either not involved in uptake or it is involved but not detected, because the blocked pathway is compensated for by activation of another pathway [262]. Eventually, endocytic pathways themselves may have been misinterpreted in the past, caveolin-positive vesicles potentially carrying ubiquitinylated caveolin targeted for degradation instead of representing a compartment type [263].

5 Preclinical and Clinical Applicability

5.1 Extracellular Barriers

In addition to intracellular barriers, extracellular barriers need to be overcome for therapeutic application (barriers summarized in Fig. 1). Only seven of 1000 delivered carriers entered a tumour in mouse models according to a comparative literature analysis spanning a time period of 10 years until 2016 [264]. Some extracellular barriers apply generally, and some are specifically relevant to cytosolic delivery of macromolecules. General factors include biodistribution, access to target tissues or target cells, in vivo stability and circulation half-lives of the drug. Specific barriers for in vivo cytosolic macromolecule delivery may be caused by in vivo effects affecting intracellular trafficking or endosomal escape. Toxicity beyond the cellular level may also occur, for example, PEG may exhibit renal toxicity at high doses [265].

The administration route influences the distribution of particles; for example, after intravenous administration, a polymer has been observed to mainly target the liver and spleen, while subcutaneous or intranodal administration resulted in accumulation in local lymph nodes [90, 266]. Particle distribution across organs is size dependent [267, 268], and small particles with diameters of 6 nm are rapidly cleared by the kidneys [64, 269].

Depending on administration route and target organs, physical barriers like mucus layers in the lung can be relevant, or the blood–brain barrier, as an obstacle to delivery to the brain [90]. Leaving the bloodstream and accessing target tissues requires overcoming endothelial layers, which have different permeability (gaps in the range of 1 nm to over 100 nm depending on tissue) [8]. A special case is tumour vasculature, which is leaky and, therefore, potentially advantageous for drug delivery. The "enhanced permeation and retention effect" (EPR effect) has been proposed as a mechanism to explain preferential accumulation of drugs in tumours [8, 270]. However, the impact of the EPR effect is not entirely clear and may be limited if only 0.7% of injected nanoparticles reach the tumour site [90, 264, 271]. An interaction of only 2% of tumour cells with nanoparticles was reported in one case [271]. In tumour microenvironments, special conditions prevail, posing additional barriers, including limited perfusion of nanoparticles into the tumour, dense extracellular matrices and degradative enzymes [90, 271–273]. Overproduction of extracellular matrix (ECM) as a dense mesh may pose a physical barrier to nanoparticle delivery [90, 264, 271], which is particularly true for cationic nanoparticles that interact with the negatively charged ECM [90].

Discrimination between target cells and non-target cells can be required for therapy. If cargo delivery is non-specific, promoters could be used for nucleic acid cargoes that allow tissue-specific cargo activity. Targeted delivery using receptor-specific antibodies can be another strategy. Targeted delivery has been attempted including brain, liver, lung or tumour tissue targeting [64].

5.2 Extracellular Barrier: In Vivo Stability

Stability issues related to in vivo conditions may include chemical and colloidal stability. Cargoes can be exposed to enzymes like, for example, RNAses in the extracellular space [274]. The in vivo environment can affect stability by affecting the nanoparticle's aggregation propensity [275]. High ionic strength in biological fluids may affect the tendency of nanoparticles to aggregate [275, 276]. For

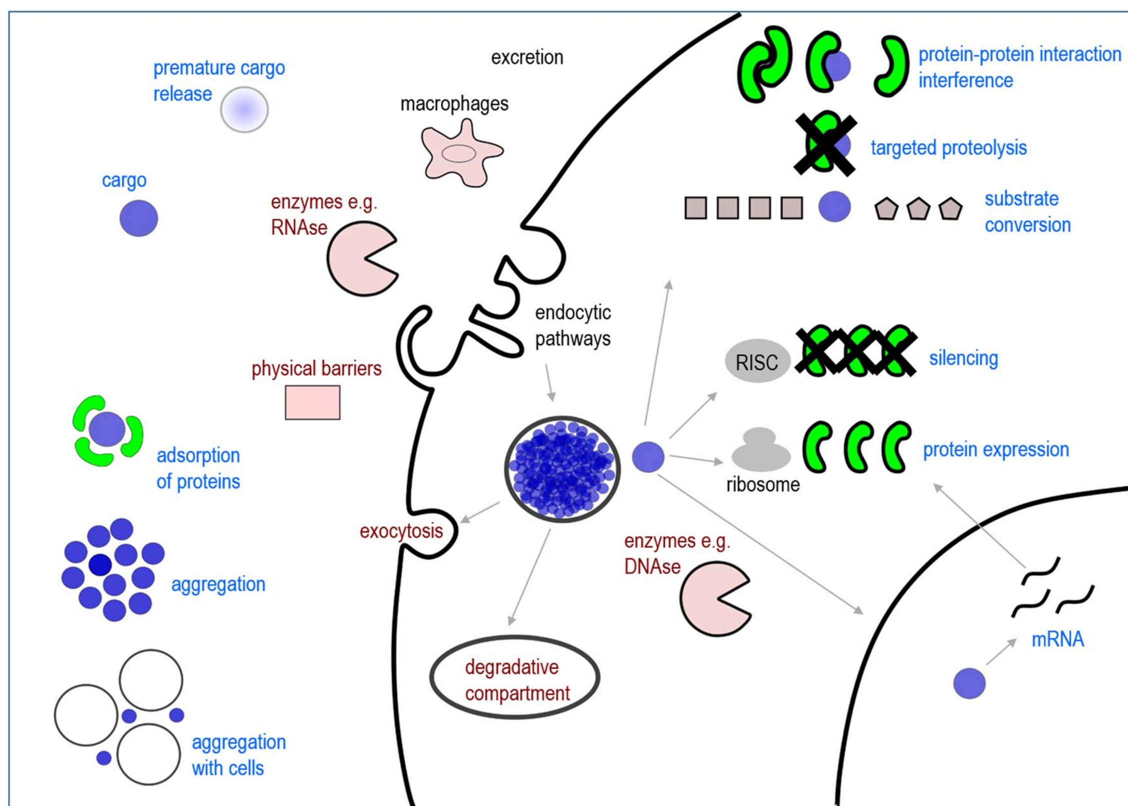

Fig. 1 Summary of intracellular and extracellular barriers. Chemicals as carriers for the delivery of macromolecules like DNA, RNA or proteins typically associate with their cargo and are internalized by the cell via one or several endocytic pathways. Intracellular barriers to delivery include endosomal entrapment, exocytosis or elimination in a degradative compartment before endosomal escape, the presence of degrading enzymes and insufficient release of the cargo from its carrier at its site of action. Extracellular barriers for cargo delivery include premature cargo release from the carrier and adsorption of proteins from biological fluids like serum proteins, which may for instance affect tissue distribution. Extracellular barriers may be posed by changed aggregation behaviour of carrier/cargo complexes in the extracellular space (e.g. aggregation of complexes to larger particles or formation of aggregates with cells in the blood), which can influence intracellular delivery and may even pose a risk for blocking capillaries. Extracellular barriers further include excretion mechanisms, elimination by the immune system or degradation of cargoes by extracellular enzymes and physical barriers. *DNAse* deoxyribonuclease, *mRNA* messenger RNA, *RISC* RNA-induced silencing complex, *RNAse* ribonuclease

example, DNA/PEI complexes aggregated at physiological ion concentrations to large particles of up to approximately a micrometre in size [275, 276]. Carriers may also form aggregates with cells in the blood stream [277]. Aggregate size may influence the endocytic uptake route taken [278], and if aggregate sizes are large enough, they embolize capillaries [8, 279]. Shear forces and the adherence of serum proteins like albumin contributed to the dissociation of polymer micelles as observed from in vitro experiments and in blood vessels of mice [262, 280].

Non-specific adsorption of biomolecules on nanoparticles from the environment can form a “corona” on the particle surface in vivo [90, 156, 275, 281, 282], and its composition depends on particle-size and particle-surface properties [283] as well as tissue-specific presence, adsorption or desorption of proteins in the environment of the particle [90, 284]. The corona can influence tissue distribution, cellular delivery efficiency and circulation times of the drug [90, 285, 286]. As an example, if apolipoprotein E (ApoE) is adsorbed to carriers or if components of the carriers mimic ApoE, this influences the distribution of carriers by targeting to low-density lipoprotein (LDL) receptors and subsequent delivery to hepatocytes [90, 286, 287]. Plasma proteins that have been reported to interact with lipid-based carriers and to influence their in vivo performance include highly abundant proteins like albumin, which may cause destabilization of particles or proteins that promote clearance by the immune system like complement proteins or immunoglobulins [288]. The immune system may contribute to rapid clearance via phagocytes if molecules in the corona act as opsonins [289, 290]. In an analysis of lipid-based carriers, it was observed that if carriers disintegrated in the presence of serum proteins with cargo release, in vivo transfection efficiency was poor, but carriers that aggregated and only slowly disintegrated upon exposure to serum proteins were efficient at transfecting cells [291].

Shielding agents like PEG have been used for carriers to prevent aggregation and to prolong blood circulation time [90, 277]. However, PEG may also induce anti-PEG antibodies and result in accelerated blood clearance [292].

Carriers are more rapidly cleared in vivo if they are cationic and have the longest half-lives if they are neutral [268, 293].

Circulation half-lives are influenced by excretion and clearance by the immune system. The optimal size of nanoparticles should be above 10 nm for preventing excretion via the kidneys, but has been suggested to ideally be not larger than 200 nm, a size that fits well through microcapillaries [156, 294].

5.3 Extracellular Barriers for Macromolecule Delivery to the Cytosol in Particular

Intracellular barriers that are specific to the in vivo environment add to the general intracellular barriers. The "biological identity" of particles that forms through adsorption of biomolecules in the in vivo environment may influence delivery if affecting endocytosis. Indeed, the corona of biomolecules on particles was reported to affect the quantity and also the mechanism of uptake. For example, protein coronas can decrease the uptake of particles and also may influence by which endocytic pathway they are mainly taken up [295–297]. A mechanism by which the endocytic pathway taken can be affected by biomolecule coronas is the promotion of receptor-mediated endocytosis via a biomolecule that is enriched in the corona and can act as a ligand for a specific receptor [297].

The formation of protein coronas can be counteracted by PEGylation, which is also beneficial in prolonging circulation half-lives, but PEGylation has been found to inhibit endosomal escape [176, 298, 299]. Another example for a strategy to enhance in vivo utility that impairs cytosolic delivery is cross-linking the surface of a polyplex to increase "lateral" stability, which increased circulation half-life, but resulted in a decreased intracellular cargo delivery as indicated by decreased transgene expression [8, 300].

5.4 Examples of Clinical Potential and Clinical Applications

The long list of approved nanoparticles, polymer based, lipid based or inorganic, demonstrates the safety and clinical utility of chemical carriers [90, 301], although mostly not applied for cytosolic delivery of macromolecules. Magnetic, radioactive or optical properties make inorganic nanoparticles suitable for diagnostics or particular therapies like photothermal therapies [90]. An approved inorganic nanoparticle is NBTXR3 (Hensify®, Nanobiotix, Paris, France, EU), 50-nm particles from hafnium oxide, which act without delivering cargoes by physically enhancing radiotherapy [302, 303]. Liposomal drugs carrying small molecule chemotherapeutics as cargoes have been approved by the Food and Drug Administration (FDA) [90, 302], and nanoparticles are used for controlled release of small molecule drugs, protecting from rapid clearance in the body and allowing drug action with prolonged duration. Delivery of a small molecule by a stimuli-responsive polymeric carrier had an enhanced effect compared to free small molecules [304, 305]. A liposomal formulation of daunorubicin and cytarabine (VYXEOS®, Jazz Pharmaceuticals, Palo Alto, CA, USA) contains 100-nm-sized bilamellar liposomes, which encapsulate the two small molecule cancer therapeutics (chemotherapeutics) [302].

Nucleic acid delivery has been performed in vivo with lipid- and polymer-based carriers [9, 90, 306], but approved gene therapies deliver DNA often with viral vectors [307], non-viral carriers for DNA delivery having reached clinical trials [64, 307]. Lipid-based carriers for nucleic acids are clinically most advanced [302, 308, 309] and have been applied in vivo for siRNA [9, 310], mRNA [10–14] and DNA [311]. Clinical trials or drugs based on RNA, including approved single-stranded antisense oligonucleotides, are reviewed, for example, by Kim, Dammes and Peer, and Roberts et al. [312–314]. A comparison of the utility of DNA, RNA and proteins for carrier-mediated delivery of cargoes is given in Table 3.

Delivery of mRNA for expressing proteins can be used to express antigens for eliciting an immune response, as known from the RNA-based coronavirus disease 2019 (COVID-19) vaccines [10–14], or it can be used with the intention to express therapeutic proteins, including antibodies [43, 315, 316].

RNA expression levels sufficient for inducing immune responses via DCs may not be sufficient for other applications [42]. Therefore, expression levels need to be evaluated for the intended application, but were favourable in pre-clinical tests for a virus-specific mRNA-encoded antibody, which has progressed to a clinical trial. Mean systemic antibody concentrations of 3 μg/ml, 10 μg/ml and 78 μg/ml were reached in mice, and the latter two concentrations were sufficient to completely protect mice with a 100% survival rate after a lethal challenge with Chikungunya virus [60]. The feasibility of mRNA-encoded antibody therapy has been demonstrated in disease models for viral infection (rabies), toxication (botulism) and cancer (lymphoma) [43, 59]. Furthermore, if in situ expression leads to a high local concentration, the total amount of expressed antibody required for an effect may be lower, [43]. So far there are only a few pre-clinical trials and a phase I clinical trial for mRNA-encoded antibodies [55, 317]. DNA-encoded antibodies have also been suggested and pursued as a therapeutic strategy [318, 319]. Advantages of delivering mRNA-encoded

Table 3 Utility of carrier-mediated delivery of cargoes as drugs in comparison

	DNA	RNA	Proteins
Storage stability	+++	−/+	+
Clinical utility	Viral vectors have often been used for approved drugs [307, 331]	Approved drugs based on carrier-mediated delivery [13, 312, 313]	Less advanced than nucleic acid delivery

+++ indicates comparably high stability and tolerance towards various conditions (e.g. tolerance to high and low temperatures or to water withdrawal)

+ indicates medium stability with potential sensitivity towards several conditions including extreme temperatures or water withdrawal

−/+ indicates a tendency for comparably high sensitivity towards ubiquitous environmental factors often requiring freezing and storage at very low temperatures or the development of strategies to improve storage stability

antibodies compared to the administration of antibodies as proteins include the much more uniform physicochemical properties of RNA compared to proteins, making laborious optimizations of production parameters and storage buffers as required for proteins unnecessary [43]. Rapid production of RNA in large quantities is possible [43, 320, 321] without lengthy process optimizations as often required for each individual protein. Disadvantages of mRNA-encoded antibodies can be immunogenicity and activation of pattern recognition receptors and inflammatory responses, which can be desirable for vaccinations, but not for all applications of mRNA-encoded antibodies [42, 43]. Another disadvantage of RNA therapeutics compared to the administration of antibodies as proteins are effects originating from the carrier, like carrier-mediated toxicity with activation of the complement system and the risk for complement activation-related pseudoallergy (CARPA) [43, 322].

Patisiran is an siRNA-based drug that is administered intravenously and was approved in 2018 in the USA and the EU as the first clinically approved RNAi drug [9, 302]. Patisiran is a formulation of siRNA with a lipid-based carrier composed of DLin-MC3-DMA (6Z, 9Z, 28Z, 31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl-4-(dimethylamino) butanoate)/DSPC (1,2-Distearoyl-sn-glycero-3-phosphocholine)/cholesterol and PEG2000-C-DMG (3-N-[(ω-methoxy poly(ethylene glycol)2000) carbamoyl]-1,2-dimyristyloxy-propylamine), see also ClinicalTrials.gov identifier: NCT01960348 and clinical study protocol ALN-TTR02-004 and Assessment Report EMA/554262/2018 , an ionisable lipid formulation that has been suggested to be composed of micellar structures of cationic lipids with RNA, which is eventually coated with polar lipids like PEG and phospholipids [323]. Compared to initial lipid-based carriers containing DLinDAP (1,2-dilinoleoyl-3-dimethylamino-propane), the effective dose of DLin-MC3-DMA-containing lipid-based carriers has with 0.005 mg/kg improved by more than three orders of magnitude, showing how carrier optimization allowed a remarkable improvement towards clinical utility [310, 323]. The ionisable lipid DLin-MC3-DMA is mostly uncharged at neutral pH and becomes cationic at low pH, with approximately 80% of lipid being charged at endosomal pH 5.5 [123, 310]. The cationic version of ionisable lipids is thought to interact with anionic lipids of the inner part of the endosomal bilayer and disrupt the membrane after changing it from a lamellar to a hexagonal phase [324], and DLin-MC3-DMA is considered to be the key component enabling endosomal escape [123]. Patisiran is indicated for the treatment of hereditary transthyretin-mediated amyloidosis (hATTR), a disease that is characterized by the accumulation of aberrant proteins forming aggregates and destroying tissues, including that of peripheral nerves and the heart. The aggregates originate from a mutated version of a protein that is predominantly produced by the liver. Therefore, targeting of the drug to the liver is an advantage [9].

Liver targeting occurred because the lipid-based carrier adsorbs ApoE from the blood, which directs the drug to hepatocytes [313, 325]. Previous research had already found siRNA delivered by a lipid-based carrier to accumulate primarily in the liver, spleen and kidney [326]. Targeting of an siRNA drug to the liver by conjugating it to a ligand for a receptor on hepatocytes is achieved by givosiran (GIVLAARI®, Alnylam Pharmaceuticals, Cambridge, MA, USA), a drug that was approved in 2019 [313]. Givosiran is an siRNA conjugated to *N*-acetylgalactosamine (GalNAc), which induces receptor-mediated internalization into hepatocytes [313].

Although naked RNA can induce an effect [42, 45], lipid-based carriers are used for the mRNA-based COVID-19 vaccines [10–14, 313]. The approved or most advanced mRNA-based COVID-19 vaccines from BioNTech, Moderna and CureVac use carriers with ionisable cationic lipids and a similar composition of helper lipids [13]. Very similar to patisiran, RNA-based COVID-19 vaccines use carriers with the formulation "ionisable cationic lipid/DSPC/cholesterol and PEG-lipid", differing in molar lipid ratios and in the exact type of ionisable cationic lipid or PEG-lipid that is used. The ionisable cationic lipid ALC-0315 (Acuitas) is used in the vaccine BNT162b2 (Comirnaty®, BioNTech,

Table 4 Examples for therapeutic application areas of approved drugs based on delivery mediated by chemical carriers

Therapeutic area	Cargo	Carrier/delivery mediator	Drug name	References
Hereditary disease (hATTR)	siRNA	Lipid-based	Patisiran	[9]
Viral infection (COVID-19 vaccine)	mRNA	Lipid-based	BNT162b2 mRNA-1273	[11–13]

COVID-19 coronavirus disease 2019, *hATTR* hereditary transthyretin-mediated amyloidosis, *mRNA* messenger RNA, *siRNA* short interfering RNA

Mainz, Germany, EU/Pfizer, New York City, NY, USA), the vaccine VCnCoV from CureVac (Tübingen, Germany) also uses an Acuitas lipid and the vaccine mRNA-1273 (Moderna COVID-19 Vaccine®, Moderna, Cambridge, MA, USA) uses SM-102 as an ionisable cationic lipid. The substances PEG-lipid ALC-0159 in BNT162b2 (2-[(polyethylene glycol)-2000]-N,N-ditetradecylacetamide, see Assessment Report EMA/707383/2020), PEG-lipid (undisclosed for VCnCoV) or PEG-DMG in mRNA-1273 (1-monomethoxy-polyethyleneglycol-2,3-dimyristylglycerol with polyethylene glycol of average molecular weight 2000, see Assessment Report EMA/15689/2021) are used as helper lipids [13, 14]. Adsorption of ApoE to lipid-based carriers could potentially also play a role in targeting cell types that are relevant for vaccination, like DCs, which also highly express LDL receptors [13, 327]. Therapeutic application areas are listed in Table 4 for approved drugs based on chemical carrier-mediated cytosolic delivery.

5.5 Examples for in Vivo Delivery of Protein Cargoes into the Cell

Intracellular delivery of proteins in vivo is challenging but desirable, as efforts towards this goal indicate. Intravenous injection of lipid carriers with protein cargoes was, for example, performed in mice. Horseradish peroxidase and a variant of avidin served as model proteins. As a strategy for complexation with the lipid-based carrier, protein cargoes had been conjugated to oligonucleotides [328].

In vivo delivery of the genome editing proteins Cre and Cas9 with a lipid-based carrier has been reported [28, 329]. Negatively supercharged Cre recombinase or Cas9:sgRNA was complexed with a redox-responsive lipid via electrostatic interaction [329]. Redox-responsive properties of a carrier might facilitate release of the cargo from the carrier in the reducing environment of the cytosol [19]. Gene expression was induced after local injection of Cre/lipid complexes into the brain of a mouse line with a Cre-excisable stop-codon that prevents the expression of the fluorescent protein tdTomato. Expression of tdTomato occurred only in a very small region around the injection site in the brain, indicating very little diffusion of the injected complexes [329]. Similarly, the delivery of anionized green fluorescent protein (GFP)-Cre or Cas9:sgRNA with a lipid-based carrier to the inner ear of mice was confined to a region close to the injection site [28, 164]. These examples demonstrate the feasibility of delivering proteins to the cytosol and nucleus in vivo. Because four Cre molecules are theoretically sufficient for recombination [148], an effect may not necessarily have required highly efficient delivery by the carrier. Because Cre alone without carrier has been reported to activate a reporter in 1.5% [48] or 17% of cells [49], the contribution of the carrier to the delivery efficiency of Cre could be questioned. However, there are also reports of experiments during which Cre was not observed to enter cells [330].

6 Conclusion, Outlook

Various mechanisms have been proposed over time to explain how macromolecules might reach the cytosol or nucleus of cells mediated by chemical carriers. A combination of several mechanisms is most likely responsible for successful delivery. Besides the search for the most appropriate model to describe the mechanism of delivery, the low efficiency of the delivery process mediated by chemical carriers has been repeatedly confirmed for macromolecules. Sufficient efficiency allows us to benefit from the advantages of chemical carriers compared to delivery vehicles that are more efficient but also have more drawbacks concerning safety and manufacturing. Strategies for how sufficient efficiency can be achieved in the future are outlined by which delivery approaches have been successful so far. Strategies may include engineering carriers to design delivery vehicles that are optimally tailored to enact a time- and location-controlled "delivery programme" based on detailed knowledge of molecular and cellular processes during the delivery process. Obtaining sufficient delivery efficiency may be achieved by using cargoes with a high potency to make up for the limit of the carrier for delivery efficiency. Cargoes with an inherent high potency could be chosen if possible or cargoes could be conceived to be engineered for higher potency. Selection of "productive" endocytosis pathways by avoiding uptake routes that do not contribute to endosomal escape could be a further strategy

to enhance efficiency in the future. Another way to render delivery efficiency sufficient is the choice of highly potent processes at the organism level, which are effective even in the presence of small amounts of cargo, like the process to induce an immune response during vaccination, as demonstrated for the RNA-based COVID-19 vaccines. Eventually, all strategies combined—carriers engineered for enhanced performance, selecting potent cargoes or engineering of cargo potency, selection of endocytic uptake routes and the choice of potent processes—could be imagined to maximize an intended therapeutic effect in the future. Indeed, optimization of carriers had a substantial impact in being beneficial to the first approved siRNA drug [323]. The clinical success with nucleic acid delivery demonstrates the feasibility of cytosolic delivery in therapy. Tailoring carriers for proteins might be one way to advance protein delivery, because previous research showed nucleic acid carriers cannot easily be repurposed for proteins. With seven commercially available nucleic acid carriers, delivery efficiency for proteins was less than 5%, and only one of 25 tested lipids efficiently delivered an antibody and beta-galactosidase into NIH3T3 cells [24]. The example of the previously hardly deliverable RNA now being used in approved drugs shows how optimization of carriers may have tremendous effects [13, 323]. Delivery of protein cargoes with chemical carriers may critically depend on the ability to cater to the physicochemical diversity of proteins, compared to nucleic acids. Attempts at engineering proteins towards more uniform cargoes have already been made, and tailoring carrier design for individual cargo properties as well as the selection of processes that are most promising to interfere with may be most relevant to advance this approach in the future. The ability to deliver proteins to the cytosol of cells could unlock a massive number of currently undruggable targets and allow interference with cellular processes in ways that are unique to proteins.

Declarations

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