Delivery Systems for In Vivo Use of Nucleic Acid Drugs

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Abstract: The notorious biotechnological advance of the last few decades has allowed the development of experimental methods for understanding molecular mechanisms of genes and new therapeutic approaches. Gene therapy is maturing into a viable, practical method with the potential to cure a variety of human illnesses. Some nucleic-acid-based drugs are now available for controlling the progression of genetic diseases by inhibiting gene expression or the activity of their gene products. New therapeutic strategies employ a wide range of molecular tools such as bacterial plasmids containing transgenic inserts, RNA interference and aptamers. A nucleic-acid based constitution confers a lower immunogenic potential and as result of the high stringency selection of large molecular variety, these drugs have high affinity and selectivity for their targets. However, nucleic acids have poor biostability thus requiring chemical modifications and delivery systems to maintain their activity and ease their cellular internalization. This review discusses some of the mechanisms of action and the application of therapies based on nucleic acids such as aptamers and RNA interference as well as platforms for cellular uptake and intracellular delivery of therapeutic oligonucleotides and their trade-offs.

Keywords: aptamers, RNA interference (RNAi), drug delivery systems, nucleic-acid-based drugs.

Introduction

The discovery of the DNA molecule was one of the most important achievements in the understanding of the fundamental basis of life and now its untapped therapeutic potential is being revealed. Therapies based on nucleic acids (NAs) including plasmids containing transgenes used in gene therapy, antisense and antigene oligonucleotides, ribozymes, DNAzymes, DNA and RNA aptamers and small interfering RNAs, have been developed over the past couple of decades (Crooke, 1998; Stull and Szoka, 1995; Patil et al. 2005; Ulrich et al. 2006). Although most NA-based drugs are in the early stages of clinical trials, this molecule class has emerged during recent years as promising drug candidates able to act in a large range of diseases including AIDS, cancer and neurological and cardiovascular disorders (Stull and Szoka, 1995; Patil et al. 2005; Ulrich et al. 2006). The sequencing of the human genome and the transcriptome and proteome projects are providing additional platforms for the advancement of NA-based therapies by supplying new targets for the design, screening and selection of drugs. One of the most significant advantages of NA-based drugs over conventional pharmaceutical drugs is its high selectivity towards its molecular targets resulting in very specific physiological action. These drugs can be used to investigate the genetic disease condition or used in prophylactic measures, thereby preventing disease progression and/or complications in its early stages. For instance, gene therapy usually involves the correction of a malfunctioning gene by the introduction and expression of its correct copy, thus resulting in a corrected protein product. Similarly, NA-based drugs involved in gene ablation turn off only selected genes, guaranteeing specific control of the disease state. Thus, at least theoretically, the use of NA-based therapies can result in null or minimal collateral effects when compared to conventional, often less specific, pharmaceutical drugs.

However, the effects of NA drugs on human exposition must be completely understood, with emphasis to unforeseen long-term effects. There is yet little knowledge about pharmacokinetics of NA-based drugs. The greatest challenge facing the therapeutic utility of NAs is to overcome the low cellular absorption inherent to their highly polar molecular structure. The innate ability of these drugs to cross membranes is minimal in normal circumstances. In addition, their low biostability

results in unpredictable pharmacokinetics. NA molecules that happen to enter the cell are subsequently subjected to intracellular degradation by nucleases effectively narrowing the drug's activity time-span.

The first NA-drug approved in November 1998 by the FDA was denominated Vitravene®, an oligonucleotide discovered by ISIS® as antisense drug for the treatment of cytomegalovirus retinitis in AIDS patients (Perry and Balfour, 1999). Vitravene® was marketed by Novartis Ophthalmics® and revealed to be a powerful AIDS medication. In preclinical studies, antisense inhibition of c-Raf kinase was associated with a reduction in the formation of new blood vessels in the eye involved in both, age-related macular degeneration (AMD) and diabetic retinopathy (Danis et al. 2003). The approval of this first nucleic-acid based drug serves as an encouragement for scientists to further use SELEX Systematic Evolution of Ligands by Exponential Enrichment and RNA interference (RNAi) approaches for drug development (see Fig.1A for a scheme of oligonucleotide action on gene expression and protein acivity).

Nucleic-acid Based Therapies
Mechanisms of action small interfering RNAs

Artificial modulation of gene expression is mainly based on the inhibition of gene transcription or mRNA degradation. The phenomenon of RNAi was first observed in the nematode worm *Caenorhabditis elegans* in response to a double-stranded RNA (dsRNA) treatment which resulted in sequence-specific gene silencing (Fire et al. 1998). Later, the same phenomenon was observed in a large variety of biological systems including several invertebrates and, more recently, also vertebrates such as *Xenopus* and mice (Nakano et al. 2000; Wianny and Zernicka-Goetz, 2000).

For induction of RNAi, small double stranded RNAs (termed small interfering RNAs – siRNAs) are produced by the cleavage of long dsRNAs (Tuschl et al. 1999; Zamore et al. 2000; Hamilton and Baulcombe, 1999; Hammond et al. 2000). The cytoplasmic, highly conserved Dicer protein, member of the family of RNase III–like enzymes, forms a characteristical 21–23-nucleotide long dsRNA duplex with symmetric two- to three-nucleotide 3' overhangs (Bernstein et al. 2001; Elbashir et al. 2001a). The duplex small-interfering

(si)-RNAs (products of the long dsRNA cleavage) are integrated into the RISC complex (RNA-induced silencing complex). The complex becomes activated by the unwinding of the duplex upon the loss of one strand of the si-RNA duplex by an RNA helicase activity. Depending on several target and si-RNA properties that are not entirely understood, RISC can either specifically cleave and degrade target mRNA (Yekta et al. 2004; Meister et al. 2004; Zamore et al. 2000; Bagga et al. 2005; Giraldez et al. 2005; Wu et al. 2006) or inhibit its translation without initiating its sequence-specific mRNA degradation process (Olsen and Ambros, 1999; Reinhart et al. 2000; Wightman et al. 1993). The target mRNA is cleaved by the RISC complex at the middle of the complementary region, ten nucleotides upstream of the nucleotide paired with the 5' end of the guide siRNA (Elbashir et al. 2001b). The cleavage reaction guided by RISC does not require ATP (Nykänen et al. 2001; Hutvágner and Zamore, 2002). However, multiple rounds of mRNA cleavage, which requires the release of cleaved mRNA products, are more efficient in the presence of ATP (Hutvágner and Zamore, 2002). When the dsRNA is of endogenous origin, the Dicer cleavage products are named micro RNA (miRNA). The miRNA-guided mechanism of translational regulation is not as well understood. Studies of mutant or transgenic *C. elegans*, showed that miRNAs inhibited target-protein synthesis without affecting mRNA levels (Bartel, 2004). The target mRNA contains three-prime untranslated regions with several binding sites for the miRNA, and both the target and the miRNA were found to be associated with polyribosomes. This suggested that miRNAs block translation elongation or termination rather than translational initiation (Olsen and Ambros, 1999; Seggerson et al. 2002).

By using protein mutants various genes involved in the RNAi process were identified, including some highly homologous helicases and other enzymes involved in the transposition of mobile elements, such as RNase D (Bertrand et al. 2002; McManus and Sharp, 2002; Scherr et al. 2003). Results like these corroborate the currently accepted hypothesis that the response to dsRNAs plays a defensive physiologic role against deleterious RNAs such as virus transcripts. Therefore miRNAs are essential for the maintenance of genome integrity in a large range of biological
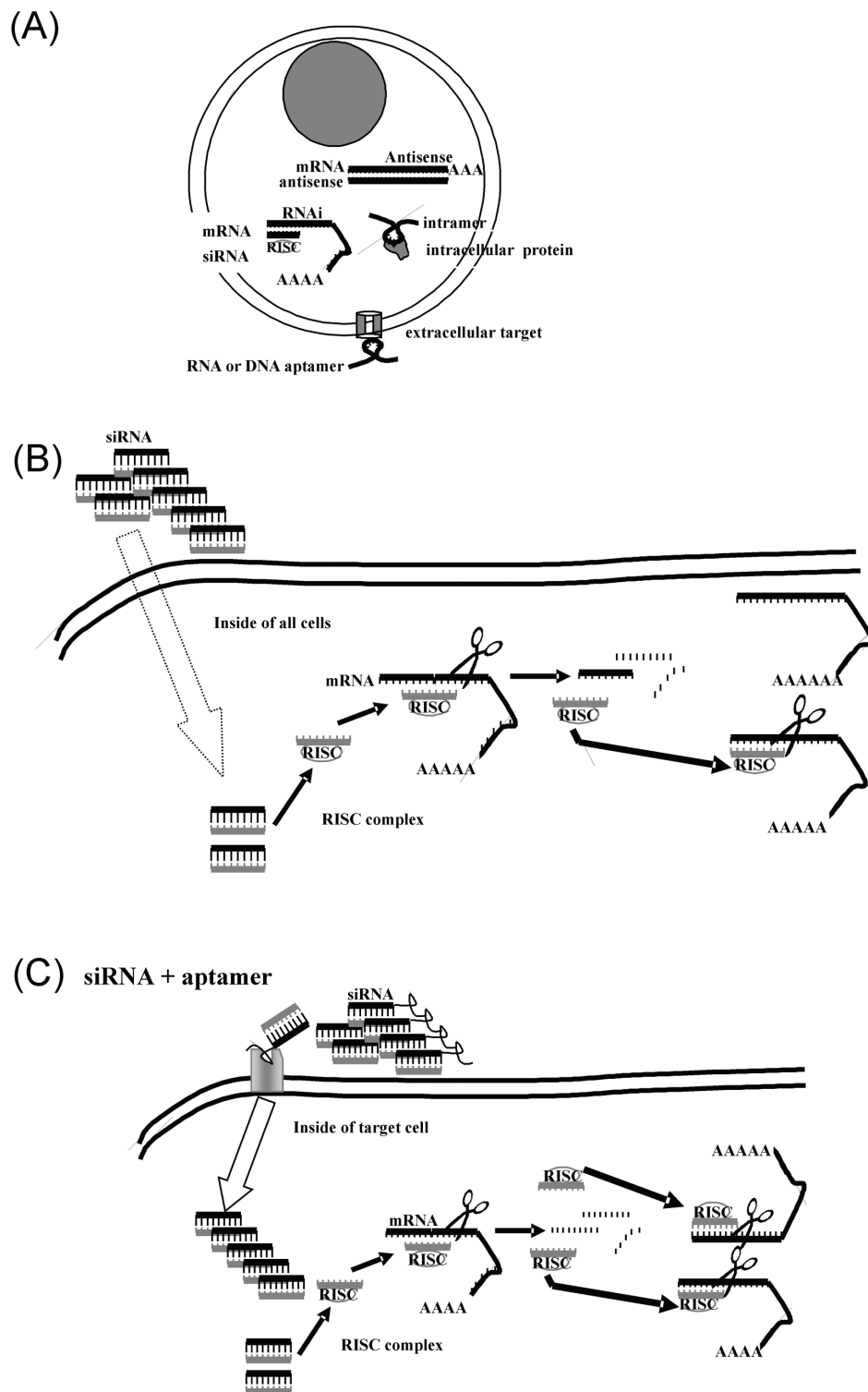
(A)

(B)

(C) siRNA + aptamer

Figure 1. Nucleic-acid based therapies. (**A**) Actions of antisense oligonucleotides, small interfering RNAs, aptamers and intramers (intracellularly acting aptamers) on target cells. The antisense technology is based on the introduction of a complementary oligonucleotide sequence to the target mRNA resulting in RNAse H activation and target RNA degradation. For RNA interference process 21–23 nucleotide-long siRNAs induced into the cell activate the RISC complex leading to degradation of target mRNA. RNA or DNA aptamers target and inhibit the products of gene expression such as intracellular or extracellular protein. (**B**) Conventional delivery of siRNA to target cells by transfection. Therapeutic applications are hindered due to poor cellular uptake and absence of a mechanism to deliver siRNAs specifically to target cells. (**C**) Aptamer-directed siRNA delivery to target cells. The si-RNA is coupled to an aptamer which specifically binds a surface epitope on target cells (i.e. prostate-specific membrane antigen), thereby possibiliting the specific down-regulation of gene expression in cancer cells. Following binding of the siRNA-aptamer chimera to its cell-surface receptor, the receptor-oligonucleotide complex is internalized followed by induction of RNA interference.

systems (Hutvágner and Zamore, 2005). The interference effect of dsRNA is non-stoichiometric in relation to homologous mRNA, since very low amounts of dsRNA cause strong interference. This suggests that the RNAi effect involves a catalytic stage and cannot be based on the titration of endogenous mRNA (Sverdlov, 2001).

Therapeutical applications of RNAi

Currently, siRNA drugs are being developed in order to inhibit cell infection by HIV (Martinez et al. 2002) and Influenza viruses (Ge et al. 2003) and to treat autoimmune hepatitis (Song et al. 2003) (Fig. 1B). RNAi has also been used as a tool to study signaling pathways involved in neurogenesis and neurodegeneration (Miller et al. 2005). As an example, the function of vascular endothelial growth factor (VEGF) in directing neurogenesis was verified via RNAi (Cao et al. 2004). Another approach uses adenoviral, lentiviral and AAV (adeno-associated viral) delivery systems (Chen et al. 2006) to treat spinocebellar ataxia (Xia et al. 2004), amyotrophic lateral sclerosis (ALS) (Ralph et al. 2005; Raoul et al. 2005), Huntington´s (Harper et al. 2005; Rodriguez-Lebron et al. 2006) and Alzheimer's disease (Singer et al. 2005).

When compared to other gene ablation tools such as antinsense oligonucleotides, siRNAs are notably superior due to their higher degree of mRNA degradation and low potential of inducting immune responses (Bertrand et al. 2002). Since siRNAs molecules are not integrated in the genome as plasmids potentially are, they cause collateral effects and thus are much more therapeutically advantageous. Small-interfering RNAs do not need to be transferred into the nucleus in order to present activity, requiring less sophisticated delivery systems. In addition, due to their relative small size, the delivery of a cocktail of siRNAs targeting the expression of multiple disease causing genes at the same time should be feasible.

VEGF gene expression was the first target for clinical trials using siRNA in the treatment of age-related macular degeneration (AMD). RNAi is also being used to combat infection by the respiratory syncytial virus (RSV) genome. The vehicle used in both cases was a saline formulation. The success of these trials may be due to the direct administration of siRNAs at the diseased organs. The advantage of the direct administration is the high concentrations of the siRNA available at the target side. A siRNA-based drug denominated

Cand6 which also suppresses VEGF gene expression is currently being tested in a phase 2 trial with patients suffering from AMD. Moreover, early in 2006, Cand5 was also tested in phase 2 clinical trials for the treatment of diabetic macular edema (http://www.acuitypharma.com/). The siRNA ALN-RSV01 developed by Alnylam Pharmaceuticals, Inc., for treatment of RSV infection has completed two phase 1 trials and now appears to be appropriate for tests in humans. Other siRNAs targeting Influenza and hepatitis C viruses (Protiva Biotherapeutics) are expected to be available for clinical use within the coming year (Protiva Biotherapeutics).

Stability of siRNA in plasma

siRNA molecules are unstable in serum as a result of degradation by serum nucleases and thus have very short half-lives *in vivo* (Soutschek et al. 2004). Stability against nuclease degradation can be achieved by introducing a phosphorothioate (P = S) backbone linkage at the 3′ end for exonuclease resistance and 2′modifications (2′-OMe, 2′-F or related) for endonuclease resistance (Vornlocher et al. 2005; Li et al. 2005; Choung et al. 2006). Moreover, siRNA molecules consisting entirely of 2′-*O*-methyl and 2′-fluoro-modified nucleotides demonstrated enhanced plasma stability and increased *in vitro* potency. Duplexes containing the 4′-thioribose modification present increased thermal stability and are 600 fold more resistant to degradation in plasma than natural RNA duplexes are (Hoshika et al. 2004). Substantial improvements in siRNA activity and plasma stability have also been achieved by judicious combination of 4′-thioribose with 2′-*O*-Me and 2′-O-methoxyethyl modifications (Dande et al. 2006).

Aptamers

Aptamers are oligonucleotides identified by an *in vitro* selection process as high-affinity binders to a given target molecule. For this purpose, a DNA library is synthesized containing an inner randomized sequence of typically 20–100 nucleotides flanked by two outer constant regions of 20–40 nucleotides. A T7 promoter site is incorporated in one of the constant sequences, if a RNA aptamer is to be selected. The chemically synthesized DNA pool is amplified by PCR in the presence of sense- and anti-sense-primers. The DNA template can now either be transcribed *in vitro* to the RNA

pool or be denatured to originate a single-stranded (ss) DNA pool to be used in the *in vitro* selection process. In many cases 2′-F-modified pyrimidines are employed in the *in vitro* transcription reaction to improve nuclease-resistance of generated RNA molecules (reviewed by Ulrich et al. 2004). Reiterative cycles of *in vitro* selection, also denominated as systematic evolution of ligands by exponential enrichment (SELEX), are carried out by incubating the target protein or another molecule of biological importance with the combinatorial DNA or RNA pool, followed by elution and amplification of target binders by RT-PCR or PCR techniques. Selection stringency is increased with the numbers of SELEX cycles. Increased stringency can be achieved by augmenting the number of DNA or RNA molecules relative to possible target binding sites as well as by extensive washing for removal of low-affinity binders. These procedures ensure that the original random pool containing 10^{13}–10^{15} different sequences becomes narrowed down to a more homogenous population of high-affinity target binders. When the binding affinity of a selected RNA or DNA library to its target cannot be any longer improved by subsequent SELEX cycles, this final pool is sequenced for identification of aptamers. At this stage one expects that similar sequence motifs have been preserved in aptamers with binding affinity to their targets and that in most cases these consensus sequences fall into conserved stem-loop motifs. These RNA or DNA molecules with unique binding characteristics, also denominated as aptamers (from latin *aptus* = to fit) can be used for basic research, clinical and diagnostic purposes. Basic research purposes include the characterization of aptamer-target protein interaction in its cellular context (Ulrich et al. 1998, 2002), the study of the mechanism of protein activation and inactivation (Hess et al. 2000), the mapping of binding sites (Shi et al. 2007), the dissection of intracellular signaling pathways (Famulok et al. 2001) as well as the inhibition of intracellular virus replication (Toulmé et al. 2003).

An example for an intracellular acting aptamer (intramer) is an RNA molecule selected as high-affinity ligand of the B52 protein. The protein B52 is expressed during *Drosophila* development and acts there in the gene-splicing process. As the exact function of this protein was not fully understood, an anti-B52 intramer was developed and expressed as a pentameric structure in developing *Drosophila* cells. The observed drastic reduction of *Drosophila*

survival in the presence of the aptamer indicated crucial functions of the B52 protein (Shi et al. 1999). Another approach in therapeutics makes use of an aptamer that binds the intracellular domain of the β−2 integrin lymphocyte function-associated antigen-1 (LFA-1). This portion mediates cell adhesion by binding to the intercellular adhesion molecule-1 (ICAM-1). The specific blockage of signaling pathways *in vivo* by intramers could potentially be applied to any signal-transduction cascade (Blind et al. 1999). The intramer against the rev protein, which is involved in the cycle of replication of HIV resulted in inhibition of the virus replication in cell culture (Good et al. 1997), and in human lymphocyte cells (Chaloin et al. 2002). These results indicate that intramers may be an alternative to RNAi by specifically suppressing the activity of gene products instead of inducing degradation of mRNAs coding for these proteins.

The ability to modulate intramer activity would drastically increase the efficiency of regulation of intracellular signaling by oligonucleotides. The activity of these aptamers could be put under the allosteric control of a second molecule (Tang and Breaker, 1997) or their expression could be allosterically regulated. The sequence coding for the intramer can be introduced in the 5′-promoter position and work as an inductive promoter. Some intramers were developed against kanamicin and tobramicin and expressed in the position of the 5′-gene promoter. The addition of those antibiotics to the cell system resulted in the shut down of the 5′-gene transcription (Werstuck and Green, 1998).

The high specificity of aptamers in acting just on an isotype or a splice-variant of a target protein makes them excellent drug candidates. In this regard, Theis and colleagues identified intramers which bind and switch off the cytohesin-2 guanine nucleotide exchanger but do not affect the homologous protein cytohesin-1 that has a different function. This effect was observed following six hours of HeLa cell transfection with the aptamer construct (Theis et al. 2004). These properties of intramers make them promising tools for development of therapeutics with applications in vaccine development, blockage of intracellular transduction pathways, in viral infection control and time-dependent gene knockdown of protein activity. The suppression of target protein activity at desired time points followed by aptamer inactivation may gain importance in the control of important

physiological functions such as blood coagulation (Rusconi et al. 2004). Inhibition of coagulation is for instance desirable in a pathological condition of high blood pressure, but must be immediately reversed in case of hemorrhage. Moreover, aptamers are very potent inhibitors of extracellular protein activity, for instance by blocking growth factor-receptor binding. An anti-$VEGF_{165}$ aptamer was developed that blocks pathological $VEGF_{165}$-receptor binding, thereby leaving other vital VEGF isoform action unaffected. An anti-$VEGF_{165}$ aptamer formulation has been recently approved for therapeutic use by the FDA and trade-named as Macugen (reviewed by Ulrich et al. 2006; Vavvas and D'Amico, 2006; Ng et al. 2006).

Another promising aptamer tested in clinical trials has been denominated REG1 (Regado Biosciences). This therapeutic aptamer targets factor IXa (drug, RB006) with anti-coagulation activity. The aptamer and its complementary oligonucleotide antidote (RB007) were tested in healthy volunteers in a Phase 1A Pharmacodynamic Evaluation (Dyke et al. 2006). There was not any significant bleeding occurrence associated with RB006 treatment, and both aptamer drug and antidote were well tolerated. The overall results of the pharmacokinetics of the two compounds in healthy volunteers indicated their safe use in humans encouraging further studies (Dyke et al. 2006).

In addition to their possible therapeutic relevance, aptamers may be used in diagnostic applications, such as differentiating between normal and tumoral vasculature (Blank et al. 2001), the pathogenic form of the prion protein from its normal conformation (Rhie et al. 2003), as well as identifying possible biohazards, such as Anthrax spores (Bruno and Kiel, 1999). The possible pharmaceutical and therapeutic importance of aptamers is mainly related to the following characteristics: (I) their affinity for their targets with dissociation constants in the nano- to picomolar range, similar to those found on monoclonal antibody-antigen complexes. High-affinity aptamer-protein interactions result from specific hydrogen-bond formation between bases and amino acids of the target proteins in addition to interactions between oligonucleotide backbones and protein secondary and tertiary structures. (II) Aptamers can be chemically modified to acquire more stability for *in vivo* applicatons, resulting in an increase of their half-life time from a couple of seconds to days (Ulrich et al. 2004). These modifications in oligonucleotide structure can be done prior or following the SELEX process. (III) Identified aptamer sequences can be truncated to their minimal sequences, represented by a single loop structure which is sufficient for binding and biological activity. (IV) Aptamers can be easily enzymatically reproduced or produced in a large scale by chemical synthesis (reviewed by Ulrich et al. 2006).

Vehicles for Oligonucleotide Delivery

The combination of target-specific drug delivery and its controlled release (Langer, 1998) is an important goal in the search for more efficient and less hazardous treatment of tumoral diseases. It is desirable that the cytotoxic drug dosage is delivered to target cells for a long time span, thereby sparing the healthy cells of the surrounding tissue. In order to attain this objective, it is critical to develop specialized vessels which encapsulate the chemotherapeutical drugs for its controlled release and that such vessels are directed to cancer cells (i.e. by presenting appropriate ligands which recognize specific cancer-cell antigens).

A wide variety of address-molecules have been investigated for their efficiency to deliver oligonucleotides to cancer cells. Humanized antibodies and single chain variable fragments generated by murine hybridomas or phage displayed, minibodies and peptides were among the tested delivery vehicles (reviewed by Weiner and Adams, 2000). For the development of vehicles, some pre-requisites must be satisfied in order to improve their chances of passing functional and clinical trials. The drug encapsulating particle-system must be composed of biocompatible, biodegradable polymers approved for clinical use by the drug regulating agencies. Moreover, the vessel particles must efficiently bind the negative charges of NA chains while minimally adverse-effecting their tridimensional folding and thus their binding properties. The delivery vessels must yet efficiently and selectively bind to target cells, as well as have a long half-life in circulation in order to reach the target before being degraded and releasing their contents. Vehicles may be classified as such (I) improving oligonucleotide pharmacokinetics by attaching a high-molecular weight lipophilic molecule to an aptamer in order to augment the half-life of a therapeutic oligonucleotide in the plasma, and as

such (II) permitting the immobilized oligonucleotide to pass physiological barriers such as the brain-blood barrier or plasma membranes to be delivered into cells. In addition to their ability to act as therapeutic drugs by themselves, aptamers can also be used as vehicles themselves to deliver another oligonucleotides to specific target cells (Farokhzad et al. 2006).

Applications for oligonucleotide-directed drug delivery

Prostate-specific membrane antigen (PSMA) is a type-2 integral membrane glycoprotein, expressed at the prostate carcinoma surface and on new vessels formed by various other solid tumors. This antigen is highly expressed in every stage of prostate cancer development (Rajasekaran et al. 2005) and, therefore, is a strong molecular target candidate for immunotherapy and prostate-cancer imaging. Previous efforts to selectively destroy cancer cells generally have made use of anti-bodies to deliver cytotoxic packages (Wu and Senter, 2005). However, aptamers developed against PSMA as protein or another molecule of biological importance potent target binders can be used instead of antibodies to deliver cytotoxic agents to cancer cells (Farokhzad et al. 2006). Gelonin (Gel) is a protein toxin with n-glycosidase activity promoting cell death by cleavage of a specific glycosidic bond of rRNA thereby promoting inhibition of protein synthesis and resulting in elimination of target cells. However, gelonin does not contain a translocation domain such as those of many other ribosomal toxins do, and is not incorporated into cells at considerable quantities (Rosenblum et al. 1999). In order to further improve its cytotoxicity, a recombinant Gel (rGel) was developed. Although this recombinant variant provoked some toxicity on target cells, membrane-permeability was not significantly improved (Rosenblum et al. 1999). This problem was solved by chemical conjugation of rGel or its genetic fusion with the delivery package's recognition molecules and by addition of cysteine residues to form antibody immuno-conjugates (Rosenblum et al. 2003; Better et al. 1994). For instance, the cytokine VEGF was coupled to gelonin and the resulting conjugate specifically killed cancer cells overexpressing the VEGF receptor FLT-1 (Veenendaal et al. 2002). A selective drug that can home in a specific target cell or tissue as therapeutical nanoparticle is the most desirable aim of any delivery system. Previously selected RNA aptamers specifically binding to PSMA were used to escort Gel to tumoral cells expressing PSMA at their surface. The conjugated toxin destroyed prostate cancer cells with an IC_{50} value of 27 nM, presenting an increase in toxicity of more than 600 times in comparison to cells which do not express PSMA (Chu et al. 2006). The extracellular domain of PSMA can now be recognized by a biocompatible and biodegradable polymeric nanoparticle encapsulated with a docetaxel (Dtxl) surface functionalized with a stable, nuclease-resistant RNA aptamer containing 2′-F-modified pyrimidine bases (Farokhzad et al. 2006). These aptamer-nanoparticle bioconjugates (Dtxl-NP-Apt) bound to PSMA proteins expressed at the surface of prostate epithelial cells LNCaP were easily incorporated by cancer cells with cytotoxic effects *in vitro*. Encouragingly, Dtxl-NP-Apt bioconjugates also presented remarkable efficacy and reduced side effects *in vivo*. These observations strongly indicate a potential therapeutic application of aptamer-nanoparticle bioconjugates to specifically target and destroy cancer cells.

Another approach with possible therapeutic applications makes use of packaging RNA (pRNA) as part of the DNA-packaging machinery of the bacteriophage Phi29. This pRNA was genetically engineered to originate chimeric RNA that forms dimers via interlocking right- and left-handed loops (Guo et al. 2005). Fusing pRNA with either receptor-binding RNA aptamers, folate, small interfering RNA (siRNA), ribozyme, or another chemical group did not disturb dimer formation or interfere with the function of the inserted moieties. Incubation of cancer cells with the pRNA dimer with one subunit harboring the receptor-binding moiety and the other containining the gene expression-silencing molecule resulted in target-cell recognition, uptake into these cells and subsequent silencing of anti-apoptotic gene expression. The chimeric pRNA complex was found to be processed into functional double-stranded siRNA by the RNA-specific endonuclease Dicer. Animal trials confirmed the suppression of tumorigenicity of cancer cells by *ex vivo* delivery (Guo et al. 2005; Khaled et al. 2005). These small-size RNA nanoparticles will allow repeated long-term administration and avoid the problems of short retention time of small molecules and will also

avoid the delivery problems of particles larger than 100 nm.

Farokhzad and collaborators (2004) synthesized a polylactic acid (PLA)-block-polyethylene glycol (PEG) copolymer with a carboxilic terminal functional group (PLA-PEG-COOH), and encapsulated rhodamine-labeled dextran inside PLA-PEG-COOH nanoparticles. These nanoparticles have negatively charged carboxilic groups on their surfaces, which minimize unspecific interactions with negatively charged NAs and, for instance, can be conjugated with amino-modified NAs. Clinical trials revealed that the presence of anti-PSMA aptamers at target cells is increased 77-fold when it is bioconjugated to a nanoparticle (Farokhzad et al. 2004). The incubation of protein-free nanoscale particles containing a receptor-binding aptamer or other ligands may result in the binding and internalization of the trivalent therapeutic particles (Dtxl-NP-Apt or PLA-PEG-COOH-Apt) subsequently modulating prostate cancer cell apoptosis. These bioconjugates were based on materials which had been priorly approved for clinical use by the FDA. Since these molecules are small, relatively stable, non-immunogenic and easy to synthesize, the translation of these bioconjugates into clinical practice is facilitated. Therefore, therapeutic and diagnostic nanoparticle-aptamer bioconjugates will be shortly developed for other important human diseases. In this regard, RNA molecules might be used as building blocks in many associations in nanotechnology.

The delivery of macromolecules (including globular proteins and aptamers) to the sclera as therapeutics in eye disease has been described in recent studies (Ambati et al. 2000a; Ambati et al. 2000b). The transport and potential diffusion of molecules to the sclera takes place through an extensive surface area containing a high percentage of water. Water is the main constituent of the extracellular matrix containing few cells and unchanging permeability during aging (Olsen et al. 1998; Olsen et al. 1995; Boubriak et al. 2000). The use of this route of administration could avoid problems and limitations of other delivery approaches in the treatment of viral and systemic diseases (Kamei et al. 1999; Dayle, 2001; Lang, 1995). Since some transscleral delivery systems may be destructive (i.e. iontophoresis) occasionally provoking retinal necrosis and gliosis (Lam et al. 1991), the ideal approach would be to develop biodegradable polimeric particles with prolonged delivery capacity. Lenghtened

protein and NA release time-spans would allow more efficient addressing of drugs to specific target tissues (Carrasquillo et al. 1999; Carrasquillo et al. 2001a; Carrasquillo et al. 2001b).

Frazza and Schmitt (1971) developed a poly (lactic-*co*-glycolic) acid (PLGA) polymer, which since then has been widely used in clinical procedures as suture for tissue engineering (Hasirci et al. 2001; Ma and Choi, 2001). PLGA may be locally applied, allowing intralesional administration of drugs while minimizing adverse systemic effects. The possibility of local application of PLGA constitutes an important pharmacological advantage (Mallery et al. 2000; Moritera et al. 1991). The employment of PLGA as a drug delivery system for the therapy of ocular diseases did not reveal any signs of ocular toxicity or inflammatory processes even during long treatment periods (Moritera et al. 1991; Giordano et al. 1995). However, adverse side effects resulted from the sclerotomy as result of the delivery procedure of small encapsulated synthetic drugs. Carrasquillo et al. (2003) developed a drug delivery system that continuously releases the EYE001 anti-VEGF aptamer (Macugen) in a controlled fashion during significant time spans when it is locally administered at the external area of the sclera. The use of the proposed delivery system for the release of Macugen illustrates a promising alternative for the tansscleral delivery of drugs for the treatment of ocular and choroidal illnesses.

Another way to successfully home aptamers at their subcellular targets is to express them in the cells of interest. In this case the expressed drugs would be useful for both the treatment of hereditary diseases as well as of viral diseases such as AIDS. The TAR region of the RNA genome of HIV-1 is an attractive target for inhibitory NA-based drugs. TAR is a 57 nucleotide regulatory element present at the 5′ end of every viral RNA particle. It exerts a crucial role on viral transcription, as it is recognized by the ternary complex composed of the Tat viral protein (*trans*-activator of transcription) and of two cellular proteins named cyclin T1 and CDK9 (Herrmann and Mancini, 2001; Richter et al. 2002).CDK9 when associated to Tat, hyperphosphorilates the carboxy-terminal of RNA polymerase II and subsequently activates the transcription machinery triggering the efficient synthesis of the entire viral RNA. A NA-based drug strongly interacting with TAR competes with the formation of the

transcription complex and consequently inhibits the trans-activation of the transcription apparatus. Thus, HIV-1 replication is compromised. Moreover, since the TAR element is located in close proximity to the 5′ end of the mature mRNA, a TAR binder could interfere with the ribosomal machinery as well.

NAs, including antisense oligonuvleotides, siRNA and aptamers were employed in many studies for targeting the TAR element (Turner et al. 2005; Yoshinari et al. 2004; Ducongé and Toulmé, 1999). The anti-TAR RNA aptamer (Ducongé and Toulmé, 1999) was optimized by chemical modifications towards improved stability regarding nuclease resistance and decreased *trans*-activation of transcription in cell nuclei extract assays (Darfeuille et al. 2002a; Darfeuille et al. 2002b; Darfeuille et al. 2004; Kolb et al. 2005; Toulmé et al. 2001). Other aptamers were also developed interfering with HIV-1 gene expression, such as the anti-HIV Rev-binding aptamer (RBEapt) (Konopka et al. 1998). Konopka and collaborators used cationic liposomes as delivery vessels carrying the association of RBEapt and a ribozyme that acts against the HIV-1 env gene inhibiting viral production (Konopka et al. 1998). These data provide strong evidence for the therapeutical potential of NA ligands as anti-HIV agents when their intracellular delivery is efficient.

Delivery systems for siRNA

Conjugation of NA terminals with lipophilic molecules has been reported to improve or direct cellular uptake. For example, siRNAs conjugated with cholesterol improved *in vitro* and *in vivo* permeation of liver cells (Lorenz et al. 2004). A number of approaches—including lipid-based formulation, TransMessenger (Niu et al. 2006) and complexation with polyethylenimine (Grzelinski et al. 2006), cholesterol-oligoarginine (Kim et al. 2006), a protamine-Fab fusion protein (Song et al. 2005) and atelocollagen (Takei et al. 2004; Minakuchi et al. 2004)—have been shown to facilitate delivery into tumor cells. Aptamer-siRNA chimeric RNAs have also been successfully used to facilitate siRNA delivery *in vivo* (McNamara et al. 2006; Chu et al. 2006) (Fig.1 C).

In 2004, Soutschek and coworkers demonstrated effective silencing of gene expression of apolipoprotein apoB by intravenous administration of chemically modified siRNA which resulted in silencing of the apoB mRNA in liver and jejunum,

decreased plasma levels of apoB protein, and reduced total cholesterol concentration in mice (Soutschek et al. 2004). Judge et al (2005) made use of siRNA duplexes formulated in stable NA lipid particles (SNALPs) to attain gene silencing of apoB in mice (Zimmermann et al. 2006). Moreover, the general applicability of SNALP formulations for hepatic delivery of siRNA has been demonstrated in animal models of HBV and Ebola virus infection (Morrissey et al. 2005; Geisbert et al. 2006).

In oncology, direct delivery of siRNAs and viral delivery of small hairpin (sh)-RNAs to tumors have been shown to successfully inhibit xenograft growth in mouse models.

Combination of siRNA and aptamers in therapeutics

Technologies that mediate targeted delivery of siRNAs are needed to improve their therapeutic efficacy and safety for use in humans. Lupold et al. (2002) identified two aptamers that bind with low nanomolar affinity to the extracellular portion of PSMA. The two aptamers, denominated as xPSM-A9 and xPSM-A10, did not share any consensus sequences and bound to different sites of PSMA. Distinct modes of inhibition suggested that each aptamer identifies a unique extracellular epitope of recombinant PSMA (xPSM). These aptamers were the first recognizing specific prostate cancer markers. Chu et al. (2006) coupled siRNAs interfering with laminin and GAPDH gene expression by a modular streptavidin bridge to the aptamer A9 which binds to prostate-cancer cells. Comparison of oligofectamine (Invitrogen) and aptamer-mediated siRNA transfection resulted in similar inhibition of target-protein expression as evaluated by real-time PCR experiments. PSMA endocytosis in LNCaP cells is thought to predominantly proceed via clathrin-coated pits. The rate of internalization has been previously measured using antibodies directed against PSMA and also in the presence of the anti-PSMA aptamer A10. Internalization was shown to take place for both types of targeting agents within hours of binding (Farokhzad et al. 2004). Inhibition of target-gene expression was detected after 72 h of siRNA-aptamer transfection.

McNamara et al. (2006) generated a chimera of A10 and a siRNA construct targeting polo-like

kinase 1 (PLK1) and BCL2 (Yano et al. 2004; Reagan-Shaw and Ahmad, 2005) as two survival genes overexpressed in most human tumors (Takai et al. 2004; Eckerdt et al. 2005; Cory and Adams, 2005). Since Dicer also acts on chimeric RNAs, aptamer-siRNAs are directed into the RNAi pathway and silence their cognate mRNAs. Aptamer-siRNA chimera–mediated gene silencing is dependent on Dicer activation and occurs via the RNAi pathway. However, inhibition of Dicer activation had no effect on transfected PLK1 siRNA-mediated silencing, as 21- to 23-nucleotide containing siRNAs have been shown to bypass the Dicer step (Murchison et al. 2005).

The chimera A10-siRNA specifically bound to PSMA on the surface of LNCaP cells but did not interact with PC-3 prostate cancer cells which do not express PSMA (see scheme in Fig.1C). The combination of shRNA and aptamer for specific gene expression control in target cells was accomplished by An et al (2006). This research group constructed a vector containing the theophylline-binding aptamer (Jenison et al. 1994; Zimmermann et al. 1997) and the loop region of the shRNA targeting the enhanced green fluorescent protein (EGFP) under U6 promoter expression. When this construct was co-transfected with a construct coding for EGFP expression (pEGFP-N1) into HEK cells, shRNA-induced EGFP gene expression silencing was dose-dependently inhibited by increasing concentrations of theophylline, as aptamer-bound theophylline interfered with the Dicer-cleavage site. This study proved the feasibility of modulation of gene expression under the control of intracellular proteins or cell metabolites (An et al. 2006).

Recent Clinical Developments

One of the fastest approvals ever obtained for a drug by the FDA was for imatinib (imatinib mesylate [Gleevec], from Novartis) for the treatment of chronic myelogenous leukemia (CML). Although the incidence of CML is low, the rate of cure with conventional treatment is poor. Gleevec is a tyrosine kinase inhibitor and affects only the leukemic cells that are caused by fusion of two genes, *bcr* and *abl*, a chromosomal shuffling between chromosomes 9 and 22. Although expensive, Gleevec, an oral drug, led to remission in 90% to 95% of CML-relapsed patients. However, it has not worked well in blast crisis.

Current activities of the CytRx Corporation are concentrated in the development of small molecule drugs, RNAi drug discovery and DNA vaccines and a delivery technology with multiple applications in the area of DNA vaccine and gene therapy. Its proprietary poloxamer compound, TranzFect, has revealed good results regarding its transfection ability, immunoadjuvant activity and toxicity profile as prerequisites for DNA-based vaccines. This company also participates in the development of gene-silencing technologies for treatment of Amyotrophic lateral sclerosis, type II diabetes, CMV retinitis, obesity, and cancer. Targeting of the epidermal growth factor (EGF) receptor by pegylated immunoliposomes carrying a plasmid coding for the shRNA prolonged the survival of mice with intracranial human brain cancer (Zhang et al. 2004). The observation that encapsulated oligonucleotides readily passed the blood-brain barrier, encourages the development of NA-based therapies for neurodegenerative diseases (reviewed by Sa, 2004).

Conclusions

In the nanotechnology field, aptamers have the potential to act as targeting molecules by directing the delivery of nanoparticles to antigens present on the surface of target cells. In general terms, therapeutic nanoparticles are components of specialized delivery vehicles of an encapsulated drug. Drug release should occur in a regulated and defined manner. Depending on the therapeutic demand, such devices are designed to ensure continuous or immediate drug release. The combination of targeted delivery and controlled release of drugs at affected tissue sites will lead to the development of "smart therapeutics", which are more effective and will have less undesired side effects than drugs available today. Several studies have shown that nanoparticles can be attached to NAs in a way that NA-binding properties to their targets are preserved, thus reducing their potentially associated deleterious side effects. These new approaches can supplement the conventional chemotherapy and radiotherapy in cancer treatment, prevent drug resistance and damage to normal tissues. In view of the impact of the genomic revolution on improving medicines and healthcare, state-of-the-art NA carriers together with highly specific oligonucleotide drugs may help in reducing the side effects of drugs during therapy.

Acknowledgments

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