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Using drug-excipient interactions for siRNA delivery ☆

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ABSTRACT

SiRNA is the trigger of RNA interference, a mechanism discovered in the late 1990s. To release the therapeutic potential of this versatile but large and fragile molecule, excipients are used which either interact by electrostatic interaction, passively encapsulate siRNA or are covalently attached to enable specific and safe delivery of the drug substance. Controlling the delicate balance between protective complexation and release of siRNA at the right point and time is done by understanding excipients–siRNA interactions. These can be lipids, polymers such as PEI, PLGA, Chitosans, Cyclodextrins, as well as aptamers and peptides. This review describes the mechanisms of interaction of the most commonly used siRNA delivery vehicles, and looks at the results of their clinical and preclinical studies.

Contents

Abbreviations: AFM, atomic force microscopy; ApoB, Apolipoprotein B; AMD, age related macular degeneration; Bp, base-pairs; bPEI, branched PEI; CDP, Cyclodextrin-containing polymers; CPP, cell penetrating peptide; D5W, 5% (w/v) glucose in water; DD, deacetylation degree; DLS, Dynamic light scattering; ds, double-stranded; IL, Interleukin; ITC, Isothermal titration calorimetry; i.v., intravenous; mRNA, messenger RNA; MW, molecular weight; N/P ratio, Nitrogen to Phosphor ratio; PAMAM, poly(amidoamine); PEG, polyethylene glycol; PEI, Polyethyleneimine; R8, octaarginine; RES, reticuloendothelial system; RISC, RNA-induced silencing complex; RNAi, RNA interference; SAXS, Small angle X-ray scattering; shRNA, small hairpin RNA; siRNA, small interfering RNA; STR-R8, stearyl octaarginine; t½, half-life; TEM, transmission electron microscopy; TLR, Toll-like receptor; TNF-α, Tumor necrosis factor-α; VEGF, Vascular Endothelial Growth Factor.

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1. Introduction

Since its discovery in the late 1990s, RNA interference (RNAi) has not only been established as an invaluable tool in basic research, but has also raised hopes in medicine to find novel therapies for previously "undrugable" targets and diseases.

The finding that RNA, in addition to its classical role as messenger from DNA encoded genetic information for protein production, can also function as regulator of gene expression, has led to a quantum leap in understanding gene regulation. RNA molecules can inhibit protein translation either by binding to complementary messenger RNA (mRNA) and thereby blocking the ribosomal action (antisense RNA or microRNA) or by triggering degradation of the mRNA molecule in a catalytic fashion (RNAi). This phenomenon was first described in 1998 in *Caenorhabditis elegans* using double-stranded (ds) RNA to degrade its complementary mRNA [1]. This discovery led to the Nobel Prize in Physiology or Medicine in 2006 for Fire and Mello. Until then, state of the art of gene silencing *in vitro*, *in vivo* and even in patients was via single-stranded antisense oligodeoxynucleotides (ODNs) [2]. Research in this area goes back over 30 years [3, 4]. These two classes of molecules are similar in size range, charge and structure, and some of the lessons learnt formulating them are also valid for siRNA formulation.

RNA interference, however, is mediated by small interfering, double-stranded RNAs ("siRNA"). It silences specific target genes by associating with a multi-component nuclease called RNA-induced silencing complex (RISC). It guides the enzyme for sequence-specific degradation of its complementary mRNA [5]. This mechanism was demonstrated first in 2001 in a range of mammalian cells *in vitro*, and showed that siRNAs are effective at concentrations several orders of magnitude lower than antisense or ribozyme RNAs [6]. This advantage over antisense technology, a mechanism discovered and utilized some 10 years earlier than siRNA (1998 FDA approval for Vitravene, an antisense based ophthalmic drug), is known as catalytic cleavage of mRNA. The siRNA is not destroyed during this process and one siRNA molecule can lead to cleavage of multiple target mRNAs.

The introduction of ds RNA longer than 30 base-pairs (bp), the endogenous precursor of siRNA, into mammalian cells, induces a strong interferon response. siRNAs on the other hand are ds RNAs of generally 21–27 bp in length and bind their target with high degree of specificity. Therefore, the potential use against any transcribed mRNA of an organism's genome has triggered excitement in the drug development field not only as research tool but also as a potential new class of drug molecule with versatility comparable to antibodies.

siRNAs can also be endogenously expressed following transfection of plasmid or viral vectors [7, 8]. Viral delivery of siRNAs has also been demonstrated *in vivo* [9]. However, concerns about competition with the native micro-RNA pathway as well as interferon response of innate immunity towards longer ds RNA limit their applicability in human [10]. Furthermore, the potential immunogenicity and oncogenicity as well as the high cost of production of viral vectors make this technology unlikely to be applied for clinical use. These will therefore not be covered in this review. Adeno-associated viral vectors (AAV) on the other hand have been shown to induce less immunogenic response and are currently under evaluation for gene therapy in several clinical trials and might have a potential as a siRNA delivery vehicle [11]. However, the toxicity issues remain to be resolved before moving this drug forward [12].

Plasmid DNA, antisense RNA and siRNA molecules have some physicochemical properties such as charge density in common. There are also fundamental differences, most importantly the size, which is why the mechanisms of transfection are not always compatible. siRNA needs to be transported to the cytosol, the location of RNAi machinery, whereas plasmids expressing shRNA (small hairpin RNA) or siRNA need to be transported to the nucleus. Therefore numerous non-viral delivery methods are under investigation for their transfection efficacy as well as toxicity properties. The specific molecular nature of nucleic acids demands special formulation strategies to utilize its therapeutic potential. RNA molecules are very labile in acidic environments such as in the stomach or in endosomes and lysosomes in cells. It can be destroyed by enzymatic degradation in the gastrointestinal tract or in the blood circulation. The large size (~13 kDa) and the negative charge of their phosphodiester backbone (~40 negative phosphate charges), leading to electrostatic repulsion from the anionic cell membrane surface, limit their permeability into cells and release from endosomes.

This article sheds light on the most promising formulation efforts of the last decade which led to the first RNA interference based clinical trials, and summarizes the mechanisms used in pursuit of turning this class of fragile molecules into a therapy.

2. Delivery of naked siRNA

Despite its properties, naked siRNA has been shown to be delivered efficiently *in vivo*, although in many cases its uptake mechanism remains unknown.

The first successful *in vivo* demonstration of siRNA triggered gene silencing was reported in 2002. Naked synthetic siRNAs as well as endogenously transcribed hairpin RNAs were delivered to the liver [13] and other organs of mice, and inhibited co-delivered reporter-genes for several days [14]. Subsequently, treatment of hepatitis as potential therapeutic application was described [15]. However, in these experiments the unmodified siRNA or shRNA expressing plasmid was delivered by quickly injecting large volumes via the tail vein (hydrodynamic tail vein injection). This technique is restricted to rodents and not applicable for higher animals or humans.

Delivery of unmodified siRNA via the portal vein, a potentially clinically more relevant method, was demonstrated as equally efficient as hydrodynamic tail vein injection *in vivo* and protected mice against virus induced liver failure [16]. Low-pressure intravenous (i.v.) delivery of naked siRNA reaches the well-perfused organs liver and kidney [17]. This report also showed that chemically stabilizing the siRNA by introducing phosphorothioate instead of phosphodiester bonds (see Fig. 1) did not alter biodistribution but increased stability both *in vitro* and *in vivo*. However, the subcellular location was not described, and the cell uptake remained unproven.

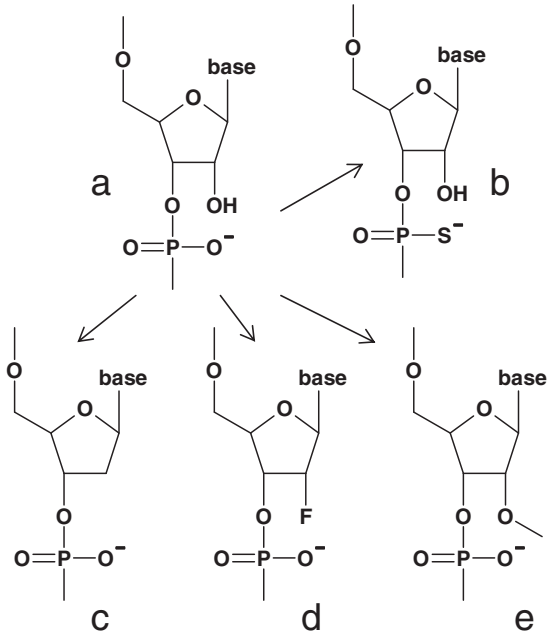


Fig. 1. Chemical modifications for stabilization of siRNA: a: phosphodiester (unmodified RNA), b: phosphorothioate RNA, c: 2′-deoxy-RNA d: 2′-deoxy-2′-fluoro (2′F-) RNA, e: 2′-*O*-methyl (2′-*O*-Me) RNA.

Naked and chemically unmodified siRNA has a very short half-life (t½) in blood or serum, even though being more stable than single stranded RNA [18]. Chemical modifications of siRNA such as 2′-F, 2′-*O*-Me or 2′-deoxy residues (see Fig. 1) and 5′ end capping with 3 ribonucleotides prolonged t½ in serum from few minutes for unmodified siRNA to 2–3 days, and in circulation from about 2 min to 49 min. In addition, the chemical modification also reduced the significant immune stimulation otherwise induced by unmodified siRNA [19].

Similarly, 2′-*O*-Me backbone modification at specific positions within the siRNA seed region abrogated some unspecific off target effects [20] and cytokine production [21], without significantly affecting silencing of the intended targets.

Not all modifications are tolerated with the same efficacy and specificity [22]. The effect of chemical modification on stability and half life is synergistically enhanced upon further encapsulation. The most commonly used chemical modifications have been reviewed recently [23].

Naked, unmodified siRNA can also be transported to the cytoplasm via electroporation, a method, which has been developed for plasmid DNA in the 1960s. It is regularly used for transfection of siRNA *in vitro* [24], but has also been described for *in vivo* transfection. Naked siRNAs were delivered using slow injection into the muscle tissue of mice followed by 8×20 ms pulses of 120 V and 1 Hz frequency. These settings proved to be mild and safe, resulting in complete repression of gene expression for up to 11 days [25].

Tumor necrosis factor (TNF)-α siRNA injected into joints followed by electroporation with 2 pulses of 200 V/cm reduced collagen-induced arthritis [26]. With specially modified "plate and fork" type of electrodes, the conditions for delivery of chemically stabilized VEGF (Vascular Endothelial Growth Factor) siRNA have been optimized to 91 V/cm without observation of any gross adverse events such as loss of body weight [27]. A correlation between the endogenous conductivity of the tumor tissue and the efficacy of delivery has also been observed. The conductivity itself was correlated with the microvascular density, and therefore with the VEGF concentration in the tumor. With this method, VEGF expression, microvascularization and tumor weight could be significantly reduced in a xenograft mouse tumor model. When electroporating the tumor after systemic siRNA administration instead of directly injecting into the tumor, a 40-fold higher VEGF-siRNA concentration was required to reach the same effect.

Due to the challenges associated with naked siRNA stability, most naked siRNA applications are focused on local administration such as to mucosal tissue or the eye. In 2004, only six years after the discovery of RNAi, the first siRNA for human therapy Sirna-027 (treatment for wet age related macular degeneration or AMD, Sirna Therapeutics Inc,) entered PhI clinical trials (see Table 1 for a summary of ongoing clinical trials). This chemically modified siRNA was injected intravitreal as a naked entity at concentrations up 1.5 mg/eye without severe adverse events. Although low amounts (100–200 μg) showed the anticipated clinical outcome of improvement of foveal thickness and visual acuity, a dose–response effect was not observed. This was thought to be due to sequence-unspecific reactions such as with TLR3 (Toll-like receptor 3) [28, 29]. Another clinical trial starting in 2004 also used free siRNA (Cand5) silencing VEGF to treat AMD as intravitreal injection up to 3 mg/eye. In 2009 this study was terminated early because the preliminary data indicated that the primary endpoint was unlikely to be met. However, other dosing regimen or delivery vehicles to improve the outcome are being considered.

Table 1
Clinical trials using siRNA as active ingredient.

Product owner	Technology originator	Clinical phase	Clinical outcome	Delivery technology	Administration	Indication	Product	Target
Alnylam Pharma Inc. (Cubist Pharma)		PhII (2007–2009)	Well tolerated, antiviral activity	Naked siRNA	Nasal, inhalation	Respiratory syncytial infection	ALN-RSV01	Respiratory syncytial virus
Alnylam Pharma Inc.	Tekmira	PhI (2009–2011) well tolerated, antitumor activity		SNALP	I.v. Infusion	Liver cancer	ALN-VSP02	Vascular endothelial growth factor, Kinesin spindle protein
Alnylam Pharma Inc.	Tekmira	PhI (2010–2012)		SNALP	I.v. infusion	Amyloidosis	ALN-TTR01	Transthyretin
Alnylam Pharma Inc./ Tekmira		PhI (2009–2010)	Terminated due to immune stimulation	SNALP	I.v. infusion	Lipid disorders	TKM-ApoB	Apolipoprotein B
Tekmira/Alnylam Pharma Inc.		PhI (2010–2012)		SNALP	I.v. infusion	Cancer	TKM-PLK1-001 (TKM-080301)	Polo-like kinase-1
Merck, Allergan Inc.	Sirna Therapeutics	PhII (2004–2007)	Well tolerated, potentially unspecific clinical effects	Naked siRNA	Intravitreal	Wet AMD	Sirna-027 (AGN-211745)	Vascular endothelial growth factor
Opko Health Inc.	Acuity Pharmaceutics	PhIII (2007–2009)	Well tolerated, clinical endpoint not met	Naked siRNA	Intravitreal	AMD	Bevasiranib (Cand5)Vascular endothelial growth factor	
Quark Pharma Inc./Atugen AG/Pfizer		PhII (2009–2011)	Well tolerated, improved vision	Naked siRNA	Intravitreal	Wet AMD	PF-655 (REDD14NP, RTP801i)	Hypoxia-inducible gene RTP801
Quark Pharma Inc./Silence Therapeutics	Atugen AG	PhII (2008–2012)		Naked siRNA	I.v. injection	Acute renal failure after kidney transplantation	AKII-5 (I-5NP, QPI-1002)	p53
Quark Pharma Inc.,		PhI (2010–2013)		Naked siRNA	Intravitreal	Eye diseases	QPI-1007	Caspase 2
Alnylam/Calando		PhI (2008–2011)	Well tolerated, accumulation in tumor, reduction of mRNA level	Rondel	I.v. infusion	Cancer	CALAA01	M2 subunit of ribonucleotide reductase
Silence Therapeutics	Atugen AG	PhI (2009–2011)	Well tolerated, stabilization of disease	atuPLEX	I.v. infusion	Cancer	Atu027	Protein kinase N3

The fact that naked siRNA is rapidly cleared via the kidney, can be utilized for kidney targeting [30]. 1 h after low pressure i.v. administration of unmodified, naked, radiolabeled siRNA in rats, the concentration in the kidneys was 40-fold increased compared to other organs. A selective downregulation of its target was also observed. Although not yet identified, several receptors have been suggested to be responsible for uptake of oligonucleotides in the kidney. This effect is also used in Quark Pharmaceuticals AKII-5 siRNA against p53, which is currently in PhI clinical trials for acute renal failure after kidney transplantation.

Local delivery of naked, unmodified siRNA and target gene knockdown has also been successful *in vivo* in lung after intranasal delivery [31]. This delivery route can result in a certain systemic exposure, as demonstrated for siRNA administered with the natural calf lung surfactant Infasurf [32]. Infasurf is a pulmonary surfactant composed of phospholipids and proteins with the ability to spread and line the alveoli. One single intranasal administration lead to an approximate 50% knockdown of the housekeeping protein GAPDH in lung, heart and kidney for up to 7 days, and did not alter liver protein levels. Other reports compared intranasal co-delivery of unmodified siRNAs with plasmid in D5W (5% (w/v) D-glucose in water) with Infasurf solution to treat SCV (SARS coronavirus) induced SARS (severe acute respiratory syndrome) in mouse and Rhesus macaque [33, 34]. In mice, the delivery with D5W was more efficient than with Infasurf. Subsequent non-human primate studies of SCV infection and siRNA-mediated knockdown were conducted with D5W only. They demonstrated the therapeutic efficacy of siRNA to relieve infection induced symptoms. Naked siRNA administered in saline has been shown effective in treatment of respiratory syncytial virus (RSV) infection, as demonstrated in Alnylams anti-viral ALN-RSV01 PhII studies [35, 36]. Although nasal administration in healthy volunteers as well as nebulized drug in lung transplant patients prevented from RSV infection, the systemic bioavailability was reported to be minimal. A PhIIb study is currently recruiting patients and is expected to conclude in 2012.

3. Excipient-siRNA interactions to tailor delivery

For indications which require systemic exposure, a formulation helping to overcome the stability and uptake challenges of siRNA is required. Which delivery system is indicated, depends on the biological environment of the targeted application sites (e. g. local/systemic). The excipient has to protect the siRNA from chemical or enzymatic degradation and exposure to TLR3 while releasing it upon arrival at the target site. If systemic administration is required, rapid renal clearance can be avoided by ensuring a particle size of >10 nm. On the other hand, the size of the nanoparticles should not exceed 150 nm, to enable extravasation and adequate tissue penetration. The charge should be high enough to prohibit self-aggregation but should not trigger nonspecific interactions with cell membranes and plasma proteins and uptake by the RES (reticuloendothelial system) [37]. Many excipients have the potential to non-covalently complex siRNA mostly due to charge-interaction, which has the advantage of using this delivery technology as modular tool, without chemically altering the drug substance, thereby maintaining biodegradability of the components and ease of production. On the other hand, these interactions are not as stable as covalently bound excipients, which may have a better chance to deliver cargo safely to its target tissue. However, covalent modifications create new chemical entities, which have to be thoroughly toxicologically characterized each time the formulation is changed. In this light, the formulation can be considered part of the drug, not just an excipient. There is a vast variety of excipient classes which provide some of these properties and are in various stages of preclinical or clinical exploration.

3.1. Lipids

The intrinsic properties of phospholipids such as their ability to self-assemble to fluid, flexible vesicles are used by nature to traffic biomolecules within as well as between cells. Lipidic systems for siRNA delivery have many advantageous features: the cell membrane consists predominantly of phospholipids (*e.g.* phosphatidylcholine) and cholesterol; therefore these natural lipids are biocompatible. They can interact with the cell membrane and deliver the payload efficiently into the cell and can be purified or synthesized in large quantities. Generally, two lipidic delivery vehicles can be considered: neutral or positively charged liposomes or lipoplexes, which have specific differences in interaction with the drug substance as well as interaction with the cell membranes.

3.1.1. Neutral liposomes

Liposomes composed of neutral lipids encapsulate siRNA in their aqueous core. They have comparably low entrapment efficiency for nucleic acids and low drug to lipid ratio as compared to cationic liposomes. These on the other hand have been shown to be more toxic *in vivo e.g.* after pulmonary application [38]. These toxic effects were induced by reactive oxygen intermediates (ROI) and correlated with the valency of the cationic lipid species.

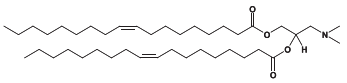
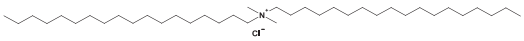
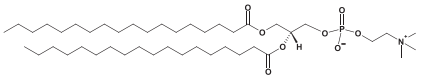
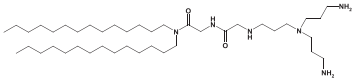
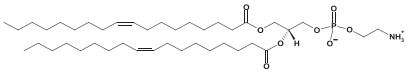
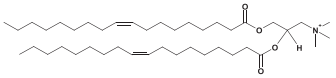
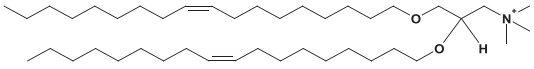
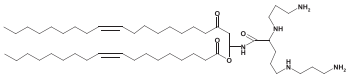
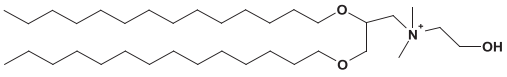
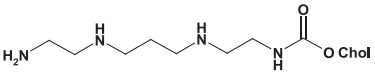
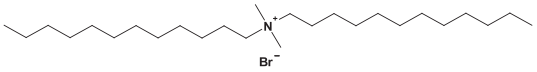
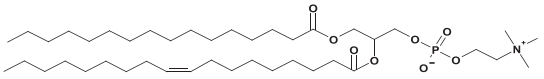
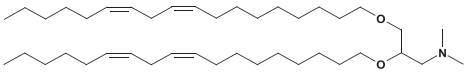
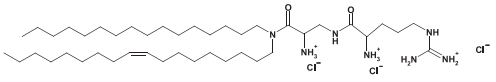
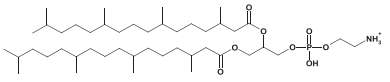
An elegant compromise between the two has been described for the encapsulation of antisense-oligonucleotides (ODNs) using the ionizable lipid DODAP (see Table 2 for chemical structure of the most common lipids) [39]. This approach allowed efficient entrapment of ODNs during formulation at low pH thanks to electrostatic interaction and subsequent adjustment of pH to neutral. This avoided some of the unfavorable biological effects of intravenously administered nanoparticles containing positively charged amino lipids such as DODAC. Increasing the amount of nucleic acid per lipid increased the lamellarity of the particles, indicating that the charge interaction was the driving force for encapsulation. A covering of polyethylene glycol (PEG) increased stability in both DODAC and DODAP liposomes and the t½ of the phosphorothioate ODNs *in vivo*. A more stable lipid anchor (C20 instead of C14) for PEG enhanced this effect.

A correlation between ethanol concentration used in the liposome preparation and entrapment efficiency has been described elsewhere [40]. High ethanol concentrations (up to 40% (v/v)), are ideal to achieve high entrapment efficiency of nucleic acids. The lipids are able to flip-flop across membranes, and regions enriched with the nucleic acid and depleted of PEG-ceramide were suggested to be the focal point of electrostatic interaction between drug substance and excipient. Subsequent rearrangement to multilamellar structures traps the nucleic acid between the lamellae. Increase of PEGylation stabilizes the liposome, resulting in an increase of ethanol required to achieve entrapment. It also increased the final entrapment efficiency. However, a large portion of the PEG gets displaced during formulation and can be separated as free PEG-ceramide and micelles. The lipid DODAP, which is cationic at the formulation pH 4, becomes neutral after buffer exchange to pH 7, where it releases all surface bound, un-entrapped nucleic acids. This method results in nucleic acid to lipid ratio of about 1:6 (mass), which is about 3 orders of magnitudes higher than with passive encapsulation.

An alternative to the low encapsulation efficiency of siRNA in neutral liposomes is to “lipophilize” the siRNA molecules by covalent conjugation to lipid molecules. Covalent conjugation of derivatives of lauric acid, lithocholic acids or cholesterol to the 5′-end of unmodified siRNA enabled siRNA delivery and knockdown *in vitro* [41].

Cholesterol modified and chemically stabilized siRNA have been shown to bind to human serum albumin (HSA) in circulation with an estimated dissociation constant of 1 μM [42]. It showed improved *in vivo* pharmacokinetic properties with a t½ of 95 min compared to t½ = 6 min for unconjugated siRNAs in mice. Unlike unconjugated siRNA, the cholesterol-conjugate was detected after 24 h in a variety of tissues (liver, heart, and kidney) and silenced apoB mRNA in liver and jejunum, resulting in decreased plasma levels of apoB protein and cholesterol.

Table 2
Lipids used for siRNA encapsulation and delivery.

DODAP	1,2-dioleoyl-3-dimethylammonium propane	
DODAC	Dioleoyldimethylammonium chloride	
DSPC	1,2-Distearoyl-sn-glycero-3-phosphocholine	
RPR209120	2-{3-[Bis-(3-amino-propyl)-amino]-propylamino}-N-ditetradecyl carbamoyl methyl-acetamide	
DOPE	Dioleoyl-L-R-phosphatidylethanolamine	
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium	
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium	
DOSPER	1,3-Di-Oleoyloxy-2-(6-Carboxy-spermyl)-propylamid	
DMRIE	1,2-Dimyristyloxypropyl-3-dimethylhydroxy ethyl ammonium	
CDAN	N1-Cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine	
DDAB	Dimethyldioctadecylammonium bromide	
POPC	1-Palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine	
DLinDMA	1,2-Dilinoleyloxy-3-dimethylaminopropane	
AtuFECT01	b-L-Arginyl-2,3-L-diaminopropionic acid-N-palmityl-N-oleyl-amide trihydrochloride	
DPhyPE	1,2-Diphytanoyl-sn-glycero-3-phosphoethanolamine	

The transport and uptake mechanism of a variety of lipophilized, chemically stabilized siRNA conjugates with cholesterol, fatty acids or bile acids were studied *in vivo* in several rodent species [43]. The conjugates interact with high-density lipoprotein (HDL) followed by uptake via HDL-receptors in the liver, gut and kidney. Binding to low density lipoprotein (LDL) targets the complexes mainly to the LDL-receptors in the liver. The ability to bind to HDL is higher for longer fatty acid chains (C18–C22), whereas short- to medium fatty acids (C12, C14) tend to bind to albumin. Furthermore, pre-assembly of chol-siRNA to HDL results in 8-15-fold higher silencing efficacy *in vivo* than cholesterol-siRNA alone.

3.1.2. Cationic lipids

Cationic lipid transfection has been described for DNA already almost 25 years ago [44]. Although siRNA or antisense oligonucleotides have similar physicochemical properties as plasmid DNA, they lack the capacity to condensate to nanoparticles upon neutralization with cationic lipids. One pDNA molecule can collapse to form a nanoparticle, whereas it takes multiple siRNAs to form a similar structure [45]. A molecule of at least 800 bp is required to trigger this condensation effect, leading to a 10,000-fold reduction in size and resulting in the favorable size range for long *in vivo* circulation time and transfection efficiency. The minimum persistence length

(measure for polymer stiffness) for ds nucleic acids is 60 nm or 26 bp. Consequently, the 21 bp siRNAs are essentially rigid rods.

DNA co-complexation with siRNA enhances the delivery of lipoplexes, although no significant structural difference between the particles with or without DNA was observed, except from the requirement of more lipid per nucleic acid to form complexes in the case of siRNA only [46]. In this study, the cationic lipid RPR209120 containing a globular spermine headgroup was used in combination with DOPE. Also other transfection reagents such as micellar RPR120535, Lipofectamine liposomes and to a lesser extend polyethylenimine (PEI) polymer nanoplexes showed increased efficacy, if co-formulated with plasmid DNA. Below the efficient ratio of cationic lipid to nucleic acid of 2–8 nmol/μg, the efficacy is diminished and above this ratio toxicity is observed. The presence of serum decreases the transfection efficiency of these lipoplexes. When the zeta potential of the complexes approaches zero and the net charge of the surface is neutral, the repulsive electrostatic forces otherwise preventing aggregation, are absent and the particle size increases. An explanation for the higher transfection efficiency may be the poorer entrapment and subsequent facilitated release of siRNA in the presence of pDNA.

The polycationic liposomal transfection reagent DOSPER (Roche) has been shown to trigger RNAi *in vitro* after co-transfection of plasmid DNA and synthetic oligonucleotide duplexes [47]. Similarly, i.v. co-injection of DOTAP-complexed, unmodified siRNA with a reporter plasmid DNA lead to efficient silencing in the target organs of liver and spleen. Intraperitoneal injection of siRNA complexes targeting TNF-α reduced TNF-α expression after lipopolysaccharide (LPS) challenge without increasing the pro-inflammatory cytokine Interleukin (IL)1-α, suggesting a therapeutic potential [48].

Another difference between siRNA and pDNA [49] or antisense RNA [50] is that synthetic siRNA usually does not enter the nucleus after transfection, but rather remains in the perinuclear region [51]. This shows that conclusions drawn from transfection experiments with plasmid or antisense DNA are not necessarily transferable to siRNA transfection, also termed "siFection" [52]. Recent studies suggest that using cationic lipids to transfect cells with unmodified siRNA leads to uptake via endocytosis. A small fraction of the siRNA lipoplexes may also be delivered via another, cholesterol-dependent route directly to the cytoplasm and may be responsible for the majority of mRNA degradation. These results may indicate that endosomal escape is a major bottleneck to functional siRNA delivery [53].

Complexes of nucleic acids and lipids can employ different states, which depend on the lipid composition, the shape of the single lipid molecules (rod or cone shaped) and their phase transition temperature. In the inverted hexagonal liquid-crystalline state (H_{II}), the nucleic acid is surrounded by lipid molecules in a rod-like fashion which are stacked in a hexagonal lattice. In the multilamellar structure (L_α), the nucleic acid is sandwiched between flat lipid monolayers [54] (see Fig. 2). The structural differences between DNA and siRNA encapsulation in cationic lipid complexes have been thoroughly investigated using X-ray diffraction and showed only minimal differences, although H_{II}-siRNA polyplexes showed higher toxicity and lower *in vitro* efficacy than H_{II}-DNA complexes [55]. Higher cationic lipid/nucleic acid molar charge ratios are required for siRNA transfection than for DNA. Furthermore, multivalent cationic lipids are more efficient siRNA transfection reagents than monovalent lipids. NMR analysis of nucleic-acid-lipid nanoparticles revealed that helper-lipids such as DOPE or cholesterol, which promote adoption of the H_{II} phase rather than lipid bilayers, enhance cationic lipid-mediated transfection of cells [56]. The ability to adopt a non-bilayer structure correlates directly with the un-saturation of the cationic lipid and a decrease of the bilayer-to-H_{II} transition temperature (T_{BH}). Consequently, a low T_{BH} indicates good transfection efficiency. Ions or molecules such as Ca^{2+} or polylysine may facilitate this transition and therefore enhance the transfection efficiency of cationic lipid nanoplexes.

Lipofectin is one of the earliest cationic lipid transfection reagents for nucleic acid transfer comprised of an equimolar mixture of the cationic lipid DOTMA and the co-lipid DOPE. It was first described for the use of antisense RNA transfection in the early 90s [57].

DMRIE-C is a 1:1 (molar ratio) liposome formulation of the cationic lipid DMRIE and cholesterol [58]. In these particles, nucleic acids associate on the outside of these preformed liposomes of about 400–500 nm through electrostatic interaction.

Numerous other proprietary lipid-based compositions of transfection agents are commercially available, mostly designed for *in vitro* cell culture. *Trans*IT TKO (Mirus) is based on lipids and polymers, forms lipoplexes with nucleic acids and is endocytosed and efficiently released in the cell.

Lipofectamine has been launched 1993 for DNA transfection and has been further optimized for siRNA transfection (Oligofectamine, Lipofectamine RNAiMAX). Lipofectamine transfected, unmodified anti-TNF-α siRNA prevented experimental colitis after rectal

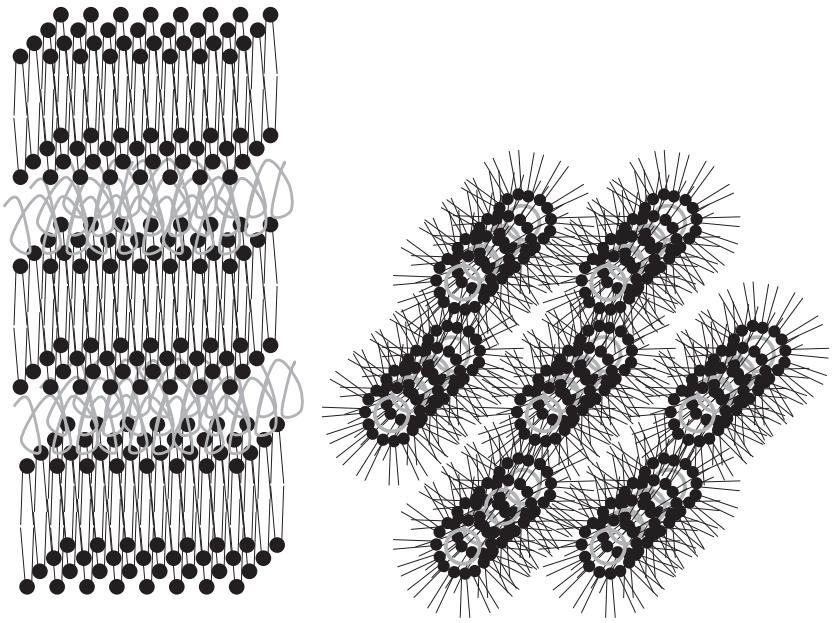


Fig. 2. Schematic representation of the structural states of siRNA–lipid complexes: multilamellar structure (L_α, left), inverted hexagonal liquid-crystalline state (H_{II}, right). Gray: siRNA, black: lipids.

administration in mice [59]. Similarly, Oligofectamine has been shown to enhance mucosal uptake of unmodified siRNA *in vivo* in vaginal tissue, protecting mice from HSV-2 (herpes simplex virus) infection [60]. Both reagents did not show toxicity in these experiments, demonstrating their feasibility of local mucosal delivery of siRNA. Oligofectamine has been efficient in reducing mouse tumors after intraperitoneal injection with unmodified β-catenin siRNA [61]. However, these transfection reagents are recommended to be used in medium without serum and no systemic administration has been described to date. Some of these commercially available transfection systems have been described to show unspecific total cell protein knockdown and toxicity *in vitro*.

Optimal specific knockdown with low toxicity was achieved using the cationic cholesterol-based polyamine lipid CDAN and the neutral helper lipid DOPE for transfection of unmodified siRNA [62]. This study underlines the differences in formulation and uptake mechanism between plasmid DNA- and siRNA-lipoplexes, as well as the formulation conditions such as salt concentration.

While it is evident, that cationic lipids have a higher affinity to nucleic acid and therefore higher loading capacity than neutral lipids, a net cationic charge of intravenously administered lipid-antisense RNA nanoparticles have been linked to rapid plasma clearance due to opsonization by the RES, distribution into lung, liver and spleen, [63]. Cationic lipids alone often exhibit liver and hemodynamic toxicities [64], and prolonged clotting times as shown with antisense oligonucleotide lipid vesicles [39]. Unmodified siRNA transfected with cationic lipid DOTAP was shown to trigger unspecific knockdown and off-target effects *in vitro* due to activation of innate immune response in a sequence- and concentration-dependent manner [65]. It appears to be dependent on TLR interaction and signaling (TLR8), since electroporation, delivering siRNA directly into the cytoplasm did not show these effects. This effect was shown for both single- and double stranded unmodified oligonucleotides just in the combination with the lipid, which on its own did not trigger immune response. Some of the immune stimulatory effects can be traced back to specific GU-rich sequences within the siRNA or other specific formats of the molecule [66]. This substantial, dose-dependent activation of the innate immune system as detected by upregulation of interferon-α (IFN-α), inflammatory cytokines including IL-6 and TNF-α was demonstrated in several mouse strains and also required the combination of vehicle and siRNA.

Meanwhile the quest for the optimal lipidic delivery vehicle for siRNA has become more systematic and a large combinatorial library of over 1200 lipid-like delivery molecules, termed lipidoids, has been generated and evaluated *in vitro* for chemically stabilized siRNA as well as antisense delivery. Several of these candidates have been shown to be safe and efficacious and proceeded to *in vivo* trials in rodents and non-human primates [67]. Unlike most other lipidic transfection agents, the best performers of this screening contained secondary amines and had more than two aliphatic chains, shorter than those of the typical C18-lipids. These lipidoids were more effective than Lipofectamine 2000 in delivering siRNA into typically hard to transfect cells such as macrophages at low siRNA concentrations. However, they displayed varying efficiency in different cell lines. In order to provide a neutral, hydrophilic shell and reduce opsonization and macrophage uptake *in vivo*, these particles contained also cholesterol and PEG-conjugates. The efficacy of the formulation was shown for liver targeting after i.v. administration as well as for pulmonary and peritoneal administration.

Liposomes also allow attachment of targeting ligands such as antibodies. Anti-DEC-205 antibodies were used to generate immunoliposomes targeted against dendritic cells to deliver unmodified CD40 siRNA *in vitro* and *in vivo* in dendritic cell rich organs, and to silence the target mRNA [68]. In the same report, the positive effect of incorporation of the positively charged lipid DDAB in an otherwise neutral liposome based on POPC on the siRNA encapsulation efficiency was demonstrated. In another report, a nanoparticle composed of the cationic lipid DOTAP, cholesterol, DSPE-PEG2000 and the polycationic peptide protamine sulfate modified with anisamide as targeting ligand delivered unmodified siRNA to sigma receptor expressing B16F10 tumor cells 4 times more efficiently than the corresponding untargeted nanoparticle [69]. *In vivo* administration of a single i.v. dose of 150 μg siRNA/kg resulted in up to 80% gene silencing with little reported immunotoxicity.

The membrane destabilizing effect of ethanol on encapsulation in conjunction with the stabilizing effect of PEG-lipids was used to generate stabilized nucleic acid-lipid nanoparticles termed SNALPs. These consist of a lipid bilayer containing a mixture of cationic and fusogenic lipids enabling cellular uptake and endosomal release of the particles payload. Due to the short lipid anchor of the PEG molecule, it can be shed after injection into circulation, resulting in a transfection-competent particle. Thereby, the t½ of the chemically stabilized siRNA in circulation was increased from 49 min to 6.5 h. When encapsulated in SNALPs, *in vivo* toxicity and interferon response of siRNA was decreased, without compromising its activity [18, 19]. In non-human primates, SNALPs based on the lipids DLinDMA, DSPC and cholesterol proved to be efficient to deliver chemically stabilized ApoB-siRNA and silence the target protein. Lower serum cholesterol and low-density lipoprotein levels were detected as early as 24 h after administration, lasting up to 11 days. It triggered even less changes in clinically relevant chemical and hematological parameters in blood than the previously published SNALP particles [70]. The preparation of the nanoparticles was adapted from a plasmid-formulation method [71]. In this ethanol dilution method, lipids, dissolved in ethanol were rapidly mixed with the aqueous siRNA solution in a T-shaped mixing chamber, which instantaneously diluted the ethanol concentration below the concentration required to support lipid solubility, resulting in a particle population of narrow size distribution. The antitumor effect of SNALP-delivered, chemically stabilized siRNA has been confirmed not only in hepatic but also in subcutaneous tumor models *in vivo* with polo-like kinase 1 (PLK1) and kinesin spindle protein (KSP) siRNA [72]. SNALP nanoparticles also proved to be more efficient than polyethylenimine (PEI) nanoparticles in protecting guinea pigs from Ebola virus infection, using the same, unmodified siRNA [73]. Table 1 gives an overview over the currently ongoing clinical trials using SNALP technology.

A new potent, liposomal system for siRNA delivery, termed AtuPLEX, has been developed at Atugen AG (Silence Therapeutics), based on the cationic lipid AtuFECT01, the neutral helper lipid DPhyPE, and 1 mol% DSPE-PEG. In these particles the 2′-*O*-methyl modified siRNAs sit on the outside of positively charged, PEGylated liposomes instead of being encapsulated such as in SNALP particles. They showed efficient siRNA delivery, endosomal siRNA escape and knockdown *in vitro* and *in vivo* [74]. This novel lipid's highly charged head group allows a stronger interaction with siRNA compared to DOTAP or DOTMA. In this study also the effect of various degrees of PEGylation was studied. While 5 mol% abolished *in vitro* transfection efficiency, 1–2 mol% PEGylation maintained RNAi and the particles could be detected in small, uniform vesicles inside the cell. *In vivo* 1 mol% PEGylation prevented unspecific toxic side-effects which were otherwise observed with un-PEGylated polyplexes. This demonstrates the delicate balance between transfection efficiency and toxicity in terms of PEGylation of siRNA nanoplexes. Based on this composition, the drug Atu027, a PKN-3 siRNA, was developed and showed antitumor-efficacy in rodents and non-human primates after systemic administration [75]. The downregulation of the siRNA target protein kinase 3 (PKN3) was most robust in lung and liver tissue due to their high vascularization. Due to the 2′-*O*-methyl-modification of the siRNA, no sequence-unrelated unspecific cytokine activation was detected. This formulation is currently evaluated in a PhI clinical trial for the treatment of advanced

solid tumors. Preliminary results show that the drug is well tolerated; nine of the 24 patients treated with Atu027 achieved stable disease after repeated treatment, out of which two experienced slight regression in tumor metastases.

3.2. Polymers

3.2.1. Polyethyleneimine (PEI)

A class of polymers widely used for nucleic acid delivery is the synthetic polymer polyethylenimine, an organic linear or branched polymer with molecular weights ranging from 1 to over 1000 kDa [76].

The capacity of PEI to bind nucleic acids and form self-assembled nanoplexes is similar to cationic lipids due to its positive charge. However, only one sixth of the protonable amino nitrogen atoms are protonated at physiologic pH, resulting in a high buffering capacity and in release of its cargo in the acidic environment of the endosome; this is referred to as proton sponge effect. The unprotonated amines in the polymer show different acid dissociation constant (pKa) values due to crowding of the amines, and display a buffering effect over a wide range of pH [77]. The buffering may protect the siRNA from degradation in the endosomal compartment during the maturation of the early endosomes into late endosomes, and their subsequent fusion with the lysosomes. The buffering capacity of PEI causes an influx of protons, chloride ions and water into the endosomes, which eventually leads to swelling and burst of the endosome due to increased osmotic pressure. This, together with the protective effect against nucleases in circulation and a relatively low *in vitro* toxicity has led to PEI being tested as nucleic acid delivery agent for plasmids and oligonucleotides already in 1995 [78]. Optimization of this drug delivery system for siRNA revealed a lower optimal ratio of Nitrogen to Phosphate (N/P ratio) for siRNA than for pDNA (5 *vs*. 10), when using linear PEI. This led to an *in vitro* target downregulation comparable to the lipidic transfection agent Oligofectamine [79].

Comparing PEIs of different degrees of branching, molecular sizes at N/P ratios showed that the transfection efficiency highly depends on the biophysical and structural characteristics of the particles. 25 kDa branched PEI (bPEI) at the N/P ratio of 6 or 8 showed specific knockdown without unspecific toxic effects *in vitro* [80]. The low efficacy of other complexes with N:P ratios of 1 was traced back to bigger particle size and overall negative zeta potential. Complexes of siRNA and 25 kDa PEI at an N/P ratio of 10 showed significant silencing efficacy in lung after i.v. injection into mice, and could prevent and also treat an already established influenza infection [81].

Toxicity of polycations can be due to interaction with the negatively charged cell or mitochondrial membranes, leading to membrane damage and charge dependent activation of the complement system. BPEI with a molecular weight (MW) of 25 kDa or higher is generally believed to have both a higher efficiency and cytotoxicity as compared to lower MW or linear PEI. Polyplexes with PEGylated PEI are generally better tolerated than those with PEI alone. One detailed *in vitro* toxicity study comparing 25 kDa PEI and PEG/PEI (polyethlylenglycol-grafted PEI) using a variety of *in vitro* assays such as cell viability, LDH release and expression of apoptosis-markers, not only confirmed the better tolerability of PEGylated PEI, but also showed that the diblock-co-polymer stimulates other pathways than PEI in various cell lines. This demonstrates the significance of assay conditions when comparing and interpreting toxicity of nanoplexes [82]. VEGF-siRNA covalently coupled via a disulfide linkage to PEG formed micelles through electrostatic interaction with a branched PEI core. The protective PEG corona is cleaved off in the reductive endosomal environment, resulting in sequence-specific gene silencing *in vitro* [83].

Intratracheal delivery of unmodified siRNA via unmodified or fatty-acid modified PEI-based nanocomplexes into mice lungs showed cytotoxicity despite efficient knockdown efficacy. PEGylation of PEI reduced the cytotoxic effect, but on the other hand increased immune response and inflammation [84]. This study demonstrated the delicate balance between the hydrophilic PEG and the hydrophobic modification with respect to cytotoxicity, immunostimulation and efficient siRNA delivery.

Water soluble lipopolymer (WSLP) based on 1.8 kD bPEI showed improved delivery and efficacy of anti-VEGF siRNA when compared to conventional bPEI *in vitro* and *in vivo* [85]. The polymer neutralized and condensed the siRNA at a w/w ratio of 1:1 (WSLP/siRNA), resulting in a zeta potential of 10 mV, reduced cell toxicity *in vitro* and efficient protection from serum degradation. Intratumoral injection of the PEI/siRNA complexes in mice resulted in reduced tumor growth compared to controls.

Substitution of 6–9 mol% of primary amines of branched 25 kDa PEI with carboxyalkyl chains of various lengths resulted in significantly reduced cytotoxicity and improved silencing efficacy *in vitro*, due to reduced surface charge and increased hydrophobic interaction [86]. Unmodified PEI showed cytotoxic effects at w/w ratios above 1:1, which is not efficient in terms of silencing, however modified PEI was tolerated and efficient at ratios up to 3:1. Excess of modification not only decreased the toxicity, but also the neutralization capacity and thereby the efficacy.

Among a variety of derivates of bPEI, including ethyl acrylate modified, acetylated or negatively charged propionic acid substituted amines, succinylation appeared to be the modification resulting in lowest toxicity and sequence specific knockdown of up to 90% *in vitro* [87]. Excess of modification led to less toxic, but also ineffective polymers.

Modification of PEI with disulfide bonds and increased degree of branching can enhance the cell-uptake, intracellular endosomal release and silencing efficiency [88]. These modified polymers complexed and neutralized siRNA at N/P ratios of 4 or higher. The hydrodynamic diameter of the polyplexes correlated directly with N/P ratio and reached a maximum of 400–500 nm at N/P ratios higher than 20. Cellular uptake as well as the toxic effect increased with higher polymer concentration. The dose dependent silencing effect however leveled off at only 45% silencing compared to 90% for the cationic transfection reagent Lipofectamine RNAiMAX.

A ketalized version of PEI showed increased cytoplasmic localization of the polyplexes followed by efficient release of siRNA by acid-hydrolysis of the amino ketal linkages *in vitro*, while unmodified PEI targeted siRNA partly to the nucleus [89]. In addition, this modified polymer also had a lower cytotoxicity and serum dependency than unmodified PEI. The complexation efficiency decreased with increasing MW and with ketalization of PEI evidenced by particle size, ethidium bromide exclusion assay and transmission electron microscopy (TEM). This was possibly a result of to the steric hindrance of interaction due to the bulky ketal side chains. However the additional primary amines from the ketal branches allowed closer interaction with siRNA and therefore better retardation on the gel at lower N/P ratios that unmodified PEI.

Branched 25 kDa PEI was used to target unmodified VEGF siRNA via integrin targeting RGD peptide to tumors [90]. The modified PEI condensed siRNA at N/P ratios as low as 2. PEGylation decreased the surface charge from 35 to about 5 mV, suggesting the localization of the RGD-modified PEG chains on the surface of the particle and preventing aggregation, which otherwise occurred with un-PEGylated PEI nanoplexes. *In vitro* only RGD-targeted or un-PEGylated siRNA/PEI nanoparticles were able to enter the cells and inhibit endogenous luciferase expression. PEGylated untargeted nanoparticles showed no effect, demonstrating the decreased interaction of the hydrophilic surface and the cells. These particles also specifically decreased tumor angiogenesis and growth after injection into tumor bearing mice.

In a similar active targeting approach, folate was employed to target PEI/siRNA nanoparticles to folate-receptor overexpressing cells *in vitro* [91]. Covalent PEGylation decreased both the zeta potential as well as the particles size due to the charge shielding effect of PEG for 43 kDa bPEI.

Covalent attachment of 5000 kDa PEG to the unstabilized siRNA via disulfate linkage has been combined with 25 kDa bPEI at a N/P ratio of 16, leading to formation of micelles of about 100 nm size. They significantly inhibited VEGF expression and tumor growth after i.v. as well as intratumoral administration in a tumor mouse model without detectable inflammatory response [92]. The disulfide linkage is efficiently cleaved by the endogenous intracellular concentration of 10 mM glutathione, releasing functional siRNA.

PEI encapsulated in yeast derived glucan allowed to target siRNA to macrophages in several organs such as lung, liver and spleen after oral administration in mice [93]. The macrophages of the intestinals Peyer's patches transported the 2–4 μm particles across the intestine to the lymphatic tissue.

Intraperitoneal injection of unmodified HER-2 siRNA complexed with linear low molecular weight PEI (JetPEI, commercially available from Polyplus transfection) led to delivery of intact siRNA to subcutaneous tumor and reduction in tumor growth in a mouse xenograft model. In comparison to DNA/PEI particles, siRNA/PEI nanoplexes were detected exclusively in the cytoplasm and not in the nucleus [94]. In another study, positively charged JetPEI/siRNA nanoparticles were efficient in delivering the unmodified siRNA to xenograft tumors resulting in knockdown of its target growth factor pleiotrophin, and tumor growth regression after subcutaneous or intraperitoneal administration without any measurable stimulation of the immune system [95]. These complexes were also injected directly into the central nervous system of a glioblastoma model and showed antitumoral effects. Atomic force microscopy (AFM) analysis of the complexes revealed that the PEI–siRNA complexes had a more spherical shape and somewhat smaller size when compared to PEI–DNA particles. The lack of free fiber-like structures sticking out of the round complexes indicated that the siRNA was completely covered by polymer. This polymeric nucleic acid delivery agent has been shown safe in clinical trials after delivery of pDNA via intravesical infusion [96] as well as for dermal application [97]. However, safe systemic administration in man remains to be proven.

3.2.2. Poly(lactic-co-glycolic acid) (PLGA)

PLGA is a water insoluble, biodegradable and biocompatible polymer, which is used for biopharmaceuticals due to its controlled release characteristics. However, swelling of the particles after introduction into an aqueous medium can lead to a release of encapsulated drug near the surface in a burst release fashion.

Its use has been described first in 2006 for siRNA encapsulation and delivery, efficiently silencing its GFP target *in vitro* [98]. The correlation between process parameters, loading efficiency and release of siRNA from PLGA nanoparticles has been investigated in a design of experiment [99]. PLGA concentration is the most important factor and increases the viscosity of the oil phase in the primary emulsion, resulting in a decrease of the leakage of siRNA into the outer water phase, resulting in encapsulation efficiencies as high as 70%. A burst release of surface-localized siRNA was observed followed by a triphasic sustained release over 2 months.

PLGA has been used also as ~40 μm microspheres in combination with 25 kDa bPEI and polyarginine to achieve sustained release of unmodified anti-VEGF siRNA. The study over 1 month *in vivo* evidenced a reduction of tumor growth after intratumoral injection in mice [100]. The interaction between the terminal carboxylic anions of PLGA and the basic amino or imino groups of arginine and PEI promoted high encapsulation efficiency and reduced burst release.

Topical administration of unmodified siRNA to mucosal tissue PLGA nanoparticles has been demonstrated as efficacious [101]. These nanoparticles of less than 200 nm encapsulated over 1000 siRNA molecules, penetrated the mucosal tissue, released siRNA in a sustained fashion over 14 days after a single topical administration to vaginal mucosa and led to 50–60% reduction of target expression with less inflammatory responses than lipoplexes. Complexation of siRNA with spermidine at an N/P ratio of 8:1 before encapsulation into PLGA nanoparticles increased the loading efficiency over 40-fold. The particles did not show any cytotoxic effects *in vitro* in concentrations up to 10 mg/ml, whereas the silencing efficiency was comparable to Lipofectamine RNAiMAX.

In summary, this polymer may not be the first choice as excipient for systemic siRNA delivery agent on its own, but could improve a formulation in combination with other excipients due to its sustained release properties.

3.2.3. Dendrimers

Cationic dendrimers such as the extensively studied poly(amidoamine) (PAMAM) dendrimer are polymers composed of multiple covalently attached branched monomers emanating from a central core. The number of branching points can be precisely controlled during synthesis and defines the generation G. A higher G will result in an increase in surface groups, a closer packing and higher charge density. Dendrimers have been used for DNA delivery due to their beneficial properties such as the defined molecular size, structural homogeneity or the high density of functional groups.

The first efficacious PAMAM dendrimer-siRNA nanoparticles have been described in 2006 [102]. Their primary amine groups facilitated nucleic acid binding, neutralized siRNA at N/P ratios as low as 2.5 and triggered a proton sponge endosomal burst similar to other polymers such as PEI. The nanosized particles of about 100 nm protected unmodified siRNA from RNase degradation and delivered siRNA into cells *in vitro*. The interaction stability also directly correlated with the generation of the dendrimer. The thermodynamics of the self-assembly process have been characterized using dynamic light scattering (DLS), small-angle X-ray scattering (SAXS), isothermal titration calorimetry (ITC) and *in silico* modeling more recently [103]. It was shown, that increasing the N/P ratio results in a decreased size with higher polydispersity, but siRNA binding did not alter the size of the PAMAM monomers of 3.3 nm. Calorimetric analysis suggested an exothermic, biphasic binding of siRNA to dendrimer and a binding enthalpy ΔH_{bind} of -870 kcal/mol at a 1:1 molar ratio. Further studies, investigating the interaction between these polymers and siRNA in detail both *in silico* and in NMR and gel retardation assays revealed that with an increasing G, the dendrimers become more rigid resulting in lower binding affinity, which is furthermore highly pH dependent [104, 105]. This effect was most pronounced for the generation G5, which therefore might be ideal for binding as well as endosomal release of its cargo. Activated PAMAM-dendrimers, commercialized by QIAGEN (Superfect and Polyfect), have been used mainly for plasmid transfection [106] and with siRNA in co-complexation with plasmid DNA [107].

Due to the toxicity of higher generation dendrimers, only few *in vivo* studies have been carried out. PEGylation has been shown to not only decrease the toxicity of dendrimer particles, but also to improve the stability of unmodified siRNA in plasma while maintaining their transfection efficacy [108]. Polypropylenimine dendrimers of the generation 5 were complexed with unmodified siRNA, stabilized by a dithiol crosslinker and shielded by PEG. This encapsulation method substantially decreased the otherwise fast release and degradation of the unmodified siRNA from the dendrimer-nanoplexes in serum [109]. Due to PEGylation, the unspecific interaction with the cell surface was decreased, while a targeting peptide triggered specific cell uptake and gene silencing both in tumor cell lines and in mouse tumor models.

In another *in vivo* study, nanoparticles based on polymerized polyglycerol dendrimers were able to stabilize unmodified siRNA and induced silencing without detecting toxic effects [110]. A dendrimer-based, lysine-containing nanoparticle with surface bound lipid chains termed iNOP stabilized ApoB siRNA and silenced its target *in vitro* [111]. After low pressure tail vein injection the particles were delivered to the liver and spleen and to a lesser extend to the lungs and

silenced ApoB *in vivo* without toxic effects. The maximum silencing effect was reached at a dose of 1 mg/kg, resulting in about 50% knockdown.

3.2.4. Cyclodextrin

Cyclodextrins are water soluble cyclic oligosaccharides, which are used in pharmaceutical formulations due to their solubilization properties, biocompatibility, and their low toxicity and immune stimulation [112, 113].

Cyclodextrin-containing polymer (CDP) based siRNA nanoparticles have been developed and biophysically characterized *in vitro* [114] [115]. The cyclodextrin was pre-mixed with an adamantane-PEG5000 conjugate at equimolar ratios, before adding nucleic acid in the appropriate ratio to reach the desired tunable charge of these self-assembled nanoparticles. Adamantane formed an inclusion complex with β-cyclodextrin with a high association constant of up to 10^5/M. The PEG-molecules were modified with transferrin to enable active targeting. The small size of the building blocks ensures renal clearance once disassembled, although no enzymatic degradation in human takes place. Imidazole groups attached on the backbone of the cyclodextrin group assisted in the endosomal release of the siRNA due to their buffering effect. The particles required a slightly positive charge ratio of 1 to 1.5 for complete complexation and protection of siRNA from serum enzymes. PEGylation significantly reduced the size compared to un-PEGylated particles, as evidenced by light scattering, AFM and TEM. Using isothermal titration calorimetry, a negative ΔH was observed, indicating release of energy upon binding of the hydrophobic adamantane and the cyclodextrin. Centrifugal filtration in combination with multiangle light scattering allowed to calculate the stoichiometry of these complex particles, and revealed a ratio of about 10,000 CDP chains, 2000 siRNA molecules, 4000 adamantane-PEG molecules and 100 transferrin-PEG molecules. The uptake in cell culture in dependence of transferrin-conjugation was confirmed by confocal microscopy and FACS analysis. *In vitro* results using different transferrin-densities on the nanoparticle surface evidenced that the multivalency of the transferrin-decorated particles increased affinity to transferrin-receptor expressing cells due to avidity effects, and led to release of functional siRNA and gene silencing. This delivery strategy has been shown to slow down tumor growth in a mouse model of metastatic Ewing's sarcoma after delivery of 8×2.5 mg/kg over 4 weeks unmodified anti-*EWS-FL11* siRNA in cyclodextrin particles in D5W via low volume tail-vein injection [116]. Furthermore, no increase in inflammation markers was detected. The observation that brain metastases were not affected suggests that these particles did not cross the blood brain barrier (BBB).

In another example of *in vivo* use of this cyclodextrin based siRNA delivery vehicle in a xenograft tumor mouse model, the accumulation of the labeled, but not stabilized luciferase siRNA in the tumor, showed no significant difference, whether the transferrin was attached to the nanoparticles or not. The luciferase activity reduction in the tumor was significantly higher in the transferrin-targeted siRNA nanoparticles, suggesting a more efficient endocytosis of the actively targeted nanoparticles [117]. A dose-escalating non-human primate study using this delivery vehicle showed mild and reversible toxic effects in kidney and liver only at a dose of 27 mg/kg of unmodified siRNA. The lower doses up to 9 mg/kg were well tolerated, suggesting the potential safe use of these formulations in human use [118]. The relatively rapid clearance of the nanoparticles from plasma was ascribed to the intended tissue targeting. Based on this multicomponent delivery vehicle, a clinical dosage form ("RONDEL") ultimately entered as first formulated and actively targeted siRNA PhI clinical trials in 2008 as CALAA-01, containing a nonmodified siRNA targeting ribonucleotide reductase subunit 2 [37]. This promising formulation can be produced under scalable cGMP conditions in a 2-vial dosage form, where the complexes are formed spontaneously upon mixing of siRNA with the excipients. It was shown, that the nanoparticles were well tolerated in 15 cancer patients at the highest dose of 0.6 mg/kg, accumulated in the tumor of the 3 melanoma patients investigated and specific and cleavage of mRNA took place for over 1 month [119].

3.2.5. Chitosan

Chitosan is a positively charged, biodegradable, linear polysaccharide composed of β-(1–4)-linked D-glucosamine and *N*-acetyl-D-glucosamine with low toxicity and immunogenicity [120]. In pharmaceutics it has been used as dietary supplement and wound healing biomaterial [121, 122]. The muco-adhesive and -disruptive properties of chitosan suggest applications for mucosal delivery. The potential for chitosan to serve as excipient for nasal delivery of high molecular weight compounds is reflected in the studies on insulin delivery [123].

The primary amines, generated by alkaline deacetylation of the natural source chitin become protonated at pH<5.5, allowing binding to nucleic acids and facilitating endosomal release through the proton-sponge effect, as described above for other positively charged polymers. The zeta potential of nucleic acid-chitosan nanoparticles is directly linked to the deacetylation degree (DD) [124]. The thermodynamic interaction between chitosan and nucleic acids has been described using isothermal titration calorimetry [125]. The enthalpies for the formation of these nanoplexes are 2–3 orders of magnitude higher than for liposomal or peptide/nucleic acid interactions [126], which may contribute to the lower biological activity of these complexes due to their high stability preventing nucleic acid release.

Chitosan for siRNA delivery has been introduced in 2006, demonstrating self-assembled nanoplexes of 40–600 nm composed of 114 kDa chitosan (84% DD) with unmodified siRNA, efficiently protecting siRNA from nuclease degradation and silencing EGFP *in vitro* in several cell lines at similar levels as the *Trans*IT-TKO siRNA transfection reagent control [127]. In contrast to DNA, siRNA has been reported to require higher MW chitosans for efficient complexation and knockdown, possibly in order to compensate the shorter size of siRNA [128]. Studying the correlation between size and DD, a higher knockdown efficacy *in vitro* was shown for higher MW chitosans and higher DD [129]. The optimal N/P ratio was determined to be 150 using 114 or 170 kDa chitosan with 84% DD, also using unmodified siRNA. Lower and higher MW chitosans decreased the cell viability. In contrast to the study discussed previously, a lower N/P ratio (50) also reduced cell viability of the cells. These conflicting results may be due to different cell lines as well as chitosan batches from different suppliers, which may react differently.

The conclusion of a study comparing different 85% deacetylated chitosan salt forms for siRNA delivery revealed that complexing capacity mainly depended on the weight ratio rather than the salt form or MW of the chitosan, whereas transfection and silencing efficacy *in vitro* was influenced by both weight ratio and MW [130]. Optimal silencing was achieved with low MW chitosan of 20 kDa at a weight ratio of 32 (excess to siRNA), demonstrating the importance of the balance of siRNA binding and release at the site of action. When compared to pDNA complexation, siRNA showed a rather weak binding with only slightly positive zeta potential, due to the rigid, exposed structure of siRNA neutralizing the positive charges, unlike supercoiled pDNA, which already forms stable complexes at a weight ratio higher than 1.

SiRNA complexed with 75–85% deacetylated chitosan of 200–300 kDa at a 50-fold mass excess of chitosan resulted in 70% reduction of FHL2 gene expression *in vitro*, similar to Lipofectamine [131]. The resulting nanoparticles of about 125 nm and a positive zeta potential of 60 mV exhibited irregular, lamellar and dendritic structures in scanning electron microscopy (SEM) and mainly spherical particles when using AFM. When comparing simple ionic complexation to the ionic gelation method, using the crosslinker tripolyphosphate to produce nanosized chitosan siRNA particles, covalently crosslinked particles showed better *in vitro* silencing efficacy [132]. The particle size correlated with

the MW of the chitosans, and complete complexation could only be achieved at high weight ratios of chitosan to siRNA of 100:1. In the case of TTP-crosslinking, the ratio of TTP to chitosan as well as the reaction pH affected the particle size.

A very low N:P ratio of only 4–8 resulted in transfection competent siRNA-nanoparticles using 4 different chitosans of fungal origin with various DD with no significant proinflammatory effect *in vitro* [133]. ITC revealed nanomolar dissociation constants. Particles of chitosan with lower molecular weight exhibited more regular shaped particles and faster cellular uptake than those with higher molecular weight chitosan, as evidenced by TEM, SAXS and confocal microscopy. This study points out that different origins (fungus *vs.* shrimp) and purification methods for chitosan may have a significant influence on the polyplex formation, and could be one of the reasons for inconsistent results throughout literature concerning efficient N:P ratios and toxicity.

Due to the instability of the complex at neutral pH, only a few *in vivo* studies using chitosan as siRNA delivery vehicle have been reported. However, intraperitoneal administration of siRNA/Chitosan nanoparticles as an alternative delivery method to avoid plasma related protein interaction and reach a macrophage-rich environment, has been described for the knockdown of TNF-α in macrophages [134], resulting in reduction of 44% of target gene expression. These nanoparticles were prepared using chemically stabilized siRNA, 114 kDa 84% DD chitosan and an N/P ratio of 63.

Further limitations of the use of chitosan *in vivo* are its poor solubility above pH 6.5, low buffering at endosomal and physiological pH (5.5–7.4), and poor cytoplasmic dissociation kinetics. Imidazole modification of the primary amines of 130 kDa and 86% DD chitosan increased transfection efficacy, buffering capacity and solubility [135]. In this report, PEGylated chitosan/GAPDH siRNA nanoparticles at an N/P ratio of 40–50 resulted in significant knockdown of its target gene in lung and liver after i.v. administration, as well as in lung after intranasal administration in mice without adverse events. The PEGylation of these 150–300 nm particles decreased their charge from 40 mV to 20 mV.

In order to enhance the *in vivo* transfection efficacy of chitosan, complexes composed of polyisohexylcyanoacrylate (PIHCA) and unmodified siRNA were coated with chitosan. These complexes efficiently delivered siRNA to subcutaneously implanted breast cancer cells after i.v. administration showing no toxic effects [136].

3.2.6. Atelocollagen

Atelocollagen is a highly purified protein derived from calf dermis by pepsin digestion removing potentially antigenic telopeptides attached on both ends of the polymer. Due to its low toxicity and low immunogenicity, it is used in medicine for applications such as wound healing or cartilage substitution. Its special physical property of phase change with temperature (liquid at 4 °C and gel at 37 °C) allows local targeting.

Its positive charge lends it to be used for plasmid DNA or antisense RNA delivery. It has been shown to stabilize siRNA when co-injected into the tumor *in vivo* in a xenograft mouse tumor model, where it facilitated transfection of the unmodified siRNA with no apparent toxicity [137]. The size of the nucleic acid-atelocollagen particles could be controlled between <200 nm and 10 μm.

Another study looked at the self assembly of 10–300 nm 300 kDa atelocollagen nanoparticles with siRNA. It protected siRNA from nuclease degradation in a reverse transfection protocol *in vitro*. It increased cellular uptake and silencing efficacy as efficient as for liposome transfection. *In vivo*, these particles enabled prolonged release of unmodified siRNA and silencing efficacy after intratesticular delivery in an orthotopic testis tumor model [138].

Systemic delivery by i.v. injection of atelocollagen/siRNA nanoparticles resulted in delivery of siRNA and gene silencing in organs such as liver, spleen, kidney and predominantly tumor in a xenograft tumor model [139]. Another report showed that after i.v. injection of atelocollagen-complexed, but otherwise nonmodified siRNA, the drug was delivered to tumors, including metastatic bone tumor, without induction of IL-12 or IFN-α associated toxicity [140]. Atelocollagen-siRNA nanoparticles delivered to mice into skeletal muscle, as well as intravenously, resulted in silencing of its target myostatin in increased skeletal muscle growth [141]. This demonstrates that atelocollagen nanoparticles may be of therapeutic use not only by utilizing the enhanced permeation and retention (EPR) effect to target tumors but also against diseases of other tissues.

3.2.7. Hyaluronic acid

Hyaluronic acid (HA) is a negatively charged, nonsulfated glycosaminogluycan polysaccharide composed of alternating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid, naturally occurring in extracellular matrix. Its biocompatibility and biodegradability led to its use in tissue engineering and drug delivery devices [142]. HA receptors such as CD44, expressed on tumor cells, can be utilized as port of entry of nanoscaled formulations [143]. Since its negative charge prohibits electrostatic interaction with nucleic acid as particle-forming force, different strategies have to be employed to physically entrap or covalently bind siRNA.

Disulfide-crosslinked HA nanogels prepared by inverse emulsion method resulted in ~200 nm particles which released siRNA upon glutathione treatment, simulating the reductive cytoplasmatic environment. These nanoparticles were taken up by HA-receptor overexpressing cells without inducing any cytotoxicity and showed similar *in vitro* gene silencing efficacy as PEI/siRNA complexes. Due to their slightly negative charge they also showed less protein binding and better *in vitro* silencing efficacy in the presence of serum compared to PEI delivered siRNA [144]. The negative charge of HA is also used in conjunction with other excipients in order to reduce toxicity and shield the charge of positively charged liposomes [145]. Using covalently HA-modified DOTAP/DOPE lipids in the ethanol-injection method, particles of smaller than 170 nm could be prepared, with improved binding, protection properties and stability of the particles compared to non HA-modified liposomes. Cytotoxicity was reduced and HA-receptor dependent uptake was improved *in vitro*.

A conjugate of branched 25 kDa PEI and 130 kDa HA self-assembled with siRNA to 21 nm nanoparticles at a weight ratio of siRNA to PEI/HA of 1:5. It was taken up via HA-receptor mediated endocytosis and displayed *in vitro* silencing efficacy with a lower toxicity profile than PEI alone [146].

A complex of PEGylated chitosan and HA was prepared by the ionotropic gelation technique, where HA is cross-linked using tripolyphosphate in the presence of PEG-chitosan followed by complexation with siRNA [147]. The resulting particles of less than 200 nm protected siRNA from serum degradation and delivered siRNA in cells *in vitro*, with a silencing efficiency comparable to Lipofectamine 2000 with low toxicity. This study also highlighted the differences in complexation and condensation of pDNA compared to siRNA, resulting in a lower siRNA encapsulation efficiency of the nanoparticles compared to pDNA.

There are also several examples of the efficient and safe use of HA in combination with other excipients for local or systemic siRNA delivery *in vivo* [148, 149, 150]. As for PLGA, the most likely application for HA in siRNA delivery *in vivo* is in combination with other excipients.

3.3. Cell penetrating peptides

Besides cationic lipids and cationic polymers, cell penetrating peptides (CPPs) as a third class of positively charged molecules have been evaluated as non-viral siRNA delivery vehicle. Like for the other positively charged polymers, the charge of these short peptides not only allows the spontaneous interaction and encapsulation with siRNA but also facilitates interaction with the negatively charged cell membranes. The idea of using peptides as carrier for drugs dates back over 20 years.

MPG was the first peptide to been shown to form non-covalent, electrostatically stabilized complexes with siRNA and deliver its cargo into cells. It is a recombinant amphipathic peptide composed of a hydrophobic fusion sequence of the HIV protein gp41 and a hydrophilic domain from the nuclear localization sequence of SV40 large T antigen [151]. MPG was initially used for pDNA delivery and therefore contains a nuclear localization signal (NLS), which was mutated to adapt this nanocarrier for the cytoplasmatic delivery of siRNA. Inhibition studies indicated that the uptake follows a receptor-independent pathway, as proposed also for other CPPs such as penetratin (from the insect *Antennapedia* homeoprotein), transportan (from the neuropeptides galanin and mastoparan) and TAT-peptide (derived from HIV-1 *trans*-activator protein).

A CPP derived from the same sequence, termed MPGα, differs from the MPG peptide described above by a mutation resulting in a partially helical conformation [152]. In this study, no difference in uptake mechanism or silencing efficacy was observed for MPGα, whether or not the NLS was mutated. Neither of the variants delivered siRNA to the nucleus, but to endosome-like cytosolic vesicles. Because the uptake was inhibited at low temperature and upon addition of several endocytosis inhibitors, it was suggested that it was driven by an endocytotic process. The amount of siRNA to achieve half-maximal inhibition of its target was 10,000 siRNA molecules per cell in case of peptide delivery, compared to only 300 molecules for Lipofectamine 2000 delivery, suggesting a 30-fold lower bioavailability for the peptide-bound siRNA. This might be due to a less efficient endosomal release of peptide delivered siRNA.

The MPG peptide has been further optimized by deleting 6 amino acids to improve its interaction with both siRNA as well as the lipidic cell membrane. In contrast to the previous studies, the resulting particles have also been studied in terms of size and charge, as well as the toxic effects on cells [153]. The so called MPG-8 peptide complexed unmodified siRNA at a 20-fold molar excess of peptide, resulting in particles of 120 nm with a zeta potential of +16 mV, and an *in vitro* silencing efficacy 30 to 60-fold higher than MPG. No cell toxicity was observed up to 20 μM, whereas 100 μM reduced cell viability by 10–15%. Intratumoral injection of the self-assembled cyclin B1 siRNA nanoparticles completely stopped tumor growth at a 0.25 mg/kg dose as a result of reduction of cyclin B1 mRNA level. In order to improve the stability of the particles in the circulation, the peptide was conjugated to cholesterol. I.v. injection of the cholesterol-modified siRNA particles resulted in 70% survival of mice *vs.* 20% for cholesterol-free siRNA formulation.

Polyarginin (POA) is based on the amino acid sequence of the protein transduction domain (PTD) of various viral proteins and has been used for delivery of dsRNA to plant cells in 2004 [154]. An optimal weight ratio of 1:2 for siRNA:POA was found to completely complex siRNA. POA has also been used conjugated to PEG and in combination with cationic lipids to encapsulate siRNA at a molar excess of 30, resulting in particles below 200 nm and with a charge between 20 and 40 mV. The downregulation of GFP protein expression *in vitro* was higher as with un-PEGylated or PEGylated liposomes without POA [155]. Cell viability after incubation with 40 nM siRNA complexes was also increased to >80% upon addition of POA compared to formulation with PEG alone. In another approach, modification of polyarginine with a 14-carbon myristic acid moiety (MPAP) increased the affinity for lipid bilayer membranes, thereby enhancing peptide entry [156]. MPAP also has the ability to cross the blood brain barrier, which can be utilized for neurological applications. A molar ratio of siRNA to MPAP of 1:5 was able to complex siRNA, protect it partially from serum degradation and deliver it into neuron cells, resulting in target knockdown *in vitro* without cytotoxicity.

Hydrophobic modification by N-terminal conjugation of cholesterol to oligo-D-arginine (Chol-R9) peptide has been utilized to synergistically increase cellular uptake of siRNA [157]. Complete complexation of siRNA was reached at an N/P ratio of 40 as compared to only 8 for pDNA. Over 90% cell viability remained at incubation with Chol-R9/DNA complexes with N/P ratios of 8 to 48 in HEK293 cells, while a higher ratio drastically increased toxicity.

The multifunctional envelope-type nanodevice MEND composed of stearyl octaarginine (STR-R8) covered by a DOPE-CHEMS (9:2 molar ratio) lipid membrane condensed siRNA to nanoscaled particles below 100 nm and transfected cells *in vitro* via macropinocytosis [158]. The N/P ratio of the complexes was 2.9. Particles containing 60 nM siRNA did not show any significant effect on HeLa cell viability. Poly-L-lysine (PLL) and protamine were also tested, but failed to condense siRNA into particles smaller than 100 nm. The condensed STR-R8/siRNA nanoparticles alone could not transfect cells, but depended on the lipid envelope, in contrast to another report achieving an RNAi effect using siRNA-STR-R8 [159].

Liposomes bearing R8 molecules attached to the liposome surface delivered unmodified siRNA *in vitro* into lung tumor cells [160]. The particles self-assembled by mixing egg PC, DOTAP, cholesterol, PE-PEG2000 and siRNA at a ratio resulting in neutralization of the positive and negative charges. Subsequent addition of R8-PEG-PE resulted in particles in the range from 50 to 200 nm composed of smaller spherical particles of about 20 nm. Reversing the order of mixing by adding siRNA after preformation of R8-PEG-PE particles resulted in weaker binding, supporting the hypothesis, that upon heating of the preformed lipid-siRNA nanoparticles, lipid molecules rearranged to small inverted hexagonal micelles incorporating one single siRNA molecule each, and subsequently arranged to form a bigger particle covered by a second layer of lipids. These particles efficiently protected siRNA from serum degradation, and delivered siRNA in various cell lines *in vitro* in a R8-dependent fashion with less toxicity than Lipofectamine 2000. Concentrations up to 10 μg/ml lipid did not affect cell viability.

HA has been used to shield some of the toxic effects of poly-L-arginine [161]. The complexes with unmodified siRNA were positively charged and <200 nm. They were taken up and led to sequence-specific silencing independent of the presence of serum. Intratumoral injection of these HA-nanoparticles also resulted in target silencing in mice, however possible toxic effects were not investigated *in vivo*.

The CPPs Penetratin and Transportan were linked via C-terminal cysteine residue to the thiol containing sense strand of siRNA [162]. It was shown, that a 5′-modification of the siRNA sense strand abrogated the silencing efficacy, whereas 3′-functionalization maintained silencing efficacy. However, very high doses had to be used to reach knockdown comparable to lipofection. Another technical complication appeared to be annealing of the two siRNA strands, after modification of the sense strand with these highly cationic lipids. In contrast, covalent conjugation of siRNA to penetratin and transportan via an acid labile disulfide bond resulted in better delivery characteristics than with cationic liposomes. These peptides delivered siRNA without endocytosis directly to the cytoplasm, where the disulfide bond was cleaved.

Another CPP used for siRNA delivery is derived from protamine, a small, arginine rich protein, endogenously involved in DNA packaging. Use of an antibody-protamine fusion protein is an elegant solution to non-covalently complex siRNA, and target it to the diseased cells or tissue [163]. A Fab fragment targeting an HIV-1 envelope antigen delivered the unmodified siRNA specifically to cells expressing the antigen *in vitro* and *in vivo* without triggering interferon response or other obvious toxic effects. Meanwhile, this strategy has been employed for several other targets such as the human integrin lymphocyte function-associated antigen-1 (LFA-1) *in vitro* and *in vivo* in a SCID mouse xenograft tumor model, where the particles specifically delivered unmodified siRNA to the target expressing cells after systemic administration [164]. In a similar approach 2.5 mg/kg protamine-condensed siRNA entrapped in a liposome decorated with Fab-modified HA reversed colitis after systemic administration in mice [165]. The condensation of unmodified siRNA with

protamine allowed a high drug load per nanoparticle (~4000 siRNA molecules), protection of the liposome against interferon production in response to the siRNA, and the active targeted uptake mechanism of the coupled Fab-fragment.

Not only electrostatic interaction, but also dsRNA binding domains have been employed to bind siRNAs, resulting in more efficient transfection *in vitro* as Lipofectamine 2000 in several cell lines including T cells and primary cells [166]. These TAT-PTD (Protein transduction domain) fusion proteins did not induce significant toxic effects on the investigated cell lines, and reduced target gene expression in a luciferase mouse model after nasal administration of 750 pmol complexed luc siRNA.

Despite some promising reports of *in vivo* applications, most studies investigate toxicity *in vitro*, which does not predict potential immune response after repeated doses of the CPPs. Appropriate *in vivo* models need to be considered, since immunodeficient mice are often used for xenograft models. Induction of unspecific toxicity and immune stimulation by peptides may limit their *in vivo* use, as evidenced for TAT and penetratin for siRNA delivery in mouse lung [167]. Although conjugates of siRNA covalently attached to TAT or penetratin peptide via disulfide enabled siRNA delivery and siRNA-specific knockdown in cell culture, intratracheal administration of the TAT-peptide reduced target mRNA expression, suggesting toxic effects of the peptide, while penetratin-siRNA conjugate activated innate immune response, indicating a different uptake mechanism as for TAT-mediated delivery.

Endosomal release of siRNA is another difficulty for siRNA delivery to the cytosol [168]. A new, synthetic cell penetrating peptide called PepFect6 promises potential for efficient siRNA transfection in several cell lines *in vitro*, as well as in a variety of organs in mouse *in vivo* [169]. The potent proton acceptor trifluoromethylquinoline was attached to the TP10 peptide and after mixing with unmodified siRNA spontaneously formed nanoparticles of approx. 100 nm and −10 mV. This acted as a proton sponge in the endosome, facilitating endosomal release. A stearic acid moiety covalently attached to the CPP increased its serum stability. Unlike other CPP based nanoparticles, these particles remained stable over several weeks. Over 50% knockdown was achieved in kidney, lung and liver with no apparent toxic and immunogenic side-effects.

3.4. Aptamers

Aptamers are synthetic nucleic acids, selected from random sequence libraries and optimized for their ability to bind to a specific target molecule at nano- to pico-molar dissociation constants. Synthesis of siRNA as an aptamer chimera allows active targeting using RNA instead of antibodies. This principle has the same versatility as antibodies, while having low immunogenicity. They can be synthesized in large quantities at relatively low cost and can be modified to increase resistance to degradation and improved pharmacokinetics *in vivo*. Their smaller size compared to antibodies (15 *vs.* 150 kDa) improves tissue penetration. They have been shown to target siRNAs to the cell surface receptor PSMA, which is overexpressed in prostate cancer cells, and reduce tumor size upon intratumoral injection of these therapeutic siRNA-chimeras [170].

The fact that these constructs are actually Dicer substrates may be a reason for the more efficient incorporation of the resulting siRNAs into the RISC complex. These fusion constructs have been optimized to increase their efficiency, the ease of production and pharmacokinetic properties for *in vivo* use, resulting in molecules that are capable of reducing tumor growth in mice even after systemic administration.

For instance, coupling of a 20 kDa PEG group increased circulation t½ from <35 min to over 30 h, decreasing the required dose to reach the therapeutic effect *in vivo* [171]. The same target PSMA has been used in another report to deliver siRNA to cancer cells using a biotin-streptavidin linker with or without cleavable disulfide linker [172]. 27mer Dicer-substrate siRNA was used and the aptamer induced sequence specific knockdown was comparable to Oligofectamine facilitated silencing without interferon response. Although the tetramer streptavidin provides 4 binding sites and therefore would enable binding of four different siRNAs, the immunogenicity of the biotin-streptavidin components still poses a hurdle for further pharmaceutical utilization of this coupling chemistry.

Another study targeting the HIV-1 glycoprotein gp120 allowed silencing tat/rev and inhibiting HIV replication *in vitro* [173]. A 27mer Dicer-substrate siRNA was linked to the aptamer, which on its own already has the potency to decrease HIV-infectivity and therefore serves as targeting device and therapeutic agent. The chimera was synthesized *in vitro* by bacteriophage transcriptions and annealed with the antisense strand RNA and did not induce any IFN response in cell culture.

4. Conclusion

SiRNAs comprise a class of therapeutic molecules which have been discovered about one decade ago, and since then have triggered a lot of excitement in the pharmaceutical area due to their intrinsic versatility and specificity.

The initial enthusiasm led many pharma-companies to start their own siRNA program. But the development of siRNA based therapies turned out to be more challenging than expected, leading to termination of some of these programs such as during the restructuring of Roche in 2010. Their specific physicochemical properties in many cases demand formulations protecting these molecules in the biological environment and facilitating delivery to the target site.

This review discussed the most common delivery siRNA strategies. Whether a specific encapsulation strategy can be applied for a particular therapy depends on whether the indication, anticipated administration and dose regimen allows the use of certain substances such as ethanol, cholesterol or fatty acids.

The list of currently ongoing clinical trials suggests that a high unmet medical need for cure of diseases, such as cancer, convinces the increasingly conservative health authorities more easily to approve novel excipients, which might not be approved for other indications. Although a number of excipients have been investigated for their potential to overcome the most challenging siRNA delivery hurdles *in vivo*, only few proceeded to human trials. Naked siRNA formulations are obviously the simplest and cheapest formats, they are however limited to local administration in the lung, eye, and also to the kidneys after systemic administration.

Lipidic formulation systems for systemic drug delivery on the other hand have a head start over some other excipients, as liposome research has been going on for many decades and their *in vivo* fate is well understood, leading to formulations such as SNALP or AtuPLEX.

The exceptional example of the cyclodextrin based Rondel formulation shows that novel and very complex formulations are legitimate options provided a certain unmet medical demand is addressed. Many other novel excipients would still have to undergo expensive and time-consuming toxicological studies to proceed to clinics.

Numerous *in vitro* and *in vivo* studies are trying to shed light on the toxicological profile of those innovative delivery systems, however in absence of systematic comparison and due to different protocols, cell lines, assays and *in vivo* models, the results are often inconsistent and controversial. Furthermore, upscaling and GMP requirements for excipients as well as manufacturing protocols are rarely taken into consideration when novel delivery technologies are being investigated. Consequently, the future of RNAi therapies will depend on the willingness of Pharma companies to invest long term, and the healthcare systems to pay the resulting price for these therapies. However, since the field of RNAi is still relatively young, the number of ongoing

clinical trials and also successful preclinical *in vivo* studies nevertheless promises a therapeutic as well as commercial potential for these molecules.

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