Published in final edited form as:

Ther Deliv. 2011 July ; 2(7): 891–905.

Nanomedicines based on recombinant fusion proteins for targeting therapeutic siRNA oligonucleotides

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Abstract

The enormous promise of siRNA technology for rational and targeted therapy can only be realized if the inherent problems in terms of pharmaceutical development are overcome. Besides liposomal and polymeric nanoparticles, fusion proteins hold great potential for cell-type specific delivery of siRNA. Consisting of a protein binder and an oligonucleotide complexing domain, fusion proteins are designed for targeted delivery to a certain tissue or organ and subsequent release of the siRNA after cellular uptake. This article focuses on the possibilities and importance of targeting and complexing domains, including polymers and dendrimers. *In vitro* and *in vivo* evaluations are discussed with an in-depth view on pharmacokinetic properties. Remaining challenges concerning specificity on the tissue and molecular levels are highlighted.

siRNA in therapy

A decade after the publication of the article that is widely regarded as the birth of RNA interference [1] as a general mechanism for gene silencing, this technology has already advanced to the early stages of clinical trials [2–4]. Despite this rapid progression, and the immense impact on scientific research, there is still a long way to go for the successful application of siRNA-mediated gene silencing in therapeutic settings. Underlined by disappointing results from the first ever Phase III trial of an siRNA, bevasiranib [5], hurdles like the lack of cellular uptake, poor stability, insufficient efficiency, sometimes questionable specificity and potential safety issues have to be overcome to ensure full exploitation of the enormous potential of the rational silencing of single genes. The fate of the initially similarly hyped concepts of gene therapy, DNA vaccines and antisense oligonucleotides are cautionary tales of neglecting proper and careful pharmaceutical development of nucleic acid-based agents.

Given the enormous possibilities for therapy of diseases such as cancer, autoimmune diseases and viral infections, it is no surprise that the biotech and pharmaceutical industry has made considerable efforts to develop the siRNA technology towards clinical applications (Table 1). Initial trials have begun a few years ago, with bevasiranib being the first substance to reach Phase III. However, the trial was terminated by OPKO because it became unlikely that the primary end point would be met. This demonstrates that careful optimization and trial design is necessary to avoid clinical failure. This and other initial trials

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Financial & competing interests disclosure

Work of the author is supported by the Austrian Science Fund (FWF): J2593-B11 and I519-B11. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

involve local administration at the eye, mirroring the development of antisense agents in the 1990s [6]. Many of the problems concerning pharmacokinetics will be circumvented by local administration, because fewer barriers need to be overcome, but for realization of the full potential against cancer and viral infections, systemic administration with adequate biodistribution is imperative.

Because of their chemical structure, size, and hydrophilic nature, siRNAs are extremely poor active pharmaceutical ingredients (APIs). They do not readily cross physiological barriers, and are quickly degraded in blood and serum. Chemical modification of the lead structure is an essential step in drug development; however, the molecular effector responsible for cleaving the targeted mRNA, the RISC, only tolerates minor aberrations in the chemical structure. Several 2'-modifications have been shown to result in retention of the potency, at the same time increasing stability against enzymatic degradation [7]. To date, no alteration has resulted in convincingly improved cellular uptake. Consequently, many efforts are concentrating on developing delivery systems for siRNA that are capable of guiding the oligonucleotide cargo to the place of action in the cytosol, where they have to release the active ingredient. Research on lipid- or polymer-based delivery systems has been carried out for decades to improve pharmacokinetic parameters of small molecules, antisense oligonucleotides, and DNA, and has been adapted for siRNA delivery [8,9]. Liposomal and stable nucleic acid lipid particle (SNALP)-based formulations are already being used in early stage clinical siRNA trials. Grafting targeting agents such as antibodies or other ligands to the surface allows a certain accumulation in the targeted tissue. A formulation of transferrin-targeted cyclodextrin nanoparticles is applied in one clinical trial (CALAA-01) [10,11].

More recently, **fusion proteins** consisting of a cell-recognizing motif and a component for oligonucleotide interaction have been developed and evaluated in preclinical settings. Clear distinction from targeted nanoparticle and liposomal delivery systems is difficult, because several fusion proteins will inevitably lead to a certain extent of aggregation, creating particles of up to several hundred kDa and up to 200 nm in size [12,13]. Generally, these fusion proteins will result in smaller and more uniform particles than liposomal and nanoparticle formulations. Compared to other delivery strategies, fusion proteins offer the advantage of simple generation and high reproducibility, because such devices ultimately consist of only two components: the fusion protein as the carrier and the siRNA as the cargo. The complexation strategy eliminates the need for covalent conjugation, resulting in easy variation of the siRNA and its target. In the vein of personalized medicine, [14] individual gene targets can be aimed at with the same delivery agent without the need for separate purification and impurity analysis that a conjugation reaction inherently brings along.

Proteins for targeting

Antibodies & antibody fragments

For efficient and selective targeting, the cell-recognizing protein needs to have a high functional affinity, low dissociation rate, appropriate biodistribution characteristics and low immunogenicity [15]. Antibodies are nature's gold standard for antigen recognition and consequently the first choice for targeting purposes. Full-length antibodies have a large size and are difficult to produce and handle because of their post-translational modifications. They can be reduced in size by only using the antigen recognition site, located in the Fab fragment, which itself consists of a heavy and a light chain, each having a constant and a variable domain. A **single-chain variable fragment antibody** (scFv) is constructed by fusing the variable regions of the heavy and light chains together with a short peptide linker, eliminating the need of combining the two components after recombinant production. Antibodies and their derivatives have themselves undergone a long development process for

therapeutic applications until their inherent drawbacks had been eliminated by careful optimization. Today, more than 20 antibodies are approved for therapy by the FDA. Consequently, the majority of researchers make use of antibody derivatives as the targeting component of fusion proteins for siRNA delivery (Figure 1 & Table 2).

In 1995, the first use of a **protamine**-fusion protein for specific delivery was reported [16]. The heavy chain Fab fragment of an HIV-1 envelope antibody was fused to a truncated protamine in order to enable complexation of nucleic acids. A plasmid encoding the bacterial toxin ETA was specifically transported into HIV-infected cells using this device. In 2001, the generation of a fusion protein consisting of a single-chain antibody specific for the tumor surface protein ErbB2, and a truncated protamine was reported [17]. This fusion protein was subsequently used to deliver a luciferase plasmid into antigen-positive cells. Later, Song *et al.* [18] used these two fusion proteins for siRNA complexation. They were the first to demonstrate that such a fusion protein is sufficient to selectively transport siRNA to the tumor tissue *in vitro* as well as in a xenograft animal model and finally results in downregulation of the targeted mRNA.

The same group later fused a scFv targeting the human integrin lymphocyte function-associated antigen (LFA)-1 to the protamine fragment for selective delivery of siRNA to lymphocytes [19]. The antibody recognized only activated lymphocytes, allowing very efficient targeting. Because in addition to blocking activated LFA-1, proinflammatory genes can be silenced with siRNA, this approach promises an additive effect in suppressing immune activation and inflammatory processes.

Protamine fusion proteins can not only target tumors or human blood cells, using an scFv-recognizing hepatitis B surface antigen, siRNA can be directed at the hepatitis B virus [20]. siRNA uptake with a single-chain antibody fragment protamine fusion protein was compared with that when using a fusion protein having an additional light-chain constant fragment (C κ) inserted before the protamine. Both constructs resulted in virtually the same siRNA uptake. The scFv-C κ -protamine construct successfully inhibited hepatitis B protein expression in an animal model.

Instead of expressing a fusion protein, Kumar *et al.* chemically conjugated a nona-arginine to the cystein-modified C-terminus of a scFv specifically binding to CD7 of human T cells [21]. Using this conjugate, a combination of siRNAs for simultaneous silencing of several viral genes and the chemokine receptor CCR5, essential for HIV docking, was targeted to T cells in a mouse HIV model.

A minibody, composed of a leukemia-selective scFv region, and the regions CH2 and CH3 of the human Fc, was fused to the nona-arginine (R9) peptide [22]. This construct enabled selective delivery to Jurkat cells to a relatively small fraction of leukemic cells in mouse bone marrow.

Recently, the hemagglutinin antigen of the avian influenza virus (AIV) was used as a recognition site of a fusion protein comprised of a specific single-chain antibody and truncated protamine [23]. Here, a plasmid DNA, not siRNA, was selectively transported into AIV-infected cells and effectively inhibited virus replication. However, the same construct can supposedly be used for siRNA-mediated gene silencing.

Instead of basic peptides, polymers can be used for oligonucleotide complexation. A conjugate of an anti-PECAM (antiplatelet cell adhesion molecule) antibody with the highly cationic polyethyleneimine (PEI) was used for targeting a DNA plasmid to pulmonary epithelium [24]. A model plasmid was successfully targeted to mouse lungs. In another example, the Her2-specific antibody trastuzumab was covalently linked to linear

polyethylenimine by introducing a disulfide tether [12]. The resulting conjugate was shown to efficiently transport plasmid DNA into tumor cells displaying the corresponding antigen.

Ankyrin repeat proteins

Designed ankyrin repeat proteins (DARPin)s are an emerging class of antigen-binding proteins [25-27]. Ankyrin repeat proteins are naturally occurring selective protein binders, comprised of several ankyrin domains. Engineered DARPins usually consist of five of these ankyrin domains, and their binding characteristics can be modulated by rational design [28] or by generating libraries and optimizing antigen binding using phage display [29] or ribosome display [30]. DARPins have some important advantages over traditional antibody-derived proteins. They have a molecular weight of less than 20 kDa, making them smaller than immunoglobulins and even scFvs. The lack of post-translational modifications and cysteines and their high stability result in high-yield recombinant production, easy handling and give comprehensive possibilities for modifications and conjugation reactions. Due to their recent emergence, little data regarding the *in vivo* properties are available and the value in therapeutic applications remains to be seen.

A fusion protein of a DARPin binding to the epithelial cell-adhesion molecule EpCAM, which is highly upregulated in tumors of epithelial origin and a *bona fide* cancer marker, with a truncated protamine sequence has been generated [31]. The construct was able to deliver the complexed siRNA only in tumor cells expressing EpCAM and finally led to downregulation of the targeted antiapoptotic tumor protein Bcl-2. This is the first example of using nonimmunoglobulin protein binders for tumor-selective targeting of siRNAs.

Other proteins

Other proteins that can be employed for protein-mediated delivery include the iron-binding plasma protein transferrin [10,32-34], or virus-derived proteins arranged as capsoids [35]. The latter can bind to sialic acids displayed at cell membranes, while transferrin receptors are found in all actively metabolizing cells. Thus, a true tissue-targeting effect is lacking for both delivery devices, but they activate receptor-mediated endocytosis. Transferrin covalently bound to cationic polymers or poly-lysine has been used to successfully transfect nucleic acids or antisense oligonucleotides [34].

Atelocollagen is produced from dermal calf collagen by pepsin-mediated cleavage of antigenic telopeptides from both ends [36]. The resulting cationic protein can be used for packaging siRNA for transfection *in vitro* and *in vivo* in tumor xenograft mouse models [37,38]. Because of the cationic charge of atelocollagen itself, there is no need to add an oligonucleotide complexing motive, but the enhancement of membrane permeation is due to the cationic charges of the protein and as such lacks any tissue specificity.

Peptides

Even small peptides can be specific ligands. A 12-mer peptide with selectivity to dendritic cells was fused to nona-arginine via a five amino acid linker for silencing of a dengue virus gene and TNF- α in virus-infected dendritic cells [39]. The fusion peptide showed selectivity for immune cells *in vitro* and its application as a targeting device for TNF- α siRNA prevented TNF- α stimulation in a mouse model.

A 29-mer peptide derived from rabies virus glycoprotein (RVG) was demonstrated to be enough for specific binding to the acetyl choline receptor. A fusion with nona-(D)-arginine was used for targeting siRNA to neuronal cells, enabling transvascular penetration of the blood-brain barrier [40]. An siRNA sequence directed at the Japanese encephalitis virus delivered with the RVG-R9 fusion peptide protected mice from viral encephalitis.

Remarkably, the same delivery system was recently used for siRNA uptake into macrophages and microglia cells expressing the nicotinic acetyl choline receptor [41]. While this demonstrates the versatility of the fusion peptide, it questions its specificity, and shows that targeted delivery is in the first place dependent on careful selection of the targeted antigen.

The tripeptide Arg-Gly-Asp (RGD) binds to $\alpha_v\beta_3$ integrin, which is expressed on endothelial cells only during angiogenesis. Several attempts [13,42,43] have been made to exploit this binding for targeting of plasmids using polymer attachment, but the results both in terms of transfection efficiency and targeting effect were modest.

A 9-mer peptide hormone, the luteinizing hormone-releasing hormone (LHRH) can be used for specific targeting to some tumor types, because the corresponding LHRH receptor is over expressed in the plasma membrane of a number of tumors, but not found in healthy tissue [44]. Attachment of this peptide to a dendrimeric carrier system was used to generate a delivery system for siRNA, with the cellular uptake being dependent on the presence of the LHRH receptor.

Cell-penetrating peptides (CPPs) such as the tat peptide or the antennapedia peptide penetratin have been widely used for enhancing cellular uptake of antisense agents, siRNAs or plasmid DNA in the preclinical setting [45]. The exact mechanism of membrane permeation is not fully understood. While early research indicated an energy-independent mechanism of direct permeation, it was soon discovered that much of these data can be attributed to fixation artifacts of fluorescently tagged CPPs. The most important route through the lipid bilayer is ostensibly an endocytotic mechanism, meaning that the transported cargo inevitably ends up in endosomes. There is some discussion whether CPPs may also be internalized in a non-endocytotic manner when applied in high concentrations [46,47]. CPP-mediated uptake involves binding to proteoglycans located at the cell surface and as such is wholly cell-type unspecific [48]. CPPs can be covalently attached to oligonucleotides or complexed to their cargo directly or via a polymer. CPPs are usually characterized by a high relative abundance of the cationic amino acids arginine and lysine. Oligo-arginines such as R9 are also often referred to as CPPs. Thus, arginine-rich peptides can both be used as complexation agent and as membrane-permeation enhancer. Combining the tat (49–57) and the cationic peptide LK15, creates a highly charged peptide that can efficiently transfect siRNA [49,50]. However, this approach lacks celltype specificity and concerns of toxicity remain.

Oligonucleotide complexing agents

Basic peptides

For charge complexation of the multiply negatively charged oligonucleotides, various basic, and thus cationic peptides can be used. Both oligo-arginine and oligo-lysine peptides have been used to condense oligonucleotides in order to mask their charge and consequently improve cell-membrane permeation [46,51–55]. Due to its diffusely distributed charge, arginine binds more tightly to the acidic phosphate backbone of nucleic acids and therefore results in stronger complexation. Protamines-1 and -2 are naturally occurring, arginine-rich peptides. They are 51 and 102 amino acids long, respectively, and replace histones for condensation of nucleic acids in sperm [56]. Protamine sulfate has not only been frequently used for aiding oligonucleotide transfection, but also in nanoparticulate structures, with or without other excipients such as albumin [57–61]. In addition, protamine has been used for over 60 years as an ingredient in retard formulations of insulin, known as NPH (neutral protamine Hagedorn) insulin [62], and also finds medical application as a heparin antagonist [57,63,64]. Consequently, a wealth of data regarding drug safety is available and there is no

doubt that protamines are appropriate and useful excipients in pharmaceutical formulations. Their inherent antigenicity is low, but can lead to adverse drug reaction in a certain number of patients. It can effectively be lowered using a low molecular weight fraction [64]. While protamine can be applied on its own for packaging oligonucleotides and enhancing cellular uptake, it lacks any targeting effect [65]. A truncated form of protamine-1, consisting of a 23 aa arginine-rich sequence, is the most frequently used complexing peptide in fusion proteins for targeted delivery of siRNA. The truncated protamine has been reported to be able to complex between four and six molecules of siRNA [18,20,31]. Depending on the type of protein used for targeting, expression of protamine fusion proteins in *E. coli* can be challenging. Because of the high number of closely located arginines, expression yields may decrease significantly. Codon optimization or the use of specially engineered bacterial strains may be necessary to avoid depletion of rare arginine codons of standard *E. coli* strains. In addition, denaturing protein purification methods usually have to be applied, because of the strong tendency of bacterial nucleic acids sticking to protamine. Refolding procedures are highly dependent on the characteristics of the targeting protein, and a certain loss of binding affinity was reported for antibodies [18,19]. Ankyrin repeat proteins can be refolded more easily without loss of activity due to the lack of cysteine disulfide bridges [31].

Oligo-arginine peptides have similar properties like the truncated protamine, and nonaarginine R9 or R15 can equally be used for charge complexation of siRNAs [22,66]. For their effect as complexing agents, the stereochemistry of arginine is unimportant, and the D-amino acids can be used with the same effect [39,41]. Literature reports indicate a slightly lower capacity of nonaarginine for siRNA binding compared with protamine, which consists of 13 non-sequential arginines distributed over a 23 aa-long peptide. Complexation ratios can be annotated in molar amounts, or in amine to phosphate (N/P) charge ratios. The latter method is commonly used in nanoparticle research and is of course dependent on the number of arginines or lysines present in the complexing peptide. Despite their high similarity, for oligo-arginines an N/P ratio between 0.5 and 1.0 is usually required for complete binding of oligonucleotides, but for the truncated protamine sequence, molar amounts of up to six molecules of siRNA were reported to be bound, which corresponds to an N/P value of only 0.05. Some of the discrepancies can be explained by the fact that different assays for cargo capacity determination were used; an electrophoretic shift assay estimates the amount of completely complexed nucleic acids [22,66], while an immunological pull-down experiment determines the amount bound after adding a surplus of the nucleic acid cargo [18]. Based on the complexation studies, most *in vitro* and *in vivo* studies with the protamine peptide used a molar ratio of protein:siRNA of 1:6, [18,19] while with nonaarginine, a five- to ten-fold surplus of the peptide is commonly applied [21,22,39]. These apparent discrepancies highlight the insufficient biochemical characterization of the complexation reaction of the various peptides. For pharmaceutical development, it will be imperative to assess the properties of the specific arginine-rich peptides in order to identify the best candidate in terms of siRNA loading capacity, stability, as well as the lowest possible toxicity and immunogenicity.

A longer, naturally derived 65 aa double-stranded RNA binding domain was fused to tat for binding and enhancing cellular uptake of siRNA [67]. Gene targets were knocked down *in vitro* and *in vivo* after treating mice intranasally. High target downregulation and low toxicity were reported, but no selectivity can be expected from such a system.

Cationic polymers

While the majority of researchers use cloning strategies to generate expression plasmids encoding such fusion proteins, enabling recombinant expression, it is also possible to employ chemical coupling methods to combine targeting and siRNA complexing of

structures. The recombinant expression of fusion proteins offers the advantages of producing and purifying the targeting device in a single step, facilitating the manufacturing and product characterization process. On the other hand, as described above, expression of highly basic peptide sequences is challenging. Chemical conjugation approaches enable the use of non-natural peptides, such as oligo-D-arginine, polymers and **dendrimers**, but result in more complex preparation procedures with the need to remove unreacted educts and side products.

Tethering cationic polymers to targeting proteins is an attractive option for introducing an oligonucleotide complexation agent. The highly basic PEI has been used for transfecting culture cells with DNA and oligonucleotides for 15 years [68]. Its efficiency is similar to lipoplex-mediated transfections, but the toxicity likewise prevents its use in therapeutic settings. PEI very effectively complexes siRNAs because the distance between amino groups is roughly the same as the space between phosphates in nucleic acids. PEI can be attached relatively easily by a maleimide reaction, if the targeting protein can be modified to contain a C-terminal cysteine. A conjugate of an anti-PECAM antibody with PEI enabled specific delivery of the plasmid cargo to lung tissue [24]. The RGD tripeptide was attached to PEI to increase cellular uptake, which was dependent on the charge (N/P) ratio [13]. With N/P ratios over 5, particle size reached 100 nm. If most of the PEI charge was neutralized by the siRNA, specific uptake was enhanced, but with a high surplus of PEI, its transfection properties accounted for unspecific uptake to the same extent as unconjugated PEI. Inserting polyethyleneglycol (PEG) between PEI and RGD abolished the effect, possibly by shielding the RGD peptide and preventing specific binding.

These reports indicate that because of its inherent membrane-permeating properties, which is tightly coupled to its toxicity, PEI is poorly suited for generating cell-specific targeting systems. The cationic polymer would have to be nearly completely neutralized to ensure selective binding mediated by the targeting moiety without interference of unspecific interactions of the basic amines with anionic components of cell membranes.

Dendrimers

Dendrimers are highly branched polymers that are prepared in a step-by-step fashion from a multifunctional core structure, from which branched arms are extended in an ordered fashion. Dendrimers are of spherical shape, and their size depends on the number of synthesis steps (called generation) [69]. Their size increases linearly with the number of synthesis steps, while the number of surface groups increases exponentially. Compared to other polymers, dendrimers have a well-defined size and structure and their surface groups can be tailor-made for particular applications. Polyamidoamine (PAMAM) dendrimers with surface amino groups are most often used for nucleic acid complexation, because of their commercial availability and their high charge density. Both transfection efficiency and toxic effects are thought to be connected to the number of surface and internal charges, and partial neutralization can lower cytotoxicity, but also limits unspecific cellular uptake [70]. For targeting approaches, dendrimers offer the advantages of relatively easy conjugation to one or several ligands using peptide bond formations or click chemistry, and high capacity for siRNA complexation.

Few attempts to employ dendrimers for targeting have been reported so far. Recently, the 53 aa EGF was attached to generation 4 PAMAM dendrimers [71]. Uptake of this conjugate was found to be dependent on the EGF receptor, and siRNA delivered in high amounts resulted in significant gene silencing *in vitro*.

The 9-mer LHRH peptide was attached as the targeting moiety to PAMAM dendrimers [72]. Here, the internal amino groups were quaternalized with methyl iodide to generate cationic

amines for siRNA binding, and hydroxyl surface groups were used instead of amino groups to lower toxicity and prevent unspecific interaction with cell membranes. Indeed, cellular uptake was found to be dependent on the targeting peptide.

Conjugates of TAT-peptides with PAMAM dendrimers were generated to enhance oligonucleotide uptake, but failed to result in increased uptake of siRNA and improved genesilencing activity [73].

Tethering the RGD peptide led to increased binding to integrin-positive cells, and intracellular accumulation of imaging agents [42]. Later, it was shown that there was no enhancement of the RGD-targeted dendrimer compared with the untargeted dendrimer in an *in vitro* cell-culture model due to domination of membrane permeation mediated by the cationic surface groups. However, tissue penetration was increased by the RGD conjugation in a 3D cell-culture model and a larger portion of the siRNA was delivered to the tumor center [43].

Dendrimers may be developed to be a suitable option for targeted delivery if sufficient specific uptake and appropriate gene-silencing effect can be demonstrated and remaining issues with cytotoxicity are resolved. Both for polymeric and dendrimeric carriers, the borders between fusion proteins and nanoparticles are diffuse, as some aggregation to multi-molecular structures can be observed [13].

Pharmacokinetics: barriers to siRNA delivery

Fusion proteins have been mainly exploited for targeted delivery to tumor tissue, and the following chapter will focus on cancer-directed siRNA delivery. Before an siRNA therapeutic can access its cytosolic molecular target, it has to overcome several biological barriers. Their intrinsic hydrophilicity, multiple net charges, and relatively small size are a serious challenge for systemic delivery. Effective siRNA carriers have to: increase circulation time by protection against nucleases and decreasing renal clearance; enhance biodistribution; facilitate cellular uptake, ideally introducing tissue selectivity, and; enable entry into the cytosol through endosomal escape and release from the carrier [5,74].

Attempts to chemically modify antisense and siRNA oligonucleotides in order to enhance their pharmacokinetic properties have not yet resulted in satisfying outcomes despite considerable progress. At least stability against enzymatic degradation can be conferred by chemical modifications of the oligonucleotide backbone or at the 2'-position. More rigorous changes of the lead structure leads to loss of efficacy due to the structural selectivity of the molecular effector RISC [75].

Stability

For systemic applications, an siRNA delivery system needs to access the target organ via the bloodstream. The siRNA needs to be protected against enzymatic degradation. Complexation to cationic carriers such as protamine or oligoarginine as well as packaging into nanoparticles or liposomes usually shields siRNA from nucleases. In addition, the use of 2'-O'-modified siRNAs is an attractive option for preventing nuclease digestion.

Clearance

Elimination by renal clearance is dependent on molecular size, and naked siRNA is rapidly excreted from the blood compartment. Also smaller antibody derivatives and proteins such as scFVs and DARPins are subject to renal excretion. Larger constructs, which can easily be generated by PEGylation of these small proteins, benefit from increased half-life rates, but on the other hand suffer from insufficient organ penetration. For example, a PEGylated

DARPin showed higher total tumor accumulation than the naked protein, but a lower tumor-to-blood ratio [26]. A molecule size of 60–120 kDa is regarded to be optimal for tumor-targeting purposes [76]. Thus, a complex of a scFv or DARPin (15–30 kDa) with three to six molecules of siRNA (12 kDa each) would fall within these size limits.

Extravasation

After reaching the target organ, the delivery systems have to extravasate from the blood stream into the extracellular matrix. Extravasation is not necessary for targeting endothelial cells or viruses in the bloodstream, but it is a major issue for tumor targeting. The extent and mode of extravasation of fusion proteins is dependent on the size of the delivery system and the targeted tissue. Endothelial tissue, capillaries, and post-capillary microvessels have pores of less than 10 nm in diameter [5], which effectively prohibits unspecific diffusion of macromolecules. In other tissues, such as kidney, liver, spleen and bone marrow, larger pores of up to 150 nm are found, and protein complexes may be able to leave the bloodstream.

Tumor microvessels have the unique feature of increased leakiness; for example, they have pores in the range of 100 nm to up to 3 μ m [77]. Therefore, macromolecules, but also liposomes and nanoparticles are able to leave the blood and enter the interstitial fluid. This tumor accumulation effect is called enhanced permeation and retention (EPR). However, pore sizes greatly vary in different tumors, and the interstitial delivery is difficult to predict. In addition, transport of large molecules in the interstitium is achieved mainly by convection, and the increased fluid pressure results in an outward flow from the tumor core to the blood vessels, thereby complicating the passage of targeting systems through the interstitial space to the tumor cells [78]. Smaller constructs are favored for interstitial transport because they benefit from diffusion, which is dependent on the concentration gradient and not the pressure-driven flow. Thus, fusion protein-mediated delivery of siRNA may exhibit superior properties compared with liposomal or nanoparticulate delivery in regard to tumor accumulation.

Experimental approaches to increase extravasation and interstitial transport include radiating or applying hyperthermia to tumor vessels, and decreasing tumor interstitial pressure [77].

Uptake: endocytosis

Once the fusion protein targeting system has successfully reached its antigen, it is localized on the cell surface. Cellular uptake is consequently afforded by receptor-mediated endocytosis. There are several different subtypes of receptor-mediated endocytosis [79]:

- The clathrin-coated pathway;
- The caveolar pathway;
- The non-caveolar, clathrin-independent pathways, which can be divided into several subgroups;
- Phagocytosis and macropinocytosis.

The last one is predominantly used by macrophages and granulocytes for ingestion of large particulates, and thus can be neglected for targeted delivery of siRNA. The majority of proteins bound to membrane receptors are taken up by clathrin-mediated endocytosis, in which upon binding of a cargo protein, a pit coated with the protein clathrin is formed.

It is difficult to assess the exact endocytotic mechanism, which may be dependent on the particular ligand used as well as its concentration [80]. The importance of distinct mechanisms for fusion protein-mediated siRNA uptake is poorly defined. Regardless of the

endocytotic mechanism, the complex of antigen–protein–siRNA ends up encased into a vesicle, and is then trafficked into the cell, leaving endosomal particles as the final major barrier.

Endosomal escape

Probably all types of siRNA delivery agents, including liposomes, polymeric carriers and polyplexes leave the oligonucleotides in the endosomal vesicle. [74,81]. First, siRNA cargoes are located in early endosomes, from where they are passed over to sorting endosomes, and late endosomes (Figure 2). Here, the pH is acidified to approximately 5–6 by membrane-bound proton pumps. Subsequently, its content is relocated to lysosomes, where the pH is further lowered, and nucleases and proteases breakdown the ingested molecules. Thus, in order to reach the site of action in the cytoplasm, the siRNA has to leave the endosomes before being transferred to lysosomal compartments and being degraded. The ligand is dissociated from the receptor at low pH values in many instances, and the receptor is often recycled back to the cell surface. Endosomal escape is thought to be the rate-limiting step in siRNA uptake, and there are several distinct mechanisms for relocation to the cytosol.

Depending on the applied agent, there are a different possibilities for endosomal escape:

- Peptides derived from viruses or bacteria can form pores in the endosomal membrane, creating exits for trapped cargo;
- Fusogenic peptides change their conformation upon a trigger such as acidification and then can fuse into the cellular membrane, rupturing the endosomes in the process;
- The proton-sponge effect that is created when compounds with a high buffering capacity lead to an excessive influx of protons and water into endosomes. This results in osmotic swelling, and finally disruption of the endosome with release of its content into the cytosol [81].

There is some evidence that small amounts of siRNA or antisense oligonucleotides can diffuse from endosomes to the cytosol, but for efficient siRNA therapy, some additional escape mechanisms need to be activated. Cationic lipoplexes and polyplexes profit from destabilization of (endosomal) membranes by membrane fusion. Cationic peptides, such as oligo-arginines, have been shown to be able to rupture endosomes by the proton-sponge effect, thereby releasing the siRNA cargo into the cytoplasm [74]. Although no detailed proof has been published, it can be assumed that most fusion proteins relying on cationic peptides or dendrimers for siRNA complexation have this effect.

Close examination of the route to the final intracellular target reveals that many features of a delivery system that are beneficial for one stage are detrimental for another. For example, an excess of cationic binding domain enhances cellular uptake and endosomal escape, but can lead to toxic effects and decrease of tissue selectivity. Similarly, PEGylation increases stability and half-life of the formulation, but is in turn membrane stabilizing and results in lower release from the endosomal compartment. In addition, it can decrease antigen selectivity by interfering with interaction of the targeting protein to its antigen. Consequently, a perfect delivery system has to find the optimal trade off of several factors to ensure the highest possible delivery efficiency, target selectivity, stability and circulation time, while at the same time having an acceptable drug safety profile and optimal retention at the target tissue. Careful structure optimization using validated and appropriate preclinical assays is necessary for selection of candidates for clinical development.

Off-target effects & toxicity

Despite being highly specific in theory, experimental data has shown that siRNAs can result in toxic effects or alter the expression of genes other than the targeted complementary one [82]. These effects can be due to the activation of the immune system, degradation of mRNAs with a mismatched sequence, or competition with endogenous miRNAs. The latter two can be avoided by careful selection of an appropriate sequence. Today, powerful design algorithms are available to avoid stretches in the seed region, which are complementary to other genes and can therefore result in **off-target effects** [83].

Activation of the immune systems is regulated by Toll-like receptors 7 and 8, which are located in endosomes [84]. Toll-like receptors subsequently activate signaling pathways that finally lead to activation of interferon and other proinflammatory cytokines. While such activation may be even exploited for therapeutic application [85], it is generally an unwanted effect that should be avoided. By using chemical modifications, activation of Toll-like receptors can be prevented. In particular, 2'-*O*-modified siRNAs such as 2'-*O*-alkylated, 2'-F or 2'-desoxy-oligonucleotides [75] do not result in activation of the innate immune response. However, these modifications may lead to decreased affinity to the RISC, thus abolishing the gene-silencing effect [86]. By careful design and validation of the selected sequence, effective and non-immunostimulatory siRNAs can be prepared. The second possibility to prevent Toll-like receptor activation is the selection of appropriate delivery systems. Because the receptor interaction takes place in the endosome, siRNAs would have to be shielded in the endosomal compartment. There is evidence that packaging siRNAs in poly-lysine can prevent Toll-like receptor activation [84], and similar effects are possible for the diverse fusion proteins.

Despite such class-related effects, off-target effects can also be inferred by chemical modifications. The example of antisense oligonucleotides effectively proved that careful evaluation of chemical modifications is necessary at early development stages to avoid failure in latter clinical stages. **Phosphorothioate** antisense oligonucleotides were among the first modifications to show high stability against nuclease degradation and reasonably good pharmacokinetic parameters. However, an apoptosis-inducing effect that is sequence independent was discovered only after a decade of clinical frustration [87,88]. Phosphorothioate-modified siRNAs result in similar off-target effects, albeit to a lower extent [89].

Comparison with other nanoparticle delivery systems

Using fusion proteins comprised of a targeting domain and an oligonucleotide complexing domain is a new approach in the quest for therapeutic delivery of siRNA oligonucleotides. No direct comparisons of fusion protein-mediated delivery and other targeted approaches in the same setting have been published. Therefore it is impossible to conclude whether the fusion protein concept will be superior to competing delivery systems. Initial data show that such fusion proteins are able to deliver siRNAs *in vitro* and *in vivo*. A short peptide consisting of as few as ten arginines is sufficient for complexing enough siRNA for effective gene silencing. Compared to lipid- or polymer-based delivery vehicles, a drastic size decrease is achieved for packaging the siRNA. The same targeting proteins can be used for producing fusion proteins and for grafting on lipid and polymeric nanoparticles, so, barring aggregation issues, the size advantages of fusion proteins remains. Consequently, fusion proteins are expected to be able to reach the targeted tissue more easily due to better interstitial transport. Targeted, multicomponental nanoparticles have a diameter of more than 100 nm and usually suffer from poor penetration into the tumor extracellular matrix despite good site accumulation due to the leaky vasculature. Complexation of siRNA with fusion proteins generally results in particles with a diameter of less than 25 nm, which

facilitates transport through the extracellular matrix. However, fusion proteins are also a heterogeneous group, and the boundaries to other targeted delivery systems are blurred. When full-length antibodies and large polymers for siRNA complexation are used, size can be as high as 200 nm [12,13].

Many different devices have been used for siRNA, antisense, or plasmid packaging [8,9]. They include micelles, liposomes, nanoemulsions and solid-liquid nanoparticles. Liposomes are engineered nanobodies formed by lipids. Liposomal delivery systems for anticancer drugs such as doxorubicin have already been approved for therapy. SNALPs are the most advanced systems for siRNA delivery and are already being used in clinical trials. They are characterized by a high payload, long circulation time, effective shielding of the cargo and good membrane-permeation abilities. Recent advances using combinatorial approaches [90] and rational design [91] have increased the *in vivo* delivery efficiency of an untargeted system. Depending on their vesicular structure, liposomes have a particle size between 100 nm and 10 μ m, and attaching targeting proteins on their surface inevitably further increases their size. For pharmaceutical development, targeting systems need to be produced in an economical way in a validated and reproducible process, which is increasingly difficult with higher complexity of the formulation.

While fusion protein-based targeted delivery devices are still a far way from being used in the clinical setting, it can be predicted that they may have technological and economical advantages over traditional liposomal and polymeric nanoparticles, because their lower complexity will likely result in easier pharmaceutical development, more facile preparation, inherent reduction of batch-to-batch variations and easier impurity profiling.

Future perspective

As noted in the distinct sections, many aspects of fusion proteins need to be carefully optimized. The targeting protein domain is responsible for tissue selectivity. A number of different possibilities have been tested, and the best targeting moiety is likely dependent on the tissue. Selecting the most appropriate antigen for binding will be essential for ensuring high selectivity. Current knowledge indicates that the importance of antigen affinity is less pronounced for tumor accumulation, and nanomolar affinity may be enough to achieve appropriate tissue accumulation.

The mode of siRNA attachment has to be optimized for increasing payload and achieving high intracellular accumulation. So far, protamine-derived peptides or oligo-arginines have been used predominantly, but seemingly rather due to the ease of preparation and availability. Other, more refined complexation strategies such as dendrimeric structures, may improve loading capacity, but at the same time, particle size will increase. Unspecific cellular uptake and toxic effects may arise when using highly charged complexing agents, and optimal size and structure have to be defined for safe and effective therapeutic use. More preclinical research is necessary in order to confirm possible advantages over competing approaches, such as structurally more complex lipid- or polymer-based targeted nanoparticles.

Finally, the siRNA structure and sequence needs to be chosen carefully. Currently, partially 2'-O-modified oligonucleotides and LNAs (locked nucleic acids) [7,92] dominate siRNA technology, but experiences with antisense oligonucleotides have shown that chemical optimization is crucial for the success of nucleic acid-based therapeutics. Meticulous testing of all chemical modifications in terms of efficiency, target selectivity, and endosomal escape is imperative prior to advancing specific structures and sequences to clinical experiments.

In the case of cancer therapeutics, a tailored nanomedicine may be designed taking expression profiles of the distinct tumor into account for selection of both the target cell-surface antigen as well as the target gene for siRNA-mediated silencing.

Acknowledgments

The author thanks Martina Stessl for critical reading of the manuscript.

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Key Terms

Recombinant fusion proteins: Proteins created by genetic engineering and joining of two proteins or peptides. For nucleic acid delivery purposes, an antigen-recognizing protein is fused to a peptide for siRNA, antisense or plasmid DNA binding.

Single-chain variable fragment: The antigen-recognizing motif of immunoglobulins, consisting of the variable regions of the heavy and light chain (V_H and V_L), connected by a peptide linker. The specificity is retained, and the smaller size makes scFv attractive to binding proteins for therapeutic targeting.

Protamine: A family of small arginine-rich peptides which replace histones during the late stages of spermatogenesis. Protamines bind nucleic acids and oligonucleotides by charge interaction between the cationic arginines and the anionic phosphates. Protamine-1 is a component of neutral protamine Hagedorn, a retard formulation of insulin, and used as a heparin antagonist in cardiac bypass surgery.

Key Term

Designed ankyrin repeat proteins: These antibody mimetic proteins are derived from natural ankyrin proteins and consist typically of five ankyrin repeat motifs. DARPins with picomolar affinity and high specificity can be selected from libraries using ribosome display. Their small size, high thermal and thermodynamic stability and the lack of cysteines in the scaffold, which enables generation of specific conjugation sites, make them promising proteins for targeted delivery approaches.

Key Term

Dendrimers: Highly branched, highly symmetric, and roughly spherical macromolecules, consisting of a core structure, and an inner and outer shell. The surface groups can be derivatized with targeting ligands, and amino groups on the surface and interior void spaces are able to complex antisense and siRNA oligonucleotides.

Key Terms

siRNA off-target effects: Despite their theoretical specificity, siRNA oligonucleotides are known to be able to trigger off-target effects. Activation of Toll-like receptors results in immune responses, interference with miRNA can affect genes other than the targeted one, and derivatization to partially complementary sequences can lead to knockdown of other than the intended gene. In addition, certain chemical modifications can trigger off-target effects by unspecific protein binding.

Phosphorothioate: A backbone modification of antisense and siRNA oligonucleotides, where an oxygen atom of the phosphate is substituted by sulfur. Antisense phosphorothioates have been widely used in clinical development, but it has been recently demonstrated that this chemical modification can cause sequence-unspecific effects.

Executive summary

- Fusion proteins for siRNA delivery are chemically well-defined molecules consisting typically of a cell-type specific binding domain and an oligonucleotide complexing domain such as a cationic peptide, polymer or dendrimer.
- Compared to nanoparticles and liposomes, the smaller size and well-defined structure of fusion proteins are expected to facilitate pharmaceutical development and may improve delivery to the targeted tissue and cellular uptake. In this regard, scFvs or other small protein binders have an advantage over full-length immunoglobulins.
- Tissue-binding properties can easily be modulated by using appropriate protein binders, and siRNAs specific to a given target and of different chemical modifications can easily be applied due to the complexation strategy. The modular approach of complexing tissue-specific protein and gene-specific siRNA paves the way for personalized medicines, where both antigen and gene target can be chosen according to genomic disease analysis.
- siRNA target specificity is a concern for the therapeutic development, but activation of the immune system and relevant off-target effects can be excluded for each sequence by choosing optimal chemical modifications. Optimization in terms of pharmacokinetic properties, immunogenicity, cellular uptake and molecular efficiency is needed before entering clinical trials.

Figure 1. Diverse fusion proteins for siRNA delivery

Antibody or antibody fragments fused to basic peptides or polymers have been used to enhance specific siRNA uptake.

DARPin: Designed ankyrin repeat protein; PEI: Polyethyleneimine.

Figure 2. Cellular uptake and endosomal processing of siRNA complexed to a targeting protein (DARPin) protamine fusion protein

After docking to the antigen on the cell surface, the complex is endocytosed, usually by a clathrin-mediated mechanism. siRNA is liberated after acidification of the endosome and reaches the cytosol, where it activates RISC, or is degraded in the endosome.

Table 1
Status of clinical trials with siRNA agents as of February 2011

siRNA	Indication	Gene target	Clinical phase	Administration (formulation)	Ref.
Bevasiranib	Wet AMD	VEGF	III (terminated)	Intravitreal	[101]
ALN-RSV01	Respiratory syncytial virus infection	N-nucleocapsid of RSV	IIb	Intranasal	[102]
AGN211745 (siRNA 027)	Wet AMD	VEGF-receptor 1	II	Intravitreal	
QPI-1002 (I5NP)	Kidney injury, delayed graft function	p53	I/II	Intravenous	[103]
PF655 (RTP801i)	Wet AMD	RTP801	I	Intravitreal	[103]
ALN-VSP	Solid tumors	Kinesin spindle protein and VEGF	I	Intravenous (SNALP)	[102]
TD101	Pachyonychia congenita	Mutated keratin	I	Local injection	[93]
CALAA-01	Solid tumors	M subunit of ribonucleotide reductase	I	Intravenous (targeted nanoparticle)	[104]
Atu027	Solid tumors	Protein kinase N3	I	Intravenous (liposome)	[105]
TKM-ApoB (Pro-040201)	Hypercholesterolemia	Apolipoprotein B	I	Intravenous (SNALP)	[106]
ALN-TTR01	Transthyretin-mediated amyloidosis	Transthyretin	I	Intravenous (SNALP)	[102]
QPI-1007	Chronic optic nerve neuropathy, optic neuropathy	Caspase 2	I	Intravitreal	[103]
TKM-PLK1	Solid tumors	Polo-like kinase 1	I	Intravenous (SNALP)	[106]

AMD: Age-related macular degeneration; SNALP: Stable nucleic acid lipid particles.

Data are obtained from clinicaltrials.gov [107] and company webpages.

Table 2
Overview of fusion protein-based siRNA delivery systems

Targeting domain	Target	Complexing domain	Remarks	Ref.
Heavy chain Fab fragment	gp120 of HIV	Protamine-1		[16,18]
Antibody	PECAM	Polyethylenimine	For DNA delivery	[24]
Single-chain Fv fragment	ErbB2	Truncated protamine-1		[17,18]
RGD-peptide	Integrin receptor	Polyethylenimine	PEGylated	[13]
Trastuzumab	Her-2	Polyethylenimine		[12]
Tat	–	G5 PAMAM dendrimer		[73]
Peptide	RVG	Arg-9	Blood–brain barrier transport	[40]
Single-chain Fv fragment	Lymphocyte integrin LFA-1	Truncated protamine-1		[19]
Single-chain Fv fragment	Hepatitis B surface antigen	Truncated protamine-1		[20]
Single-chain Fv fragment	CD7	Arg-9	Chemical conjugation	[21]
Tat	–	dsRNA binding domain (65 aa)		[67]
LHRH (9-mer peptide)	LHRH receptor	G4 PAMAM dendrimer		[72]
RGD-peptide	Integrin receptor	G5 PAMAM dendrimer		[42,43]
Ankyrin repeat protein (DARPin)	EpCAM	Truncated protamine-1		[31]
Tat	–	LK15		[49,50]
RVG-peptide	Nicotinic acetylcholine receptor	D-Arg-9		[41]
Minibody	JL-1	Arg-9		[22]
DC3 (12-mer peptide)	Dendritic cells	Arg-9		[39]
Single-chain Fv fragment	Hemagglutinin of avian influenza virus	Truncated protamine-1	For DNA delivery	[23]
EGF (53-mer peptide)	EGF receptor	G4 PAMAM dendrimer		[71]

DC: Dendritic cell targeting peptide; EpCAM: Epithelial cell-adhesion molecule; JL: Human T cell surface molecule; LFA: Lymphocyte factor-associated antigen; LHRH: Luteinizing hormone-releasing hormone; PAMAM: Polyamidoamine; PECAM: Platelet endothelial cell-adhesion molecule; RGD: Arginine-glycine-asparagine; RVG: Rabies virus glycoprotein.