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Chapter 10

# Non-viral siRNA and shRNA Delivery Systems in Cancer Therapy

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/62826

**Abstract**

RNA interference represents a promising therapeutic strategy for the silencing of specific target genes in cancer therapy. Small interfering RNAs and DNA-based vectors encoding short hairpin RNAs provide sequence-specific post-transcriptional gene silencing by binding to its complementary RNA. For the therapeutic use of siRNA in cancer, efficient intracellular delivery is necessary. The efficient cancer therapy with RNAi is not still accomplished because of internalization and intracellular trafficking problems such as low transfection efficiency, enzyme degradation, inappropriate subcellular localization, and endosomal trapping of siRNAs in cells. Cancer is a complex disease including multiple genes and pathways. The most important benefits of siRNA therapy are high target specificity and non-toxicity compared with chemotherapy. The uptake of siRNA by cells without a carrier system is possible, but naked siRNA is mostly degraded with nucleases and activates the immune responses. Development of appropriate delivery systems is an important issue in the use of siRNA-based therapeutics. Non-viral delivery systems have great potential for safe and effective delivery of siRNA therapeutics to tumor cells. Nanocarriers such as nanoplexes, lipoplexes, nanoparticles, and liposomes have been commonly used for siRNA delivery. This chapter highlights the importance of non-viral delivery systems *in vitro* and *in vivo* cancer therapy.

**Keywords:** Cancer, non-viral vectors, RNAi, siRNA, shRNA

## 1. Introduction

RNA interference (RNAi) is a conserved endogenous cellular process for post-transcriptional regulation in sequence-specific gene silencing. The regulatory RNA molecules include small interfering RNAs (siRNAs), and short hairpin RNAs (shRNAs) provide the specific degradation of target mRNA in mammalian cells [1]. siRNAs are the products of long double-stranded

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RNA (dsRNA) molecules in cells, which are expressed transgenically or delivered exogenously. Synthetic siRNAs can be transfected into cells that specifically silence the expression of target genes. In the RNAi pathway, dsRNA (over 100 nt) molecules are cleaved into 21- to 23-nucleotide duplexed RNAs, termed as siRNA duplex, by endoribonuclease Dicer or RNAse III-type enzyme. The cleaved siRNA duplexes contain 5′-phosphate and two-base 3′ overhangs. siRNAs are incorporated into the endogenous RNA-induced silencing complex (RISC). One of the two strands of siRNA duplex is guide (antisense) strand, and the other is passenger (sense) strand. siRNA duplex is unwinded by RNA helicase activity. While the guide strand binds to the RISC, the passenger strand is degraded. The activated RISC binds to mRNA with base-pairing for sequence-specific degradation of complementary mRNA. The mRNA fragments cleaved by Argonaute (Ago) proteins are released from RISC and degraded by other endogenous nucleases. After mRNA degradation, the active RISC is rebuilt and can participate in another RNAi pathway [2, 3]. In the event, RNAi process decreases specific mRNA levels, and thus decreases target gene expression.

Among the nucleic acid–based drugs, siRNAs as potential novel drug candidates are offered a highly promising strategy in cancer therapy. The knockdown of abnormal gene overexpression, occuring in cancer by using siRNA, has been used in therapeutic applications [2]. Targets of chemical drugs are limited to certain classes of receptors, ion channels, and enzymes. In the treatment with siRNA, known sequence of any gene of interest is sufficient and its target choice is unlimited [4].

The potential advantages of siRNA treatment compared with other treatment methods are that siRNAs [5–7] (i) provide sequence-specific gene therapy; (ii) can specifically target many undruggable genes or downregulate gene products; (iii) are considered the safe therapeutics; (iv) are potent and efficient molecules and possess high gene silencing activity; and (iv) can be easily designed for any disease.

RNAi might be a promising new pharmaceutical area for treatment of incurable and severe diseases such as cancers and infections. RNAi applications have been recently achieved by using synthetic siRNAs and vector-based siRNA expression systems or short hairpin RNAs (shRNAs) synthesized within the cells by vector-mediated production. The expressed shRNAs from plasmid and viral vectors in nucleus are cleaved by Dicer in cytoplasm and siRNAs are formed. There both strategies have advantages and disadvantages. Vector-based siRNA expression systems have several advantages for applying RNAi compared to synthetic siRNAs. Both permanent and transient transfection with vector-based systems can be achieved, and thus vector-based system increases the period of siRNA-mediated inhibition of gene expression [8]. In addition, shRNA constructs are more stable than siRNAs [9]. Low amount (nM) of siRNA and less than five copies of shRNA are sufficient for stable transfection and for acheiving gene silencing effect [10]. The synthetic siRNAs can be easily synthesized in large amounts and chemically modified to improve stability, permeability, efficacy, and transfection control; however, the modified siRNAs are highly expensive [11]. siRNAs are not integrated into host genome. The modification of vector-based shRNA systems is difficult, but shRNA expression systems can be regulated or induced by appropriate promoters and termination sequences. Choice of promoter, loop structure of shRNA, length and arrangement of sense and

http://dx.doi.org/10.5772/62826

antisense strands, and orientation of restriction enzyme regions are important for shRNA expression cassette preparation. Similar to the various RNAi applications for targeted gene silencing, the chimeric expression cassettes of siRNA and shRNA in the same expression unit might also be made [12].

Design of optimal siRNA sequence plays a key role for successful siRNA therapy. The choice of potent and specific siRNA sequences is important for minimization of immune responses and off-target effects. siRNAs are 19–27 base pairs in length, but mostly preferred to be 21 nt of siRNAs with a structure of 19 nt duplex region and two nucleotide overhangs at the 3′ end, usually TT and UU, which are important for recognition by the RNAi machinery. Increasing the length of the dsRNA may enhance its potency, dsRNAs with 27 nucleotides are up to 100 times more potent than the siRNAs containing 21 nucleotides. The longer (25–30 nt) duplexes act as a substrate for Dicer (Dicer-subtrate siRNAs). This Dicer-substrate siRNAs are more efficiently loaded into the RISC over the siRNAs with 21 bp and newly produced siRNAs from the long dsRNAs directly incorporated into the RISC complex. Thus, gene silencing mechanism can be facilitated [13, 14]. On the other hand, dsRNAs longer than about 30 bp can lead to interferon response, which is the defense mechanism against viral infection. The activation of interferon pathway causes non-specific mRNA degradation and apoptosis [15, 16]. The long dsRNAs activates innate immune response by interaction with protein kinase receptor (PKR) and Toll-like receptors (TLR7 and 8 are activated by ssRNA; TLR9 activated by unmethylated CpG; and TLR3 activated by dsRNA). The activation of these receptors induces interferon (IFN) and proinflammatory cytokines [10, 17].

In addition to immune responses, another important problem for efficient RNAi therapy is off-target effects of these molecules. The cause of off-target effects are the suppression of undesired or unpredicted genes other than the desired target genes. The absence of homolog sequences between siRNA and its target mRNA can cause cleavage of non-targeted mRNA regions. Off-target effects of siRNA can lead to problems in the interpretation of gene silencing studies, serious and unwanted side effects such as potential toxicity and even cell death [18]. There are many factors and mechanisms leading to occurence of off-target effects. These factors are the length of dsRNA or siRNA, the length and position of siRNA-target sequence mismatch, and coding sequences and untranslated regions in genes [19]. The mechanisms leading to off-target effects of siRNAs are (i) the regulation of unwanted transcripts by seed sequence homology to the 3′ UTR of cellular mRNAs, (ii) the saturation of RISC by affecting cellular miRNA activity of siRNAs in large amounts, (iii) the function of miRNAs as siRNAs or shRNAs because of similarities in gene silencing pathway, (iv) non-specific distribution by non-targeting systemic delivery, and (v) immunostimulatory motifs in siRNA sequences [14, 17, 20].

Many preclinical studies with siRNA indicated that it is a hopeful molecule for clinical research of various diseases. Up to date, at least 22 RNAi-based drugs have been evaluated in clinical trials [21]. The first clinical trial with siRNA was made in 2004 by Opko Health. The clinical studies of Bevasiranib, that is, siRNA targeting vascular endothelial growth factor (VEGF) to suppress ocular neovascularization in patients with age-related macular degeneration (AMD), continued to the phase III trial, but the clinical trials was terminated in 2009 because of its poor efficacy and causing vision loss. Allergan company has terminated the phase II clinical trials

of siRNA AGN-745, targeting VEGF because of its off-target effects [21]. The clinical translation of RNAi can be possible with development of safe and efficient RNAi delivery systems that lack of off-target effects [7, 21].

Although siRNAs are used as the potential therapeutic molecules in cancer and other diseases, the most important challenge in the development of RNAi-based drugs is efficient and safe delivery of appropriate doses to target cells and tissues. Therefore, the development of siRNA-based viral and non-viral delivery systems are required to have an enhanced efficacy, improved stability, and minimized non-specific gene silencing such as off-target effects and immune responses. This chapter focuses on recent improvements in the non-viral siRNA delivery systems in cancer therapy.

## 2. RNAi in cancer therapy

Cancer is a multistep genetic disease, which develops as a consequence of changes in the control of cell proliferation and differentiation. In the transformation of normal cells to tumor cells, the affected cells undergo mutations such as downregulation of tumor suppressor genes and overexpression of oncogenes. RNAi-based therapeutics have been extensively used for knockdown of cancer-associated genes. The *in vitro* and *in vivo* studies with siRNA and shRNA have shown that silencing of genes related to tumor cell growth, invasion, angiogenesis, metastasis, and chemoresistance in various types of cancer [17]. RNAi technology, as a new approach for cancer therapeutics, offers many advantages over conventional cancer treatment strategies. Advantages of gene silencing by siRNA and shRNA are the high degree of specificity to target tumor cells and tissues, a capacity to inhibit target gene expression, a simple and rapid design, and synthesis [22]. While non-specific chemotherapy leads to death of cancer cells, it significantly damages the surrounding healthy tissues and organs, causing extensive systemic toxicity. The side-effects of chemotherapeutic drugs can be minimized with siRNA treatment.

Cancer cells have the ability to develop a resistance to chemotherapeutic drugs. RNAi-based therapeutics can simultaneously target multiple genes in cancer signaling pathway. The simultaneous silencing of multiple genes in cancer therapy have importance in terms of minimizing the multiple drug resistance caused by small chemical molecules given in high dose [23, 24]. This therapy inhibits survival signals and pathways that take part in the development of multi-drug resistance in cancer cells.

Oncogenes, mutated tumor supressor genes, survival and apoptotic genes, causing tumor initiation and progression, are major targets for RNAi-based therapy. Simultaneous suppression of one target gene or multiple genes has provided a significant advantage in cancer therapy. siRNAs can be designed for effective gene knockdown by targeting any gene or multiple genes in cells [25]. siRNAs are likely to be more effective than other antisense approaches because of many properties such as a highly specific mRNA degradation, cell-to-cell spreading of gene silencing effect, long silencing activity, improved stability *in vivo*, and their efficiency in lower concentrations [26, 27]. A single therapeutic strategy is insufficient for

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the inhibition of cancer growth and progression; RNAi as a new therapeutic strategy may be used as well as with chemotherapy, immunotherapy, anti-hormone therapy, and radiotherapy for achieving synergistic therapeutic effect.

In clinical trials, the most of siRNAs have been given by local administration. When siRNAs are delivered to target tissue locally, lower siRNA doses can be used for pharmacologic effect (e.g., saline-based formulation, or excipiants such as 5% dextrose) and any drug delivery approaches (e.g., liposome, nanoparticle, and complexes) [28]. However, systemic drug administration by intravenous injection is required for cancer diseases [7]. In systemic effect, siRNAs must encounter several extra- and intra-cellular barriers until it reaches the target cell and tissue. siRNAs cannot freely cross physiological and cellular barriers because of their high molecular weight and negative charge. The significant challenges of using siRNA are their poor cellular uptake, degradation by serum nucleases, and rapid elimination. These factors and barriers reduce therapeutic effect of siRNA. Therefore, efficient *in vitro* and *in vivo* delivery of siRNA-based therapeutics in cancer is dependent on the development of appropriate delivery systems. siRNA delivery systems should (a) protect siRNAs against degradation enzymes and serum proteins, (b) prolong the circulation time of siRNA, (c) provide siRNA stability in blood serum, (d) avoid sequestration in the reticuloendothelial system (RES), (e) avoid aggregation in serum, (f) minimize non-specific tissue and cellular uptake, (g) achieve target-specific siRNA delivery, (h) allow for immune evasion, (i) resist rapid renal clearance, (j) enhance vascular permeability to reach cancer tissues, (k) promote trafficking to the cytoplasm and uptake into RISC, and (l) have low or non-toxicity [7, 29, 30].

## 3. Delivery strategies of RNAi-based therapeutics

siRNAs have large molecular weight (~13 kDa) and are polyanionic nature (~40 negative phosphate charge) and are easily degraded by enzymes in cells, tissues, and bloodstream. In addition, siRNAs cannot easily cross the cell membrane [29]. The naked siRNAs are readily degraded by serum endonucleases. The half-life of circulating naked siRNA is less than 10 minutes because of its rapid clearance by the kidneys, so that they cannot reach to target cell efficiently. The gene silencing activity of unmodified or uncomplexed siRNAs is little or absent [31]. To solve this problem, two strategies are used: chemical modifications and conjugation of siRNA molecules or use of gene delivery systems for increasing efficiency of RNAi-based therapeutics.

Chemical modification is the major approach to overcome *in vivo* siRNA delivery problems. Chemical modifications of naked siRNAs have been performed to (i) enhance siRNA stability, (ii) protect siRNA from degradation, (iii) avoid recognition by the innate immune system and minimize immunostimulatory responses, (iv) minimize off-target effects, (v) reduce required dose for gene silencing, (vi) improve pharmacodynamic properties, (vii) increase delivery to target cells, and (viii) allow the delivery by systemic administration. The sugar, backbone and nucleobase modifications of siRNA, can significantly protect siRNA in both serum and cytoplasm. The commonly used chemical siRNA modifications are the incorporation of locked

nucleic acids (LNA), phosphorothioate linkages, and 2′-o-methyl, 2′-amine, 2′-fluoro groups [7, 32, 33]. Chemical modifications must increase the stability of siRNA without affecting its gene silencing activity [23]. However, these substitutions may lead to off-target effects, cytotoxicity, reduced RNAi activity, and impaired biological activity [17, 34].

Other chemical strategies for siRNA are cholesterol, folate, and aptamer conjugation and peptide modification. siRNAs can associate with aptamers, ligands, and antibodies by electrostatic interaction or direct conjugation. The conjugation of these functional groups provides cell- or tissue-specific targeting and efficient delivery. As a result, the efficacy of silencing can be increased [1].

Viral and non-viral vectors have been extensively used in the siRNA-based therapy. Viral vectors encoding shRNA have a high gene transduction and gene silencing effects. Adeno-associated viral vectors, lentiviral vectors, and adenoviral vectors have been extensively used in gene knockdown studies [35]. The transfering of shRNA-encoding vectors into the nucleus of cells have obtained high and long-term shRNA expression. In addition, viral vectors can integrate the host genome [14]. Although viral vectors have a high gene transfection efficiency, the challenges such as inflammatory reactions, strong immunogenicity, insertional mutagenesis, and oncogenic transformation of viral vectors can cause important safety concerns. In addition, some viral vectors have low capacity for transgene insertion. To overcome these problems, non-viral vectors have been developed and used in siRNA delivery. Compared to viral vectors, non-viral vectors have several advantages such as lack of immunogenicity, low or no integration into genome, large-scale production, and use of wide variety of nucleic acids size [36]. However, the transfection efficiency of non-viral vectors is not as high as the viral vectors.

### 3.1. Non-viral vectors

The non-viral delivery of siRNA and shRNA therapeutics to target tumor cells is a multi-step process. To achieve efficient delivery and therapeutic gene silencing, siRNAs should be stable in biological fluids and must have above mentioned properties [37, 38]. The circulating siRNAs after systemic administration must be evaded from the reticuloendothelial system (RES). Negatively charged siRNAs gain the positive charge after complexed with cationic charged polymers. This positive charge facilitates cellular internalization of siRNAs; however, the cationic charge increase non-specific interactions by non-target cells, negatively charged serum proteins, and extracellular matrix. As a consequence of these non-specific interactions, clot-like accumulations or aggregations are formed. Complexes are entrapped in the endothelial capillary bed or taken up by RES recognition. While RES organs such as spleen, liver, and bone marrow uptake the major part of injected dose, the minor part of this reaches to tumors [25, 37, 39].

Non-viral delivery vectors prolong the biological half-life and mean residence time of siRNA, and they enhance accumulation of siRNA molecules in tumor tissues. siRNA therapeutics can be accumulated into cancer tissue by enhanced permeability and retention (EPR) effect as a result of discontinuous vasculature (permeation) and poor lymphatic drainage (retention) in

http://dx.doi.org/10.5772/62826

the abnormal tumor blood vessels compared to the normal blood vessels. Tumor endothelium allows penetration of macromolecules [37, 38].

The other challanges of RNAi-based therapeutics delivery to the tumor tissues after systemic circulation are crossing of cellular membrane, intracellular traffic into the cells with endosomal/lysosomal compartments, release of siRNA or shRNA from carriers, and nuclear transport for vector-based siRNA/shRNA therapeutics and entry to cytoplasm for siRNA-based therapeutics. The cell membrane is an important extracellular barrier for siRNA uptake. The average size of a single siRNA molecule is less than 10 nm. Despite their small size, polyanionic nature and hydrophilicity of siRNA make crossing of biological membranes difficult [18]. To overcome this problem, the complexation of negatively charged siRNA with cationic polymers or lipids are performed. The net positive charge of this formulations facilitates binding to negatively charged cell membranes, following internalization by adsorptive pinocytosis. For cell-type specific delivery, targeting ligands, antibodies, and aptamer-binding non-viral vectors pass through the cell membrane by receptor-mediated endocytosis [1]. After crossing from the cell membrane, siRNAs and vector-based siRNAs/shRNAs encounter several intracellular barriers that include the endosomal trafficking, unpackaging of siRNA, and nuclear traffic. The intracellular traffic of endosomal content is important for succesful siRNA delivery. When siRNA released from the carrier reaches cytosol, RNAi mechanism is induced inside the cells. However, for the onset of RNAi effect, transfer of vector-based siRNA/shRNA to the nucleus is required. In the delivery process, early release of siRNA from endosome is required. If siRNA remains inside the endosome for long time, it will be degraded. Therefore, different agents (fusogenic protein) conjugated with polymers disrupt the endosomal membrane. In addition, polymers possess proton-sponge effect (polyethyleneimine, PEI), which have been used to induce osmotic swelling and subsequent disruption of the endosome [15].

### *3.1.1. Polymer-based RNAi delivery system in cancer therapy*

Negatively charged siRNAs or shRNAs can readily bind to cationic polymers or load to the nanocarriers by ionic interactions. Nanosized complexes or polyplexes by electrostatic interactions and nanoparticle formulations by encapsulation have been developed for efficient siRNA/shRNA delivery. Thus, siRNAs can be protected from nuclease attack and cellular uptake of siRNAs via endocytic pathway faciliated. Many natural and synthetic polymers are used for gene delivery, such as polyethyleneimine (PEI), poly-l-lysine (PLL), chitosan, protamine, gelatin, atelocollagen, cationic polypeptides, cyclodextran polymers, dendrimers, poly-lactide-co-glycolide (PLGA), and polydimethylaminoethylmethacrylate (PDMAEMA) [34]. In addition, polyethyleneglycol (PEG) is widely used as a linker between polymer and ligand or nucleic acid or for binding of siRNA onto nanocarrier surface [40].

#### *3.1.1.1. Chitosan*

Among the non-viral vectors, chitosan or its derivatives are attractive where chitosan has been shown to be biodegradable, biocompatible, non-toxic, mucoadhesive, and non-inflammatory and has low cost of production. Chitosan is a cationic polysaccharide, consisting of *N*-acetyl-d-glucosamine and d-glucosamine units. In addition, chitosan has been designated as "Gen-

erally Recognized As Safe (GRAS)" by the FDA [41]. It has been widely used in *in vivo* siRNA and shRNA delivery applications because of positively charged amines, allowing electrostatic interactions with negatively charged nucleic acids to form stable complexes. The protonated amine groups allow transportation to cellular membranes and subsequent endocytosis into cells. Moreover, the high amounts of chitosan in siRNA complexes may lead to increase cellular accumulation of siRNA molecules and facilitate release of siRNA from endosomes to cytosol under high osmotic pressure in the endosomes of cells [42].

Chitosan-based nanocarriers are prepared by three different methods. These include simple complexation, ionic gelation (siRNA entrapment), and adsorption of siRNA onto the surface of chitosan nanoparticles [42]. The molecular weight and degree of deacetylation of chitosan influence its solubility, hydrophobicity, charge density, and thus the interaction ability with nucleic acids. The N/P ratio (ratio between chitosan nitrogen per siRNA phosphate) of chitosan/siRNA nanoplexes is an important factor for optimization of complex properties (size and zeta potential), transfection, and gene silencing efficiency. Increasing the N/P ratio not only helps to obtain a high transfection efficiency but also enhances toxicity. The excess of free chitosan in the formulations can interact with cell membrane and cellular process, and thus, may reduce cell viability [41].

Chitosan has a great potential in siRNA-based cancer therapy studies, because it can be safely and efficiently delivered to cancer cells. It is reported that chitosan or modified chitosan nanoplexes and nanoparticles as delivery system exerted antitumoral effects in different cancers [43–48].

**Studies with chitosan formulations in different cancers**

Howard et al. [43] developed chitosan nanoparticles using polyelectrolyte complexation method. The size of nanoparticles was between 40 and 600 nm. The endogenous enhanced green fluorescent protein (EGFP) silencing efficiency with nanoparticles was found to be 77.9 and 89.3% in human lung carcinoma cells (H1299) and murine peritoneal macrophages. The siRNA/chitosan nanoparticles reduced EGFP expression (43%) compared to untreated control in transgenic EGFP mice. They suggested that this chitosan-based system can be used in the treatment of systemic and mucosal diseases.

Salva and Akbuga [44] studied silencing effect of chitosan/*VEGF* shRNA nanoplexes in breast cancer cell lines. A significant *VEGF* gene silencing (60%) was obtained after nanoplexes application in MCF-7 cells. Salva et al. [44, 45] demonstrated the successful application of chitosan/siRNA or shRNA *VEGF* nanoplexes in *in vivo* breast cancer models. After intratumoral and intraperitoneal injection, comparison was made and higher tumor inhibition was obtained with intratumoral injection. qRT-PCR and Western Blot analysis showed that *VEGF* mRNA and protein expression was significantly reduced by chitosan nanoplexes.

Salva et al. [46] also studied the IL-4 encoded plasmid (pIL-4) to improve the therapeutic efficacy of siRNA targeting *VEGF* because of the anti-angiogenic effect of IL-4 molecule. Researchers prepared chitosan nanoparticles containing shRNA *VEGF* and pIL-4, and they have reported that co-delivery of shRNA *VEGF* and pIL-4 into chitosan nanoparticles caused additive effect on breast tumor cell growth in rat model (97% inhibition) [46].

In another study, Salva et al. [47] obtained enhanced silencing effect by using siRNAs targeting to *VEGF* and *HIF-1α* in different breast cancer cell lines such as MCF-7, MDA-MB-231, and MDA-MB-435. Two siRNAs were encapsulated into liposome coated with chitosan, and the co-delivery of siRNA *VEGF* and *HIF-1α* into liposomal form have significantly inhibited *VEGF* (89%) and the *HIF-1α* (62%) [47].

Yang et al. [48] reported that chitosan/siRNA *VEGF* nanoparticles prepared by complex coacervation method showed spherical morphology with a mean diameter of 110–200 nm and positively charged surface (20 mV). Chitosan nanoparticles were effectively transfected to mouse melanoma cells (B16-F10), and they have investigated 40% of the *VEGF* gene silencing efficiency in cells without any cytotoxicity.

Wang et al. [49] prepared the chitosan-TPP (tripolyphosphate) nanoparticles by ionic gelation method for the delivery of shRNA expressing vector to the human rhabdomyosarcoma (RD) cell line and for the inhibition of *TGF-β1* expression. Suppression of *TGF-β1* gene by chitosan nanoparticles containing shRNA has resulted in decrease of RD cell growth *in vitro* and tumorigenicity in nude mice.

Huang et al. [50] studied the effect of chitosan/shRNA *VEGF* nanocomplexes on angiogenesis and tumor growth in hepatocellular carcinoma (HCC). The administration of low molecular weight chitosan/shRNA *VEGF* complexes by intratumoral or intravenous injection demonstrated more effective suppression of tumor angiogenesis and tumor growth in the different HCC models. They showed that LMWC could effectively deliver shRNA into tumor tissue. shRNA *VEGF* concentrations in tumor tissue dramatically increased after intravenous administration of chitosan/shRNA *VEGF* complexes.

**Studies with chitosan derivatives and conjugation with other polymers and ligands in different cancers**

In order to increase the transfection efficiency of chitosan, different modifications are made on the structure of chitosan. Modified forms of chitosan such as carboxymethyl or trimethyl chitosan, trisaccharide-substituted chitosan oligomers, and succinated or galactosylated chitosan are formed. Chitosan is also conjugated with folic acid or PEG [51].

Jere et al. [52] used chitosan-graft-polyethylenimine (CHI-g-PEI) copolymer for delivery of shRNA *Akt1* expressing plasmid in lung cancer cells. The formed complexes were silenced *Akt1* onco-protein and significantly reduced the survival, proliferation, and growth progression of lung cancer cell. *Akt1* silencing induced apoptosis in cancer cells. The suppression of *Akt1* oncoprotein decreased A549 cell malignancy and metastasis. The therapeutic efficiency of CHI-g-PEI-shRNA *Akt* was found higher than PEI25K-shRNA *Akt* compared to carrier.

Noh et al. [53] prepared a copolymer containing additional cationic moieties linked with chitosan to enhance the cationic charge of chitosan. Therefore, chitosan derivation with poly-l-arginine (PLR) and polyethyleneglycol (PEG) (PLR-grafted CS) polyplexes were used for *in vitro* and *in vivo* delivery of siRNA *RFP*. PLR alone can be cytotoxic, thus conjugation of PLR to chitosan both decreased cytotoxicity of PLR and enhanced siRNA delivery efficiency. The pegylation of cationic polymers reduces the charge of polymers and limits the interaction with

cell membranes. PEG-CS-PLR did not significantly reduce the cellular delivery of siRNA. Three intratumoral injections of 120 μg of PEG-CS-PLR/siRNA *RFP* complexes to B16F10-RFP tumor-bearing mice had decreased *RFP* expression at 90% level in tumor tissues. It is indicated that PEG-CS-PLR can be a useful carrier for delivery of oncogene-specific siRNAs.

Fernandes et al. [54] investigated folate conjugation to improve gene transfection efficiency of chitosan. When chitosan was conjugated with folate, the folate-chitosan-siRNA complexes have increased gene silencing efficiency because of promoted uptake in HeLa and OV-3 cell lines, which are known to have high folate receptor expression. Higher transfection efficiency and lower toxicity of folate-chitosan complexes are reported in folate receptor–positive cells.

Cell penetrating peptide-based systems may improve cellular uptake and gene silencing efficiency of siRNAs without side effects. Protamine is a cationic, non-toxic polypeptide that has membrane translocation and nuclear localization activities because of its arginine-rich amino acid sequences. In addition to its stabilization enhancing properties, protamine is known to exhibit cell penetrating activity and is an important compound for several cancer targeting systems [55].

Salva et al. [46] have developed ternary nanoplexes of chitosan/protamine/siRNA targeting *VEGF* in breast cancer cell lines for efficient siRNA uptake and inhibition effect. Ternary nanoplexes showed the highest cellular uptake than binary nanoplexes.

Erdem-Cakmak et al. [56] reported that addition of protamine to chitosan complexes increased the silencing of *VEGF* genes after using chitosan/shRNA/protamine nanoplexes. In terms of the gene silencing and transfection, when the molecular weight of chitosans were compared at the different cell lines including HEK293, HeLa, and MCF-7, low molecular weight chitosan (70 kDa) proved more efficient than medium molecular weight chitosan. Gene inhibition values in cell lines after transfection of binary and ternary complexes followed the rank HEK293>HeLa>MCF-7. In addition, any cytotoxicity was not found after the complexes.

Song et al. [57] used protamine/antibody fusion protein to deliver siRNAs targeting *c-my*, *MDM2*, and *VEGF* specifically to HIV envelope-expressing B16 melanoma cells and envelope-expressing subcutaneous B16 tumors. The positively charged protamine served as binding partner for negatively charged siRNA and showed cell internalization and release of the siRNA cargo. The antibody-protamine delivery system can target siRNA specifically to cells.

Choi et al. [58] reported that complexes prepared with low molecular weight protamine (LMWP) inhibited cell growth by suppressing *VEGF* expression in hepatocarcinoma cancer cells. In tumor tissues, the expression of *VEGF* was inhibited through the systemic application of peptide complex, thereby suppressing tumor growth.

#### *3.1.1.2. Polyethylenimine*

Polyethylenimines (PEIs) are water-soluble cationic synthetic polymers. They can be synthesized in different lengths and different molecular weights such as branched (bPEI) or linear (lPEI) and low molecular weight (<1000 Da) or high molecular weight (>1000 kDa). PEI has a high cationic charge density because of the protonation of its primary, secondary, and tertiary

amine groups positioned at every third nitrogen [59]. While in linear PEI all nitrogen atoms are protonable, in the branched form, two-thirds of nitrogens can be charged. PEI can lead to proton accumulation in endosome, which was brought in by endosomal ATPase with an influx of chloride anion. Proton accumulation in endolysosome counteracts pH decrease, inhibits nucleases and unbalances endosome osmolarity depend on CI concentration and results in osmotic swelling of endosome. This effect of PEI is named as "proton sponge effect". PEI may enhance intracellular delivery by facilitating endosomal escape and induce lysosomal disruption, endosomal release, and DNA/siRNA protection from lysosomal degradation by buffering endosomes [60].

The molecular weight of PEI is important in the development of gene delivery and level of cytotoxicity in cells. The high molecular weight PEI has higher transfection efficiency than low molecular weight PEIs. PEI has a high electrostatic capacity, which can provide strong electrostatic interactions with the siRNA and contribute to cell membrane binding and internalization. Especially, the 25 kDa bPEI is one of the most effective non-viral vectors in gene silencing because of efficient endosomal escape. However, the high positive charge of bPEI leads to severe cytotoxicity and non-specific interactions with serum proteins [61, 62]. The cytotoxicity of PEIs can be decreased with modification of free amine groups or conjugation of cell binding and targeting ligands. Therefore, graft copolymers have been usually preferred as a delivery system.

Schiffelers et al. [63] prepared PEGylated PEI nanoplexes with Arg-Gly-Asp (RGD) peptide ligand containing siRNA targeting *VEGFR-2* and investigated the effect of angiogenesis and tumor growth in tumor-bearing mice. This study indicated that nanoplexes containing siRNA *VEGFR-2* reached tumor tissues after systemic administration. This delivery system has sequence-specific inhibition effect and reduced the tumor growth.

Jiang et al. [64] studied anti-*VEGF* siRNA/PEI-HA complex prepared by PEI-hyaluronic acid (PEI-HA). Complexes at the dose of 4.5 μg of siRNA/mouse were applied intratumorally to C57BL/6 mice by daily injection for 3 days. At 24 hours post-injection, the siRNA *VEGF* formulations were distributed mainly in the tumor, spleen, lung, heart, liver, and kidney. This study suggested that siRNA *VEGF*/PEI-HA complexes can be used for the treatment of cancer in the tissues having HA receptors such as the liver and kidney.

Park et al. [61] synthesized siRNA/(PEI-SS)-b-HA complexes and, after characterization, applied to *in vitro* and *in vivo* gene silencing for target-specific tumor treatment. This delivery system demonstrated an excellent *in vitro* gene silencing efficiency (50–80%). siRNA *VEGF*/(PEI-SS)-b-HA complexes were administrated intratumorally to colorectal tumor bearing mice every 3 days. After the treatment of tumor, *VEGF* gene silencing and reduction in tumor growth were seen.

### *3.1.2. Other non-viral delivery systems*

Among cationic polymers, poly (l-Lysine) (PLL) is one of the mostly studied polymers used for nucleic acid delivery. It formed complexes with DNA smaller than 100 nm. Its complexes can target different cells after binding suitable ligands. PLL can be easily produced in large

scale and is physiologically stable and biosafe [65]. PLL may protect siRNA from degradation effect of nucleases. However, PLL has some hurdless that impade its clinical application. PLL does not have the proton buffering ability to enhance lysosomal release of transported siRNA. It can be modified also by addition of ligands [66].

The ternary copolymer mPEG-b-PLL-g(ss-IPEI) was used for siRNA delivery to SKOV-3 ovarian cancer treatment. Nanocomplexes were administered to SKOV-3-implated Balb/c mice and tumor growth inhibition was observed [67].

Dendrimers are highly branched spherical and synthetic multifunctional macromolecules. The surface functional groups of dendrimers can be modified to enhance biocompatibility and decrease toxicity. Polycationic dendrimers such as poly(amidoamine) (PAMAM) and poly(propyleneimine) (PPI) dendrimers, because of the high density of positive charges on the surface, are highly attractive for delivery of negatively charged pDNA, antisense oligonucleotide (AsODN), and siRNAs. PAMAM dendrimers have primary amine groups on their surface and tertiary amine groups inside. Their amine groups are complexed with siRNAs. Thus, compact structure promote cellular uptake of siRNA and tertiary amine groups initiate the proton sponge effect to enhance endosomal release of siRNA [68, 69].

Waite et al. [70] conjugated cationic PAMAM dendrimers with RGD targeting peptides to enhance the delivery efficiency of siRNA to glioma cells. They suggested a promising strategy of RGD-conjugated dendrimers for siRNA delivery to solid tumors.

Liu et al. [71] investigated *in vitro* characterization and anticancer effect of PAMAM dendrimer-mediated shRNA against human telomerase reverse transcriptase (*hTERT*) in oral cancer. Dendriplexes had 110 nm size and +30 mV zeta potential which were favorable parameters for escape from the vasculature and intracellular delivery. shRNA *hTERT* dendriplexes were applied by intratumoral administration to tumors. Dendrimer-mediated shRNA *TERT* resulted in cell growth inhibition and apoptosis *in vitro* and tumor growth inhibition *in vivo* in the xenograft model. In addition, expression of *HTERT* and *PCNA* proteins was reduced in tumors.

Atelocollagen, which is produced from bovine type I collagen, has biomaterial properties such as high biocompatibility, biodegredability, and low immunogenicity. Atelocollagen forms a helix of three polypeptide chains and has positive charge, which enable its binding to nucleic acid molecules [72]. At low temperature, atelocollagen exists in liquid form (2–10°C), therefore, it can be easily mixed with nucleic acid solutions [72, 73]. Thus, atelocollagen can increase cellular uptake, nuclease resistance, and prolong release of nucleic acids. The size, charge, and sustained release of atelocollagen/siRNA complexes can be altered by ratio of siRNA to atelocollagen [74, 75].

Takei et al. [76] first studied anti-tumoral effect of atelocollagen complexes containing siRNA *VEGF in vitro* and *in vivo*. They showed that siRNA *VEGF* with atelocollagen inhibited tumor angiogenesis and tumor growth in PC-3 cell lines *in vitro* and xenograft tumor *in vivo* model.

Koyanagi et al. [77] reported that siRNA targeting vasohibin-2 (*VASH-2*) using atelocollagen complexes siginificantly inhibited ovarian tumor growth and angiogenesis in ovarian cancer

xenograft model. The knockdown of *VASH2* with atelocollagen/siRNA *VASH2* complexes exerted a significant antitumor effect and helped in tumor vascularization.

PLGA has been widely used as gene delivery system because of its biodegredability, biocompatibility, and non-toxic properties. FDA has approved PLGA as a pharmaceutical excipient. PLGA nanoparticles enter the cells efficiently by specific and non-specific endocytosis. Nanoparticles can release the encapsulated drug slowly leading to sustained drug effect [25].

Murata et al. [78] investigated anti-tumor effect of long-term sustained release of PLGA microspheres encapsulating anti-*VEGF* siRNA. The release of siRNA from microspheres was sustained for over one month. Intratumoral injection of PLGA microspheres containing siRNA *VEGF* inhibited tumor growth.

Su et al. [79] prepared PEI-coated PLGA nanoparticles loaded with paclitaxel and *Stat3* siRNA. PLGA-PEI nanoparticles more rapidly released *Stat3* siRNA than paclitaxel. Thus, decrease of *Stat3* expression by siRNA in human lung cancer cells (A549) and A549-derived paclitaxel-resistant A549/T12 cell lines reduced resistance of cell to paclitaxel. The released paclitaxel from nanoparticles killed the cancer cells that induce microtubule aggregation. In summary, inhibition of *Stat3* expression decreased cell viability, increased apoptosis, and reduced cellular resistance to paclitaxel.

### *3.1.3. Lipid-based siRNA delivery systems in cancer therapy*

Cationic lipids are used as carrier for siRNA delivery. Liposomes and lipoplexes, as lipid-based delivery systems, have been widely used in local and systemic siRNA or shRNA delivery. Liposomes are microscopic vesicles that consist of single or multiple lipid bilayer, form in a sphere with an aqueous core. Nucleic acids can either be entrapped in the aqueous core of liposomes or attached to the lipid surface for delivery. The advantages of liposomes as delivery system include a high gene transfection efficiency, enhanced stabilization, easy penetration into cell membranes, efficient *in vivo* delivery, and flexible and versatile physicochemical properties. The disadvantages of liposomes are the short half-life in serum, lack of tissue specificity, rapid liver clearence, and cell toxicity [17]. Three different liposomes, such as neutral, anionic, and cationic liposomes, are used in the siRNA delivery studies [22]. Cationic liposomes for siRNA delivery can easily cross the cell membrane, promote escape from the endosomal compartment, and reach the target genes with good biocompatibility. However, cationic lipids can induce an interferon response and cause unwanted interactions with negatively charged serum proteins because of its high cationic charge density [32, 67]. Interferon responses can lead to not only change in gene expression but also show dose-dependent cytotoxicity and pulmonary inflammation [80, 81]. The toxicity and transfection efficiency of cationic lipids depend on length and structure of hydrocarbon chains of lipids [82].

Neutral lipids lead to less cellular toxicity and do not induce immune responses without the down-regulation of gene expression. However, neutral liposomes have shown low transfection efficiency because of their lack of surface charges [17]. The commonly used cationic lipids for siRNA delivery include 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP) and 1,2-di-o-octadecenyl-2-trimethylammonium propane (DOTMA) have combined with neutral

lipids such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). This combination can enhance transfection efficiency. Because neutral lipids facilitate fusion to the host cell's membrane, cationic lipids can facilitate electrostatically complexation with siRNA to obtain more stable formulation and entry to cells more easily [18]. Liposomes are usually more stable than lipoplex in biological fluids [5].

Lipid-based siRNA delivery strategies have shown as promising in cancer therapy. Tumor-targeting approaches have been used to enhance antitumor efficacy of these delivery systems. Specific delivery to target cells can be achieved by conjugation of ligands or molecules such as transferin or PEG on the surface of liposomes [20]. The targeting and prolonged circulation half-life of liposomes allow for the enhanced permeability of tumor vasculature, increased delivery to tumor tissue, and reduced side effects [34, 82]. Cationic liposomes containing siRNA targeting tumor-associated genes have been used to inhibit tumor growth and proliferation, induce apoptosis, and enhance the radiosensitivity of tumor cells [83–85].

Cationic lipids can interact with negatively charged siRNA by ionic interactions. Thus, self-assembly formed lipoplexes protect siRNA from enzymatic degradation, enhance cellular uptake of siRNA by endocytosis, enhance the release of siRNA from endosomal/lysosomal entrapment, and thus, promote siRNA accumulation in the cytosol [16]. Commercially available cationic lipid formulations such as Lipofectin®, Lipofectamine® (Invitrogen), Dharmafect® (Dharmacon), RNAifect® (Qiagen), and TransIT TKO® (Mirus) have been studied as tranfection reagents for siRNA delivery *in vitro* [86]. The ratio of lipid and siRNA (lipid/siRNA ratio) affects the colloidal properties of the lipoplexes (particle size and zeta potential). Lipid/siRNA or shRNA ratio is important to facilitate the cellular internalization of lipoplexes and to dissociate the nucleic acids in the cytosol. Lipid/siRNA ratio can be optimized in terms of biological activity [16]. Developing a lipid-based delivery system, choice of lipids, and appropriate formulations are essential to decrease cytotoxicity and increase the transfection efficiency of formulation.

To overcome the drawbacks of lipoplexes and liposomes, different nanostructures such as neutral lipid-based nanoliposomes, stable nucleic acid lipid particles (SNALP), and solid lipid nanoparticles (SLN) have been developed as siRNA delivery system. SNALPs are composed of cationic, neutral, and fusogenic lipid mixture. SNALPs increase cellular uptake and endosomal release of siRNA [4]. PEG-conjugated SNALPs represent exciting lipid-based systemic RNAi. The PEG-lipid conjugate improves the retention time to >10 hours [87]. Recently, Tekmira Pharmaceuticals [88] has developed siRNA-based drugs that are encapsulated in the SNALPs for delivery of siRNAs to target tissue by intravenous injection. SNALP-encapsulated siRNA targeting *PLK1* initiated phase I trial in December 2010. Alnylam Pharmaceuticals [89] has developed first dual-targeted siRNA drug, SNALP-encapsulated siRNAs targeting *VEGF,* and kinesin spindle protein (*KSP*) for the treatment of hepatocellular carinoma. Phase I trial was initiated in April 2009 [90].

Tekedereli et al. [91] indicated that knockdown of *Bcl-2* by intravenously administered nanoliposomal-siRNA *Bcl2* (150 μg siRNA/kg) twice a week lead to antitumoral activity in breast tumors of orthotopic xenograft models. In addition, nanoliposomal-siRNAs have enhanced the efficacy of chemotherapeutic agents in the breast cancer therapy.

Landen et al. [92] studied neutral nanoliposomes incorporating siRNA targeting *EphA2* in orthotopic mouse model of ovarian cancer. Three weeks of treatment with *EphA2*-targeting siRNA nanoliposomes (150 μg/kg twice weekly) reduced tumor growth. The combination therapy with paclitaxel reduced tumor growth.

Salva et al. [47] investigated the effect of co-delivery of siRNA *HIF1-α* and *VEGF* in liposomal form in the breast cancer cell lines. Chitosan-coated liposomal formulation for co-delivery of siRNA *VEGF* and *HIF1-α* were developed. The co-delivery of siRNA *VEGF* and *HIF1-α* was greatly enhanced *in vitro* gene silencing efficiency in the breast camcer cell lines (95%). In addition, chitosan-coated liposomes showed 96% cell viability. Salva et al. has suggested that siRNA-based therapies with chitosan-coated liposomes may have some promises in cancer therapy [47].

In conclusion, siRNA-based therapeutics are new and potential targets in cancer studies. In cancer, different mechanisms including angiogenesis, and cell growth were studied as target pathways. However, siRNAs have different hurdles in treatment because of their short biological life in blood, instability, and poor cellular internalization. In order to overcome these hurdles two solutions are present: one is modification of siRNA and the other is use of suitable siRNA delivery system. In cancer treatment, viral and non-viral delivery systems are evaluated as siRNA delivery. Although limited information is available related to *in vivo* delivery, more papers are present in literature. Viral delivery systems have serious problems. Therefore, non-viral systems are more attractable than viral systems for siRNAs. Cationic lipids, liposomes, and polymers such as chitosan, PEI, PLL, and PLGA are used as non-viral siRNA delivery system. However, more suitable carriers are needed for siRNA delivery systems.

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