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Current Challenges in Delivery and Cytosolic Translocation of Therapeutic RNAs

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RNA interference (RNAi) is a fundamental cellular process for the posttranscriptional regulation of gene expression. RNAi can exogenously be modulated by small RNA oligonucleotides, such as microRNAs (miRNAs) and small interfering RNAs (siRNAs), or by antisense oligonucleotides. These small oligonucleotides provided the scientific community with powerful and versatile tools to turn off the expression of genes of interest, and hold out the promise of new therapeutic solutions against a wide range of gene-associated pathologies. However, unmodified nucleic acids are highly instable in biological systems, and their weak interaction with plasma proteins confers an unfavorable pharmacokinetics. In this review, we first provide an overview of the most efficient chemical strategies that, over the past 30 years, have been used to significantly improve the therapeutic potential of oligonucleotides. Oligonucleotides targeting and delivery technologies are then presented, including covalent conjugates between oligonucleotides and targeting ligand, and noncovalent association with lipid or polymer nanoparticles. Finally, we specifically focus on the endosomal escape step, which represents a major stumbling block for the effective use of oligonucleotides as therapeutic agents. The need for approaches to quantitatively measure endosomal escape and cytosolic arrival of biomolecules is discussed in the context of the development of efficient oligonucleotide targeting and delivery vectors.

Keywords: oligonucleotides, miRNA, siRNA, Shiga toxin B-subunit, immunotherapy, checkpoint inhibitor

Introduction

Groundbreaking discoveries of the past 20 years revealed that RNA molecules not only serve as carriers of genetic information, but also have central roles in regulating gene expression. Indeed, noncoding RNAs intervene in a multitude of cellular functions [1]. RNA molecules, such as double-stranded small interfering RNAs (siRNAs) and microRNAs (miRNAs), are able to trigger RNA interference (RNAi). RNAi represents a fundamental cellular process used for the posttranscriptional regulation of gene expressions [2], and as defense mechanism against foreign RNA molecules (eg, viral double-stranded RNA) [3]. Interestingly, RNAi provided the scientific community with a powerful and versatile tool to turn off individual genes. miRNAs, synthetic siRNAs, and antisense oligonucleotides (ASOs) have in common a great therapeutic potential for a wide range of diseases, since genes and related products now represent druggable targets [4].

Human cells express more than 400 different miRNAs, which are involved in the regulation of at least one-third of all human protein-coding genes. Biogenesis of miRNA starts in the nucleus, where RNA polymerase II transcribes miRNA genes into primary miRNAs (pri-miRNAs). Complementarity in pri-miRNAs sequences confers to these single-stranded molecules a hairpin structure that is capped at the 5′ end, and polyadenylated at the 3′ end. pri-miRNAs maturation starts in the nucleus, where cropping occurs at specific sites, followed by export to the cytosol [5].

miRNA precursors are cleaved in the cytosol by the Dicer enzyme to form a proper double-stranded miRNA of 22 bp in length. Mature miRNA is then loaded onto the RNA-induced silencing complex (RISC). One of the RISC proteins, Argonaute, cleaves and thereby discards one of the miRNA strands, whereas the remaining single-stranded (antisense) molecule serves as guide for RISC to recognize and bind target messenger RNAs (mRNAs) [6]. Depending on the extent of base pairing with the RISC/miRNA complex, mRNAs can either be cleaved by the Argonaute protein and degraded by endonucleases, or moved to a cytosolic structure called processing bodies (P-bodies), where RNA-degrading enzymes proceed with mRNA destruction [7]. Following degradation of the mRNA, the RISC, still associated with the guide-strand miRNA, can seek for further mRNAs and catalyze their degradation.

siRNAs are double-stranded RNA molecules that typically are 19–23 nucleotides in length. Once in the cytosol, siRNAs directly associate with the RISC machinery. Similarly to miRNAs, one strand is cleaved by the Argonaute protein, whereas the other one serves as RISC guide strand [8].

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ASOs have also been extensively studied as potential therapeutic agents. ASOs are single-stranded oligonucleotides, typically 20 bp in length that promote degradation of complementary mRNA by engaging ribonuclease H (RNase H) enzymes [9]. RNase H hydrolyzes the RNA in RNA/DNA heteroduplexes [10]. Similar to the catalytic mechanism triggered by siRNAs and miRNAs, ASOs are released after mRNA degradation, and induce the degradation of further mRNA molecules. ASOs can also arrest ribosomal activity by steric hindrance, or interfere with mRNA maturation [11].

High specificity, potency, versatility, and design simplicity hold out the promise to transform small oligonucleotide molecules into therapeutic solutions against gene-associated pathologies, from cancer to autoimmune diseases, genetic disorder, and viral infections. However, unmodified nucleic acids are highly instable molecules in biological systems by being exposed to ubiquitous nucleases. Furthermore, small size and negative charge result in weak interaction with plasma proteins and in fast kidney excretion, leading to unfavorable pharmacokinetics. This represents a major hurdle for unmodified nucleic acids to reach their molecular targets, and therefore to be used as systemic drugs.

In this review, we first provide an overview of the most efficient chemical strategies that, over the past 30 years, have been used to significantly improve the therapeutic potential of oligonucleotides, yielding nuclease-resistant oligoRNAs with increased plasma stability and favorable pharmacokinetic profiles. Oligonucleotide targeting and delivery technologies are then presented, including covalent conjugates between oligonucleotides and targeting ligand, and noncovalent association with lipid or polymer nanoparticles. Finally, we specifically focus on the endosomal escape step, which represents the major stumbling block for the effective use of oligonucleotides as therapeutic agents. The need for efficient approaches to quantitatively measure endosomal escape and cytosolic arrival of biomolecules is discussed in the context of the development of efficient oligonucleotide targeting and delivery vectors.

This review does not cover replicative oligonucleotide delivery technologies such as viruses, bacteria, and bacteriophages. The reader is referred to recent reviews on these approaches [12–15].

Chemical Modifications

Chemical modification of the nucleotides' backbone increased its stability toward nucleases, while maintaining, or even improving, affinity and specificity for target RNAs. This resulted in more favorable pharmacodynamics and pharmacokinetic profiles for oligonucleotide therapeutics. New classes of chemically modified oligonucleotide also afforded reduced immunostimulatory signaling, thereby decreasing off-target effects and increasing circulation time. Such longer half-life in circulation is directly related to a stronger interaction with plasma proteins. Excellent recent reviews [16–18] provide a detailed description of chemical nucleotide modifications. In this section, we will therefore limit our discussion on the main chemically synthesized nucleic acids, and the most common modifications of the RNA backbone (Fig. 1a).

Phosphorothioate

Because of its instability toward nucleases, the phosphodiester bond has been one of the most modified linkages within the oligonucleotide backbone. The first successful modification, which to date is still the one that is used most often, consisted in the replacement of one of the nonbridging oxygen atoms of the phosphate group with a sulfur atom, thereby generating a phosphorothioate (PS) bond (Fig. 1b). The sulfur atom confers to PS oligonucleotides an excellent resistance toward nucleases [19], and favorable pharmacokinetic profiles. Indeed, sulfur atoms increase the hydrophobicity of the nucleotide backbone, leading to an increased interaction with plasma proteins. This modification, together with the 2-O′ sugar modification, has been extensively used in the development of new therapeutic ASOs [11,20]. Besides the improved stability and the longer circulation time, PS-ASOs remain substrates for RNase H and, even more importantly, enhanced hydrophobicity favors membrane translocation to the cytosol to reach mRNA targets [21–23]. The mechanisms by which the plasma membrane (or internal membranes) is crossed remain poorly understood. The capacity of membrane translocation has represented a key advantage for PS-ASOs' development, as no delivery/transfection agents are required [21].

The PS modification has also been used extensively in the design and synthesis of nuclease-resistant siRNAs. However, full PS modification of siRNAs reduces their silencing effect, whereas partially substituted siRNAs retain silencing activity [24,25]. The reduced silencing activity of fully PS-modified siRNAs is probably a consequence of an alteration of the double-stranded A-form structure. In fact, the A-form's major groove in siRNA–mRNA duplexes is essential for RISC recognition and activation, and its alteration affects the RNAi machinery. PS modification is better tolerated at the ends of the strands, leaving the major groove unaltered [26].

2OH′ modification

Changes in the ribose 2′ OH group are generally well tolerated by the RNAi machinery. In fact, this hydroxyl group is not involved in the catalytic activity of RISC [26]. The 2′-O-Me, 2′-F, and 2′-O-(2-methoxyethyl) (MOE) represent the most commonly used ribose modifications (Fig. 1c). In particular, the biophysical propriety of 2′-O-Me and 2′-F groups are similar to the ribose 2′ OH group, which allows to preserve the siRNA A-form, or at least a RNA-like conformation. Indeed, these modifications confer excellent stability toward nucleases [27], and prevent the activation of the immune system [3,16,28]. It must be taken in account that the 2′ modification can lead to a shift in terms of RNAi activity [24]. Furthermore, extensive studies have been performed to understand how 2′OH modification influences RNAi activity while improving endonuclease resistance [29,30]. The trick appears to be to find the right siRNA sequence for a given gene in combination with the best 2′ modification pattern to induce a long-lasting silencing effect, while increasing RNA stability [31]. siRNA design often relies on the use of rational algorithms followed by alignment studies and gene expression analysis to ensure target specificity [31,32]. Fully 2′-modified siRNAs have been shown to retain RNAi activity and long duration of RNAi responses, when compared with unmodified siRNA [33,34]. Indeed, most of the therapeutic siRNA currently in clinical trials contain 2′F and 2′OMe modifications [35,36]. Several ASO bearing 2′ modification have also entered clinical trials [37–39], and two drugs, Mipomersen [40] and Nusinersen [41], have received FDA approval [20].

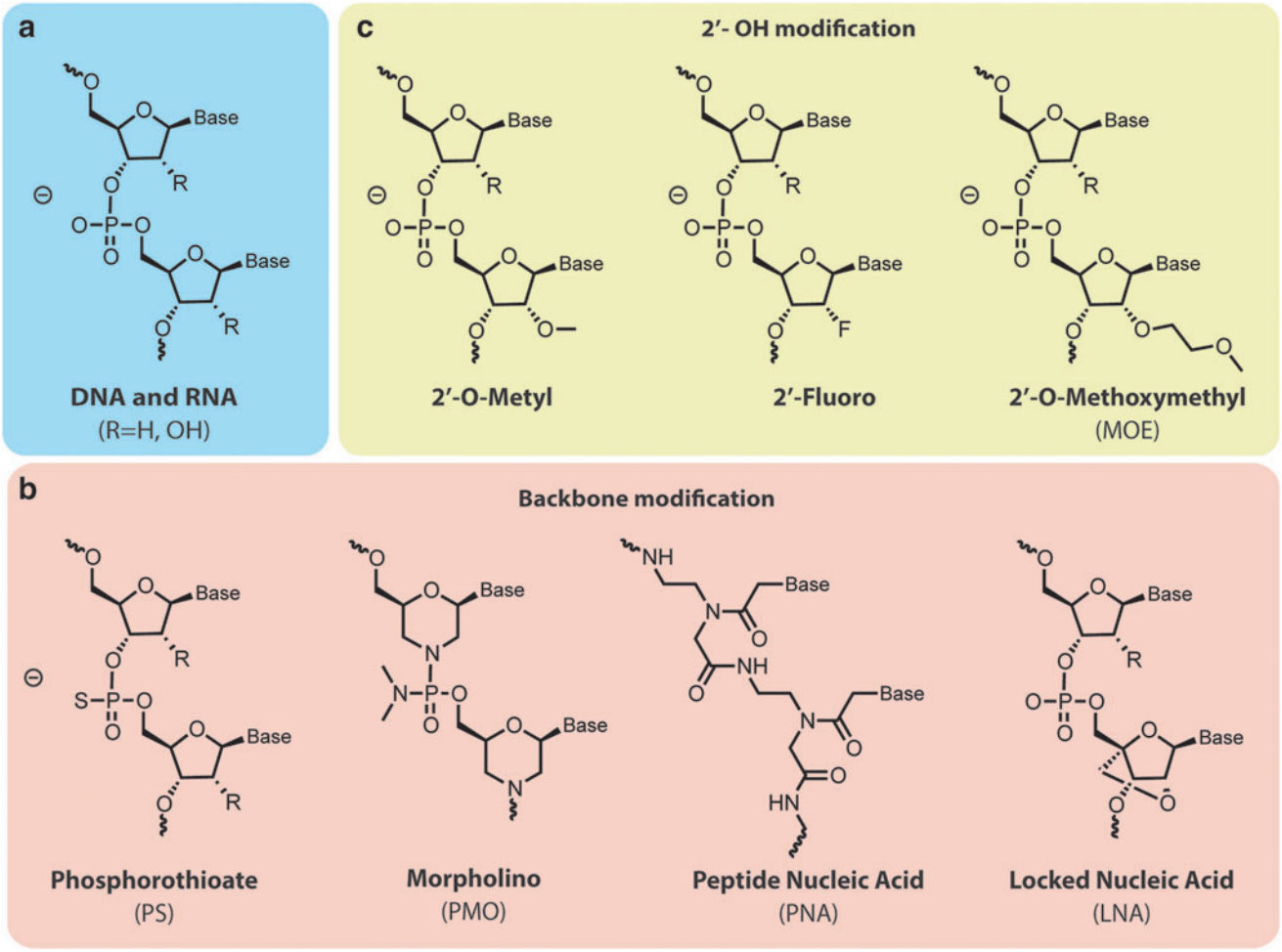

FIG. 1. Modifications of native RNA structure **(a)** have been developed to improve oligonucleotide stability and their drug-like properties. **(b)** Phosphorothioate was the first group used as a bioisosteric replacement of the phosphodiester backbone. Many other stable chemical groups, such as phosphorodiamidate morpholino oligonucleotides, peptidic nucleic acids, and locked nucleic acids have since been developed. **(c)** The majority of modifications involve changes at the 2′-OH position. 2′-O-Me, 2′-F and 2′-O-(2-methoxyethyl) (MOE) represent the most commonly used ribose modifications.

Other modifications

Many other nucleotide modifications have been explored for the development of therapeutic ASOs, in addition to PS and 2′-OH. Indeed, locked nucleic acids (LNAs), often referred as inaccessible RNAs, are nowadays widely used. Their main advantages are stability against nucleases, and increased oligonucleotide hybridization and binding properties [42]. In LNAs, the 2′ oxygen is covalently bridged to the 4′ carbon (Fig. 1b). This confers a locked conformation to the molecule [43], which results in enhanced base stacking and backbone preorganization. The phosphorodiamidate morpholino oligonucleotide (PMO) and peptide nucleic acid (PNA) modifications exhibit high stability toward nucleases, and provide an uncharged backbone [44] (Fig. 1b). The constrained structure of PMO and low electrostatic repulsion result in an improved interaction between synthetic oligoRNAs and target mRNA, even when compared with the DNA/RNA interaction [45]. The sugar rings in PMOs are replaced by morpholino rings linked to each other through a phosphorodiamidate bond. Eteplirsen is a 30-nucleotide phosphorodiamidate morpholino oligomer, neutrally charged at physiological pH that has been the first FDA-approved drug for Duchenne muscular dystrophy (DMD). DMD is a fatal neuromuscular disorder characterized by progressive muscular deterioration due to a loss-of-function mutation in the gene coding for dystrophin. Eteplirsen promotes dystrophin production by restoring its translation reading frame through specific skipping of exon 51 in defective gene variants [46]. However, even if the FDA granted an accelerated approval for eteplirsen, its efficacy still remains controversial.

In PNAs, the sugar backbone is entirely replaced by repeated N-(2-aminoethyl)-glycine bridges linked to each other by amide bonds [17]. Even if PNAs are a radically different class of oligonucleotides, they still maintain the ability to Watson–Crick base pair with complementary RNA and DNA [47]. However, both PMOs and PNAs do not support RNase H activity [27,47].

Throughout the last decade, the phosphodiester bond has successfully been replaced by amides [48,49], hydroxylamines [50], and acetals [51], preserving good binding affinity and stability. However, better results as those obtained with the PS backbone have not yet been achieved.

Delivery Issues

Chemical modification of ASOs has afforded significant improvements in terms of nucleases stability, low immunogenicity, increased efficiency of base pairing, and reduced off-target activity. However, to exploit the full potential of oligonucleotide-based therapeutics, some additional difficulties have to be overcome. In fact, ASOs and siRNAs are large and highly negatively charged biomolecules, with suboptimal bioavailability, and in most cases, an ineffective delivery onto target cells. In the context of a systemic administration of oligonucleotide-based drugs, many biological hurdles have to be overcome, ranging from vascular impediments to internalization into target cells and endosomal escape to the cytosolic compartment.

Vascular endothelial cells are strongly tied together by adherence and tight junctions, forming a highly regulated barrier between blood and surrounding tissues. Passing this vascular barrier for therapeutic purposes through paracellular or transcellular transport routes remains highly challenging [52]. In some organs, such as liver and spleen, and in particular pathological condition, such as inflammation and certain types of cancer, endothelial fenestrations are large enough to allow egress of oligonucleotides or nanoparticles [53]. In fact, for nontargeted siRNAs or nanoparticles, liver and kidney have been shown to be the major accumulation sites [54,55]. Therefore, it is not surprising that many siRNAs and miRNAs *N*-acetyl galactosamine (GalNAc) conjugates and nanoparticles that have entered the clinics have been developed to treat liver-associated disease (see Sehgal *et al.* [56]).

Internalization into cells by endocytosis and translocation to the cytosol further constitute major problems encountered in the development of RNA drugs. Indeed, because of their polyanionic nature, RNA oligomers are not able to spontaneously cross cellular membranes. They often remain trapped in endosomal compartments, leading to lysosomal degradation or recycling to the plasma membrane [21,57].

These problems represent important obstacles for the development of RNA scaffolds as a new class of therapeutics. Indeed, still only a limited number of RNA-based formulations have advanced to clinical testing, despite numerous preclinical reports on the optimization and development of novel tools for oligonucleotide delivery. Hereafter, the main technologies for RNA delivery are presented.

Cationic peptides

The capacity to interact and condensate negatively charged RNAs, the ease by which they can be synthesized, and their tunable physicochemical properties have made cationic peptides a widely used carrier for oligonucleotides [58]. Cationic peptides have been exploited in different approaches, such as direct conjugation to RNA strands, noncovalent complexation with negatively charged oligonucleotides, and use as adjuvants in polymeric or lipidic carriers [59].

However, many problems emerged with the use of polycation RNA complexes. Indeed, such formulations show poor long-term stability with the tendency to form aggregates. Apart from the loss in transfection efficiency, aggregation entails substantial hurdles that would impede manufacturing of marketable pharmaceutical products [60]. While *in vitro* experiments show promising results, the administration of cationic polymer- and peptide-based nanoparticles cause major adverse effects [61]. Lastly, high variability of freshly prepared injection solutions would represent an unacceptable risk for the patients. Therefore, the reader should be aware that aggregation and toxicity are two main concerns that still need to be addressed conclusively with the use of cationic peptides and proteins.

Poly(L-lysines). Poly(L-lysines) (PLLs) were one of the first oligonucleotide carriers [62]. Their polyamino acidic nature made PLLs an attractive biodegradable polymer for drug delivery purposes. The molecular weight of PLLs can vary from a few hundreds of Da to more than 100 kDa. However, PLLs have been shown to be toxic *in vivo*, and their toxicity increases with chain length. PLLs with a molecular weight between 2 and 30 kDa have been preferably used for siRNA and DNA delivery [58,63]. Poly-lysine polymers themselves do not exhibit any particular transfection capacity and are susceptible to proteolytic degradation, opsonization, and rapidly excretion through kidneys [64]. To overcome these limitations and to improve their delivery capacity and half-life, PLLs have been extensively functionalized throughout the years. Conjugation with endosomal-disrupting agents and fusogenic peptides to promote endosomal escape and cytosolic arrival was among the most important modifications [59]. Polyethylene glycol (PEG) functionalization has been widely exploited to reduce opsonization and kidney filtration, thus extending circulation time [65]. Furthermore, PLL nanoparticles are disassembled in serum, thereby limiting the gene silencing efficacy of corresponding siRNA complexes.

Protamine. Protamines are a family of arginine-rich proteins involved in spermatid genome condensation in many animal and plant cells [66]. Taking advantage of this natural function, protamines have been extensively used for oligonucleotide delivery. Native protamine has been exploited to condense long mRNA molecules into nanoparticle complexes [67], and for the delivery of small RNAs. For instance, cyclin D1(CyD1), a cell cycle-regulating protein that governs proliferation of normal and malignant cells, has been significantly depleted in cells and mice using protamine-condensed CyD1 siRNAs that were efficiently incorporated in β7-integrin targeting liposomes (Table 1) [68]. In another study, protamine has been used to condense a multicomponent siRNA–biotin–streptavidin complex to produce small nanoparticles characterized by efficient cellular uptake [69].

Probably the most innovative and most widely used approach concerns antibody–protamine fusion proteins that exploit antibody-mediated targeted delivery. Many groups over the past few years have successfully produced antibody–protamine fusion protein to complex siRNA and to specifically downregulate protein expression in target cell lines or organs (Fig. 2a). For instance, HIV-1 envelope protein expression was significantly reduced in HIV-infected cells using a protamine-Fab fragment fusion protein complexed with anti-gag siRNA (Table 1) [70]. Similarly, antibody-recognition of prostate-specific membrane antigen (PSMA), a cell surface glycoprotein mainly expressed on prostate cancer cells, has been exploited to selectively deliver protamine-complexed siRNAs against Notch1. The Notch pathway is involved in several cellular processes. Its abnormal activation has been observed in many cancers, including prostate cancer [71]. Targeted delivery of siNotch1 was successfully achieved in

Table 1. Targeted Oligonucleotide Systems: Targeting Ligands Are Paired with Oligonucleotide Formulations to Afford Specific Gene Silencing

Targeting moiety	*Formulation*	*Disease/application*	*Targeted gene*	*References*
Monoclonal antibody against β7 integrin	Liposome containing protamine-condensed siRNA	Inflammation	CyD1	[68]
Fab fragment of an HIV-1 envelope antibody	siRNA condensed to antibody–protamine fusion protein	HIV	gag	[70]
Anti-PSMA antibody	siRNA condensed to antibody–protamine fusion protein	Cancer	Notch1	[72]
Single-chain antibody against DEC205	siRNA encapsulated within lipid nanoparticles	Immunosuppression	CD40, CD80, CD86	[119]
Folic acid	DNA nanoparticles	Cancer	Luciferase assay	[111]
Folic acid	PEI nanoparticles containing siRNA	Cancer	VEGF	[123]
Apolipoprotein E	siRNA encapsulated within lipid nanoparticles	Amyloidosis	Transthyretin	[108]
RGD	SSO conjugate	Cancer	Luciferase assay	[127]
RGD	Chitosan nanoparticles containing siRNA	Cancer	Periostin	[128]
Cyclo RGD	siRNA conjugate	Cancer	VEGFR2	[129]
GalNAc	siRNA conjugate	Amyloidotic, cardiomyopathy	TTR	[130]
GalNAc	siRNA conjugate	β-Thalassemia	Tmprss6	[130]
Aptamer against PMSA	siRNA chimera	Cancer	PLK1	[133]
Aptamer against gp120	siRNA chimera	HIV	HIV RNAs	[134]

GalNAc, *N*-acetyl galactosamine; PEI, poly(ethylenimine); PLK1, polo-like kinase 1; PSMA, prostate-specific membrane antigen; RGD, arginine–glycine–aspartate; siRNA, small interfering RNA; SSO, splice-shifting oligonucleotide; TTR, transthyretin; VEGF, vascular endothelial growth factor.

the LNCaP prostate cancer cell line. Notch1 downregulation led to cell cycle arrest and apoptosis (Table 1) [72].

Another group has taken advantage of the epidermal growth factor receptor family member ErbB2 (HER2), which is overexpressed in Her2+ breast cancers to deliver polo-like kinase 1 (PLK1) siRNAs, both in primary Her2+ breast cancer cells, and in xenograft models [73]. Selective and efficient siRNA delivery led to the depletion of the targeted genes, resulting in reduced cell proliferation and the induction of apoptosis.

Many other studies confirmed the potential of protamine–antibody fusions as delivery tools for nucleic acids [74]. Interestingly, siRNA conjugates with unmodified antibodies showed lower cellular uptake compared with siRNAs that were complexed with protamine–antibody fusion proteins [70]. It is likely that the charge balance between positively charged protamine and negatively charged siRNAs favors internalization and endosomal escape.

Protamine has been widely used for siRNA delivery and other biomedical applications [75,76], despite the fact that cardiovascular and respiratory toxicity and adverse immune reactions were observed after intravenous injection [77]. To decrease toxicity, low-molecular-weight protamines (LMWPs) have been developed [78,79]. LMWPs are produced through enzymatic digestion of the native protamine with thermolysin. They contain short sequences of about 2 kDa that are enriched in arginine. LMWP are poorly immunogenic and do not cause hypotensive or toxic responses [77,80]. LMWPs have been successfully used for siRNA delivery [80,81]. Choi *et al.* showed that a stable LMWP/siRNA complex was efficiently taken up by hepatocarcinoma cells. Significant downregulation of the targeted vascular endothelial growth factor (VEGF) led to cell growth inhibition and apoptosis. Experiments in mice confirmed the therapeutic potential of this formulation [80]. Because of their peptidic nature and physicochemical similarity, LMWPs are often considered as cell-penetrating peptides (CPPs).

Cell-penetrating peptides. CPPs are peptides of 5–30 amino acids in length, often positively charged that are capable of passing through tissue barriers and cell membranes without interacting with any specific receptor [82]. CPPs are internalized by endocytosis, or translocated by passive diffusion directly across the plasma membrane. Endocytic uptake may ultimately also favor membrane translocation to the cytosol. The molecular details of processes by which the lipid bilayer is breached are not yet fully established [83].

The transactivator of transcription (TAT) and penetratine were the first CPPs to be described. It has then been shown that CPPs are capable of transporting a variety of biologically active payloads, such as peptides, proteins, and oligonucleotides inside cells [84]. siRNAs can either be covalently conjugated to CPPs, or noncovalently associated with CPPs through electrostatic interactions, yielding complexes or nanoparticles [27]. For instance, Endo-Porter is an amphipathic CPP that was shown to be capable of delivering noncovalently bound siRNAs [85] and morpholino-RNAs [86] through an energy-independent mechanism into cells. Covalent conjugation of CPPs with small oligo-RNAs affords a well-defined macro-biomolecule with a one-to-one CPP/siRNA ratio that is stable in circulation. Such well-defined molecular entity facilitates the drug development process [45].

The direct conjugation of siRNAs to CPP may result in neutralizing the charges on CPPs, which would lead to a

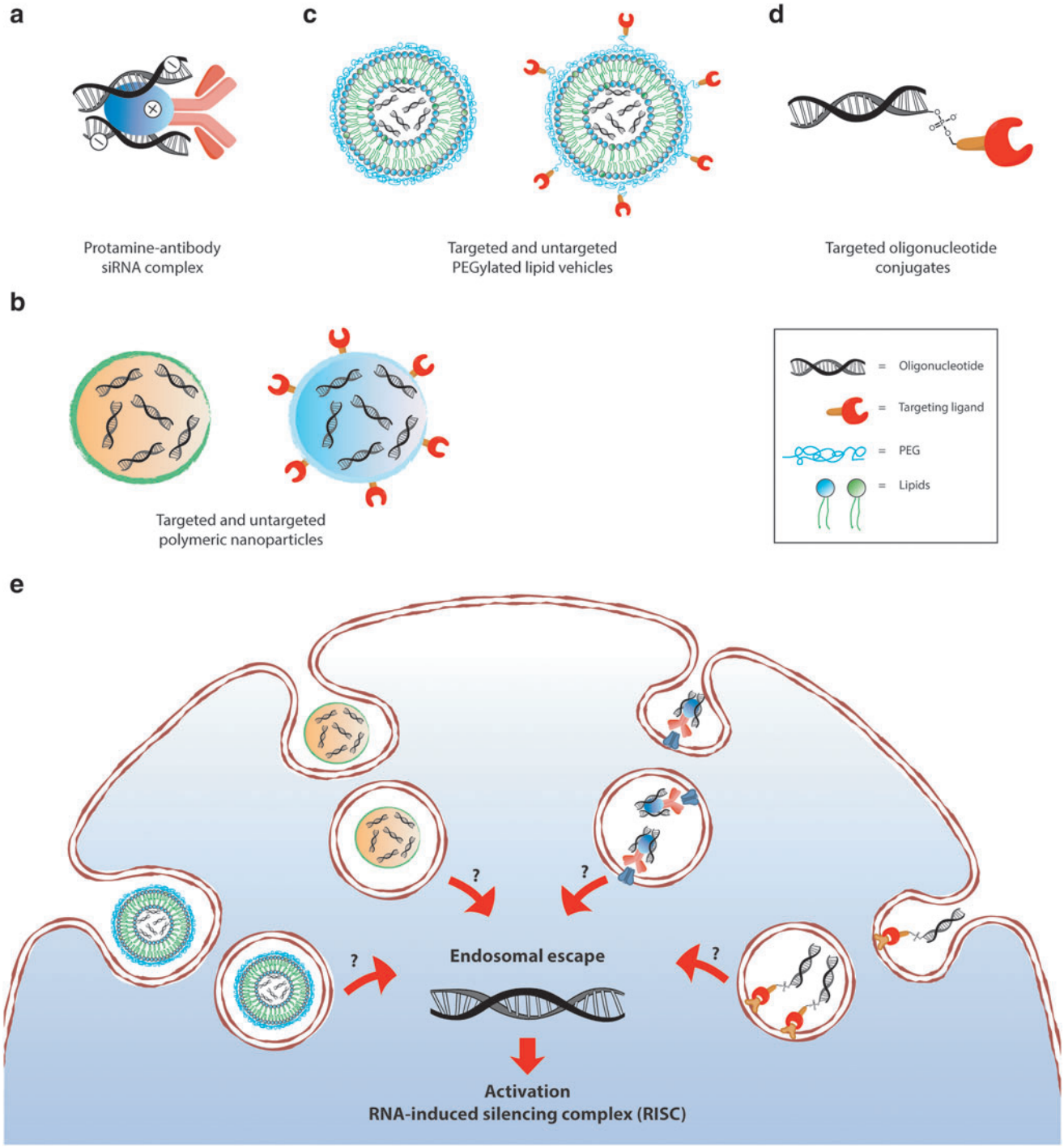

FIG. 2. Common modalities for oligonucleotide delivery. **(a)** Protamine–antibody fusion protein complexed with oligoRNAs. **(b)** Example of polymeric nanoparticles containing siRNA molecules. **(c)** Schematic illustration of PEGylated lipidic nanoparticles comprising siRNA. **(d)** Direct conjugation of siRNAs with a targeting ligand. **(e)** Despite great progress in vector development, most of internalized molecules accumulate in endosomes. Only a small fraction reaches the cytosol, where the RISC machinery is located. The mechanisms of endosomal escape still remain poorly understood. PEG, polyethylene glycol; RISC, RNA-induced silencing complex; siRNA, small interfering RNA.

reduced efficiency of membrane translocation [58]. To circumvent this problem, nucleic acid analogs with reduced charges, such as PNA and PMO, have been used in conjugation with CPPs [87,88]. In studies on cells in culture and on animals, such as mice and nonhuman primates, it was shown that conjugation to CPPs much improved the cellular uptake and efficiency of PMOs for the treatment of DMD [89,90]. Systemic injection of a cocktail of CPP-conjugated PMOs rescued muscular dystrophin expression in canine model of DMD [91]. Leaky muscle fibers and weakened muscular cell

membranes that are characteristic of muscular dystrophies likely favor cellular uptake and translocation of PMOs into the cytosol [92].

Nanoparticles. Incorporation into nanoparticles or nanocapsules is another popular strategy to protect oligonucleotides from degradation, to assist their delivery onto target cells, and to promote their cellular uptake and subsequent endosomal escape, the latter probably being the biggest challenge for the development of oligonucleotide-based therapeutics [18].

Polymeric nanoparticles. Because of their tunable chemical properties, polymers are among the most common materials that are used in nanoparticle production, followed by lipids and lipid-like materials (Fig. 2b). Since they need to condense nucleic acids, cationic polymers are preferred over neutral or negatively charged ones. Next to PLL, poly(ethylenimine) (PEI) represents another well-explored polymer for RNA delivery [12]. Besides its nucleic acid condensation properties, the many protonable secondary amines of PEI likely promote endosomal escape through the proton-sponge effect [16]. According to this hypothesis, the high proton buffering capacity of PEI would lead to high osmotic pressure within organelles of the endocytic pathway, thereby causing the rupture of endosomes and/or lysosomes, and release of oligonucleotides to the cytosol [93]. However, this mechanistic proposal has recently been called into question, and it even seems unclear whether this proton sponge effect actually exists [94].

The molecular weight of PEI is critical to balance toxicity and transfection efficiency of PEI/siRNA complexes. Yet, apparently conflicting results have been reported concerning the transfection capacity of PEI in function of the molecular weight and polymer branching [95]. It seems clear, however, that even though low molecular weight confers to PEI a more favorable toxicological profile [12], transfection efficacy of PEI is strongly correlated with cytotoxicity. Indeed, because of this toxicity, PEI is now largely excluded as delivery tool for RNAs. Other polymers such as poly(amidoamine) (PAMAM) [96] and the natural chitosan [97] have also been used with variable levels of success for siRNA delivery.

Lipid nanoparticles. Lipid nanoparticles (LNPs) represent probably the most popular approach for siRNA delivery (Fig. 2c). Of note, LNP–siRNA complexes are already used in clinical trials [98,99], and are about to become the first FDA-approved siRNA drug. Synthesis of LNPs for oligonucleotide delivery usually involves four to five components, always including cationic lipids, which promote RNA packing and nanoparticle formation, and cholesterol as a stabilizing agent [99]. Simple LNPs represent an efficient cell culture transfection agent. *In vivo*, they are rapidly opsonized, however, and cleared by phagocytic cells of the reticuloendothelial system (RES) [100]. To overcome this problem and to prolong their circulation half-life, LNP surfaces are often coated with PEG. The hydrophilic and flexible PEG chains form a protecting shell around the nanoparticles, thereby preventing their interaction with opsonins and RES activation (stealth effect) [1,101,102]. LNPs are 100–200 nm in size and, upon systemic administration, can extravasate only at sites where the endothelium is fenestrated. By taking advantage of the discontinuous and highly permeable hepatic vasculature, LNPs represent one of the main options to target liver disease [103]. Alnylam Pharmaceuticals has successfully completed a Phase 1 clinical trial with ALN-VSP LNPs to treat advanced cancer with hepatic metastasis [104]. ALN-VSP comprises two siRNAs that target VEGF, which is critical for the growth of new blood vessels [105], and kinesin spindle protein (KSP), involved in cell division [106]. Upon parenteral administration, ALN-VSP passively accumulates in the liver, where it mediates anticancer activity. ALN-VSP is well tolerated. Interestingly, the formulation is also found in extrahepatic metastatic tumor biopsies. This is most likely due to a leaky vasculature and a lack of lymphatic drainage at cancer sites.

The passive accumulation of nanoparticles on cancerous tissue is known as the enhanced permeability and retention (EPR) effect, which is nowadays strongly debated. EPR occurs efficiently in subcutaneous tumor models in mice, whereas human tumors show a different growth environment and vasculature. The extent of EPR can notably vary among tumors, and from patient to patient. Indeed, tumor size, type and location, the composition of the extracellular matrix, vascular permeability, and tumor perfusion make of EPR a heterogeneous phenomenon that needs further investigation to effectively foster nanoparticle drug delivery [107]. As described above, the vascular architecture of the liver has been extensively exploited for nanoparticle delivery. A further improvement in hepatic delivery was achieved by coating the LNP surface with hepatocyte-targeting domains [1]. For instance, Patisiran is an LNP developed for the delivery of siRNAs that target transthyretin (TTR) mRNA. This LNP is in phase 3 clinical trials for the treatment of hereditary transthyretin-mediated amyloidosis (ATTR), which is a progressive, life-threatening disease. ATTR is caused by accumulation of misfolded TTR, predominantly produced in the liver [98]. To further promote liver uptake, the LNP surface of Patisiran is coated with apolipoprotein E (ApoE), which binds to the ApoE receptor that is overexpressed on hepatocytes and mediates LNP internalization (Table 1) [108]. In a previous phase 1 trial, a significant and durable TTR knockdown was achieved in the absence of serious adverse side effects [103].

DNA and RNA nanoparticles. DNA and RNA nanotechnology has exponentially grown in the past 20 years in recognition of its potential in the field of nanomedicine [109,110]. By exploiting the base-pairing capacity of DNA and RNA, nanostructures of different complexities, shapes, and dimensions can be obtained. Indeed, the ability to precisely control size and shape in one, two, or three dimensions makes DNA and RNA nanostructures attractive and unique tools with promising delivery capacity. Modular RNA or DNA building blocks can be connected using linkers and structural junction motifs, offering a plethora of new opportunities for the bottom-up assembly of drug delivery carriers [110]. Moreover, oligonucleotide strands can be programmed such as to functionalize nanoparticles with targeting moieties whose spatial orientations and densities are precisely controlled. An additional benefit in the use of oligonucleotide-based carriers comes from the possibility of hybridizing therapeutic RNAs onto them. For instance, DNA tetrahedron nanoparticles have been developed for siRNA delivery (Table 1) [111]. A specific DNA overhang design allowed

incorporation of six 2′-OMe-modified siRNAs per nanoparticle, providing a homogeneous population of nanocarriers in terms of size and shape. Twenty-eight different ligands, from peptides to small molecules, have been conjugated to DNA nanoparticles. Cationic peptides caused nanoparticle aggregation, due to electronic interactions with negatively charged nucleotide strands, whereas folic acid conjugates afforded a dose-dependent gene-silencing effect in HeLa cells, and in xenografted mice.

RNA nanostructures are thermodynamically more stable than DNA nanostructures and possess high structural flexibility and versatility [112,113]. For instance, phi29 promoter-associated RNA (pRNA)-based vectors can be used as vehicles for siRNA delivery. To produce such RNA nanoparticles, chimeric RNA complexes were developed by fusing siRNAs with bacteriophage phi29 motor pRNA [109,110,114]. pRNA-derived nanoparticles are small (20–40 nm) and homogenous in size. Furthermore, they can be functionalized with target-specific ligands. For instance, a folate-conjugated pRNA construct has been developed for the delivery of siRNAs into folate receptor-overexpressing nasopharyngeal carcinoma cells. It was shown that the pRNA/siRNA fusion was successfully processed by Dicer, leading to specific suppression of gene expression [115].

Targeting Strategies

The remarkable advances over the last two decades in the development of ASO and siRNA vectors have not yet been accompanied by a comparable clinical success. A major limitation originates from the difficulty to deliver a therapeutic dose of oligonucleotides at the target site, particularly for extrahepatic tissues, such as cancers or immune cells. Therefore, as already introduced above, targeting oligonucleotides to specific cell types and organs appears to be of fundamental importance to enhance cellular uptake and to gain selective extrahepatic delivery. Cell specificity is achieved by combining therapeutic oligonucleotides with targeting ligands that bind receptors on the surface of target cells, and mediate internalization of conjugates (Fig. 2d).

Among the many available targeting ligands, antibodies have emerged as one of the most promising options [116]. However, following the initial excitement for oligonucleotide–antibody conjugates, many difficulties have been encountered [3]. The main problems reside in antibody production and conjugate stability.

Antibody–siRNA conjugates have shown weak silencing capacity due to poor endosomal escape of these large (180 kDa) and hydrophilic molecules [27,117]. Coating the surface of LNPs with antibodies also represents an important challenge. The main difficulty resides here in finding on optimal ratio for coating with PEG and antibody molecules, while maintaining efficient antibody-binding capacity and long circulation time [118]. Interestingly, the efficient targeting of dendritic cells was recently demonstrated, using LNPs coated with a single-chain variable fragments (scFv) (Table 1) [119]. An efficient siRNA-mediated knockdown of different costimulatory surface antigens was afforded on cells in culture, and in animals.

Other nonantibody targeting moieties have been explored for oligonucleotide delivery. For instance, folate receptor shows limited expression on healthy tissues, while being overexpressed, among others, on colon, lung, prostate, throat, and brain cancers [120]. Therefore, conjugation to folate has emerged as a valid option for siRNA targeting to cancer cells [111,121]. A folate-modified PEI nanocarrier successfully delivered VEGF siRNAs, resulting in a strong transfection efficiency on cells in culture, and an inhibition of tumor growth in mice (Table 1) [122].

Another example of targetable receptors is integrins. These α/β heterodimers form a family of transmembrane proteins that regulate cell adhesion and migration, through interaction with extracellular matrix components such as with fibronectin and collagen [123]. Certain integrins, for example, αvβ3 and αvβ5, play an important role in tumor cell proliferation and migration [124], and in angiogenesis [57]. Integrin overexpression has therefore been exploited for oligonucleotide delivery, using the arginine–glycine–aspartate (RGD) peptide from fibronectin [125]. RGD-mediated targeting has successfully been used for ASO and siRNA delivery *in vitro* (Table 1) [126] and *in vivo* (Table 1) [127,128].

Another interesting and widely used targeting moiety is the triantennary GalNAc, which binds with high affinity to trimeric asialoglycoprotein receptors (ASGPRs); these lectins are strongly expressed on hepatocytes [129]. Alnylam Pharmaceuticals and Dicerna made important improvements on a series of siRNA–GalNAc conjugates, which are currently tested in preclinical and clinical trials for the treatment of liver-related pathologies, such as hepatitis B and D, hypertriglyceridemia, familial amyloidotic cardiomyopathy, and fibrotic liver diseases (Table 1) [129].

Aptamers are single-stranded oligonucleotides that can be selected on the basis of their specific binding to given receptors [130], similar to antibodies. Aptamers can be engineered, and produced by chemical synthesis [131]. High affinity, target specificity, low immunogenicity and toxicity, low production costs, and reproducibility from batch to batch qualify aptamers as promising targeting ligands for oligonucleotide delivery. Aptamers–siRNA chimeras have been used to target antiapoptotic genes in prostate cancer cells expressing PSMA receptors (Table 1) [132]. Furthermore, an aptamer specific for PSMA receptor was generated and conjugated to therapeutic siRNAs against PLK1 and BCL2, two survival genes that are overexpressed in most human tumors. Silencing of the targeted genes resulted in decreased proliferation, induced apoptosis *in vitro*, and tumor regression in xenograft models of prostate cancer [132]. Another aptamer was developed for specific recognition of HIV-1 envelope protein (gp120) (Table 1) [133]. This aptamer was linked to siRNA-targeting proteins that regulate HIV replication. Interestingly, the aptamer itself already provided potent antiviral activity in humanized mice, which was further enhanced when coupled to siRNAs.

Endosomal Escape

Upon binding to the cell surface, oligonucleotide carriers are internalized by one of the multiple endocytosis processes. Clathrin-dependent and independent micropinocytosis and macropinocytosis have been reported to be implicated, to different extents, in oligonucleotide uptake into cells [57]. Moreover, targeting siRNAs or ASOs to specific cell surface receptors remarkably impacts the initial internalization pathway and the subsequent intracellular trafficking [134].

Another crucial aspect to achieve RNAi machinery activation is the process of endosomal escape [135]. Indeed, despite significant achievements in vector development, most internalized oligonucleotides accumulate in the endocytic pathway, and only very few molecules reach the cytosol, where the RISC machinery is located (Fig. 2e). In the past few years, the cellular process of endosomal escape has become a focus of interest, and its low efficiency now appears as the main obstacle for an effective use of oligonucleotides as therapeutic agents. However, even though inefficient endosomal escape is unanimously recognized as an important stumbling block, only very few technologies and strategies have been described that can efficiently promote the translocation of oligonucleotides from endosomes to the cytosol.

Enhancing endosomal escape

The most common approaches rely on the use of endosomolytic agents that either are natural products, or deliberately designed and produced by chemical synthesis [136].

Fusogenic peptides, proteins, and lipids. Naturally evolved membrane translocation mechanisms have attracted the attention of many researchers in the delivery field [137–139]. The identification and full understanding of mechanisms used by viruses, bacteria, or toxins to escape from endosomes to the cytosol has become an intense field of investigation for the development of oligonucleotide delivery agents.

Peptides that perturb endosomal and/or lysosomal membranes, referred to as fusogenic or endosomolytic peptides, have been used to develop delivery systems for antibodies [140], proteins [141], siRNAs [142], and LNPs [143,144]. For instance, synthetic HA2 domain derivatives of hemagglutinin from influenza virus have been used for siRNA delivery [145]. In the slightly acid pH of endosomes, HA2 domain residues are protonated, upon which the anionic hydrophilic coil of HA2 undergoes a conformational rearrangement into a hydrophobic helix that destabilizes endosomal membranes [145,146].

The translocation domain (T domain) of diphtheria toxin is another well-studied fusogenic peptide. Diphtheria toxin is an exotoxin secreted by *Corynebacterium diphtheria*. It exploits the pH-dependent conformational change of its T domain to cross the endosomal membrane and to mediate the cytosolic translocation of the catalytic domain [147]. The T domain was incorporated into PEI polymer complexes for improved plasmid DNA delivery [148]. The conjugation resulted in a significant enhancement of transfection efficiency, when compared with unconjugated PEI complex. This observation also suggests that the proton sponge effect, which was mentioned earlier in the context of PEI polymers, has probably only a small impact on endosomal escape.

Following a specifically designed synthetic approach, short sequences of hydrophobic amino acids, referred as endosomal escape domains (EEDs), were identified that, when conjugated to CPPs, strongly enhanced the cytosol arrival of peptidic biomolecules. Improved endosomal escape most likely results from a high dose of EEDs that locally insert into endosomal membranes, thereby affecting membrane integrity, leading to the cytosolic release of the payload [149].

Incorporation of fusogenic lipids, such as DOPE and DOTAP, into LNPs and liposomes has also been used to improve endosomal release of encapsulated nucleic acids [150,151]. Incorporation of DOPA into targeted lipopolyplexes for plasmid DNA delivery afforded greater transfection efficiency, both on cells in culture and in murine models, when compared with lipopolyplexes without such lipids [152]. The improved cytosolic translocation of the genetic cargo has been attributed to the tendency of DOPE to adopt an inverted hexagonal phase structure, which represents a nonbilayer lipid organization that is thought to destabilize endosomal membranes [153].

Endosomolytic polymers. Endosomal acidification has also been exploited for the development of endosomolytic polymers. In this case, the slightly acidic pH of endosomes is used to induce the gradual protonation of anionic groups distributed along polymer chains. The resulting hydrophilic-to-hydrophobic change facilitates polymer partitioning into endosome membranes, leading to their destabilization. For instance, copolymers were developed that consist of positively charged dimethylaminoethyl methacrylate (DMAEMA) to condense siRNAs, and an endosomal release block made of DMAEMA, propylacrylic acid, and hydrophobic butyl methacrylate residues [154]. Endosomal protonation of propylacrylic acid groups causes polymer rearrangement into a hydrophobic polycation that is capable of disrupting endosomal membranes by interfering with lipid packing.

Small molecules. Chloroquine [135] and other small molecules, identified by high-throughput screening [155–157], have been used *in vitro* to promote cytosolic translocation of endocytosed nucleotides and other biomolecules. However, these small molecules have a general effect on endosomes, and are not selectively at sites of internalized cargoes, and therefore result in significant toxicity. Photosensitive molecules are another class of small compounds that are successfully used to enhance the translocation of siRNAs from the endosomal to the cytosolic compartment [158,159]. Photosensitizers for endosomal escape are amphiphilic compounds that once internalized mainly localize in endosomes where, upon light stimulation, they generate highly reactive singlet oxygen. Reactive oxygen species affect endosomal membrane integrity and trigger the cytosolic release of the endosomal content. The localized production of damaging species and their short halftime guarantee a local effect without altering other cellular functions [160].

Although these strategies enhance the cytosolic translocation of RNA-based therapeutics, most internalized molecules still remain trapped into endosomes until being degraded. Estimates indicate that only 0.1%–2% of endocytosed biomolecules escape to the cytosol before other intracellular pathways, such as recycling to plasma membrane or fusion with lysosome occurs and prevents further endosomal escape. It is also worth mentioning that endosomal escape varies between cell types. For instance, endosomal escape is more prominent in antigen-presenting cells (APCs), when compared with other cell types. APCs naturally transfer exogenous antigens to the cytosol where they are processed by proteasomes, loaded onto major histocompatibility complex class-I (MHC-I) molecules in the endoplasmic reticulum, and subsequently crosspresented at cell surface. As an example, TAT-conjugated proteins are efficiently taken up by various cell lines, but only in the case of dendritic cell a cytosolic distribution of the endocytosed carriers could be observed

[161]. Similarly, it was also demonstrated that endosomal escape of a lectin–saporin conjugate was more efficient in APCs than in cancer cell lines [162]. The reasons underlying efficient endosomal escape in APCs are still not understood at this stage. One might invoke the existence either of a dedicated protein machinery, or of a particular lipid composition that would make endosomes of APCs leakier.

Quantification of cytosolic arrival

It has been reported that 2,000–10,000 molecules of cytosolic siRNAs per cell are needed to achieve maximal depletion efficiency [163,164]. Therefore, keeping in mind that only 0.1%–2% of cargoes actually reach the cytosol, it is evident that the number of molecules that need to be endocytosed is strikingly high. For instance, even if 1 million receptors were available at plasma membrane for oligonucleotide targeting onto a given cell, a synchronized saturation of all available binding sites and their concomitant internalization would be needed to afford the endosomal levels that would possibly lead to cytosolic amounts compatible with a robust interference effect. This ideal situation is unlikely to be met in the complex tissue environment in which target cells are localized within living organisms, and it is thereby obvious that efficient endosomal escape technologies could tremendously impact the development of oligonucleotide therapeutics. One of the reasons for slow progress in developing efficient strategies to foster endosomal escape resides in the difficulty to quantify the cytosolic translocation of biomedical cargoes. To this end, several approaches have been proposed, based on fluorescence microscopy, subcellular fractionation, or pH-sensitive probes [165].

Measurement of cell- and organelle-associated fluorescence is surely a fast and straightforward method. However, the differentiation between organelle-associated and cytosolic signals can only be achieved if massive amounts of marker are found in the cytosol, which is almost never the case.

Another method is based on the use of fluorescence correlation spectroscopy (FCS) [166]. FCS is a powerful analytical tool that allows to measure very small numbers of fluorescent molecules in tiny volumes (femtoliter), providing concentration values in the picomolar range. This method can be used to reliably measure the total number of internalized molecules per cell. For the quantification of the cytosolic fraction, cells must be homogenized, cytosol isolated by ultracentrifugation, and then analyzed by FCS. Such procedure is prone to experimental artefacts, however, especially when, upon translocation, the oligonucleotide delivery tool remains associated with the cytosolic leaflet of endosomes.

A biotin ligase-based assay also allows to measure the cytosolic arrival of biological therapeutics. For this, the prokaryotic biotinylation enzyme BirA is expressed in the cytosol of a eukaryotic target cell of choice. A short peptide sequence, the avi tag, is fused to a cargo whose translocation to the cytosol is to be analyzed. Upon arrival in the cytosolic compartment, the avi tag is biotinylated by the BirA enzyme. After cell lysis, cargo biotinylation is detected and quantified by western blotting, using fluorescent streptavidin or anti-biotin antibodies. The former is more sensitive, but produces significant background levels, whereas the latter in less sensitive, but with less background noise [167]. In both cases, the need for western analysis renders the method cumbersome, and the background levels make the detection of small amounts of translocated materials very difficult.

The split-complementation endosomal escape assay allows direct visualization of cytosolic delivery of biomolecules at biological relevant concentrations [168]. The success of this robust assay, used to measure endosomal escape rates and to screen endosomolytic agents [149], resides in its low background values and adaptability to stable or transient cellular expression. The assay exploits the superfolder GFP protein [169]. The removal of 16 amino acids, the GFPβ11 fragment, from the N-terminus of superfolder GFP leads to a nonfluorescent soluble protein, referred to as large GFP fragment. However, when a synthetic GFPβ11 fragment binds onto the large GFP fragment, the superfolder GFP is reconstituted and its fluorescence is restored. The GFPβ11 fragment is not fluorescent alone, and it is too big to enter cells spontaneously. It can therefore be coupled onto delivery vectors to detect their cytosolic translocation. The 1:1 ratio of both fragments that is needed to reconstitute superfolder GFP allows a direct correlation between numbers of translocated GFPβ11 and fluorescence intensity.

Besides these approaches that aim at a true quantification of absolute levels of cytosolic materials, different comparative assays have also been developed, as reviewed by Martens *et al.* [170]. Probes that are sensitive to pH or to reducing environments have been synthesized [171]. For instance, naphthofluorescein is a pH-sensitive dye that changes its fluorescence intensity when translocating from the slightly acidic pH of endosome to the neutral cytosolic pH [172].

Certain toxins exert their cytotoxic activity specifically in the cytosolic compartment. For instance, pseudomonas exotoxin [173,174], alpha-sarcin, and saporin [140,162], once in the cytosol, lead to protein biosynthesis inhibition. Combinations of these toxins with vectors or endosomolytic agents have been used to monitor arrival in the cytosol. Indeed, evaluation of protein biosynthesis levels, using radiolabeled amino acids, such as [^{3}H]-leucine or [^{35}S]-methionine, is used as an indicator of the presence of the toxin in the cytosolic compartment.

Beta-lactamase and Cre-recombinase are alternatives to these toxins. Beta-lactamase catalyzes the cleavage of a cephalosporin-derived fluorescence reporter that specifically accumulates in the cytosol of target cells. Hence, beta-lactamase is a valid tool to assess membrane translocation of vectors to which the enzyme is coupled [162]. Cre-recombinase is another enzyme that, upon reaching the cytosol, catalyzes the expression of a reporter protein, which is often the enhanced green fluorescent protein (eGFP). Cre-recombinase has been used in conjugation with CPPs [141] and antibodies [140].

Another method exploits the activation of a cytosolic glucocorticoid receptor (GR) to induce transcription of a reporter gene [175]. In such assay, any delivery vector that is tagged with glucocorticoid ligands (such as dexamethasone) induces, upon reaching the cytosol, GR activation and its nuclear translocation, leading to the expression of the reporter gene.

Conclusions

Oligonucleotide stability in biological media and their uptake into cells has been a focus of interest in medicinal chemistry. Targeted delivery of oligonucleotides allowed to improve their pharmacokinetics, and to reduce off-target

effects. However, to promote RNAi-based therapies for a wide range of gene-related diseases, other problems have to be overcome. Notably, it is essential to fully understand mechanisms underlying endosomal escape. For this, the availability of universal, reliable, and robust assays to quantitatively measure arrival in the cytosol is a key condition. We expect that a molecular understanding of endosomal escape will lead to the development of enabling technologies to boost membrane translocation and cytosolic arrival of oligonucleotide therapeutics. In the meantime, RNA-based drug candidates are already in the clinics in favorable indications. GalNac–siRNA conjugates are probably the closest to entering the market [129]. Their success in the liver is due to a unique combination of favorable elements. First, high perfusion of the liver and advantageous tissue structure allows for rapid cellular uptake, and subsequent renal excretion of this conjugates is poor. Second, ASGPR expression in hepatocytes is high, and its turnover at the plasma membrane is very dynamic. Even with an endosomal escape rate of <0.1%, the number of cytosolic siRNAs that is required for an efficient interference effect is reached within a day [3]. The exact mechanisms by which these GalNAc-dependent delivery systems reach the RNAi machinery remain unknown, and at the moment, a comparable situation at extrahepatic sites has not been reported. However, with increasing numbers of oligonucleotide-based therapeutics that enter clinical trials, and the recognition of endosomal escape as being the most limiting aspect for their success, a substantial intellectual and financial investment can be expected that likely will lead to ground-breaking new discoveries.

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