Review Article
Tumor-targeted RNA-interference: functional non-viral nanovectors

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Abstract: While small interfering RNA (siRNA) and microRNA (miRNA) have attracted extensive attention and showed significant promise for the study, diagnosis and treatment of human cancers, delivering siRNA or miRNA specifically and efficiently into tumor cells *in vivo* remains a great challenge. Delivery barriers, which arise mainly from the routes of administration associated with complex physiochemical microenvironments of the human body and the unique properties of RNAs, hinder the development of RNA-interference (RNAi)-based therapeutics in clinical practice. However, in available delivery systems, non-viral nanoparticle-based gene/RNA-delivery vectors, or nanovectors, are showing powerful delivery capacities and huge potential for improvements in functional nanomaterials, including novel fabrication approaches which would greatly enhance delivery performance. In this review, we summarize the currently recognized RNAi delivery barriers and the anti-barrier requirements related to vectors' properties. Recent efforts and achievements in the development of novel nanomaterials, nanovectors fabrication methods, and delivery approaches are discussed. We also review the outstanding needs in the areas of material synthesis and assembly, multifunction combinations, proper delivery and assisting approaches that require more intensive investigation for the comprehensive and effective delivery of RNAi by non-viral nanovectors.

Keywords: Nanoparticles, RNAi, siRNA, miRNA, cancer therapy, tumor-targeting

1. Introduction

RNAi-based therapeutics hold tremendous promise as novel and potentially more effective treatments for cancer. Small Interfering RNA (siRNA) and microRNA (miRNA) are two main types of interferencing small RNAs that modulate gene expressions and are believed by some investigators to be potential "superdrugs", thereby extensive investigation efforts have been directed to impel siRNA and miRNA into clinical therapeutic applications, and many clinical trials are ongoing.

The mechanism of RNA interference has been extensively studied. RNAi is the highly specific, homology dependent suppression of gene expression by small double-stranded RNA (dsRNA). Upon entering a cell, dsRNA is cleaved by the enzyme Dicer, into fragments of 21-mer mature siRNA to silence its target gene expression [1-5]. Available RNAs for gene interference might expand to other endogenous short RNAs that are becoming clear in mechanism and also show the potential for RNA interference such as piRNAs and esiRNAs [6].

The potent, sequence-specific gene silencing by RNAi has become a powerful tool in biomedical research and holds significant potential as novel molecular therapeutics for cancer [5, 7] and other diseases such as hepatitis C virus infection and myocardial disease [8-10]. RNAi can be induced in mammalian cells by the introduction of synthetic double-stranded siRNAs or by plasmid and viral vector systems that express double-stranded short hairpin RNAs (shRNAs), which are subsequently processed

into siRNAs by cellular machinery [5]. RNAi selectively downregulates its target pathological proteins, without being associated with the limitation the conventional therapeutic approaches, such as extensive systemic toxicity [11]. siRNA is reported to be more potent than conventional anti-sense strategies in inhibiting target gene expression and seems less toxic *in vivo* [12].

However, the promise of RNAi as cancer therapeutics is hampered by difficulties in the delivery of the siRNA molecules to the target cells *in vivo* because these molecules are extremely hydrophilic, sensitive to RNAse degradation and comparatively large [3, 5, 13, 14]. Low transfection efficiency, poor tissue penetration, and nonspecific immune stimulation by siRNAs administered *in vivo* have hindered therapeutic applications. Success of RNAi as therapeutics against diseases such as cancer hinges on the availability of a delivery vehicle that is tumor-specific and can be administered systemically, safely and repeatedly. Currently, three different kinds of RNAi delivery systems have been explored: modified naked RNA, viral vectors and non-viral vectors. Among these delivery systems, modified naked RNA best avoids an immune response and increases uptake by cells compared to naked RNA, but general chemically modified naked RNA lacks tumor targeting and specificity, thus a large amount of the RNA is needed to reach high efficiency [15]. Viral vectors show high gene transfer efficiency but are deficient in their ability to target specific cells. Their residual viral elements can also be immunogenic, cytopathic, or recombinogenic [16]. Non-viral vectors are constructed with biocompatible materials such as polymers, liposomes, peptides and proteins, and polysaccharides using innovative fabrication approaches that aim to safely transport RNA for increased transfection efficiency [16, 17]. However, applications of non-viral delivery systems are still constrained by those problems such as: low packaging efficiency, low colloidal stability, target cell internalization, endosomal escape, and comparatively low gene transfer efficiency.

Hence, for both viral and non-viral vectors, the three main difficulties associated with utilizing RNA-based therapeutics for clinical treatment remain to be the "delivery, delivery, and delivery" [18]. The challenge derives mainly from the complexity of the physiological environment in tissues and cells, combined with the unique properties of RNAs. These barriers exist and vary from case to case because of the different microenvironments of human tissues, the diversity of RNA species, and the specific approaches of administration. So far, great efforts have been directed towards overcoming the issues associated with delivery for RNAi. While some significant achievements have been made, there remains a huge gap between current progress and the ideal systemic delivery of RNAi. To gain insights and improve the efficiency and specificity of non-viral delivery system, extensive research is on-going aiming to overcome the RNAi delivery barriers one by one.

Development of current pharmacology technology has advanced many new drugs into clinical applications. Drug delivery systems have gained extensive achievements with great improvement of the drug efficiency aided by diverse carriers which have been widely reviewed [19-22]. RNA vectors share the same basic requirements with other drug delivery carriers such as biocompatibility, long-time stability remaining in body, and targeted delivery, therefore the general advancements of drug carriers in overcoming delivery barriers could also benefit the fabrication of RNAi vectors. However, specific properties of RNA pose unique requirements and these problems need to be carefully addressed when designing non-viral nanovectors for RNAi-based therapies.

Up to now, various kinds of vectors have emerged and many reviews have referred to the advancement of vectors from different points of view [23-25]. In this review, we summarize the existing delivery barriers together with the requirements to overcome these problems, and focus on the current progress and future direction of the non-viral nanovectors serving in tumor-targeted RNAi for cancer therapy.

2. Administration Routes and Associated Barriers

Barriers to RNA delivery are highly dependent on administration routes [26]. Different barriers might be encountered in respect to different administration routes, thus it would be the primary consideration on current different administration approaches possible for RNA vector delivery when crossing these delivery barriers. A number of general administration routes have been attempted for gene delivery including local

injection [27], intranasal delivery [28], intrathecal injection [29, 30], oral delivery [31], intraperitoneal injection [32] and intravenous injection [27, 33]. Other administration routes have also been demonstrated such as intravitreal injection [34], intraventricular injection [35], and intraperitoneal injection [36]. Typical routes of administration are discussed as below.

2.1. Local injection

Local injection is the easiest way to achieve a high concentration of vectors to the disease tissue such as a tumor and also minimizes complications in the circulatory system compared to other delivery routes. Other advantages include easy manipulation and time-saving, both of which are attractive for RNAi delivery. However, local injection suffers from the limited ability to apply this administration route only to certain accessible tissues, for example, skin cancer or head and neck cancer.

2.2. Intranasal delivery

The intranasal route is an easier, inexpensive, and especially non-invasive delivery approach. The nasal mucosa provides advantages as a target route for drug delivery, including large surface area for delivery, rapid drug onset, potential for central nervous system delivery, and no first-pass metabolism. Furthermore, the intranasal route shows potential to overcome the brain-blood-barrier due to of the special association between nasal mucosa and the brain. Direct instillation of RNA through intranasal route is incomparably superior for application to tissues within the respiratory system, such as enabling direct contact with lung epithelial cells, which play a significant role in lung diseases and infections, including cystic fibrosis, asthma, influenza and the common cold, and potentially lung cancer [24].

2.3. Oral delivery

Oral delivery is a preferred way to transport RNA vectors to various tissues of the body. Because of its versatility, ease of use, and non-invasive properties, the oral delivery approach is given priority in clinical therapeutics and drug development. However, orally delivered vectors require time to circulate the body before entering target sites. Furthermore, vectors in the oral pathway are directly exposed to the stomach microenvironment and must be designed to remain stable in acid fluid and avoid degradation by digestive enzyme such as pepsine and gastric lipase. Vectors that are absorbed through the intestine must be designed to penetrate across the mucus barrier and withstand first-pass metabolism, avoiding a significant decrease in the vector concentration at the disease tissue site due to its absorption by the liver.

2.4. Intravenous delivery

Compared to other administration routes, intravenous injection rapidly delivers nanovectors to the most tissues throughout the whole body. Although invasive, it is the most popular way employed in clinical treatment due to its rapidness and bioavailability. However, vectors transported through intravenous injection still encounter a fair amount of barriers; from the moment the vectors are injected into the vessels, challenges arise from the contents in the blood, organs of the circulatory system, epidermis cells or mucus cells, tissue junction, and immune cells.

2.5. Intraperitoneal injection

Intraperitoneal injection is one of the most common substitutions for intravenous injection. Certain chemotherapy drugs have been applied in intraperitoneal injection. A recent study indicates that siRNA injected through this approach accumulate more in the spleen and liver but less in the kidneys [26].

2.6. Intrathecal injection

Drugs injected into the spinal canal avoid the blood brain barrier present with the intrathecal injection and are able to directly interact with nerve system tissues and cells. However, drugs and RNA vectors used in the intrathecal injection must be specially treated to eliminate preservative or other poisonous contaminants which could provoke serious adverse effects specifically to the nerve system. Thus, intrathecal injection requires a higher level of vector and RNA material purity and safety than other administration routes. Currently, some anti-tumor drugs such as methotrexate, cytarabine (a.k.a. Ara-C) and hydrocortisone have been used for intrathecal injection, and others such

as vincristine might cause serious side effects, hence the intrathecal injection of RNAi vector should be cautious.

Generally, the delivery barriers for all administrative pathways can be categorized into four segments: long-time stability, penetration into the tissue, targeting of the desired site and entering the cells, release of the drugs and interference function. While each of these four basic barriers exist in each administrative route, the level with which each barrier presents itself varies by route.

Firstly, long-term stable dispersion requires the nanovectors be protected from kidney filtration, uptake by phagocytes, aggregation with serum proteins, evoking of T cells or B cells (except intentionally), and enzymatic degradation.

Secondly, penetration into the tissue needs to overcome the vascular endothelial barrier primarily. Besides the capillary endothelium, other adhesive surface coating molecules still need to be addressed. For example, the mucus layer is a highly viscoelastic barrier designed to trap foreign molecules and then move the drugs out with mucus clearance mechanism [37]. Capillary endothelium typically allows molecular penetration for molecules below 5nm in diameter and most of big molecule can not across until they are cleared out of the body. Only a few tissues such as tumor sites, liver and spleen allow the penetration of large molecule up to 200 nm in diameter. In general, extensive efforts are needed to address this barrier before whole-body treatments can be achieved.

Thirdly, after penetration across the capillary endothelium, RNAi nanovectors should target certain cells for specific cell transfection. Targeted delivery of drugs or RNAi minimizes unwanted uptake by other cells, and thus increases the drug concentration in the target tissue even in cases where drugs are circulating throughout the body. It also lessens adverse effects from treatment and allows for both high transfection efficiency and quick transfection function. It may also be possible to track free tumor cells dispersed in the blood through targeted delivery. The overall process depends on the nanovector recognizing and attaching to certain cell surfaces. To fulfill the assignment of siRNA and miRNA, vectors penetrate through the cell membrane with the assistance of some molecules or by endocytosis of cells, which is recognized as membrane transportation.

When nanovectors are internalized into cell via the endosome pathway, the nanovectors must break from the endosome before the endosome changes into a lysosome in a lower pH environment. Not doing so would degrade the vector or RNA.

In addition to the above mentioned requirements, there are still some other issues regarding nanovector delivery that need to be addressed such as the adverse effects of certain RNA and the toxicity of vector's nanomaterials.

3. Efforts and Solutions towards Barriers

3.1. Long-term stability of RNAi nanovectors *in vivo*

One of the biggest problems associated with the use of RNAi nanovectors is the inability of any delivery system to guarantee that all cancer cells take up the nanovectors. No single vector or method reported so far can transfect all target cells, and this could be a potential problem for some clinical treatments. For example, in a tumor, cancer stem cells, which account for only about 1% of the total cancer cells, can differentiate into progenitor and mature tumor cells; and the tumor will continue to grow as long as those minority cancer stem cells are left unharmed [38-40]. Thus the current goal is to elongate the vectors' bioavailability and life span, and to increase targeted delivery of the nanovector as much as possible. Currently, long-term bioavailability acts against the body's defense system which includes serum protein discrimination and agglutination, enzyme degradation, defense of immune system and metabolism system.

3.1.1. Avoiding protein agglutination, enzyme degradation and macrophage uptake: Polymer modification

Regardless of the administration route employed, the RNAi nanovectors should be physically and chemically stable towards serum protein agglutination and enzymatic degradation in the circulatory system until RNAs are released to function in cells. Basically, protein agglutination or enzymatic degradation involves the molecule recognition of the RNAi nanovectors.

The physicochemical properties of the nanovectors' surface, including particle size, surface charge and surface functionality, have an important effect on the bioavailability of the nanovectors. To decrease the possibility of unspecific protein and enzyme agglutination, various polymer coating materials have been tested and the most popular polymer material to avert unwanted protein disturbance is PEG modification [41-43]. PEG-coated particle have shown to decrease hemolytic activity, platelet aggregation and activation, and complement activation as reviewed [44]. Surface polymer coating such as PEG demonstrated the ability to significantly decrease recognition by immune cells and could prolong nanoparticle circulation in the blood, decreasing uptake by resident phagocytes in spleen and liver [45, 46]. Surface PEG coating sterically hinders the interaction and binding of blood components with the vector surface and could also prevent drug carrier opsonization and capture [41, 47, 48].

Currently, non-viral micelles nanovectors prepared with amphiphilic polymer generally employ PEG as a hydrophilic chain, which is commonly recognized as an effective way to avoid protein interaction. Diverse PEG-modified amphiphilic copolymers have been employed to construct amphiphilic hydrogel, micelles, and nanoparticles [49-51]. Recent development of recombinant polypeptide showed potential substitution for PEG coating to increase the half-life when shielding peptide and protein. Schellenberger et al. [52] demonstrated a recombinant polypeptide containing 864 amino acids that could effectively prolong the plasma half-life of peptides to 139 hours. The bioengineering with various length of amino acid chain could have an impact on the half-life of the linked protein and peptides.

3.1.2. Fight against kidney filtration and hepatobiliary processing

During their suspension in the circulatory system, nanovectors are likely to be cleared from the body by two main mechanisms. One is the filtration by the kidneys into urine and the other is hepatobiliary processing into bile. To avoid the kidney filtration, certain criteria should be followed with respect to the physiochemical properties of the nanovectors, including the particle size and shape, surface charge and surface chemistry [53]. The kidney clears the waste depending on the diameter of particles,

and the particles with a diameter less than 8 nm could be easily subjected to the filtration process. Nanovectors which do not undergo kidney filtration may subject to hepatobiliary processing. Some research has referred to the clearance of nanoparticles by metabolism system. Polymers and dendrimers less than 8nm primarily undergo renal clearance. Quantum dots (QDs) with less than 5.5 nm and zwitterionic coatings demonstrated a rapid renal clearance [54]. Liposome-based nanovectors primarily undergo hepatobiliary clearance. Metal-containing nanoparticles are cleared primarily through hepatobiliary clearance. Small complexes containing an oligonucleotide with a molecular weight less than 5000 kDa would be easily ultrafiltered by renal glomerulus and cannot be re-uptaken [55]. Nanovectors injected into the body should be engineered to fight against kidney and hepatobiliary processing prior to RNA release, while biodegradable nanovectors should be designed to be excreted by the body after RNA interference and degradation of vectors.

3.2. Cross the vascular endothelial and mucus barrier

The capillary endothelium sets up the first tight physical barrier as the RNAi nanovectors circulating from the circulatory system to the tissue. From observation, the largest molecule able to penetrate the capillary endothelium is less than 5nm in diameter and most big molecules cannot across. Only a few tissues, such as those of the liver, spleen and some tumor sites, allow penetration by a large molecule up to 200 nm in diameter. In these tissue sites, the endothelial barrier does not present as a problem, but in other organs, nanovectors with a diameter of more than 5nm should be designed to cross the vascular endothelial. In solid tumors, preferential accumulation of macromolecules such as siRNA complex may be contribute to enhanced permeability and retention (EPR) effect, which is attributed to the abnormal tumor blood vessel and some factors are known to assist in this effect, such as vascular endothelial growth factor, bradykinin, and peroxynitrite, etc.

Extracellular matrix, the dense networks of polysaccharides and fibrous proteins can create serious resistance against the transportation of macromolecules and nanoparticles. These matrix also create a layer of viscoelatic and adhesive gel that exists widely in the lungs, gastroin-

testinal system, vagina, eye and other mucosal surfaces. Nanoparticles designed to penetrate the mucus layer in these areas must avoid adhesion to mucin fibers and be small enough to avoid significant steric inhibition. Nanoparticles have been engineered to penetrate the mucus by channeling through low viscosity pores (refer to the recent reviews on mucus properties and corresponding mucus-penetrating drug delivery systems [37, 56]). Some studies shows that short chain and dense PEG-coated particles have improved transport rates, which has important implications for the development of therapeutics and imaging applications *in vivo* [57, 58]. In addition to PEG-modification, polysaccharide chitosan, a deacetylated derivative of chitin that contains cationic glucosamines, also appears to facilitate mucoadhension [59] and mucopermeation [60].

3.3. Target tissues and cells

Tumor cell targeted delivery of RNAi nanovectors may administer RNAi therapy specifically to tumor cells, minimizing adverse side effects and improving the efficiency of RNAi. Targeted delivery involves three main mechanisms: enhanced drug concentration on target tissues, molecule-molecule recognition for function on certain cells, and external field-guided bio-distribution.

3.3.1. Enhanced drug concentration in certain tissues

Enhanced drug concentration in certain tissue can be achieved based on the characteristic of the tissues or the administration approach. One example is the blood vessels in a tumor, where one could observe the enhanced penetration of nanoparticles across the abnormal capillary endothelium with high drug concentration in tumor sites, which is termed as enhanced permeability and retention (EPR) effect or the so-called "passive target" of the tumor. EPR can also be observed in an inflammatory site. This phenomenon is greatly related to bradykinin and some molecules such as nitric oxide and prostaglandins could also facilitate vessel permeability. The difference between inflammation and tumor is the latter prolongs retention time due to failure of the lymphatic drainage system. Biocompatible macromolecules accumulate at much higher (more than six times) concentrations in tumor sites than other tissues and could be contained there for weeks. EPR effect is observed for molecules larger than 45kDa, and

even molecules larger than 800kDa have penetrated the tumor vessles [61-63]. Other examples of enhanced drug concentration on certain tissues are related to certain administration routes, such as the intranasal and intratracheal injection. Both routes enable direct and enhanced drug contact with lung epithelial cells [64], and intrathecal injection of nanovectors allow them to immediately function in the nervous system in the spinal canal and brain.

3.3.2. Molecule-molecule recognition

Diverse molecules and delivery strategies have been investigated for their capability to target different kinds of cells. Specific cell characteristics, such as specific or nonspecific molecules on the cells or charge properties of cell surface, have the potential to be target sites. For example, the cationic surface of the polymer-RNA complex facilitates binding to cellular anionic proteins and uptake by non-specific endocytosis [65]. An enormous database of molecule-molecule recognition could assist the selection and design of target molecules by providing analyses and predictions of protein-protein and protein-chemical interactions [66].

However, some small molecules may bind to their target through unspecific spatial interactions with structurally similar proteins that may not be suitable for target cells, potentially resulting in undesirable side effects such as the inhibition of catalytic sites or membrane surface receptors.

A wide variety of cell surface ligands are available for targeted delivery, including antibodies, polypeptides, and ligands for diverse surface receptors. So far there are no widely accepted tumor-specific antigens or markers available. However, some antigens and receptors are not universally expressed on all cell surfaces but may be over-expressed in certain tumor cells. Further investigation of the uniquely expressed proteins or markers would greatly improve the specificity of tumor-targeting.

3.3.3. Examples of typical molecule-molecule interaction

3.3.3.1. Antigen-antibody

Antigens and antibodies have assisted in molecule recognition for decades. The advantage of antigen-antibody recognition is specificity and

high affinity that may be exploited for efficient and specific RNAi delivery. Currently, more and more antigens and antibodies are being discovered for specific labeling and have been denoted in RNA delivery [67-69]. Available antibody-based proteomics for human tissue profiling have been reviewed [70-72]. Furthermore, monoclonal and polyclonal antibody technology provides huge opportunities for the generation of commercial antibodies. Antibody engineering has improved the fabrication of smaller recombinant antibody fragments and other engineered variants, including diabodies, triabodies, minibodies, and single-domain antibodies, which are promising alternative candidates to monoclonal antibodies. These recombinant fragments still retain the targeting specificity of the whole antibodies and hold great potential for multivalent and multispecific reagents in tumor cell recognition.

3.3.3.2. Aptamer

Aptamers are oligonucleic acids or peptide molecules that bind to specific target molecules/receptors. Aptamers have the advantage of being chemically synthesized, environmentally stable, and less immunogenic. Available aptamers could be classified into two categories: (1) DNA or RNA aptamers typically constructed of short strands of oligonucleotides and (2) peptide aptamers consisting of a short variable peptide domain attached to a protein scaffold at both ends. Levy et al. demonstrates a streptavidin bridge used to bind siRNA to an aptamer at the prostate-specific membrane antigen expressed in prostate cancer cells and the vascular endothelia of tumors [73]. The use of aptamers as the targeting molecules has been discussed by recent nice reviews [74, 75], and there are multiple other examples [76, 77]. A database of aptamers is also currently available online.

3.3.3.3. Cell penetrating peptides (Cpp) and protein transduction domains (PTD)

PTDs/Cpps are generally peptides less than 30 amino-acids in length that are able to trigger the movement of various biomolecules (plasmid DNA, oligonucleotide, siRNA, PNA, protein, peptide, liposome, nanoparticle) across the cell membrane into the cytoplasm to improve intracellular routing, thereby facilitate interactions with the target cells. The chemistry and actions

of Cpps have been extensively reviewed [78-82], and a few key points are listed here.

Cpps such as Tat, oligo-Arg, transportan and penetratin need to be covalently linked to their cargoes, and are therefore internalized by cells along with their cargoes through endocytosis. Cpps that do not need to covalently bind or crosslink are able to form stable nanoparticles with their cargo due to their amphipathic properties. Efforts have been directed towards using non-covalently linked Cpps carriers to undergo non-endosomal pathways and allow the targeted release of cargo into appropriate, desired subcellular organelles. Different types and mechanisms of Cpps peptide-nanoparticles have been reported [83, 84]. RGD [85] motif is abbreviated for arginine- glycine- aspartic acid sequence and has been recognized for receptor-ligand interactions occurring between peptides and cell surface integrins specific for tumor neovesculature. Circular RGD can enhance the tumor targeting and transfection of gene vectors. RGD-nanoparticle conjugates have also been shown to increase capacity to target the delivery of nanoparticles to certain tumor tissues [2, 86, 87]. Sugahara et al. [88] proposed a strategy of using a tumor-homing peptides termed as iRGD (internalizing RGD, with a R/KXXR/K sequence at C-terminal position) to deliver compounds and nanoparticles into tumor tissue. These iRGD motifs go through two molecule-dependent processes: integrin-dependent tumor cell binding and peptide proteolysis, and neuropilin-1-mediated tissue and cell penetration and show a significant increase in the tumor cell internalization effect compared to the conventional RGD sequence.

3.3.3.4. CpG moiety

CpG oligonucleotides could be efficiently internalized by various immune-response cells such as dendritic cells, macrophages and B cells through Toll Like receptor 9, thereby arouse the innate and adaptive immune responses. Kortylewski et al [89] showed that immune response could generate anti-tumor microenvironment and have tumor therapeutic effects, and some examples have shown that targeting lymph node by CpG oligonucleotides could generate anti-tumor immunity [90, 91].

3.3.3.5. Cell surface receptor

Cell surface receptors are glycoproteins on the

surface of a cell which provide specific binding sites for target molecules such as cytokines, hormones, growth factors, neurotransmitters, and adhesion molecules. Molecules specifically bind to a cell surface receptor to generate biological signals for cellular responses such as prolifereation, differentiation, apoptosis, and degranulation.

Different cells have specific receptors which modulate the bioprocess of molecule transportation, and recent studies have explored cell-specific receptors for targeted delivery in RNAi delivery [89, 92, 93]. Using different targeting ligands, various cell surface receptors were targeted for delivery of nanoparticles, including Toll-like receptor [89], asialoglycoprotein receptor [92] and epidermal growth factor receptor [93]. These ligands can be proteins, antigens, agonists of oligonuclietides, and other specific molecules such as sugar [92], urokinase-type plasminogen activator [94], and deslorelin [95].

Some specific ligands for the transferrin receptor (TfR) or folate receptor have been used for attachment onto long-circulating RNAi nanovectors and have shown that they can increase the transfection efficiency *in vitro* and *in vivo* [96, 97]. TfR is over-expressed on the surface of the tumor cells, and antibodies against TfR as well as Transferrin protein (Tf) itself are among the popular ligands for targeting various nanoparticles to tumors and internalization into tumor cells [95, 98-101]. We have developed tumor-specific, ligand-targeting, self-assembled nanoparticle-DNA lipoplex systems designed for the systemic gene therapy of cancer (US Patents No. 6,749,863 and 7,479,276) [100, 101]. These nanovector systems employ transferrin or scFv against transferrin receptors as tumor-targeting ligands [100, 101]. When using Tf as a targeting ligand, we obtained the self-assembled nanovectors at the sizes of 50-90nm, with highly compact structure and favored surface charge [100]. These nanovectors have novel nanostructures that resembles a virus particle with a dense core enveloped by a membrane coated with Tf molecules spiking on the surface [100]. This nanovector system shows promising efficiency and specificity in targeted delivery of various genes and anti-sense oligonucleotides to cancer but not normal tissues *in vivo*. In the AACR 101th Annual Meeting, Washington, DC, April 17-21, 2010, at the Late-breaking Oral Presentation session on clini-

cal trials, Pirollo *et al* reported the success of a first-in-man, Phase I trial of this nanovector, TfRscFv-nano-p53 (SGT-53, NCT00470613, ClinicalTrials.gov). The nanovectors are well tolerated in humans and already showed early responses. The exogenous p53 expression was observed in human cancer tissues in a SGT-53 dose-dependent manner, but *not* in normal tissues. The study demonstrates that the nanovectors are safe and effective to deliver gene therapeutics to both primary tumors and metastatic lesions. These unprecedented findings in cancer gene therapy trial subjects represent a major breakthrough in the field and suggest that delivery of genes to tumors with selectivity is indeed possible (Pirollo, *et al*, LB-172, www.aacr.org). These results should have applicability to the tumor-targeting delivery of other therapeutics including siRNA, miRNA, small molecules and chemotherapeutics.

In a recent report [102] of a Phase I study on patients with solid cancers, Davis and co-workers provide the first clinical evidence of tumor-targeted RNAi using a targeted nanoparticle RNAi delivery system. These nanoparticles were surface-decorated with PEG to increase their circulation time in the body, and with human Tf as a targeting ligand displayed on the exterior of nanovectors to engage TfR on the surface of cancer cells. This report demonstrates that siRNA-nanoparticles administered systemically to human can produce a specific target gene inhibition [102].

In addition to TfR, folate receptors were also studied to target tumors [103, 104]. Lectins are proteins that recognize and bind to carbohydrate moieties of protein molecules (glycoprotein) on the extracellular side of the plasma membrane [105]. Cancer cells often express different glycoproteins compared to their normal counterparts. Therefore, lectins could be used as targeting molecules to direct drugs specifically to the desired target cells and tissues [106]. Other cells surface markers such as CD44, EpCAM, CD133 have been identified for targeting cancer stem cells, and CA125 and PSCA have been successfully used to target for colon and prostate cancer cells [107-109].

3.4. Internalization into the cell

Cells are able to uptake exterior substances via different pathways including phagocytosis, pino-

cytosis, macropinocytosis, and endocytosis. Understanding substance internalization pathways can facilitate the design and engineering of RNA vectors. In most cases, nanoparticles, nanovectors and other RNA-molecule conjugates are transported into cells through endocytosis.

Different endocytosis routes need to be addressed to understand the cell internalization mechanisms in the intracellular delivery of oligonucleotides. Basically, endocytosis includes three types: 1) The clathrin-coated pathway. Some proteins are found to assist in this process such as epsin and dynamin. Examples are transferrin receptor and lipoprotein receptor mediated endocytosis. In this pathway, endosome encapsulates vectors and goes through series pH change to lysosome from pH 5.9-6.0 in early endosome, pH 5.0-6.0 in late endosome to pH 5.0-5.5 in lysosome. 2) The caveolae-mediated pathway: it contains receptors such as glycosyl phosphatidylinositol-anchored proteins (GPI-APs) and Siman viruses 40(SV40) that could assist in the formation of caveolae. GPI-APs could be transported to the Golgi complex and SV40 is delivered to endoplasmic reticulum by special vehicles termed as caveosomes. 3) In clathrin- and caveolin-independent endocytosis, GPI-APs are internalized and sent to the Gogli complex or recycling endosomes [110].

Different internalization pathways for nanovectors uptake are highly related both to the vectors physical and chemical properties and to the delivery system. For example, cationic-lipid-DNA complex are internalized via clathrin-mediate endocytosis [111] while PEI polyplexes have been shown to involve clathrin and caveolin-mediated endocytosis [112].

The physicochemical properties respective to the determination of the endocytosis pathway include particle size and surface charge. 200nm latex nanoparticles could be internalized by clathrin-mediated endocytosis while lager particles rely on the caveolae-dependent pathway [113]. 50nm nanoparticles might be the most suitable size for cell internalization [113-115].

Surface charge of the nanovectors may interact with the charged cell surfaces and different outcomes may result depending on the cell surface [116]. Generally, cationic vector surfaces enhance interaction with negatively charged cell membranes and increase the transfection efficiency. Different cell lines also have different internalization pathways with the same nanovector and thus may lead to different transfection efficiencies [117].

In addition to the above mentioned pathways, studies have revealed other internalization routes, such as fluid phase endocytosis [118], and data shows that Cpps were internalized by certain cells independent of the endosomal pathway [119, 120]. The interaction between certain Cpps and the lipid membrane is mediated by the hydrophobic domain, which assists in the insertion into the lipid membrane and thus decreases the possibility of interaction with proteoglycanes for a endosomal pathway [112].

3.5. Escape from endosome to avoid lysosomal destruction: "proton sponge" effect

Upon endocytosis, nanovectors get into endosomes and would be subjected to degradation when the endosomes are subsequently fused with lysosomes. Escaping from the endosome before it fuses with a lysosome is an essential step for nanovectors to avoid enzyme degradation. In the clathrin-coated pathway, pH values of the endosome gradually change from 5.9-6.0 to 5.0-5.5 after fusion with the lysosome. Behr and others introduced the concept of the "proton sponge" and hypothesized that polymers with buffering capacities between 7.2 and 5.0, such as polyethylenimine (PEI), peptides containing lysine, arginine and histidine, could buffer the endosome and potentially induce its rupture [121, 122]. The polycationic material that has "proton sponge" effect could absorb the protons generated by the endosomes' fusion with lysosomes and thus avoids the low pH environment and subsequently leads to the collapse of the endosome/lysosome to release the nanovectors and their contents into the cytosol.

The proton sponge effect from amino acid of lysine, arginine, and histidine is derived from the cationic amino group (lysine and arginine) and imidazole group (histidine). Amphipathic proteins containing arginine-rich peptides [123], histidine rich peptides [124] and poly-lysine, such as GALA, MPG, have been used to condense RNAs for endosome escape [125, 126].

Besides proton sponge, other molecules also have the capacity to destabilize the endosomal membrane to assist the nanovector escape. Lipids such as L-α-dioleoyl phosphatidyl choline (DOPC), L-α-dioleoyl phosphatidyl ethanolamine (DOPE), and their analogues have been combined into lipid nanovectors to destabilize the endosome and enhance transfection efficiency [127, 128].

4. RNAi Nanovector Imaging and Exterior Physical Energy Guiding

In addition to the above mechanisms of nanovector transportation, exterior energy guided target delivery has also shown powerful influence on the host-nanovector interactions and thus increases delivery efficiency. The available exterior energy facilitating nanovector delivery includes fluorescent and MRI contrast material for image guiding, ultrasonic assisted cell uptake, and magnetic field directed delivery. At the same time, environmentally sensitive nanovectors that respond to interior or exterior environment changes also demonstrate a controlled release effect.

4.1. Fluorescent imaging facilitated delivery nanovectors

Fluorescent signals facilitate the imaging and sensing of RNA vectors, and some fluorescent materials have been used to prepare non-viral nanovectors to gain insight into the biodistribution and biometabolism of the delivery system. Multifunctional imaging and therapeutic nanovectors have opened new opportunities for the treatment of diseases including cancer. Organic fluorescein has been used for fluorescent imaging for decades and is commercially available. Quantum dots (QDs) are advanced fluorescent materials that have recently attracted significant attention. A number of QD-based RNAi nanovectors have been tested [44, 129], and in the construction of RNAi nanovectors, QDs usually serve as a nanoparticle scaffold for attachment and condensation of RNAs [44, 130, 131]. Gold nanoparticles are reported to effectively quench conjugated fluorophores through fluorescent energy transfer [132], and have been used to detect the fluorescence change and release. Furthermore, gold nanoparticles have also been used to study the hybrid nanostructure of the vectors, and gold nanoparticle-based RNAi nanovectors have

shown high transfection efficiency [133-136].

4.2. Magnetic guiding and imaging

In addition to moleculer recognition-based targeting, the effect of exterior energy on the enhancement of targeted delivery and transfection efficiency of RNAi nanovectors at the desired sites has also been tested. Magnetic particles have been extensively employed for targeted delivery of pharmaceuticals through magnetic drug targeting [137, 138]. Recently, efforts have been made to use biocompatible magnetic nanovectors for gene delivery in vivo [27, 139, 140]. In these systems, therapeutic or reporter genes are attached to magnetic nanoparticles, which are then transported to the target site/ cells via high-field/high-gradient magnets. The use of magnetic particles requires an external magnetic field to get a fast targeting of magnetic nanoparticles to reach the target sites. Alternatively, magnetic field could be applied to trigger the drug release when the drug is encapsulated in temperature-sensitive hydrogels.

The most important feature of the magnetic nanoparticles is that they could reduce the time needed for a successful targeted transfection of tumor in the presence of a magnetic field compared with other non-viral nanovectors. Recently, Yoshihisa et al [27] demonstrated the application of exterior and interior magnetic fields with respect to the magnetic source outside and inside the body for the enhancement of transfection efficiency. The study strongly supports the application of magnet field to enrich nanovectors at the targeted locations and thus obtain a high transfection efficiency.

Another aspect of using magnetic nanoparticles is their magnetic resonance (MR) imaging function. MR imaging is one of the most powerful non-invasive imaging modalities and is widely used in clinic. MR imaging is based on the property that hydrogen protons will align and process around an applied magnetic field [137, 141, 142]. MR imaging can be used to monitor the tumor targeting of magnetic nanoparticles *in vivo*.

4.3. Environment-sensitive nanovectors

Environment-sensitive nanoparticles have been developed to be able to release their contents based on their environmental change, for the

controlled drug release. These environment-sensitive properties basically involve pH, redox, temperature, light, or ultrasound response. Some environmental changes can be generated by certain abnormalities of pathological sites, which provide the opportunity for the controlled drug release in the disease site. Changes, such as the decreased pH environment at the tumor or inflammation sites could trigger the release of drugs from a pH sensitive carrier in these sites.

"Proton-sponge" of pH sensitivity is necessary for nanovectors to escape from lysosomes if nanovectors are taken up by cells through the clathrin-assisted endocytosis. PEG coating may protect nanovectors from macrophage and protein agglomeration, however, a PEG coating that is too tight will reduce the potential for RNA release and function, thus decreasing transfection efficiency. The pH-sensitive, biodegradable PEG-coating on RNAi nanovectors may overcome this drawback. Here the polycations and PEG are linked via acylhydrazides or pyridylhydrazines, and the pyridylhydrazone prepared from polylysine and propionaldehyde-PEG has shown the greatest acid-dependent hydrolysis. Using a polyplex shielded with bioreversible PEG conjugates, a 100-fold higher *in vitro* gene expression than the conventional polyplexes was achieved with the analogous stable PEG shields [143].

Besides pH-sensitive carriers, nanovectors sensitive to other environmental factors, such as temperature and redox conditions, have also been investigated. Ultrasound has been applied to enhance transfection efficiency via microbubbles, and recent reports on cationic lipids and polymers indicate that they could also be triggered by ultrasound. Compared with microbubbles, cationic lipids and polymers have smaller diameters and more stable formulations due to their solid matrix structures. In the case of micellar drug delivery, ultrasonic cavitation events create transient holes in the cell membrane, increasing the passive diffusion of micelles and drugs into the cells. Clumakova et al [144] demonstrated the 300 nm PLGA nanoparticle gene vector assisted by 5 min ultrasonic treatment produced a significantly greater expression of the reporter gene in the tumor than that without ultrasound. Liu et al [145] investigated light-responsive cationic vesicles for photo-assisted gene delivery, making use of the different azobenzene concentrations in the for-mation of vesicles, thus these vesicles could collapse upon UV illumination and release of the delivered gene therapeutics.

4.4. Multifunctional nanovectors

Ideal multifunctional RNAi nanovectors should be able to: 1) achieve efficient and targeted delivery of RNAi to the target cells; 2) track nanovectors localization; 3) validate targeting by a reporter whether a target is hit; and 4) indicate RNAi efficiency with report/readout of the outcome. Multifunctional nanovectors hold promise for overcoming current barriers and may open a new avenue for RNAi-based therapeutics. Combining the multi-functionality including the protection on nanovectors and RNAs, tissue and cell targeting, imaging, external energy-assisted approaches and the controlled release of RNAi would greatly improve the *in vivo* RNAi efficiency and efficacy, and would also be a powerful tool to gain insights into the gene regulation process. Until recently, several multifunctional nanovectors have been designed to solve specific problems, however, the optimum organization of the functional moieties requires delicate design since some of these functional parts might interfere with each other and affect their functions.

5. Fabrication of Gene-Nanoparticle Complex

5.1. Basic requirements for RNA or DNA complex formulation

The RNAi nanovector complex could be constructed in many different ways. Generally speaking, RNA-vector interaction is mediated by electrostatic interaction, and materials such as polycationic lipids, polymers and lipid-polymer complexes have been demonstrated to condense negatively charged DNA/RNAs to form lipoplexes, polyplexes and lipopolyplexes, respectively, and could also enhance the cellular uptake at the negatively charged cell membrane. Cationic polymers such as polyethyleneimine (PEI), polyamidoamine, and cationic dendrimers have been tested for RNAi delivery. Cationic peptides, poly-lysines, and arginine-containing peptides have been successfully used as RNAs condensing and packaging vehicles. However, cationic polymers such as polylysine and polyethyleneimine have shown to activate the complement system, and the increased polycation length and surface charge

density leads to higher complement activation and/or cytotoxic effects due to electrostatic interactions with the negatively charged cell membrane.

5.2. Possible adverse effects of therapeutic RNAi and ways to avoid them

With commonly used cationic lipoplexes or polyplexes, RNAs were commonly attached onto the surface of the vector, putting the RNAs in contact directly with environment and potentially increasing immune response or other adverse effects. A recent study [146] reported the unexpected side effects of delivering siRNA *in vivo*. These adverse side effects include the induction of type I interferon response and saturation of endogenous RNAi pathway components. The RNAi commonly used therapeutically is usually about 21 nucleotides [147, 148], and research have shown that siRNA with a length longer than 30 nucleotides could activate the host immune system when administered at high concentration [149, 150].

These adverse effects are presumably initiated by the toll-like receptors and the helicases RIG-1 and Mda5. In addition, protein kinase R also plays an important role in the recognition of siRNA by the immune system. Other undesirable side effects may arouse from cross reactions with the endogenous miRNA pathway, in which siRNA acts as miRNA and can interact with the 3'UTR of mRNAs by partial homology, inhibiting their translation without triggering their degradation [151, 152]. The dsRNAs could stimulate the interferon response when the sequence is longer than 30 nucleotides [153]. Researchers have attempted several approaches to avoid these adverse responses. In the RNAi sequence, modifications on siRNA could minimize the immune response. Incorporation of 2'–O-methyl in the sugar structure, with both sense and antisense strands, has been shown to repress endonuclease activity. Other modifications such as the introduction of a phosphorothioate backbone linkage at the 3'-end of the RNA strand, also reduce susceptibility to exonucleases.

6. Conclusions and Future Direction

Non-viral RNAi nanovectors have tremendous potential as effective carriers to overcome current gene/drug delivery barriers. Significant efforts have been directed towards solving specific problems and have attained huge achievements. However, a comprehensive analysis and engineering of the nanovectors with respect to the tumor-targeted delivery and function process has yet to be demonstrated. The ultimate goal of the RNAi delivery system is to transport the relevant RNAi to all target cells safely and effectively, and thus trigger target gene regulation. Comprehensive consideration and optimization of RNAi nanovectors requires understanding of both bodily function mechanisms and physical microenvironment properties. Multifunctional nanovectors able to identify location, indicate efficiency, remain sensitive to the environment, and target delivery with high efficiency would be a new generation of non-viral nanovectors and a carrier for diverse RNAi molecules. Development in material engineering would further accelerate RNAi nanovectors fabrication. Biocompatible, non-toxic, functional and intelligent materials have demonstrated superiority and provide guidelines for material application in RNAi nanovectors. Exterior-energy controlled delivery of RNAi nanovectors have shown brilliant prospects and more and more delivery control systems are being developed to overcome current barriers. However, although the mechanisms of the RNA interference are becoming clearer, it is still far from fully understood and other physiological phenomena also deserve further investigation to present a more detailed solution of the barriers.

7. Acknowledgements

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9. References

[1]   Oliveira S, Storm G and Schiffelers RM. Targeted delivery of siRNA. J Biomed Biotechnol. 2006; 2006(4): 63675.

[2]   Kim DH and Rossi JJ. Strategies for silencing human disease using RNA interference. Nat

Rev Genet 2007; 8:173-184.

[3] Juliano R, Alam MR, Dixit V and Kang H. Mechanisms and strategies for effective delivery of antisense and siRNA oligonucleotides. Nucleic Acids Res 2008; 36: 4158–4171.

[4] Kurreck J. RNA Interference: From Basic Research to Therapeutic Applications. Angew Chem Int Ed Engl 2009; 48: 1378-1398.

[5] Hede K. Blocking cancer with RNA interference moves toward the clinic. J Natl Cancer Inst 2005; 97: 626-628.

[6] Watanabe T, Totoki Y, Toyoda A, Kaneda M, Kuramochi-Miyagawa S, Obata Y, Chiba H, Kohara Y, Kono T, Nakano T, Surani MA, Sakaki Y and Sasaki H. Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. Nature 2008; 22;453:539-543.

[7] Sioud M. siRNA Delivery In Vivo. Methods Mol Biol 2005; 309: 237-250.

[8] Thum T, Gross C, Fiedler J, Fischer T, Kissler S, Bussen M, Galuppo P, Just S, Rottbauer W and Frantz S. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature 2008; 456:980-984.

[9] Xiao C and Rajewsky K. MicroRNA control in the immune system: basic principles. Cell 2009; 136: 26-36.

[10] Lanford R, Hildebrandt-Eriksen E, Petri A, Persson R, Lindow M, Munk M, Kauppinen S and Orum H. Therapeutic Silencing of MicroRNA-122 in Primates with Chronic Hepatitis C Virus Infection. Science 2010; 327:198-201.

[11] Bumcrot D, Manoharan M, Koteliansky V and Sah DW. RNAi therapeutics: a potential new class of pharmaceutical drugs. Nat Chem Biol 2006;2:711-719.

[12] Zender L and Kubicka S. SiRNA based strategies for inhibition of apoptotic pathways in vivo--analytical and therapeutic implications. Apoptosis 2004; 9: 51-54.

[13] Tanaka T, Mangala LS, Vivas-Mejia PE, Nieves-Alicea R, Mann AP, Mora E, Han HD, Shahzad MM, Liu X, Bhavane R, Gu J, Fakhoury JR, Chiappini C, Lu C, Matsuo K, Godin B, Stone RL, Nick AM, Lopez-Berestein G, Sood AK and Ferrari M. Sustained small interfering RNA delivery by mesoporous silicon particles. Cancer Res 2010; 70: 3687-3696.

[14] Whitehead KA, Langer R and Anderson DG. Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov 2009;8:129-138.

[15] Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Rohl I, Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliansky V, Limmer S, Manoharan M and Vornlocher HP. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 2004; 432:173-178.

[16] Pirollo KF, Xu L and Chang EH. Non-viral gene delivery for p53. Curr Opin Mol Ther 2000; 2:168-175.

[17] Xu L, Pirollo KF and Chang EH. Tumor-targeted p53-gene therapy enhances the efficacy of conventional chemo/radiotherapy. J Control Release 2001;74:115-128.

[18] Perkel JM. RNAi therapeutics: a two-year update. Science 2009; 326: 454-456.

[19] Cho KJ, Wang X, Nie SM, Chen Z and Shin DM. Therapeutic nanoparticles for drug delivery in Cancer. Clin Cancer Res 2008;14:1310-1316.

[20] Torchilin V. Multifunctional and stimuli sensitive pharmaceutical nanocarriers. Eur J Pharm Biopharm 2009;71:431-444

[21] Subramani K, Hosseinkhani H, Khraisat A, Hosseinkhani M and Pathak Y. Targeting Nanoparticles as Drug Delivery Systems for Cancer Treatment. Current Nanoscience 2009; 2: 135-140.

[22] Haley B and Frenkel E. Nanoparticles for drug delivery in cancer treatment. Urol Oncol 2008; 26:57-64.

[23] Reischl D and Zimmer A. Drug delivery of siRNA therapeutics: potentials and limits of nanosystems. Nanomedicine 2009;5:8-20.

[24] De Fougerolles AR. Delivery vehicles for small interfering RNA in vivo. Hum Gene Ther 2008; 19:125-132.

[25] Itaka K and Kataoka K. Recent development of nonviral gene delivery systems with virus-like structures and mechanisms. Eur J Pharm Biopharm 2009;71:475-483.

[26] Larson SD, Jackson LN, Chen LA, Rychahou PG and Evers BM. Effectiveness of siRNA uptake in target tissues by various delivery methods. Surgery 2007;142:262-269.

[27] Namiki Y, Namiki T, Yoshida H, Ishii Y, Tsubota A, Koido S, Nariai K, Mitsunaga M, YanagisawaS, Kashiwagi H, Mabashi Y, Yumoto Y, Hoshina S, Fujise K and Tada N. A novel magnetic crystal-lipid nanostructure for magnetically guided in vivo gene delivery. Nat Nanotechnol 2009;4:598-606.

[28] Bitko V, Musiyenko A, Shulyayeva O and Barik S. Inhibition of respiratory viruses by nasally administered siRNA. Nat Med 2005;11:50-55.

[29] Christoph T, Grunweller A, Mika J, Schafer MKH, Wade EJ, Weihe E, Erdmann VA, Frank R, Gillen C and Kurreck J. Silencing of vanilloid receptor TRPV1 by RNAi reduces neuropathic and visceral pain in vivo. Biochem Biophys Res Commun 2006;350:238-243.

[30] Luo MC, Zhang DQ, Ma SW, Huang YY, Shuster SJ, Porreca F and Lai J. An efficient intrathecaldelivery of small interfering RNA to the spinal cord and peripheral neurons. Mol Pain 2005;1:29.

[31] Akhtar S. Oral delivery of siRNA and antisense oligonucleotides. J Drug Target 2009;17:491-

495.

[32] Sorensen DR, Leirdal M and Sioud M. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. J Mol Biol 2003;4;327: 761-766.

[33] Zhang Y, Boado RJ and Pardridge WM. In vivo knockdown of gene expression in brain cancer with intravenous RNAi in adult rats. J Gene Med 2003;5:1039-1045.

[34] Tolentino MJ, Brucker AJ, Fosnot J, Ying GS, Wu IH, Malik G, Wan SH and Reich SJ. Intravitreal injection of vascular endothelial growth factor small interfering RNA inhibits growth and leakage in a nonhuman primate, laser-induced model of choroidal neovascularization. Retina 2004;24:132-138.

[35] Thakker DR, Natt F, Husken D, Maier R, Muller M, van der Putten H, Hoyer D and Cryan JF. Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. Proc Natl Acad Sci U S A 2004;101: 17270-17275.

[36] Geisbert TW, Hensley LE, Kagan E, Yu EYZ, Geisbert JB, Daddario-DiCaprio K, et al., editors. Postexposure protection of guinea pigs against a lethal Ebola virus challenge is conferred by RNA interference. 13th International Congress of Virology; 2005 Jul 23-28; San Francisco, CA: Univ Chicago Press.

[37] Lai SK, Wang YY and Hanes J. Mucus penetrating nanoparticles for drug and gene delivery to mucosal tissues Adv Drug Deliv Rev 2009;61:158-171.

[38 DeSano JT and Xu L. MicroRNA regulation of cancer stem cells and therapeutic implications. AAPS J 2009;11:682-692.

[39] Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF and Simeone DM. Identification of pancreatic cancer stem cells. Cancer Res 2007;67:1030-1037.

[40] Wicha MS. Cancer stem cells and metastasis: lethal seeds. Clin Cancer Res 2006;12:5606-5607.

[41] Chonn A, Semple SC and Cullis PR. Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes. J Biol Chem 1992;267:18759-18765.

[42] Maruyama K, Okuizumi S, Ishida O, Yamauchi H, Kikuchi H and Iwatsuru M. Phosphatidyl-Polyglycerols Prolong Liposome Circulation in vivo. Int J Pharm 1994;111:103-107.

[43] Takeuchi H, Kojima H, Toyoda T, Yamamoto H, Hino T and Kawashima Y. Prolonged circulation time of doxorubicin-loaded liposomes coated with a modified polyvinyl alcohol after intravenous injection in rats. Eur J Pharm Biopharm 1999;48:123-129.

[44] Dobrovolskaia MA, Aggarwal P, Hall JB, McNeil SE, editors. Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle

biodistribution. 7th International Symposium on Polymer Therapeutics; 2007 May 26-28; Valencia, SPAIN: Amer Chemical Soc.

[45] Paciotti GF, Myer L, Weinreich D, Goia D, Pavel N, McLaughlin RE and Tamarkin L. Colloidal gold: A novel nanoparticle vector for tumor directed drug delivery. Drug Deliv 2004;11: 169-183.

[46] Zahr AS, Davis CA and Pishko MV. Macrophage uptake of core-shell nanoparticles surface modified with poly(ethylene glycol). Langmuir 2006;22:8178-8185.

[47] Woodle MC. Surface-Modified Liposomes - Assessment and Characterization for Increased Stability and Prolonged Blood- Circulation. Chem Phys Lipids 1993;64:249-262.

[48] Torchilin VP, Omelyanenko VG, Papisov MI, Bogdanov AA, Jr., Trubetskoy VS, Herron JN and Gentry CA. Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. Biochim Biophys Acta 1994;1195:11-20.

[49] Kakizawa Y, Furukawa S and Kataoka K. Block copolymer-coated calcium phosphate nanoparticles sensing intracellular environment for oligodeoxynucleotide and siRNA delivery. J Control Release 2004;97:345-356.

[50] Kataoka K, Itaka K, Nishiyama N, Yamasaki Y, Oishi M and Nagasaki Y. Smart polymeric micelles as nanocarriers for oligonucleotides and siRNA delivery. Nucleic Acids Symp Ser (Oxf) 2005;49:17-18.

[51] Segura T and Hubbell JA. Synthesis and in vitro characterization of an ABC triblock copolymer for siRNA delivery. Bioconjug Chem 2007;18:736-745.

[52] Schellenberger V, Wang CW, Geething NC, Spink BJ, Campbell A, To W, Scholle MD, Yin Y, Yao Y, Bogin O, Cleland JL, Silverman J and Stemmer WP. A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tunable manner. Nat Biotechnol 2009;27:1186-1190.

[53] Pramanik D. Principles of physiology. Academic publishers, 2007.

[54] Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Ipe BI, Bawendi MG and Frangioni JV. Renal clearance of quantum dots. Nat Biotechnol 2007;25:1165-1170.

[55] Brenner BM, Deen WM and Robertson CR. Determinants of Glomerular-Filtration Rate. Annu Rev Physiol 1976; 38: 9-19.

[56] Cone RA. Barrier properties of mucus. Adv Drug Deliv Rev 2009;61:75-85 .

[57] Wang YY, Lai SK, Suk JS, Pace A, Cone R and Hanes J. Addressing the PEG Mucoadhesivity Paradox to Engineer Nanoparticles that "Slip" through the Human Mucus Barrier. Angew Chem Int Ed Engl 2008;47:9726-9729.

[58] Lai SK, O'Hanlon DE, Harrold S, Man ST, Wang YY, Cone R and Hanes J. Rapid transport of large polymeric nanoparticles in fresh undi-

luted human mucus. Proc Natl Acad Sci U S A 2007;104:1482-1487.

[59] Soane RJ, Frier M, Perkins AC, Jones NS, Davis SS and Illum L. Evaluation of the clearance characteristics of bioadhesive systems in humans. Int J Pharm 1999;178:55-65.

[60] Chen WY, Lu ZL, Viljoen A and Hamman J. Intestinal Drug Transport Enhancement by Aloe vera. Planta Med 2009;75:587-595.

[61] Iyer AK, Khaled G, Fang J and Maeda H. Exploiting the enhanced permeability and retention effect for tumor targeting. Drug Discov Today 2006;11:812-818.

[62] Maeda H, Fang J, Inutsuka T and Kitamoto Y. Vascular permeability enhancement in solid tumor: various factors, mechanisms involved and its implications. Int Immunopharmacol 2003;3:319-328.

[63] Torchilin VP. Drug targeting. Eur J Pharm Sci 2000; 11: 81-91.

[64] Thomas M, Lu JJ, Chen J and Klibanov AM. Non-viral siRNA delivery to the lung. Adv Drug Deliv Rev 2007;59:124-133.

[65] Elouahabi A and Ruysschaert JM. Formation and intracellular trafficking of lipoplexes and polyplexes. Mol Ther 2005;11:336-347.

[66] Shoemaker BA, Zhang D, Thangudu RR, Tyagi M, Fong JH, Marchler-Bauer A, Bryant SH, Madej T and Panchenko AR. Inferred Biomolecular Interaction Server--a web server to analyze and predict protein interacting partners and binding sites. Nucleic Acids Res 2010;38(Database issue):D518-524

[67] Sioud M. RNAi therapy: antibodies guide the way. Gene Ther 2006;13:194-195.

[68] Ikeda Y and Taira K. Ligand-targeted delivery of therapeutic siRNA. Pharm Res 2006;23: 1631-1640.

[69] O'Neill MM, Kennedy CA, Barton RW and Tatake RJ. Receptor-mediated gene delivery tohuman peripheral blood mononuclear cells using anti-CD3 antibody coupled to polyethylenimine. Gene Ther 2001;8:362-368.

[70] Uhlen M and Ponten F. Antibody-based proteomics for human tissue profiling. Mol Cell Proteomics 2005;4:384-393.

[71] Persson A, Hober S and Uhlen M. A human protein atlas based on antibody proteomics. Curr Opin Mol Ther 2006;8:185-190.

[72] Uhlen M, Bjorling E, Agaton C, et al. A human protein atlas for normal and cancer tissues based on antibody proteomics. Mol Cell Proteomics 2005;4:1920-1932.

[73] Chu TC, Twu KY, Ellington AD and Levy M. Aptamer mediated siRNA delivery. Nucleic Acids Res 2006;34(10):e73.

[74] Farokhzad OC, Karp JM and Langer R. Nanoparticle-aptamer bioconjugates for cancer targeting. Expert Opin Drug Deliv 2006;3:311-24.

[75] Cho EJ, Lee JW and Ellington AD. Applications of Aptamers as Sensors. Annu Rev Anal Chem (Palo Alto Calif) 2009;2:241-264.

[76] Farokhzad OC, Jon S, Khademhosseini A, Tran TN, Lavan DA and Langer R. Nanoparticle aptamer bioconjugates: a new approach for targeting prostate cancer cells. Cancer Res 2004;64:7668-7672.

[77] Smith JE, Medley CD, Tang Z, Shangguan D, Lofton C and Tan W. Aptamer-conjugated nanoparticles for the collection and detection of multiple cancer cells. Anal Chem 2007; 79:3075-3082

[78] Patel LN, Zaro JL and Shen WC. Cell penetrating peptides: Intracellular pathways and pharmaceutical perspectives. Pharm Res 2007;24:1977-1992

[79] Meade BR and Dowdy SF. Exogenous siRNA delivery using peptide transduction domains/ cell penetrating peptides. Adv Drug Deliv Rev 2007;59:134-140.

[80] Gupta B, Levchenko TS and Torchilin VP. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. Adv Drug Deliv Rev 2005;57:637-651.

[81] Morris MC, Deshayes S, Heitz F and Divita G. Cell-penetrating peptides: from molecular mechanisms to therapeutics. Biol Cell 2008;100:201-217.

[82] Heitz F, Morris MC and Divita G. Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. Br J Pharmacol 2009;157:195-206.

[83] Deshayes S, Morris M, Heitz F and Divita G. Delivery of proteins and nucleic acids using a non-covalent peptide-based strategy. Adv Drug Deliv Rev 2008;60:537-547

[84] Zhang K, Fang HF, Chen ZY, Taylor JSA and Wooley KL. Shape effects of nanoparticles conjugated with cell-penetrating peptides (HIV Tat PTD) on CHO cell uptake. Bioconjug Chem 2008;19:1880-1887.

[85] Schiffelers RM, Ansari A, Xu J, Zhou Q, Tang QQ, Storm G, Molema G, Lu PY, Scaria PV and Woodle MC. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. Nucleic Acids Res 2004;32:e149.

[86] Cressman S, Dobson I, Lee JB, Tam YYC and Cullis PR. Synthesis of a Labeled RGD-Lipid, Its Incorporation into Liposomal Nanoparticles, and Their Trafficking in Cultured Endothelial Cells. Bioconjug Chem 2009;20:1404-1411.

[87] Murphy EA, Majeti BK, Barnes LA, Makale M, Weis SM, Lutu-Fuga K, Wrasidlo W and Cheresh DA. Nanoparticle-mediated drug delivery to tumor vasculature suppresses metastasis. Proc Natl Acad Sci U S A 2008;105: 9343-9348.

[88] Sugahara KN, Teesalu T, Karmali PP, Kotamraju VR, Agemy L, Girard OM, Hanahan D, Mattrey RF and Ruoslahti E. Tissue-Penetrating Delivery of Compounds and Nanoparticles into

Tumors. Cancer Cell 2009;16:510-520.

[89] Kortylewski M, Swiderski P, Herrmann A, Wang L, Kowolik C, Kujawski M, Lee H, Scuto A, Liu Y, Yang CM, Deng JH, Soifer HS, Raubitschek A, Forman S, Rossi JJ, Pardoll DM, Jove R and Yu H. In vivo delivery of siRNA to immune cells by conjugation to a TLR9 agonist enhances antitumor immune responses. Nat Biotechnol 2009;27:925-932.

[90] Bourquin C, Anz D, Zwiorek K, Lanz AL, Fuchs S, Weigel S, Wurzenberger C, von der Borch P, Golic M, Moder S, Winter G, Coester C and Endres S. Targeting CpG oligonucleotides to the lymph node by nanoparticles elicits efficient antitumoral immunity. J Immunol 2008;181:2990-2998.

[91] Pan X, Chen L, Liu S, Yang X, Gao JX and Lee RJ. Antitumor activity of G3139 lipid nanoparticles (LNPs). Mol Pharm 2009;6:211-220.

[92] Maruyama K, Iwasaki F, Takizawa T, Yanagie H, Niidome T, Yamada E, Ito T and Koyama Y. Novel receptor-mediated gene delivery system comprising plasmid/protamine/ sugarcontainingsugarcontaining polyanion ternary complex. Biomaterials 2004;25:3267-3273.

[93] Lee H, Kim TH and Park TG. A receptor mediated gene delivery system using streptavidin and biotin-derivatized, pegylated epidermal growth factor. J Control Release 2002;83:109-119.

[94] Yang L, Peng XH, Wang YA, Wang X, Cao Z, Ni C, Karna P, Zhang X, Wood WC, Gao X, Nie S and Mao H. Receptor-targeted nanoparticles for in vivo imaging of breast cancer. Clin Cancer Res 2009;15:4722-4732.

[95] Sundaram S, Roy SK, Ambati BK and Kompella UB. Surface-functionalized nanoparticles for targeted gene delivery across nasal respiratory epithelium. FASEB J 2009;23:3752-3765.

[96] Qian ZM, Li HY, Sun HZ and Ho K. Targeted drug delivery via the transferrin receptor mediatedendocytosis pathway. Pharmacol Rev 2002;54:561-587.

[97] Pirollo KF and Chang EH. Does a targeting ligand influence nanoparticle tumor localization or uptake? Trends Biotechnol 2008; 26:552-558.

[98] Hu-Lieskovan S, Heidel JD, Bartlett DW, Davis ME and Triche TJ. Sequence-specific knockdownof EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. Cancer Res 2005;65:8984-8992.

[99] Xu L, Tang WH, Huang CC, Alexander W, Xiang LM, Pirollo KF, Rait A and Chang EH. Systemic p53 gene therapy of cancer with immunolipoplexes targeted by anti-transferrin receptor scFv. Mol Med 2001;7:723-734.

[100] Xu L, Frederik P, Pirollo KF, Tang WH, Rait A, Xiang LM, Huang W, Cruz I, Yin Y and Chang

EH. Self-assembly of a virus-mimicking nanostructure system for efficient tumor-targeted gene delivery. Hum Gene Ther 2002;13:469-481.

[101] Xu L, Huang CC, Huang W, Tang WH, Rait A, Yin YZ, Cruz I, Xiang LM, Pirollo KF and Chang EH. Systemic tumor-targeted gene delivery by anti-transferrin receptor scFv-immunoliposomes. Mol Cancer Ther 2002;1: 337-346.

[102] Davis ME, Zuckerman JE, Choi CH, Seligson D, Tolcher A, Alabi CA, Yen Y, Heidel JD and Ribas A. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature 2010;464:1067-1070.

[103] Benns JM, Maheshwari A, Furgeson DY, Mahato RI and Kim SW. Folate-PEG-folate-graftpolyethylenimine- based gene delivery. J Drug Target 2001;9:123-139.

[104] Ohguchi Y, Kawano K, Hattori Y and Maitani Y. Selective delivery of folate-PEG-linked, nanoemulsion- loaded aclacinomycin A to KB nasopharyngeal cells and xenograft: effect of chain lengthand amount of folate-PEG linker. J Drug Target 2008;16:660-667.

[105] Zheng T, Peelen D and Smith LM. Lectin arrays for profiling cell surface carbohydrate expression. J Am Chem Soc 2005;127:9982-9983.

[106] Allen TM. Ligand-targeted therapeutics in anticancer therapy. Nat Rev Cancer 2002;2:750-763.

[107] Pardal R, Clarke MF and Morrison SJ. Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 2003;3:895-902.

[108] Bast Jr RC, Badgwell D, Lu Z, Marquez R, Rosen D, Liu J, Baggerly KA, Atkinson EN, Skates S and Zhang Z. New tumor markers: CA125 and beyond. Int J Gynecol Cancer 2005; Suppl 3:274-281.

[109] Zhou BBS, Zhang H, Damelin M, Geles KG, Grindley JC and Dirks PB. Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. Nat Rev Drug Discov 2009;8:806-823.

[110] Perret E, Lakkaraju A, Deborde S, Schreiner R and Rodriguez-Boulan E. Evolving endosomes:how many varieties and why? Curr Opin Cell Biol 2005;17:423-434.

[111] Rejman J, Conese M and Hoekstra D. Gene transfer by means of lipo- and polyplexes: Role of clathrin and caveolae-mediated endocytosis. J Liposome Res 2006;16:237-247.

[112] Rejman J, Bragonzi A and Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther 2005;12:468-474.

[113] Rejman J, Oberle V, Zuhorn IS and Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin- and caveolaemediated endocytosis. Biochem J 2004;377(Pt 1):159-169.

[114] Chithrani BD and Chan WCW. Elucidating the mechanism of cellular uptake and removal of protein-coated gold nanoparticles of different sizes and shapes. Nano Lett 2007;7:1542-1550.

[115] Chithrani BD, Ghazani AA and Chan WCW. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. Nano Lett 2006;6:662-668.

[116] Villanueva A, Canete M, Roca AG, Calero M, Veintemillas-Verdaguer S, Serna CJ, Morales MD and Miranda R. The influence of surface functionalization on the enhanced internalization of magnetic nanoparticles in cancer cells. Nanotechnology 2009;20:115103.

[117] Douglas KL, Piccirillo CA and Tabrizian M. Cell line-dependent internalization pathways and intracellular trafficking determine transfection efficiency of nanoparticle vectors Eur J Pharm Biopharm 2008;68:676-687.

[118] Hufnagel H, Hakim P, Lima A and Hollfelder F. Fluid Phase Endocytosis Contributes to Transfection of DNA by PEI-25. Mol Ther 2009;17: 1411-1417 .

[119] Rothbard JB, Jessop TC, Lewis RS, Murray BA and Wender PA. Role of membrane potential and hydrogen bonding in the mechanism of translocation of guanidinium-rich peptides into cells. J Am Chem Soc 2004;126:9506-9507.

[120] Terrone D, Sang SLW, Roudaia L and Silvius JR. Penetratin and related cell-penetrating cationic peptides can translocate across lipid bilayers in the presence of a transbilayer potential. Biochemistry 2003;42:13787-13799.

[121] Behr JP. The proton sponge: A trick to enter cells the viruses did not exploit. Chimia 1997;51:34-36.

[122] Pack DW, Putnam D and Langer R. Design of imidazole-containing endosomolytic biopolymers for gene delivery. Biotechnol Bioeng 2000;67:217-223.

[123] El-Sayed A, Futaki S and Harashima H. Delivery of Macromolecules Using Arginine-Rich Cell-Penetrating Peptides: Ways to Overcome Endosomal Entrapment. AAPS J 2009;11:13-22.

[124] Kichler A, Leborgne C, Marz J, Danos O and Bechinger B. Histidine-rich amphipathic peptide antibiotics promote efficient delivery of DNA into mammalian cells. Proc Natl Acad Sci U S A 2003;100:1564-1568.

[125] Pack DW, Hoffman AS, Pun S and Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov 2005;4:581-593.

[126] Midoux P, Pichon C, Yaouanc JJ and Jaffres PA. Chemical vectors for gene delivery: a current review on polymers, peptides and lipids containing histidine or imidazole as nucleic acids carriers. Br J Pharmacol 2009;157:166-178.

[127] Im Hafez NM and Cullis PR. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. Gene Ther 2001;8:1188-1196.

[128] Zuhorn IS, Bakowsky U, Polushkin E, Visser WH, Stuart MCA, Engberts J and Hoekstra D. Nonbilayer phase of lipoplex–membrane mixture determines endosomal escape of genetic cargo and transfection efficiency. Mol Ther 2005;11:801-810.

[129] Klein S, Zolk O, Fromm MF, Schrodl F, Neuhuber W and Kryschi C. Functionalized silicon quantum dots tailored for targeted siRNA delivery. Biochem Biophys Res Commun 2009; 387:164-168.

[130] Yezhelyev MV, Qi LF, O'Regan RM, Nie S and Gao XH. Proton-sponge coated quantum dots for siRNA delivery and intracellular imaging. J Am Chem Soc 2008;130:9006-9012.

[131] Derfus AM, Chen AA, Min DH, Ruoslahti E and Bhatia SN. Targeted quantum dot conjugates for siRNA delivery. Bioconjug Chem 2007;18:1391-1396.

[132] Fan CH, Wang S, Hong JW, Bazan GC, Plaxco KW and Heeger AJ. Beyond superquenching: Hyper-efficient energy transfer from conjugated polymers to gold nanoparticles. Proc Natl Acad Sci U S A 2003;100:6297-6301.

[133] Han G, You CC, Kim B, Turingan RS, Forbes NS, Martin CT and Rotello VM. Light-regulated release of DNA and its delivery to nuclei by means of photolabile gold nanoparticles. Angew Chem Int Ed Engl 2006;45:3165-3169.

[134] Giljohann DA, Seferos DS, Prigodich AE, Patel PC and Mirkin CA. Gene Regulation with Polyvalent siRNA-Nanoparticle Conjugates. J Am Chem Soc 2009;131:2072-2073.

[135] Sandhu KK, McIntosh CM, Simard JM, Smith SW and Rotello VM. Gold nanoparticle mediated Transfection of mammalian cells. Bioconjug Chem 2002;13:3-6.

[136] Rosi NL, Giljohann DA, Thaxton CS, Lytton-Jean AKR, Han MS and Mirkin CA. Oligonucleotide- modified gold nanoparticles for intracellular gene regulation. Science 2006; 312:1027-1030.

[137] Sun C, Lee JSH and Zhang MQ. Magnetic nanoparticles in MR imaging and drug delivery. Adv Drug Deliv Rev 2008;60:1252-1265.

[138] Talelli M, Rijcken CJF, Lammers T, Seevinck PR, Storm G, van Nostrum CF and Hennink WE. Superparamagnetic Iron Oxide Nanoparticles Encapsulated in Biodegradable Thermosensitive Polymeric Micelles: Toward a Targeted Nanomedicine Suitable for Image-Guided Drug Delivery. Langmuir 2009;25:2060-2067.

[139] Kievit FM, Veiseh O, Bhattarai N, Fang C, Gunn JW, Lee D, Ellenbogen RG, Olson JM and Zhang MQ. PEI-PEG-Chitosan-Copolymer-Coated Iron Oxide Nanoparticles for Safe Gene Delivery: Synthesis, Complexation, and

Transfection. Adv Funct Mater 2009;19(14): 2244-2251.

[140] Mykhaylyk O, Vlaskou D, Tresilwised N, Pitha-yanukul P, Moller W, Plank C, editors. Magnetic nanoparticle formulations for DNA and siRNA delivery. 6th International Conference on the Scientific and Clinical Applications of Magnetic Carriers; 2006 May 17-30; Krems, AUSTRIA: Elsevier Science Bv.

[141] Song HT and Suh JS. Cancer - Targeted MR Molecular Imaging. J Korean Med Assoc 2009;52:121-124.

[142] Sosnovik DE, Nahrendorf M and Weissleder R. Magnetic nanoparticles for MR imaging: agents, techniques and cardiovascular applications. Basic Res Cardiol 2008;103:122-130.

[143] Walker GF, Fella C, Pelisek J, Fahrmeir J, Boeckle S, Ogris M and Wagner E. Toward synthetic viruses: Endosomal pH-triggered deshielding of targeted polyplexes greatly enhances gene transfer in vitro and in vivo. Mol Ther 2005;11:418-425.

[144] Chumakova OV, Liopo AV, Andreev VG, Cicenaite I, Evers BM, Chakrabarty S, Pappas TC and Esenaliev RO. Composition of PLGA and PEI/DNA nanoparticles improves ultrasound mediated gene delivery in solid tumors in vivo. Cancer Lett 2008;261:215-225.

[145] Liu YC, Le Ny ALM, Schmidt J, Talmon Y, Chmelka BF and Lee Jr CT. Photo-Assisted Gene Delivery Using Light-Responsive Catanionic Vesicles. Langmuir 2009;25:5713-5724.

[146] De Veer MJ, Sledz CA and Williams BRG. Detection of foreign RNA: Implications for RNAi. Immunol Cell Biol 2005;83:224-228.

[147] Bernstein E, Caudy AA, Hammond SM and Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001;409:363-366.

[148] Elbashir SM, Lendeckel W and Tuschl T. RNA interference is mediated by 21-and 22-nucleotide RNAs. Genes Dev 2001;15:188-200.

[149] Persengiev SP, Zhu X and Green MR. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). RNA 2004;10:12-18.

[150] Sledz CA, Holko M, de Veer MJ, Silverman RH and Williams BR. Activation of the interferon system by short-interfering RNAs. Nat Cell Biol 2003;5:834-839.

[151] Doench JG, Petersen CP and Sharp PA. siRNAs can function as miRNAs. Genes Dev 2003;17:438-42.

[152] Saxena S, Jonsson ZO and Dutta A. Small RNAs with imperfect match to endogenous mRNA repress translation. Implications for offtarget activity of small inhibitory RNA in mammalian cells. J Biol Chem 2003;278:44312-44319.

[153] Manche L, Green SR, Schmedt C and Mathews MB. Interactions between Double-Stranded-RNA Regulators and the Protein Kinase DAI. Mol Cell Biol 1992;12:5238-5248.