

Review

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Recent advances in siRNA delivery

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Abstract: In the 1990s an unexpected gene-silencing phenomena in plants, the later called RNA interference (RNAi), perplexed scientists. Following the proof of activity in mammalian cells, small interfering RNAs (siRNAs) have quickly crept into biomedical research as a new powerful tool for the potential treatment of different human diseases based on altered gene expression. In the past decades, several promising data from ongoing clinical trials have been reported. However, despite surprising successes in many pre-clinical studies, concrete obstacles still need to be overcome to translate therapeutic siRNAs into clinical reality. Here, we provide an update on the recent advances of RNAi-based therapeutics and highlight novel synthetic platforms for the intracellular delivery of siRNAs.

Keywords: lipid-based carriers; nanocarriers; polymeric; siRNA; stimuli-sensitive.

Introduction

The goal of RNA interference-based therapy is to silence the expression of ‘uncontrollable’ genes involved in human diseases that are un-targetable by conventional drugs. siRNAs mediate gene silencing by inducing sequence-specific cleavage of complementary messenger RNA (mRNA). Gene silencing can be induced by chemical synthesized short double-stranded RNA, including

siRNA and microRNA (miRNA), and double-stranded hairpin RNA (shRNA) transcribed *in vitro* or from viral vectors and plasmids. In the endogenous pathway, siRNA is incorporated into the RNA-induced silencing complex (RISC), a ubiquitous process in mammalian cells, where it seeks and binds perfect or nearly perfect complementary regions of target mRNA and causes translational repression and suppression of its expression (1, 2). Then, when the cleaved mRNA is released, the siRNA-RISC complex binds to another mRNA and starts a new round of cleavage (3, 4). The potency of siRNAs in knocking down the expression of specific targets has been widely demonstrated *in vivo* for the treatment of different diseases, such as hepatitis B virus (HBV) (5), human papilloma virus (6), ovarian cancer (7) and hypercholesterolemia (8).

However, in order to have a concrete impact on the health of patients, promising strategies should be proposed to translate therapeutics siRNAs into the clinical setting. Naked siRNAs are unstable in human plasma and too large and negatively charged to cross the cellular membranes. Chemically modified siRNAs have been used extensively to enhance the stability of siRNA in biological fluids. Chemical modifications on the backbone, sugar moiety and nucleobase of the siRNA, are usually introduced to the 5′ or 3′ end of the terminal regions of the sense-strand (non-guide strand) (9). This approach definitely increases the stability of siRNA in human blood often without affecting the silencing activity.

Nevertheless, the silencing effect of chemically modified siRNA has been shown only in certain body tissues, such as brain and lung (10, 11). Wider systemic delivery of siRNA to various organs and tissues possesses its unique advantages as well as challenges that deserve special focus. Using the rational siRNA design, nuclease resistant siRNA molecules could be synthesized (12, 13), however, then the longevity in the circulation becomes the new challenge. One of the main siRNA elimination pathways is through the kidneys and the pore size of the glomerular filtration limit is around 8 nm. Free siRNA with the average diameter less than 10 nm can accumulate in the kidney and pass through the filtration to urine within 5-to-10 min of administration (14, 15). This obstacle can be overcome by using a nanosized siRNA carrier system,

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which is designed to be larger than 20-nm size to avoid the renal clearance. At the same time, to exploit the leaky vasculature of the tumors and benefit from the enhanced permeability and retention (EPR) effect, the size of the carriers should be limited to 200–400 nm, depending on the various cut-off sizes of different tumors (16, 17). However, when a carrier has been used, the prevention of phagocytosis becomes crucial by using materials to form a steric barrier around the siRNA carrier, such as polyethylene glycol (PEG) (18). Following extravasation, siRNA and its carrier must overcome multiple diffusion barriers through the extracellular matrix to reach the target tissue or cells and activate the RNAi. Cellular uptake of siRNA or its carrier by the target cells is the next roadblock along the journey of successful siRNA delivery. It should also be noted that PEG, that provides vital benefits until this step, could also hinder the cellular interaction by a phenomenon called ‘PEG dilemma’. Various strategies that could be implemented to enhance the cellular uptake of the carrier systems do not guarantee the effective protein silencing because the siRNA still needs to escape endosomal encapsulation and subsequent lysosomal degradation. Endosomal escape has become the new point of focus for successful *in vivo* siRNA delivery since the nanocarrier based approaches helped to avoid most of the obstacles mentioned above in the last decade.

Realization of the challenges of *in vivo* siRNA applications makes clear that without siRNA delivery systems, the true potential of RNAi as a therapeutic approach cannot be fulfilled. Delivery strategies include physical methods, conjugation methods, and viral or non viral drug delivery systems. The physical approaches increase the siRNA uptake in certain target tissues using the gene gun, electroporation, pressure, sonoporation, but are generally difficult to apply in humans or unrealistic for a commercial translation. Modified viruses such as retrovirus, lentivirus, adeno-virus and adeno-associated viruses (AAV) are widely used to harness the endogenous pathway of RNAi and so far almost 70% of advanced clinical trials use viral vectors for gene therapy. The AAV-based gene therapeutic Glybera (alipogene tiparvovec) which contains the human lipoprotein lipase gene variant *LPLS447X* in a vector, has been approved for use in Europe to treat adult patients diagnosed with familial lipoprotein lipase deficiency.

Although there have been many advances in this field, several limitations are associated with the use of viral vectors including carcinogenesis (19), immunogenicity (20), non-selectivity (21), and difficulty of scale-up (22). The design of an effective and non-toxic delivery system that is able to selectively accumulate siRNA in the tissue of interest remains the greatest hurdle to

widespread the therapeutic application of RNAi of all the diseases. The systemic delivery of siRNA has been extensively investigated in the recent past (23–26). The number of publications on siRNA delivery strategies has steadily increased in the last decade. Different siRNA delivery approaches have been proposed, including conjugated or modified siRNAs and several delivery vehicles such as lipid nanoparticles (27–29), polymer-conjugates (30, 31), inorganic nanoparticles (32, 33), aptamer-based approaches (34–36), peptide-mediated delivery (37–39), and other non-viral systems (40).

To date several clinically tested siRNA therapeutics are administered intravenously by synthetic carriers. Among them, six use cationic liposomes, including stable nucleic-acid lipid particles (SNALPs), carriers developed by Tekmira (Barnaby, Canada), and siRNA-lipoplex known as AtuPLEX™ developed by Silence Therapeutics (London, UK), currently in phase IIa trial for the treatment of advanced solid tumors. In contrast to cationic liposomal carriers, Calando Pharmaceuticals (Pasadena, CA, USA) developed cyclodextrin-based polymer nanoparticles as a drug delivery system for siRNA, CALAA-01. Moreover, another siRNA drug that uses polymeric carriers includes a biodegradable polymeric matrix known as LODER.

Here, we review a selection of non-viral siRNA delivery systems. In this review, we highlight and provide recent advances on non-viral siRNA delivery systems as well as underline the established strategies used for successful siRNA applications.

siRNA conjugation technology

Covalent conjugation of siRNAs with other ligands and molecules can be used to increase the internalization of siRNA in specific cells as well as to increase its stability *in vivo* (41). The ligands can be placed at the 3′ or 5′ end of the sense or passenger strand without affecting the silencing activity of the siRNA. To date, the most advanced therapeutic program engaging siRNA conjugation strategy is Alnylam’s (Cambridge, MA, USA) Phase II ALN-TTRsc (USA clinical trial NCT01981837; <http://clinicaltrials.gov/show/NCT01981837>). The clinical trial targets a siRNA against transthyretin (TTR) to the liver hepatocytes by conjugating the siRNA with a N-acetylgalactosamine (GalNAc) ligand, which mediates the cell uptake by the asialoglycoprotein receptor, for the treatment of TTR amyloidosis, an inherited and fatal disease. TTR is a primarily liver-expressed tetrameric protein. Following mutations

of the *TTR* gene, the TTR protein destabilizes in tetramer with amyloid formation and it accumulates abnormally in tissues, including heart, causing amyloidosis. The results from the Phase I study showed a robust silencing of the TTR levels in the serum ($\geq 90\%$) in an encouraging non-toxic profile (<http://investors.alnylam.com/releasedetail.cfm?ReleaseID=814002>). In a recent *in vivo* study, a similar concept has been used to covalently conjugate a VEGFR2 siRNA to a cyclic arginine-glycine-aspartate (RGD) peptide (cRGD), known to bind $\alpha v\beta 3$ integrin receptors. $\alpha v\beta 3$ integrin is significantly up-regulated in tumor blood vessels, as well as in invasive tumor cells of many cancer types (42). To evaluate the potential of the developed cRGD-siRNAs conjugate as an anticancer agent, its effect on the tumor development and angiogenesis was investigated in a mouse model of tumor. Interestingly, in mice xenografted with A549 luciferase-expressing cells, the systemic delivery of the tumor-targeted conjugate resulted in a significant down-regulation of the mRNA and protein levels of the targeted gene into the tumors together with an overall reduction of tumor volume (43).

Another approach is to conjugate siRNA to natural ligands, such as cholesterol, lipids and long fatty acids in order to improve the stability of the siRNA against nuclease as well as to increase the cell uptake. These lipophilic siRNAs usually interact with lipoprotein particles and harness the RNAi machinery without needing a delivery system (13). Kubo et al. (44) developed a simple method to covalently conjugate fatty acids, such as palmitic and lauric acids, at the 5'-end of the sense strand of siRNAs via amide linkage. By using a very simple conjugation method, the gene-silencing activity, membrane permeability, and nuclease resistance were significantly enhanced *in vitro* in HeLa cells. Moreover, the lipophilic siRNA-conjugates exhibited strong RNAi potency in absence of any transfection reagents, such as Lipofectamine 2000.

Aptamer-based delivery systems

In this review it is also worth mentioning the very recent advances in aptamer-based siRNA delivery systems. Aptamers are single stranded, short, synthetic DNA or RNA molecules with high affinity and specificity to various targets (45). The target can be small molecules, proteins, cell surface receptors or even bacteria and viruses. Aptamers are selected from a pool by a process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) (46, 47), an *in vitro* step-by-step selection process with increasingly stringent conditions that ensures the resulting molecules have the highest selectivity against their

target. Easy modifications of the *in vitro* synthesis process, very high selectivity against the target structure and dissociation constant (K_d) values in the nanomolar range (48, 49) make them a promising technology for siRNA delivery area. They could be used as a drug on their own, as in the example of first FDA approved aptamer-drug Pegaptanib (Macugen) by Eyetech (Palm Beach Gardens, FL, USA) that targets VEGF 165 for ocular vascular disease (50) or AS1411, an anti-nucleolin aptamer drug in clinical trials which prevents nucleolin from binding to and stabilizing mRNA of the anti-apoptotic Bcl-2 (51). There are two main strategies for aptamer-based siRNA delivery; (i) using aptamers as the targeting ligands on the surface of nanocarriers, (ii) attaching siRNAs directly to aptamers in which aptamers directly deliver the siRNA both as a carrier and as a ligand. In recent years, aptamer-siRNA conjugates (chimeras) have gained a lot of attention and evaluated for drug hypersensitivity (52), HIV treatment (53, 54) and cancer therapy (35, 55–57).

Giangrande and colleagues were the first to describe a first generation aptamer-siRNA chimera (36). In this study, an aptamer that specifically bound the prostate-specific membrane antigen (PSMA), a receptor over-expressed in prostate cancer, has been covalently linked to the passenger strand of siRNAs. The conjugate was able to significantly inhibit the mRNA expression of targeted pro-survival genes, *PLK1* and *Bcl2* (of 80% and 90%, respectively) *in vitro* and when injected intratumorally, the tumor volume of xenografted mouse model of prostate cancer was significantly decreased. After few years, they improved both physiochemical properties and the *in vivo* stability in order to use the chimera for systemic therapy (35). In particular, to facilitate the synthesis of the chimera in a large-scale production, the length of the aptamer was significantly decreased (from 71 nt to 39 nt) without affecting the binding and targeting affinity. In addition, the introduction of a PEG moiety on the antisense strand of the siRNA increased significantly the circulation time (from 30 min to 30 h) as well as the immunogenicity was significantly reduced, making the second-generation chimera suitable for systemic therapy. This approach seems to possess minimal immunogenicity together with the possibility to easily synthesize the platform in large quantities at a relatively low cost. More recently, Lai et al. used the aptamer-siRNA chimera approach to simultaneously deliver two different siRNAs to lung cancer *in vitro* and *in vivo* (58). They reported that the combined treatment of nucleolin aptamer conjugated snail family zinc finger 2 (SLUG) siRNA (aptNCL-SLUGsiR) and neuropilin 1 (NRP1) siRNA (aptNCL-NRP1siR) synergistically suppress lung cancer cell invasion, tumor growth and angiogenesis.

While the exciting studies proved the potential of successful *in vivo* siRNA delivery by aptamer-siRNA chimeras (summarized in Table 1), the challenge of endosomal escape and intracellular fate remains unclear. In very recent years, using aptamers as highly specific targeting ligands for siRNA delivery began to attract attention. Li et al. (59) combined the advantages of PEGylated long-cationic liposomes as effective siRNA carriers and an aptamer shows the specific binding to nucleolin, a phosphoprotein overexpressed in various cancer types. Liposome formulations, consist of 3 β -[N-(N',N'-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol), cationic lipid dioleoylphosphatidylethanolamine (DOPE) and maleimide-PEG₃₄₀₀-DOPE at a mole ratio of 1:1:0.1 molar ratio, was surface modified with aptamer AS1411 via disulfide linkage. Aptamer-targeted liposomes containing anti-BRAF siRNA fully protected siRNA at N/P ratio of 20 and selectively uptaken only by cancer cells. This resulted in up to ~34% BRAF mRNA downregulation in A375 melanoma cells *in vitro*, while the liposomes modified with scrambled aptamer caused only 4% mRNA downregulation. The *in vitro* downstream protein downregulation and proliferation tests confirmed the *in vitro* results. More importantly, the aptamer targeted liposomes accumulated in tumors within 3–6 h after injection in a xenograft model of A375. *In vivo* gene silencing was investigated by real time PCR and BRAF mRNA levels decreased ca. 45% in aptamer-targeted liposomal siRNA delivery group. The study supported that siRNA delivery with aptamer modified liposomes for melanoma treatment is a very promising option with strong specificity to cancer cells and reduced overall toxicity.

Another recent study that underlines the advantages of successful *in vivo* siRNA delivery by using aptamers as the targeting ligands was very recently published by Liang et al. (60). Following the screening by cell-SELEX, the authors have chosen CH6 aptamer that targets both rat and human osteoblasts for developing CH6 aptamer-functionalized lipid nanoparticles (LNPs). The researchers encapsulated osteogenic pleckstrin homology domain-containing family O member 1 (plekho1) siRNA in LNPs that have ~84 nm size with ca. 80% siRNA encapsulation efficiency and ~83% aptamer loading efficiency. Shorter sequences and satisfactory secondary structure of CH6 aptamer compared to other osteoblast specific aptamers investigated made it easier to conjugate to LNPs. CH6-modified LNPs were internalized selectively by the osteoblast cells, as confirmed by siRNA co-localization with osteoblast markers, via macropinocytosis and clathrin-mediated endocytosis and were able to escape from lysosomes. The aptamer LNPs (CH6-LNPs-siRNA) silenced the target mRNA *in vitro* in a siRNA dose dependent manner with up to 90% at 80 nM siRNA without any cytotoxicity to the osteoblast cells. Successful *in vitro* results are translated into *in vivo* effectively using Sprague-Dawley rat model. It has been shown that the CH6-LNPs-siRNA accumulated in the bone significantly higher even after 12 h following injection while the liver and kidney accumulations were significantly lower compared to non-aptamer targeted LNPs. Gene silencing indicated more than 60% *in vivo* plekho1 downregulation. This osteoblast-specific silencing promoted bone formation, improved bone microarchitecture, increased bone mass and proved that

Table 1: Recent aptamer-siRNA conjugate systems (chimeras) for successful *in vivo* siRNA delivery.

Aptamer	siRNA	Model	Outcome	References
CTLA4, targeting tumor-associated T cells	STAT3	Athymic <i>nu/nu</i> mice engrafted with Karpas299 human lymphoma cells	Promoted tumor cell apoptosis and tumor growth inhibition, activation of tumor antigen-specific T cells, reduced tumor associated Tregs	(61)
gp120, targeting HIV-1 glycoprotein gp120	Three different Dicer substrate siRNAs (as a cocktail) against; Anti- <i>tat/rev</i> , anti-CD4, Anti-TNPO3	Humanized BALB/c-Rag2 ^{-/-} γ c ^{-/-} infected with HIV-1	Knockdown of all three target mRNAs, potent inhibition of HIV-1 replication, protection against viral induced CD4 ⁺ T-cell depletion, long-term suppression of HIV-1 viral loads	(62)
4-1BB, targeting CD8 ⁺ T cells following TCR stimulation	mTOR complex 1 (mTORC1)	Transgenic OT-1, C57BL/6 mice	Enhanced memory CD8 ⁺ T cell development, superior cytotoxic effector functions compared to pharmacological agents, enhanced vaccine induced protective immunity and rejection of subsequent tumor challenge	(63)
EpCAM, targeting EpCAM ⁺ epithelial cancers and their tumor-initiating cells	PLK1	MDA-MB-468 (Basal A triple-negative breast cancer/TNBC) nude mice xenografts	Selective uptake by only EpCAM ⁺ tumor cells but not by normal tissues, inhibition of tumor initiation by EpCAM ⁺ luminal and basal A TNBC cell lines, selective accumulation in EpCAM ⁺ Her2 ⁺ and TNBC tumors and suppression of tumor growth	(64)

the aptamers are indeed highly effective targeting ligands for cell type specific *in vivo* siRNA delivery.

Lipid-based nanocarrier delivery systems

In the late 1987 Felgner and colleagues introduced the term ‘lipofection’ for a very efficient method to deliver both DNA and RNA into eukaryotic cells by using cationic lipids (65, 66). Cationic liposomes for gene delivery, the so-called lipoplexes, are simply complexes between cationic lipids and negatively charged nucleic acids. Lipoplexes are routinely used for gene transfection *in vitro*. Although the success of cationic liposomes in efficiently take up nucleic acids, the low silencing activity *in vivo* and the high systemic toxicity strongly limited their clinical applicability. The introduction of additional compounds to the lipoplexes, such as PEG-lipids, helper lipids as well as targeting moieties (transferrin, anisamide ect), has been proposed for systemic translation of the siRNA-based complex and it has been shown to improve the pharmacokinetic profile and supposedly to target the siRNA to specific tissues. Silence Therapeutics developed a novel liposomal siRNA formulation, AtuPLEX[®], based on cationic lipids containing neutral fusogenic and PEG-modified lipid components to target the endothelial cells of the vascular system. In previous pre-clinical studies, they have shown a selective inhibition of both mRNA and protein levels of the targeted gene *in vivo* together with therapeutic efficacy, with not significant toxic effects, in xenograft mouse tumor models (67). Then, a siRNA specifically targeting the kinase N3 protein (PKN3) was formulated in the same liposomes, the so-called Atu027, for the treatment of advanced cancer (68). Atu027 uses AtuPLEX[®] technology to target the expression of the protein PKN3 which is involved in cancer progression and metastasis. From *in vivo* studies, Atu027 was able to restrict tumor growth, local invasion and both, lymph node as well as pulmonary metastasis in mouse xenograft models. A significant down-regulation of the expression of PKN3 has been shown in mice, rats and non-human primates. To date, Atu027 is being tested in a Phase IIa trial in patients with advanced solid tumors, with the aim to evaluate its safety. In patients with advanced solid tumors, the treatment with Atu027 was well tolerated and, in a significant percentage of patients (of about 50%) Atu027 caused stabilization of the disease. Interestingly, the treatment with Atu027 elicited decreases of

sFLT1 (sVEGF-R1), suggesting a potential of Atu027 as a new vascular stabilizing agent (69).

Shen et al. (70) have recently proposed a multistage vector based on the 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) neutral liposomes loaded in micro-porous silicon particles to create a nanoparticle-in-microparticle delivery system for siRNA. They described the potential of silicon particles in modulating the circulation and the interaction with the tumor vascular area without using the well known enhanced permeability and retention (EPR) effect. In particular, once in circulation, the round silicon microparticles with large surface area interact with the tumor vasculature effectively where the porous silicon degrades and the incorporated nanotherapeutics get released. The sustained release of siRNA into the tumor tissues achieved a significant reduction of the tumor growth in animal model of ovarian cancer. In addition, the treatment with the multistage vector strongly sensitized the tumors to chemotherapeutic agents, such as paclitaxel (PTX), resulting in a complete regression of the tumor growth. Same group reported various studies involving successful application of EphA2 siRNA-lipid conjugates *in vitro* and *in vivo* (71–73). Their approach granted them a Phase I clinical study (NCT01591356) for advanced cancers in 2014.

In 1998 Huang and co-workers have engineered new self-assembled nanoparticles, the so-called LPD, a cationic liposomes-polycation-DNA complex. Briefly, oligonucleotide or siRNA are first mixed with a calf thymus DNA to increase the negative charge of the nucleic acids, and then complexed with protamine, a highly positively charged peptide (74). Then, the resulting complex is coated with cationic liposomes to obtain LPD. Over the years, the LPD has been upgraded by introducing PEG-lipid moiety for sterical stabilization. For tumor target therapy, LPD have been functionalized with a specific ligand, anisamide, which targets the sigma receptor overexpressed in many human cancer cells, including lung cancer cells. A significant improvement of the *in vivo* silencing activity of the targeted LPD has been shown in a tumor model of lung cancer (75). Moreover, from biodistribution studies, tumor targeted-LPDs were preferentially accumulated in the tumor (76). Interestingly, the same lab has recently proposed an alternative platform, LCP (lipid-calcium-phosphate NPs), a mixture of lipids and calcium phosphate NPs (77). The presence of a pH sensitive CaP core allows to incorporate and to deliver more efficiently the nucleic acids in a biocompatible, biodegradable and low cost profile (78). In addition, compared to the LPD formulation, the LCP NP releases more siRNA into the cytoplasm, leading to a significant (~40-fold *in vitro* and

~4-fold *in vivo*) improvement of the siRNA delivery. Another strongly promising platform for intravenous injection of siRNA are the stable nucleic acid lipid particles (SNALP). SNALP are, right now, one of the most potent delivery technologies for gene knockdown in the liver. Many examples of siRNA efficiently delivered by SNALPs are reported and many of them are in different stages of clinical trials. Zimmermann et al. reported that a single injection of ApoB-specific siRNAs encapsulated in SNALP dramatically knocked-down the mRNA levels of ApoB in the livers of both mice and non-human primates. Moreover, the silencing effect lasted at least 11 days (79). Significant silencing activity has been shown by using the same formulation for the delivery of siRNA targeting hepatitis B virus (HBV) (5). Tekmira (Barnaby, Canada) started in 2015 a Phase II clinical trial to evaluate the efficacy of siRNA containing SNALP for the treatment of Ebola virus (TKM-100201). In previous monkey studies, a 0.5 mg/kg dose of siRNA SNALP achieved complete protection. However, this dose was the one that in the previous clinical trial (TKM-HBV) caused idiosyncratic immune stimulations and other side effects and the last results from the Phase II trial showed a not significant therapeutic benefit of TKM-100201 in Ebola virus infected patients (TKM-Ebola-Guinea). If successful, this study will strengthen the position of SNALP as the most potent nucleic acid based therapeutics technology for gene knockdown in the liver, for Ebola treatment, and supposedly for cancer treatment.

Polymer-based nanocarriers

Although many of the polymeric carriers were initially designed for plasmid DNA delivery, their usage extended to cover siRNA delivery in the recent years. Different nanocarriers composed of mainly polymeric materials and that do not fall into lipid-based nanoparticles (such as liposomes or solid lipid nanoparticles) are included in this section. Polymeric systems for effective siRNA delivery must comply with several important factors such as having cationic charge to bind and condense the negatively charged siRNA but at the same time having low toxicity of the cationic polymer, steric stabilization for a longer time in the circulation in cases of systemic delivery and successful endocytosis followed by endosomal escape. Among the variety of polymer-based nanoparticles, recent advances with organic and inorganic synthetic delivery systems based on polyethyleneimine, cyclodextrin, dendrimer and mesoporous silica nanoparticles were summarized in here.

Polyethyleneimine

Polyethyleneimine (PEI), in both its linear and branched form, is one of the most commonly used polymers in siRNA delivery systems (80, 81). Due to its proton-accepting amino groups, cationic PEI and its derivatives have a high positive charge density that enables effective condensation of siRNA by electrostatic interactions into a more compact state, referred to as a 'complex' or 'polyplex'. High molecular weight PEI is a popular gene transfection agent, both *in vitro* and *in vivo* due to its relatively high efficiency. Moreover, transfection ability is also associated with its ability to avoid lysosomal encapsulation and thus degradation of its siRNA cargo. Upon PEI mediated entry into the cell, the endosomes polymer acts as a sponge that adsorbs protons due to its high buffering capacity and primary, secondary and tertiary amine groups. This protonation causes an influx of H⁺ and Cl⁻ ions and water into the endosome, and eventually leads to swelling and bursting of the endosomes because of the osmotic pressure (82–84). The escape ability of PEI from the endo/lysosomal pathway is hypothesized by the 'proton-sponge effect'. Due to the given advantages, in recent years PEI-based siRNA delivery systems were used alone or linked to a variety of targeting moieties such as cell penetrating peptides (CPPs), folate or galactose (85–87). However, additional studies in the last years investigated further the possible intracellular trafficking mechanisms of PEI polyplexes and pointed out the possibility that the proton-sponge effect is not the dominant factor of endosomal escape (88–90). Moreover, they found for PEI polyplexes that, only a very small fraction of complexes were able to escape from endosomes (90). Recent findings indicate that endosomal escape, even for PEI polyplexes, is still a very important barrier for PEI-based siRNA delivery and optimization for more effective systems needs to be continued.

In general, high molecular weight PEIs provide high transfection efficiency but they also cause interaction with blood components and opsonization, leading to rapid clearance from the blood circulation. As a result, the PEI complexes are cleared from circulation in a few minutes and accumulate mainly in RES organs such as liver and spleen (91). Moreover, the positive charge of the high molecular weight PEI is usually linked to high non-specific toxicity (92, 93). Low molecular weight PEIs are more biocompatible but much less efficient.

To improve the balance between efficacy and toxicity of PEI, recently a series of phospholipid-modified PEI-based nanopreparations for siRNA-mediated gene silencing was introduced. Navarro et al. covalently conjugated phosphatidyl choline (PC), dipalmitoyl phosphoethanolamine

(DPPE) and dioleoyl phosphoethanolamine (DOPE) to two different molecular weight PEI backbones (1.8 kDa and 25 kDa) and investigated the structure/activity relationships for the optimization of these phospholipid-PEI amphiphiles (94). They found that although the physico-chemical properties and siRNA complexation capacity of conjugates were the same, their cellular interaction and silencing potency varied dramatically depending on the choice of lipidation. Modification of PEI with DOPE and DPPE produced di-block amphiphiles able to self-assemble into micellar aggregates with critical micelle concentrations of 97 $\mu\text{g/ml}$ and 72 $\mu\text{g/ml}$, respectively, whereas PC-PEI showed no micellization. The micelle forming ability of DPPE-PEI and DOPE-PEI completely changed PEI's interaction with the cell membrane and increased the cellular internalization up to 80% in the first hour of incubation with GFP-overexpressing cells. Among the conjugates, DOPE-PEI displayed a more effective GFP silencing (60%) compared to DPPE-PEI (30%) and PC-PEI (5%). Use of DOPE as a helper lipid for fusogenic functionality increased the overall efficacy of the lipid-conjugates. Moreover, the cytotoxicity of low-molecular weight PEI conjugates was 10 times less than that of 25 kDa PEIs. Using this structure/activity relationship information, Navarro et al. (95) formulated a novel siRNA carrier, micelle-like nanoparticle (MNP), based on the combination with DOPE-PEI and PEG-lipid/lipid mixtures for improved bioavailability (Figure 1). While DOPE-PEI polyplexes had positive surface charge (31 ± 2 mV) the developed MNPs consisted of DOPE-PEI/POPC/cholesterol/PEG-PE (4:3:3:0.3 mol/mol) had neutral zeta potential due to the shielding effect of PEG and lipids. They showed that MNPs effectively delivered FAM-labeled siRNA to B16F10 cells with more than 70%

siRNA-positive cells. The potential of MNPs were demonstrated by 20% GFP downregulation on C166-GFP cells without any cytotoxicity (95).

More recently, MNPs were used for therapeutic protein downregulation targeting p-glycoprotein (P-gp) (96). The presence of P-gp on the surface of resistant cells decreased after treating cells with DOPE-PEI or MNPs loaded siMDR-1. This P-gp downregulation was translated into an effective inhibition of doxorubicin (DOX) efflux activity and P-gp downregulation mediated by DOPE-PEI and MNPs significantly improved DOX toxicity in multi-drug resistant cells.

Another very recent approach for modification of low molecular weight PEI with different carbon length lipids was introduced by Akinc et al. (97). They have used a synthesis method based on conjugate addition alkyl-acrylates or alkyl-acrylamides to primary or secondary amines. They synthesized a total of 1200 different lipid-like siRNA delivery nanoparticles (LNP) and evaluated their efficacy using *in vitro* and *in vivo* models, including cynomolgus monkeys and created a library of these particles. Chen et al. (98) combined this combinatorial library approach with a microfluidic formulation method to produce siRNA-LNPs in a microliter scale and a high-throughput production need. They were able to create LNPs with 70 nm size, named as C12-200, which consisted of a cationic lipid from the library, cholesterol, distearoyl phosphatidylcholine and a methoxyPEG-modified lipid. The latest successful example of this approach was reported by Dhalman et al. (99) to demonstrate *in vivo* endothelial siRNA delivery using the 7C1 formulation. The lipid-like structure of 7C1 was synthesized by reacting C_{15} epoxide-terminated lipids with low molecular weight PEI (PEI_{600}) at 14:1 molar

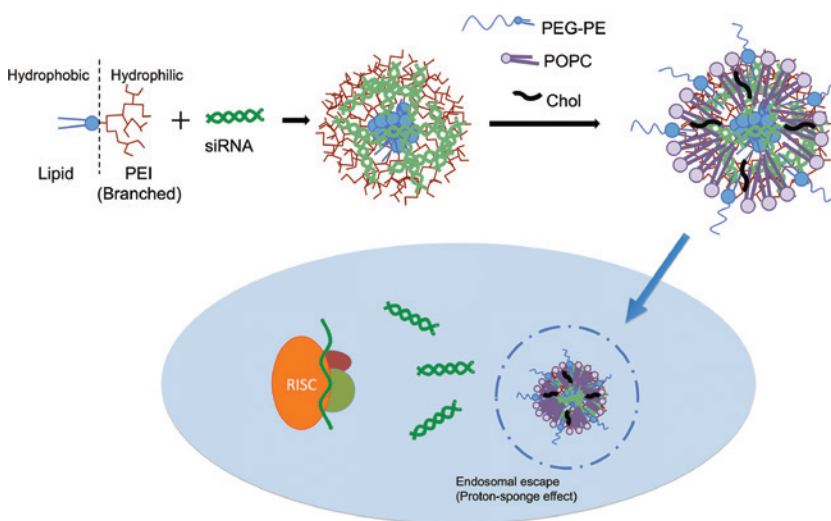


Figure 1: Schematic representation of RNA interference by self-assembled micelle-like nanoparticles (MNPs).

ratio. While the conjugate itself was effective alone, like the C12-200 example, investigators preferred the MNP approach and formulated the polymer with $C_{14}PEG_{2000}$ to increase the colloidal and *in vivo* stability. They showed 90% and 50% of *in vivo* target gene expression silencing in lung endothelial cells at doses of 0.1 mg/kg and 0.02 mg/kg. The 7C1 was able to transfect endothelial cells selectively at low doses, but more importantly it did not cause gene knockdown in hepatocytes, peritoneal immune cells, pulmonary epithelial cells or pulmonary immune cells.

Cyclodextrins

Cyclodextrins (CD) are natural polymers, which can form water-soluble inclusion complexes with small and large molecules (100). CDs are short polycationic polymers (typically $n=5$) with amide functional groups that assemble with siRNA via electrostatic interactions (101, 102). The most important aspect of using CDs in siRNA delivery is that they are the first systemically administered siRNA in clinical trials and with direct evidence of RNAi in phase I study (103). CALAA-01 by Calando Pharmaceuticals (Pasadena, CA, USA) was developed using a linear CD-based polymer with adamantane (AD)-PEG chains for *in vivo* stabilization and efficacy. It incorporates a duplex of synthetic, non-chemically-modified siRNA directed against the M2 subunit of ribonucleotide reductases (RRM2), a critical component in the proliferation of cancer cells. To increase the cellular uptake that has been reduced by PEG shielding, transferrin receptor (CD71) targeting introduced to the system using AD-PEG-Tf conjugate allowing multivalent binding to the cancer cells (104). Patients with solid tumors refractory to conventional therapies received these nanoparticles systematically in two 21-day cycles. The phase I trial (NCT00689065) showed successful delivery of CD nanoparticles by biopsies and knockdown of target protein by qRT-PCR. 5'-RLM-RACE analyses were also confirmed that the delivered siRNA engages the RNAi machinery (103). This first reported clinical trial of siRNA is additionally important due to the explanation of the factors needed to be evaluated for successful delivery strategies such as low cytotoxicity of the main cationic polymer, steric stabilization for improved circulation times and protection, using a ligand for multivalent binding and effective internalization. Moreover, using imidazole end groups in the main polymer for buffering of endocytic vesicles proved the necessity of effective endosomal escape mechanisms. The trial that started in 2008 was completed in 2013, however, currently there are no active phase II clinical trials related to CALAA-01 or other cyclodextrin-based siRNA delivery systems.

In recent years, using CD in the non-viral siRNA delivery systems as combinatory component started to become more and more popular and common. Some examples of these combination structures are with PEI (105–107), poly-L-lysine (108) and dendrimers (109–111). In the last years, PEG is one the most used modification/combination option for CD nanoparticles. Direct modification of CD with PEG chains has proved to be a challenging area with most of the research focused on PEG conjugation on the primary side of CDs. Secondary side PEG modification of CDs could be performed using copper(I)-catalyzed 'click' chemistry to form the polar secondary side (112). O'Driscoll's group has adopted the ABCD approach (112–114) introduced by Kostarelos and Miller (115) to prepare PEG conjugated CD polymers for developing co-formulations blended with CD:siRNA complexes. In these systems 'A' donates the siRNA and 'B' the amphiphilic CDs. 'C' is the stability enhancer of the formulation, which is PEG conjugated to modified 2'-hydroxyl position of β -CD by click chemistry. PEGylation significantly improved the stability of CD:siRNA formulations in high salt concentration and lowered the surface charge and reduced the cytotoxicity significantly. However, compared to non-PEGylated CD:siRNA complexes, gene silencing dramatically reduced due to the steric and charge hindrance of cationic CDs in different cell lines *in vitro* (112, 113) including the neuronal *mHypoE N41* and non-neuronal Caco-2, but stayed significantly higher than naked siRNA. The translation of this approach to *in vivo* was investigated to reduce the Huntingtin (*HTT*) gene in Huntington's Disease (HD) using the R6/2 mouse model. The silencing of *HTT* was achieved after single direct injections and after repeated brain injections, alleviation of motor deficits was observed without toxicity (116). To enhance the efficacy of these 'ABC' type systems, a targeting moiety was introduced by O'Mahony et al. and insertion of a cell penetrating peptide (CPP) octaarginine (R8) conjugated to PEG-DSPE resulted in a complete 'ABCD' delivery system (114) with 20% more cellular internalization of FAM-labeled siRNA over the non-R8 modified CD delivery system. They reported the recovered silencing effect of luciferase reporter gene by 80% knockdown and endogenous GAPDH by 40% knockdown at mass-ratio of 20 (MR20) in *mHypoE N41* mouse embryonic hypothalamic cell line as a model for neuronal cells (114).

Dendrimers

Dendrimers are highly branched synthetic macromolecules that represent another promising siRNA delivery platform (117). Dendrimers depict a structure that consists

of a central core molecule that acts as a root, from which a number of highly branched, tree-like arms originate in a symmetrical manner. The repetition of these symmetrical tree-like units during their synthesis is defined by the term 'generation'. Their unique structural properties such as precisely controlled radial symmetry, tunable size, easily accessible functional groups and high cargo encapsulation in a nanometer size surely increase this potential. For better complexation with negatively charged siRNA, cationic dendrimers have found applications as successful non-viral siRNA delivery vectors. Among them poly(amidoamine) (PAMAM) and poly(propyleneimine) (PPI) dendrimers were of interest. The elucidation of the complexation mechanism of the PAMAM-siRNA dendriplex self assembly revealed that generation four (G4) and G7 dendrimers displayed equal efficiency for dendriplex formation, while G1 with much less positive charge density, lacked the siRNA condensation (118). However, PAMAM dendrimers demonstrated immunogenicity and cytotoxicity associated with surface charge (119, 120). To avoid these disadvantages, Minko et al. introduced internally cationic dendrimers, where the system has a neutral surface for low cytotoxicity while the internal cationic charge of the dendrimer allows siRNA complexation (120, 121). Established strategies such as PEGylation to extend circulation time and attachment of targeting peptides at the distal ends of PEG to increase the specificity could also be performed effectively (122, 123). PEG is also served as a di-functional or mono-functional core for dendrimers (124, 125) and very recently cationic dendrimers with 4-arm PEG-core were synthesized using an accelerated AB_2/CD_2 dendritic growth approach by Albertazzi et al. (126) for DNA transfection with promising results and approach for translation into siRNA delivery.

In recent years, PAMAM dendrimers gained attention for siRNA delivery to the lung; an application that mostly faced important challenges based on oral inhalation device compatibility. Conti et al. (127) developed 4th-generation PAMAM dendrimer-siRNA dendriplexes and incorporated them in chitosan-g-lactic acid or mannitol microparticles for the preparation of hydrofluoroalkane (HFA)-based pressurized metered-dose inhalers (pMDI). They reported that siRNA formulated as dendriplexes in pMDI was stable and intact during the particle formation process and even after long exposure times to HFA. Respirable fractions up to 77% and 85% recovery of total siRNA with chitosan-based formulation indicates that dendrimers could be used for developing pMDIs to deliver siRNA to deep parts of the lungs.

The versatility of the dendrimers can be increased by surface modification using ligands such as CPPs, for better

intracellular siRNA delivery by enhancing the cellular uptake. Liu et al. developed arginine-terminated generation-four PAMAM dendrimers for enhanced cellular uptake of siRNA to silence heat shock protein 27 (Hsp27) in various cell lines including prostate cancer PC-3 and C4-2 cells as well as breast cancer MDA-MB-231 cells (128, 129). More interestingly, Zeng et al. designed a biodegradable and multifunctional dendronized polypeptide (denpol) platform that combines the multivalency of dendrimers and the conformational flexibility of linear polymers. While the aromatic residues in the polymer enhanced the cellular uptake, the buffering capacity of histidine facilitated the endosomal escape for successful siRNA delivery (130).

Recently Biswas et al. developed a triphenylphosphonium (TPP)-conjugated, acetylated, fluorescently-labeled generation-five PAMAM dendrimer (131). Neutralization of the surface charge of the dendrimer by acetylation prevented the non-specific interaction and binding of the molecule with cell organelles and membrane, and thus reduced the cytotoxicity while the fluorescent labeling allowed researchers to follow the dendrimers intracellularly. The cation TPP has lipophilicity due to the presence of three phenyl groups and a stable delocalized positive charge, properties that give it the targeting ability to mitochondria (132). The association of two TPP molecules on the surface of a single dendrimer helped them to reach the mitochondria of HeLa cells compared to parent and acetylated dendrimer, confirmed by confocal laser scanning microscopy.

The unique micellar-dendrimer system, where a newly synthesized construct, a PEG-DOPE modified generation-four PAMAM dendrimer ($G(4)$ -PAMAM-PEG_{2k}-DOPE) (MD) was utilized to prepare a 1:1 mixed micellar formulation with PEG_{5k}-PE (MDM) (133). It was shown that MD and MDM delivered significantly higher amounts of Cy3-labeled GAPDH siRNA than the parent G(4) dendrimers (G(4)-D), approx. 3 and 2-fold, respectively. The GFP silencing was approx. 10% with the G(4)-D, 22% for MD and 18% for MDM. Lipid modification of the dendrimer surface resulted in higher cellular association and facilitated the cell membrane penetration of the system. Moreover, MDM proved superior properties for MDR1 siRNA and anticancer drug loading confirmed by the higher co-delivery of DOX and siRNA into the cancer cells simultaneously, which makes the system a true multifunctional nanocarrier for siRNAs.

Successful *in vitro* results obtained with dendrimers were put to the challenge for *in vivo* siRNA delivery by many groups. One of the recent examples published by Zheng et al. (134) described the G5 PAMAM dendrimer-modified selenium nanoparticles (G5Se) for P-gp downregulation in cisplatin (DDP) resistant A549/DDP tumors. Nude mice bearing A549/DDP tumors were

injected with G5Se loaded with *mdr1* siRNA and cisplatin (G5Se-DDP-siRNA) daily for 2 weeks. The authors showed that the G5Se dendrimers increased the endosomal escape efficiency compared to positively charged G5-NH₂ dendrimers without selenium at the cellular level and yielded significantly higher downregulation of cyclin D1, c-myc and P-Akt. The dendrimers containing both DDP and siRNA suppressed the tumor volume effectively *in vivo* compared to only DDP containing dendrimers, without any apparent toxicity.

Finlay et al. targeted the *in vivo* breast cancer cell metastasis by downregulating TWIST1, a transcription factor activates the epithelial-mesenchymal transition (EMT), using third generation amphiphilic PAMAM dendrimer YTZ3-15 complexed with TWIST1 siRNA (135). The formulation was able to accumulate and remain in the nude mice xenograft orthotopic TNBC tumors, however, *in vivo* silencing of the target proteins was not investigated. Rajasekaran et al. (136) developed PEGylated G5 PAMAM dendrimers complexed with astrocyte elevated gene-1 (*AEG-1*) siRNA and loaded with all-*trans* retinoic acid (ATRA) to counteract hepatocellular carcinoma (HCC). Further modification of distant PEG chains on the dendrimers with lactobionic acid (Gal) allowed the formulation to interact with asialoglycoprotein receptors that are overexpressed in liver hepatocytes. Athymic nude mice implanted with QGY-7703 cells treated with ATRA resulted in low efficacy due to high *AEG-1* levels. Dendrimers delivering *AEG-1* siRNA caused significant decrease in *AEG-1* levels *in vivo*. Mice treated with Gal-PEG-PAMAM dendrimers delivering siRNA and ATRA simultaneously showed increased necrosis, inhibition of proliferation and increased apoptosis. The study marks the importance of successful *in vivo* siRNA delivery by dendrimers by increasing the efficacy of an approved anticancer agent.

Mesoporous silica nanoparticles

Among various inorganic nanoparticles that have been used for siRNA delivery such as carbon nanotubes (137–142), iron oxide (143–146), quantum dots (147–150) and gold (32, 151–155), mesoporous silica nanoparticles (MSN) emerged as a very promising choice in very recent years. Even they have and still do draw attention as efficient drug carriers in the early development years (156–160), the advantages like large surface area, ordered pore structures, tunable pore size and volume, easy surface modification and ability to encapsulate both small and large molecules make them an excellent candidate for effective

siRNA delivery platform (161). In the scope of this review, recent advances related to MSN-based siRNA delivery is summarized in this section.

The initial strategy for negatively charged siRNA loading/complexation to MSNs involves coating or modification of the MSN surface with polycationic polymers such as poly-L-lysine and PEI (162) followed by a charge driven adsorption of siRNA on the particle surface. Hom et al. developed MSNs with surface conjugated PEI at a nanoparticle:siRNA ratio of 1:25 by mass and reported better GFP silencing compared to Lipofectamine 2000 and significant down-regulation of Akt by Western blotting (163). Bhattarai et al. (164) used the well-established PEGylation and surface modification with polycation followed by siRNA complexation on the surface of the particles. They reported low cytotoxicity of the particles and high RNA interference but also the loss of mesoporous structure after incubation in cell culture media. The adsorption/complexation of siRNA on the surface of MSNs indeed limit MSNs advantages over non-porous nanoparticle-based systems that uses the same loading strategy by depleting the surface amino groups and preventing further modifications. With this in mind, in the last years several groups developed MSNs, which encapsulates siRNA in the mesopores. If the MSNs pore size is bigger than the siRNA diameter (~2.6 nm), siRNA molecules could enter the pores if they adopt the necessary conformation (165). The shape of both the pores and siRNA is cylindrical so if the inner surface of the mesopores could be modified with positive charge, the interaction area would be much more higher than the surface adsorption. Moreover, after siRNA encapsulation the pores could be capped with PEI, which facilitates the cellular internalization and endosomal escape of the system. It was shown that if the ultra-large pore sizes are used, siRNA encapsulation increases significantly without any cytotoxicity of the system. Na et al. exploited this hypothesis and created MSNs with 23 nm pore sizes and encapsulated 5800 siRNA molecule per particle (166) to obtain significant VEGF downregulation *in vivo*. However, without any steric stabilization and tendency to aggregate, the system was injected intratumorally instead of systematically. Successful *in vivo* attempts have been reported very recently by different groups using modifications that allow the systemic administration such as magnetic MSNs that have PEI coating and fusogenic peptide (KALA) on the surface of MSNs (167) to silence GFP *in vivo*. *In vivo* therapeutic siRNA (PKM2) silencing with cyclodextrin and PEI functionalized MSNs were also reported the year previous to this review with very promising results (105).

Co-delivery of siRNA and anticancer drugs

The combination therapies act generally in two ways; one agent may increase the action of another drug or two drugs may combine to exert effects that are distinct from either individual compound (168). In general, single drug therapy is typically not effective in cancer treatment and thus, combination therapy has found a wide usage in this area. Even though combination chemotherapy regimens were developed during the mid-19th century, after the development of RNA interference technology, new windows are opened in this area. Combination of siRNA with anticancer small molecules benefit both individual active substances. Tumors are highly prone to genetic mutations, which may hinder the effectiveness of siRNA as a single agent in the treatment of malignancies. Moreover, conventional anticancer agents also suffer from limitations like multidrug resistance (MDR), which hampers cancer therapy significantly. There are number of studies reporting that the pre-treatment of cancer cells with siRNAs followed by conventional anticancer small molecules could sensitize/re-sensitize the cells towards the drugs and enhance the efficacy of treatment (96, 169–171). However, they can be considered as early attempts and strategies for siRNA and small molecule combination therapy. To gain the maximum effect from both siRNA and drug *in vivo*, they must be delivered simultaneously to the same cancer cell following systemic administration for maximal cooperation. Here, we summarize the recent developments and approaches related to siRNA and small molecule combination therapy for cancer treatment involving co-incorporated nanocarrier systems.

Several studies were reported for P-gp specific siRNA and anticancer drug combinations in the past few years using different nanocarriers. Meng et al. (172) used MSNs to incorporate DOX and P-gp siRNA simultaneously to overcome drug resistance in squamous carcinoma cell line KB-V1, which has >1000 times higher expression of P-gp compared to its drug sensitive counterpart KB-31. They encapsulated positively charged DOX into the negatively charged 2–2.5 nm diameter pores' inner surface and modified the outer surface of MSNs (100–120 nm size) with different molecular weight PEI for siRNA complexation. They reported full complexation of siRNA at N/P ratios of 80, 80 and 10 for PEI 1.8 kDa, PEI 10 kDa and PEI 25 kDa, respectively. While the MSN formulation with 1.8 kDa PEI was not effective for P-gp siRNA delivery and knockdown, the 25 kDa PEI formulation was considerably cytotoxic due to proton sequestration by unsaturated PEI amines in the

lysosomal compartment. The DOX loading into the inner pores did not alter the siRNA loading and PEI coating did not affect the DOX release either. They confirmed by confocal microscopy that while the cellular internalization of DOX is increased significantly by PEI-coated MSNs over non-coated MSNs, its nuclear localization stayed the same. Only siRNA-DOX co-loaded MSN formulation resulted in significantly higher nuclear DOX localization and thus, lower IC_{50} values (~10 $\mu\text{g/ml}$). However, the authors could not overcome resistance and restore the sensitivity to the level in KB-31 cells (~0.2 $\mu\text{g/ml}$).

More recently, the same group adopted a systematic approach and silenced different targets by siRNA including P-gp, MRP1, ABCG2, Bcl-2, cMyc and PXR (173) to define the optimal siRNA to be delivered together with DOX. They found that P-gp knockdown resulted in highest DOX cytotoxicity at siRNA dose range of 0.002–1 $\mu\text{g/ml}$ and DOX dose of 0.0066–3.3 $\mu\text{g/ml}$, with siRNA:MSN mass ratio of 1:100. To demonstrate the *in vivo* efficacy of the MSN formulations, they modified the surface of MSNs with the PEI-PEG copolymer with 1.8 kDa PEI and 5 kDa PEG. At a dose of 4 mg/kg DOX and 1.2 mg/kg siRNA every 3–6 days for 30 days, P-gp siRNA-DOX co-loaded MSN showed significantly higher tumor growth inhibition (up to 80%) over free DOX (62%) or MSN loaded with DOX only (59%), due to the synergistic effect. Moreover, PEI-PEG modified MSN showed decreased MPS uptake. Li et al. (174) reported L-Arg or L-His coupled β -CD modified CdSe/ZnSe quantum dots to simultaneously delivery DOX and MDR-1 siRNA for multidrug resistance reversal of HeLa cells. Co-delivery of siRNA and DOX was confirmed by confocal microscopy as well as TEM imaging. Seventy-two hours after treatment of the MDR HeLa cells P-gp levels were significantly reduced due to successful siRNA delivery and enhanced accumulation of DOX resulted in enhanced apoptosis levels.

Another important target for RNA interference is Bcl-2, an anti-apoptotic protein that is over-expressed in MDR cancer cells (175, 176). Cheng et al. studied Bcl-2 and DOX co-delivery in a rat model with an *in situ* C6 glioma implant (177). The nanocarrier system consisted of electrostatic folic acid (FA) conjugated poly(ethylene glycol)-block-poly(glutamic acid) (FA-PEG-PGA) coating on the surface of cationic poly(ethyleneimine)-block-poly(ϵ -caprolactone) (PEI-PCL) that is preloaded with DOX and siRNA. This targeted co-delivery system induced significant apoptosis *in vitro*. In the animal studies folate-targeted co-delivery caused significant downregulation of Bcl-2 and also up-regulated the pro-apoptotic *Bax* gene. The synergistic effect of DOX resulted in effective tumor growth inhibition and prolonged survival time over treatment with non-targeted or single agent loaded system.

This system was further optimized by Zou et al. using PEG-PEI-PCL which resulted in better stability properties due to covalent binding of PEG on the surface rather than electrostatic interaction (178).

In recent years, star-shaped co-polymers consisting of a CD core and cationic arms have attracted attention due to their ability to co-load small molecules and siRNA and rose as an alternative system to micellar nanocarriers. They include a CD core (α , β or γ) and poly(amidoamine) dendron arms (179, 180), poly(glycidyl methacrylate) derivative arms (181) and non-targeted (182) or folic acid targeted (183) oligoethylenimine arms. In 2014, Lie et al. developed a copolymer with a β -CD core and poly(L-lysine) dendron arms for co-delivery of docetaxel and MMP-9 siRNA plasmid (108). The system (with a size around 125 nm) could encapsulate hydrophobic docetaxel in the CD core and positively charged arms complexed siRNA at N/P ratios 10 and higher. The system silenced the MMP-9 mRNA and protein in HNE-1 cells as confirmed by RT-PCR and Western blot, with the efficacy of 50% compared to control. At doses of 2.5 $\mu\text{g/ml}$ siRNA and 0.33 $\mu\text{g/ml}$ docetaxel, co-delivery of compounds resulted in significantly higher apoptosis increase and polymer itself was shown to have better blood compatibility and lower cytotoxicity compared to PEI-25 kDa.

A phase II study was recently initiated by Silenseed Ltd to evaluate the efficacy of a siRNA targeted against KRAS mutations, a driving oncogene in most of human pancreatic cancer cases, in combination with Gemcitabine or Folfirinox in patients with unresectable locally advanced pancreatic cancer (NCT01188785). The siRNA is formulated in LODER, a miniature biodegradable polymeric matrix, designed to continuously release the siRNA for 4 months within the pancreatic tumor. From previous pre-clinical studies, the so-called siG12D LODER was able to effectively induce cell death, to decrease the KRAS levels as well as halting the tumor growth of human pancreatic tumor cells and prolonged mouse survival (184). Encouraging preliminary results were recently noted from a phase I/IIa study, where a single dose of siG12D LODER together with Gemcitabine or Folfirinox, were administered in patients with non-operable, locally advanced pancreatic cancer. A high safety profile and an inhibition of the tumor progression were observed in all the treated patients, suggesting that the combination of siG12D-LODER and chemotherapy was well tolerated.

Stimuli-responsive siRNA delivery

Stimuli-sensitive or responsive carriers can release siRNAs in response to a given stimulus that is characteristic of the

area of interest. For all siRNA delivery platforms, one of the major focus is on preventing siRNA degradation from the time it is introduced in the systemic circulation until it reaches the RNAi machinery in the cytoplasm (185). This main goal can be realized by using multifunctional and stimuli-sensitive siRNA delivery systems and longevity in the circulation is one of the necessities. Although PEGylation have clear advantages on that manner, paradoxically PEGylation also causes a steric hindrance for the vectors that are used for endosomal escape and prevents them to freely interact with the endosomal/lysosomal membranes. Thus reduced interaction causes insufficient endosomal escape and eventually can decrease the intracellular delivery of the system and siRNA. This effect of PEG is known as the ‘PEG dilemma’. Most ideal peptide and protein carrier system should have the PEG coating layer for long circulation times and decreased opsonization *in vivo*, but this PEG layer should also dissociate from the carrier surface at the right place and time based on different abnormalities in the tumor microenvironment such as acidic pH, altered redox potential, upregulated proteins and hypoxia as stimulus (Figure 2).

Matrix metalloproteinases (MMPs), especially MMP2, are known to be involved in cancer invasion, progression and metastasis (186). In a recent study, Zhu et al. (187) reported the development of MMP2 sensitive polymeric micelles for siRNA and paclitaxel (PTX) co-delivery. They used an MMP2-sensitive self-assembling copolymer, PEG-pp-PEI-PE, consisted of branched PEI (1800 Da), DOPE and a synthetic peptide (GPLGIAGQ) for PEG (2000 Da) linkage. In the presence of MMP2 enzyme, the peptide linker between the PEG and PEI is being cleaved and this cleavage caused ‘de-shielding’ due to PEG chain removal from the rest of the polymer. Following the PEG

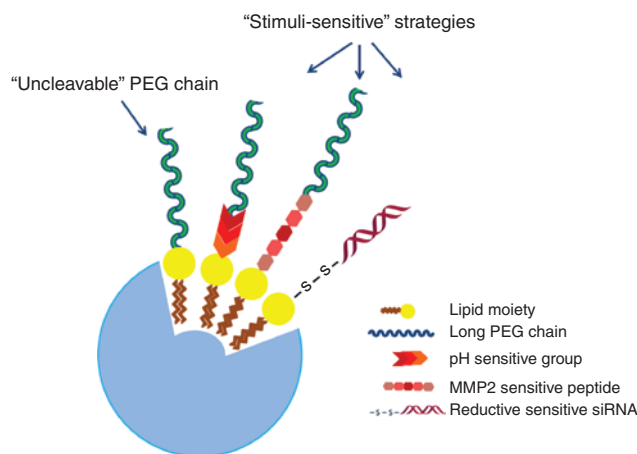


Figure 2: Schematic representation of stimuli-sensitive approaches used for the preparation of NPs for siRNA delivery.

chain removal, the remaining PEI-DOPE micelles could be internalized by the cells effectively due to exposure of the high positive charge of PEI. Moreover, after the internalization of the PEI-DOPE-siRNA complexes, both of the components of the system help endosomal escape, PEI by proton-sponge effect and DOPE by endosomal membrane destabilization. The same peptide have been used by researchers previously with both liposomes (188) and micelles (189) for MMP2-sensitive tumor targeting. PTX was loaded into the hydrophobic PE core of the polymer with 2.3 wt% incorporation efficiency and polymeric micelles were capable of forming complexes with different siRNAs and protected them from RNase degradation. The MMP2-sensitive micelles significantly silenced the GFP in copGFP A549 cells by around 55% only after one administration. Three consecutive administrations resulted in more pronounced GFP silencing up to 65%. The survivin silencing was significantly higher in the MMP2 sensitive group in multidrug resistant A549 T24 cells. PEG-pp-PEI-PE/PTX micelles significantly increased the cytotoxicity of PTX in both drug sensitive and resistant A549 cells compared to free PTX or non-sensitive micelles prepared with an uncleavable peptide. Simultaneous co-delivery of anti-survivin siRNA and PTX was confirmed with FACS and confocal studies and resulted in a synergistic effect. The IC_{50} of PTX in PEG-pp-PEI-PE micelles significantly decreased to 15 nM from 96 nM for free PTX and 28 nM for only PTX loaded PEG-pp-PEI-PE micelles. Moreover, MMP2-sensitive micelles showed a 2.4-fold increase of PTX and siRNA co-internalization than that of non-sensitive micelles and about 14.4% of total cells in tumor internalized both compounds simultaneously (187).

pH-sensitive siRNA delivery nanocarriers are another class of stimuli-sensitive systems designed to enhance the effectiveness of siRNA delivery. However, there have been different approaches and thus strategies involved in pH-sensitive delivery of siRNA. The first approach involves pH-sensitive systems to exploit the low endosomal pH for enhanced endosomal escape of internalized siRNA delivery systems. For lipid-based systems it can be achieved by an amine-rich group in the cationic structure. Following their encapsulation in the endosomes-lysosomes, these cationic lipoplexes fuse with the membranes and disrupt them. In recent years, various studies adopted this approach to develop new lipids and siRNA carrier systems for enhanced pH-dependent endosomal escape. Malamas et al. synthesized new amphiphilic cationic siRNA carriers consisted of protonable amine-based ethylenediamine head group, a hydrophobic group containing two mono-unsaturated oleic acid tails and a histidine-cysteine amino acid based linker (190). They reported higher luciferase

silencing in HT29 cells than lipofectamine RNAiMax with members of this new lipids from their library. Their findings suggest that the increased number of amines in the protonable head group and removal of histidine along with the increasing degree of unsaturation on the lipid tails resulted in improved siRNA delivery into the cytoplasm. The Harashima group introduced a novel pH-sensitive cationic lipid, YSK05, and incorporated this lipid in the multifunctional envelope-type nano device (MEND) (191). They found that YSK05-MEND has higher ability for endosomal escape than other MENDs containing conventional cationic lipids such as DOTAP and DODAP in the endosomal pH range, while showing no membrane disruption in the physiological pH. The PEGylated version of this carrier further demonstrated higher *in vivo* polo-like kinase 1 (PLK1) downregulation compared to DOTAP modified MENDs, however, the injections were intratumoral. Combining stearylated-octaarginine as cationic polycation, stearylated-octahistidine (STR-H8) as a pH responsive polycation in the MEND system, Toriyabe et al. achieved controlled siRNA to the cytoplasm (192). They used R8 and GALA, a pH-sensitive fusogenic peptide, backbone structure to prepare MENDs that shows enhanced cellular uptake and loaded STR-H8/siRNA complexes into these MENDs for decondensation of siRNA in the cytoplasm. While the above-mentioned systems focused on endosomal escape via pH-dependence, they rely first on accumulation and second on internalization of the siRNA carriers as a first step.

The second approach in the pH-sensitive siRNA delivery systems involves low pH in the tumor microenvironment as the stimulus. While these kind of multifunctional stimuli systems were successfully used earlier for mainly small molecule delivery (193, 194), recently Sawant et al. used PEI-lipid conjugate-based pH sensitive micellar nanocarriers for gene delivery (195). They incorporated PEI-DOPE in low-pH-degradable PEG-hydrazone-PE micelles. These PEGylated systems have better stability characteristics due to PEG shielding but also stimuli-sensitive PEG detachment in the relatively acidic tumor microenvironment, which shows promise as a site-specific siRNA delivery applications.

Another stimuli used for siRNA delivery systems is the higher intracellular concentration of glutathione (GSH) (~2–10 mM) compared to extracellular (~2–10 μ M) (196). Moreover, tumor microenvironment was found to be more reductive compared to healthy tissues (197). Musacchio et al. (198) previously reported bio-reductive polymeric micelles for siRNA delivery based on siRNA conjugated to phosphothioethanol (PE) via disulfide linkage. Very recently, using this reversibly phospholipid

modified siRNA approach; Salzano et al. (199) synthesized anti-survivin siRNA-S-S-PE conjugate by using SPDP-activated siRNA and PE-SH. After purification by a desalting column, resulted siRNA-S-S-PE conjugate incorporated in PEG₂₀₀₀-PE micelles encapsulating PTX. While in the non-reductive environment, the polymeric micelles and siRNA conjugate reported to be highly stable. Polymeric micelles consisted of 1:750 weight ratio of siRNA-S-S-PE/ PEG₂₀₀₀-PE resulted in approx. Twenty-five nanometer sized micelles with 50% siRNA and 70% PTX loading efficiency. However, in the presence of GSH, such as intracellular compartments, the disulfide linkage between the siRNA and lipid is cleaved and siRNA is released into the cytosol. The cell growth inhibition effect of survivin siRNA-S-S-PE incorporated micelles on the growth of different human cancer cell lines, including MDA-MB-231, A2780, SKOV-3 and PTX-resistant SKOV-3TR was investigated after treatment of cells with 200 nM survivin siRNA for 48 h. While formulations caused approx. 30% survivin silencing in all cell lines as confirmed by ELISA, cell growth inhibition was only achieved in drug sensitive cells and not in the drug resistant SKOV-3TR cell line. However, simultaneous delivery of PTX and survivin siRNA-S-S-PE at different ratios in polymeric micelles to SKOV-3TR cells led to a significant inhibition of cell growth compared to free PTX and survivin siRNA-only micelles. This synergistic effect was able to reverse drug resistance in an aggressive cell line in a stimuli-sensitive manner. Another report on the GSH dependent siRNA delivery system published by Zhao et al. (200) for herceptin targeted docetaxel and PLK1 co-delivery. The authors conjugated siPLK1 to vitamin E TPGS (TPGS) using the disulfide bond and further modified the system with herceptin conjugated TPGS for targeting which resulted in effective internalization and cytotoxicity against cancer cells.

Perche et al. (201) developed hypoxia-responsive siRNA nanocarrier using PEG₂₀₀₀, azobenzene, PEI (1.8 kDa) and DOPE where azobenzene imparts hypoxia sensitivity and specificity. The resulting polymer (PEG-azobenzene-PEI-DOPE), which is able to complex siGFP and form micelle-like structures in the aqueous environment, showed hypoxia-selective PEG detachment in hypoxic conditions. This detachment exposed the highly cationic PEI-DOPE/siRNA complexes to the cells and resulted in higher cellular internalization. Significantly higher GFP downregulation was achieved in various GFP overexpressing cancer cells under hypoxic conditions compared to normoxic conditions. Hypoxia insensitive polymer did not cause any GFP downregulation due to PEG shielding. The authors also reported enhanced spheroid penetration and siRNA delivery under hypoxic conditions using 3-D cell culture models.

Moreover, *in vivo* efficacy and selectivity of the system was investigated using mice bearing GFP expressing B16F10 and A2780 tumors. A two-fold increase in tumor-cell-associated fluorescence intensity was observed only with rhodamine-labeled hypoxia-sensitive polymer while the hypoxia-insensitive polymer was not found to accumulate in the tumors. Substantial GFP downregulation was detected after intravenous injection hypoxia-sensitive formulation by *ex vivo* imaging and by flow cytometry. This study is the first of its kind with a hypoxia-activated siRNA nanocarrier achieving silencing *in vivo*.

Expert opinion

Here we reviewed recent siRNA delivery strategies that have proven to be successful and effective. The summarized systems exhibit a wide diversity from simple conjugates to organic and inorganic nanoparticle carriers with different size, surface charge, chemistry and preparation methods. But regardless of this overwhelming variety, some of the basic guidelines became available in recent years thanks to the increasing number of *in vivo* studies and clinical trials. We know that the nanoparticle-based systems should have a size range between 20 nm and 200–400 nm, PEGylation or similar surface modification is necessary for longevity in circulation and shielding of positive charge (in case of complexation-based systems) to prevent non-specific interaction and toxicity at the off-site target is required for successful systemic application. However, after delivering the siRNA to the target site, following internalization by the target cells, an additional mechanism, which grants the endo/lysosomal escape, is another must-have. The use of targeting ligands, despite the cost and regulatory hurdles associated with the targeting strategy, still is a big motivation and the benefits are undeniable (202). While some think that combining all these characteristics in one-for-all system seems utopic, the introduction and fast-paced development of multifunctional and stimuli-sensitive siRNA delivery systems hold great possibilities in an area that is so young and needs specific requirements every day (185, 203).

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