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REVIEW ARTICLE

Overcoming obstacles in microRNA delivery towards improved cancer therapy

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Abstract MicroRNAs (miRNAs) are small noncoding RNAs found to govern nearly every biological process. They frequently acquire a gain or a loss of function in cancer, hence playing a causative role in the development and progression of cancer. There are major obstacles on the way for the successful delivery of miRNA, which include low cellular uptake of the RNA and endosomal escape, immunogenicity, degradation in the bloodstream, and rapid renal clearance. The delivered miRNA needs to be successfully routed to the target organ, enter the cell and reach its intracellular target in an active form. Consequently, in order to exploit the promise of RNA interference, there is an urgent need for efficient methods to deliver miRNAs. These can be divided into three main categories: complexation, encapsulation, and conjugation. In this review, we will discuss the special considerations for miRNA delivery for cancer therapy, focusing on nonviral delivery systems: lipid, polymeric, and inorganic nanocarriers.

Keywords Drug delivery systems · Cancer therapeutics · Combination therapy · RNAi

Introduction

Oligonucleotides in the postgenomic era

Antisense oligonucleotides (ASOs), ribozymes, and RNA interference (RNAi) are the three main mechanisms that are currently used to inhibit the expression of a target gene. ASOs are short pieces of DNA or RNA complementary to messenger RNA (mRNA) sequences, which function by hybridizing with the messenger RNA thus inhibiting its translation. Ribozymes are catalytically active RNAs composed of three helices, which cleave single-stranded regions of their own or other RNAs by trans-esterification or hydrolysis. Small interfering RNAs (siRNA) are small RNA molecules that inhibit gene expression by causing the destruction of specific mRNA molecules. In recent years, RNAi has emerged as an important tool in molecular medicine and is rapidly becoming the new paradigm for gene downregulation [1].

RNAi Biogenesis pathway

RNAi causes silencing of a gene by the use of double-stranded RNA (dsRNA) with homologues sequence of the target gene. siRNAs are generated by the cleavage of endogenous as well as exogenous dsRNA molecules with the help of the RNase III enzyme Dicer [2, 3]. The exogenous siRNA can also be introduced to the cell in its cleaved form as short duplexes (19–22 nucleotides) with symmetric 2-base 3′-overhangs composed of a sense and a complementary antisense strand. Then, the antisense strand is incorporated into the RNA-induced silencing complex (RISC), leading to cleavage of the complementary mRNA [4]. MiRNAs are first transcribed in the nucleus as primary miRNAs (pri-miRNAs), and then undergo catalysis by the RNase III Drosha to create precursor miRNAs (pre-miRNAs). Pre-miRNAs are then exported to the cytoplasm. Once in the cytoplasm, pre-miRNAs are recognized and processed into their mature ~22 nt form by Dicer. As in siRNA, the antisense strand of the miRNA is incorporated into RISC and leads to translational repression of the target mRNA (Fig. 1) [5]. Unlike siRNA, which is specific for a single gene, and its complementarity to the target gene is incomplete, miRNA regulates diverse multiple genes. It should be noted that

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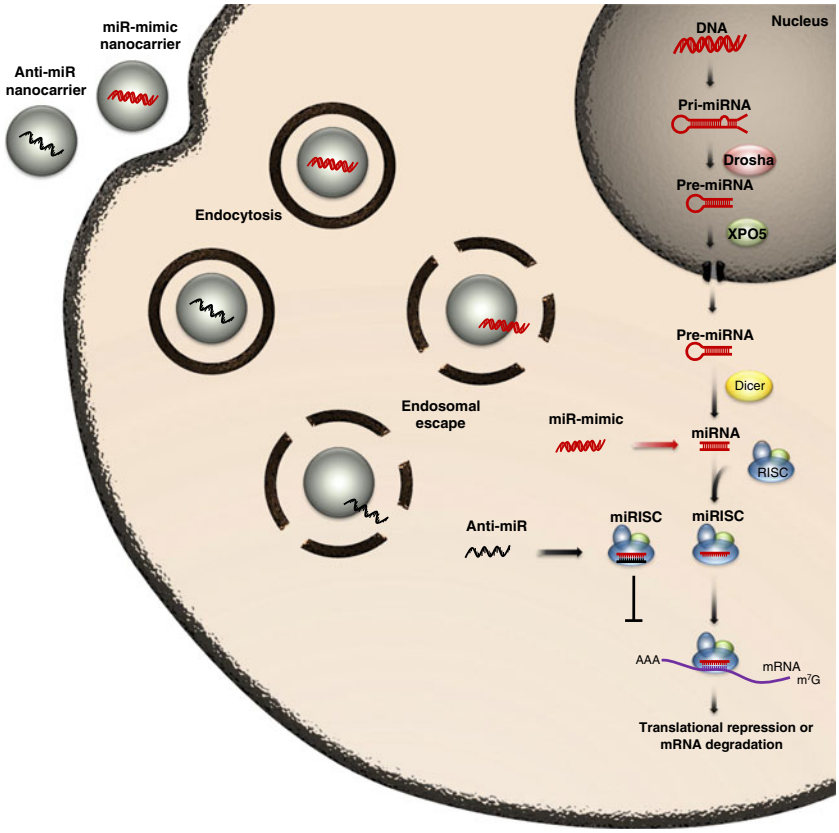

Fig. 1 Endogenous, miRNA mimic, and anti-miRNA biogenesis. miRNAs are first transcribed in the nucleus as primary miRNAs (*pri-miRNAs*) and undergo catalysis by the RNase III Drosha to create precursor miRNAs (*pre-miRNAs*). Pre-miRNAs are then exported to the cytoplasm. Once in the cytoplasm, pre-miRNAs are recognized and processed into their mature ~22 nucleotides form by Dicer. miRNA mimic involves the reintroduction of a tumor-suppressor miRNA to restore a loss of function, while the anti-miRNAs trap the endogenous miRNA in a configuration that is unable to be processed by RISC or, alternatively, might lead to degradation of the endogenous miRNA

siRNA can also work on partial complementary targets, causing "off-target" effects. Currently, a major effort in academia and industry is focused on the development of techniques and nucleotide modifications to reduce these off-target effects.

miRNAs are endogenous RNAi, found to govern nearly every biological process. They have numerous functions in physiology: from cell differentiation, proliferation, and apoptosis to the endocrine system, hematopoiesis, and fat metabolism [6–8]. They display different expression profiles from tissue to tissue, which reflects the diversity in cellular phenotypes and, as such, suggest a role in tissue differentiation and maintenance.

Modified synthetic anti-miRNA oligonucleotides (AMOs) are useful tools for the specific inhibition of individual miRNAs, thereby helping to unravel the function of miRNAs and their targets. Similar to antisense-based oligonucleotides, anti-miRNAs may contribute to the prioritization of pharmaceutical targets and have the potential to eventually evolve into a new class of therapeutic agents [9]. The anti-miRNAs can be complementary to either the mature miRNA or its precursors [10]; however, the most direct and apparently most effective anti-miRNAs assessed are complementary to the mature miRNA, designed to block its function in RISC (Fig. 1) [11]. miRNA replacement therapy is technically very similar to siRNA therapeutics. It involves adding to cells naturally occurring miRNAs to orchestrate typically a range of processes that will result in a therapeutic effect. miRNA oligonucleotide mimics are RNA duplexes consisting of the guide (driver/anti-sense) strand that is identical to the mature miRNA sequence and is designed to “mimic” the function of the endogenous miRNA aiming to restore its loss of function as a tumor suppressor. The other strand of the duplex (passenger/sense strand) is partially or fully complementary to the guide strand [12]. Same chemical modifications, as used for the anti-miRNA, are made to the oligonucleotide to reduce degradation. It must be noted though, that anti-miRNAs are more permissive to chemical modifications, while miRNA mimics still need to be able to be incorporated to the RISC, hence is limited in the possibilities for modification.

Although both siRNA and miRNA have potential use for therapeutic purposes, in this review, we will focus on miRNA.

miRNAs in cancer

Cancer can be looked at as a genetic disease, as it is caused by alterations in a number of oncogenic pathways, resulting in cancer cell transformation. Investigations on gene expression profiles have shown a number of alterations, confirming the fact that many genes and pathways play a prominent role in carcinogenesis [13]. Emerging evidence suggests that

miRNAs can function as diagnostic biomarkers and therapeutic targets for a wide range of diseases, including human cancers [14]. Numerous functional studies using cultured cancer cells and mouse models of cancer have identified miRNAs that function as conventional tumor suppressors or oncogenes [15–17]. About 50 % of the miRNA genes are frequently located in cancer-associated genomic regions or fragile sites [18].

miRNAs frequently acquire a gain or a loss of function in cancer, hence playing a causative role in its development and progression [19]. Aberrant regulation of miRNAs is manifested by differential expression in the tumor tissue relative to the normal adjacent tissue. Altered expression of miRNAs is apparent in virtually all tumor types and can be found in blood-borne malignancies as well as in solid tumors. Examples of miRNAs with oncogenic activity are miR-155 and the miR-17~92 cluster; in contrast, miR-15a, miR-16, miR-34a, and let-7 were reported to have tumor-suppressive activity [19, 20]. Other miRNAs have specifically been implicated in early tumorigenesis or metastasis, representing unique opportunities for therapeutic intervention that will depend on the context and requirement of therapy [21].

Tumor angiogenesis, the formation of new blood vessels from pre-existing ones, is a crucial step in tumor development, as it provides oxygen and nutrients to the growing mass of tumor tissue [22]. A role of miRNAs in tumor angiogenesis was shown in several studies, either as angiogenic stimulators such as the miR-17~92 cluster [23] or as angiogenic inhibitors like miR-15b, miR-16, miR-20a, and miR-20b, which target the vascular endothelial growth factor (VEGF) for repression [24]. Since miRNAs are stable in formalin-fixed paraffin-embedded tissues and can be routinely quantified relatively easily, they are quickly entering clinical laboratories as important tools for diagnostics and prognostics. Importantly, the cell and cancer-type specificity of miRNA expression profiles also holds a promise for the efficient identification of metastatic cancers of unknown primary origin [14, 25]. The discovery of miRNAs in body fluids, such as serum, urine, and colostrum, has intensified investigations into the use of miRNAs as noninvasive biomarkers of disease and therapeutic response in a range of cancers [26].

miRNA delivery considerations

Efficient delivery of miRNA for therapeutic purposes is extremely challenging. Low cellular uptake of RNA (attributed to its high molecular weight and negative charge), degradation in the bloodstream, and rapid renal clearance are significant obstacles on the way for the successful delivery of miRNA [27]. The delivered miRNA needs to be routed to the target organ, enter the cell, and reach the intracellular target in its active form (Fig. 2).

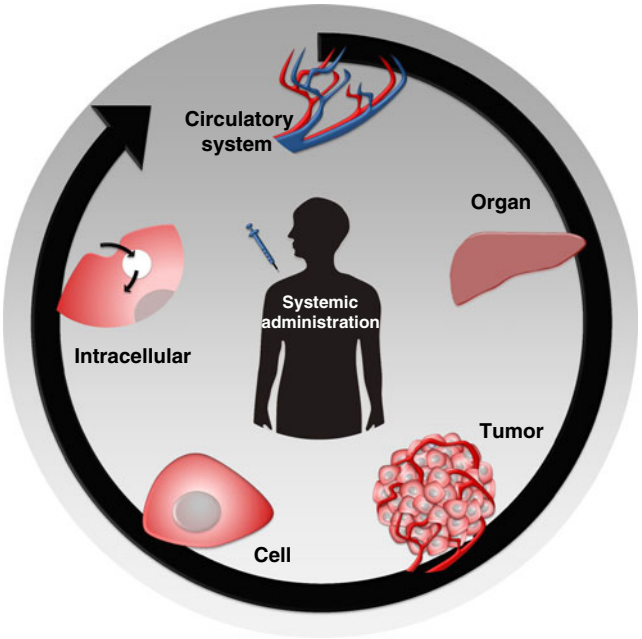

Fig. 2 Biological barriers. The delivered therapeutic-miRNA should be active, nonimmunogenic and stable. It has to overcome RNases in the blood stream, should be successfully targeted to the right organ, enter the tumor cells, and finally, be released in its active form

RNA molecules such as single-stranded RNA (ssRNA) and siRNA duplexes induce Toll-like receptor (TLR)-mediated immune stimulation after intracellular delivery [28]. Robbins et al. have previously shown that selective incorporation of 2′-*O*-methyl (2′-*O*Me) residues into siRNA abrogates cytokine production without reduction of gene silencing activity. Moreover, they showed that 2′-*O*Me modified RNA acts as a potent inhibitor of RNA-mediated cytokine induction in both human and murine systems. This activity does not require the direct incorporation of 2′-*O*Me nucleotides into the immunostimulatory RNA or that the 2′-*O*Me nucleotide-containing RNA be annealed as a complementary strand to form a duplex. Their results indicate that 2′-*O*Me RNA acts as a potent antagonist of immunostimulatory RNA [29].

Modifications to stabilize miRNA mimics and anti-miRNA to nuclease degradation and to improve affinity for target RNA are necessary for activity in cell culture and animals. It was shown that oligonucleotides with 2′-sugar modifications [including 2′-*O*Me, 2′-*O*-methoxyethyl (2′-MOE), 2′-fluoro (2′-F) and locked nucleic acid (LNA)] as well as phosphorothioate backbone modification are considered effective stabilizers of anti-miRNA. The first anti-miRNA reported to inhibit miRNA activity in vitro was 2′-*O*Me modified, which is commercially available from a variety of sources [11]. All the aforementioned modifications confer nuclease resistance and increase the binding affinity of anti-miRNA oligonucleotides to their cognate miRNAs [30]. Lennox and Behlke [31] reported that many of the chemistries and modification patterns studied in AMOs were

also tested for function when incorporated into the passenger strand in siRNA. Interestingly, Davis et al. [32] found an inverse correlation of siRNA activity with anti-miRNA activity for each modified passenger strand/AMO. Both studies concluded that those chemistries that performed well in AMOs performed poorly when used in siRNAs and vice versa [31, 32]. In general, anti-miRNA molecules usually do not require a delivery system thanks to the possible chemical modifications (e.g., anti-miR-122 currently in phase II for HCV by Santaris); however, in most cases where miRNA mimic is the entity in need, a delivery system is paramount.

In the last several years, various methods have been developed in order to promote a successful delivery of RNAi molecules in vivo. These are based mainly on incorporation into lipid- or polymer-based nanoparticles. An ideal delivery system should be biocompatible, biodegradable (or at least be excreted renally), and nonimmunogenic. Natural and synthetic lipids and polymers [e.g., phosphatidylcholine, poly(lactic-co-glycolic acid) (PLGA), chitosan, etc.], which can undergo biodegradation into products absorbed by the natural biochemical pathways of the body, are the most common nanocarriers for therapeutics delivery. Other requirements of the delivery system include being stable in the circulation, arrive at the target site, facilitate cellular uptake, avoid lysosomal degradation, enable endosomal escape, and bypass rapid renal clearance.

As outlined above, the bloodstream is a great obstacle for miRNA systemic delivery, since rapid degradation by RNases and renal clearance significantly shorten the half-life of naked RNAs in the circulation. Protection against RNase digestion can be achieved by chemical modifications of the RNA molecule. However, systemic administration of naked RNAs induces an immune response, which results in their accumulation in the reticulo-endothelial system (RES), i.e., lymph nodes, spleen, and liver, where they are digested and cleared by macrophages. The downsides of these chemical modifications include off-target effects, reduced activity, and toxicity caused by the changes of the therapeutic agent [33]. Packaging the miRNA in a delivery system can prolong its circulation half-life, protect it from degradation, and reduce RES uptake. Many delivery systems complex or encapsulate the miRNA molecule into nanoparticles (discussed in detail below), thus protecting it from nuclease degradation. In order to protect the miRNA from RES recognition, water-soluble polymers such as poly(ethylenglycol) (PEG) are used as shielding agents [34, 35].

Newly formed, angiogenic blood vessels of the tumor are characterized by a defective endothelium with wide pores and fenestrations. Moreover, tumors are usually characterized by impaired lymphatic function. These two factors lead to a phenomenon known as the enhanced permeability and retention (EPR) effect, by which macromolecular drugs accumulate selectively at tumor tissues [36]. The actual size of the gaps in the tumor vasculature is dynamic and varies greatly between different tumor types and between vessels of the same tumor. Usually, a molecular weight range of 20–200 kDa is used to take advantage of the EPR effect and to avoid rapid renal excretion. Particle size range of 20–100 nm was found to be optimal for prolonged circulation, accumulation in tumor tissue, and enhanced diffusion within tissue [37–39]. Use of nanosized carriers enables passive targeting of miRNAs to tumors, minimizing nonspecific targeting to healthy organs and lowering the amount of miRNA that needs to be administered to reach the desired therapeutic effect.

Because of their hydrophilicity and negative charge, miRNA molecules are unable to cross biological membranes. Therefore, an effective miRNA delivery is usually accomplished by the incorporation of the negatively charged nucleic acid with cationic lipids or polymers. The result of this electrostatic interaction is a net positive charge of the nanoparticles, which enables interaction with the negatively charged cell membrane. Cell internalization is then usually continued by fluid phase pinocytosis. Another way to improve cellular uptake is an addition of a targeting moiety [40]. In this case, once the drug arrives at the tumor site, it is directed to a specific target on tumor cells or tumor microenvironment cells (e.g., endothelial cells, fibroblasts, and immune cells) and then enters the cell via receptor-mediated endocytosis.

Subsequent to cell internalization, either by receptor-mediated endocytosis or by fluid phase pinocytosis, the miRNA nanocarriers must escape from the early endosome, in order to avoid fusion with the lysosome and ultimately elimination via the Golgi system [38, 41, 42]. The transition from the early endosome to the late endosome and finally fusion with the lysosome for degradation is accompanied by a rapid acidification, from neutral to approximately pH 6 in the early endosome and pH 5–5.5 in the lysosome, which can be harmful to the RNA. Several mechanisms were suggested for this endosomal escape:

- The leading hypothesis is *the proton sponge effect* [43]. According to this approach, amine groups in the polymeric backbone have the ability to absorb protons under acidic pH conditions and thereby prevent acidification of the endosomal vesicles. This leads to increased proton and chloride influx, osmotic swelling, endosomal membrane rupture, and eventually leakage of the polymer–nucleic acid complex into the cytosol [44].
- Another way to escape from the endosome is by *disruption of the endosomal membrane*. Cell penetrating peptides are short sequences of amino acids, usually cationic and/or amphipathic, which are able to translocate through biological membranes. Under acidic conditions, they fuse into the lipid bilayer of the

endosomal membrane with consequent destabilization of the vesicle, thus discharging the vesicles content into the cytoplasm.

- *Pore formation* is an additional way to achieve endosomal escape. In the acidic endosomal environment, certain peptides can undergo a conformational change into an amphipathic alpha-helical structure. In this conformation, the peptides are incorporated into the lipid bilayer, where they aggregate to form membrane pores and release the genetic material into the cytosol [45].
- Finally, cationic liposomes can facilitate endosomal escape by *ion pairing* (also known as the "flip-flop mechanism"). Once inside the endosome, the cationic liposome form charge-neutralized ion pairs with the anionic lipids of the endosomal membrane, resulting in the release of nucleic acids directly into the cytoplasm [46–48].

miRNA delivery systems

There are several approaches for nonviral miRNA delivery by nanocarriers. These can be divided into three main categories: complexation, encapsulation, and conjugation. The specific characteristics of these diverse strategies result in a unique biocompatibility, targeting capability, specificity, intracellular trafficking, and miRNA release/activation mechanism for each system. The different delivery systems currently used to deliver miRNA to cancerous tissues are summarized in Fig. 3 and Table 1.

Complexation

The vast majority of nonviral delivery systems for miRNA to date are based on formation of electrostatic complex between the negatively charged miRNA and the positively charged vehicle. The materials for complexation are varied and include lipids, polymers, proteins, and inorganic nanoparticles. Interestingly, most endogenic circulating miRNAs in the bloodstream were also shown to be stabilized by complexation with a protein, Argonaute 2 [49]. This method is fast and easy since miRNA only needs to be mixed and incubated with the appropriate carrier. The final complex usually remains positively charged, which enables it to interact with negatively charged biological membranes, but might also result in its toxicity.

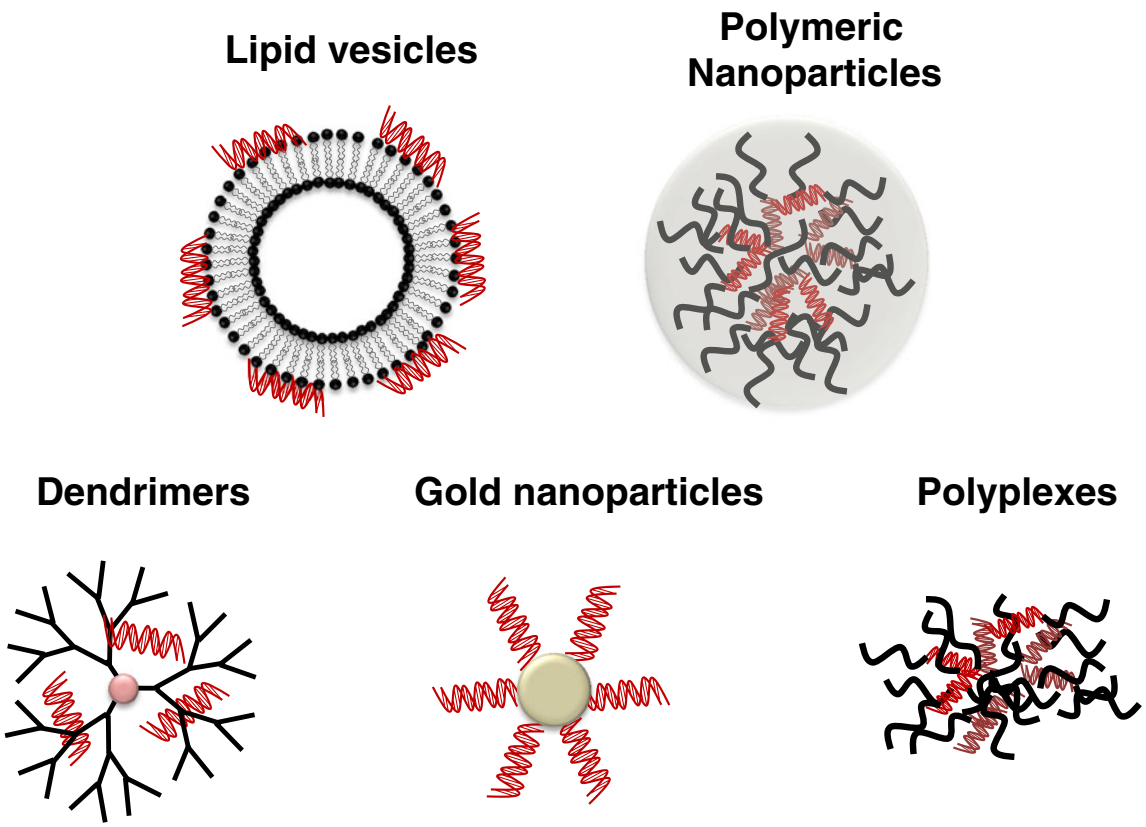

Fig. 3 miRNA main delivery systems. Several vehicles can be used for miRNA-based delivery. The addition of the miRNA to the vehicle can be done using three different methods. The main delivery systems are lipid vesicles (complexation), polymeric nanoparticles (encapsulation), dendrimers (complexation), gold nanoparticles (complexation/conjugation), and cationic polymeric polyplexes

Table 1 Summary of delivery systems and additional moieties for miRNA-based cancer therapeutics

Delivery system	Additional moiety	miRNA	miR type	Cancer	Reference
Complexation					
Lipid vehicle					
Cationic lipoplex		29b	Mimic	Lung	[52]
Cationic lipoplex		133b	Mimic	Lung	[53]
Liposome-polycation-hyaluronic acid	PEGylated modified with cyclic RGD motif	296	Anti-miR	Angiogenesis	[62]
Lipid-based nanoparticles		34a; 143-145 cluster	Mimic	Pancreas	[54]
Liposomes	$\alpha_v\beta_3$-targeted	132	Anti-miR	Breast	[59]
Lipid nanoparticles	Lacosylated gramicidin	155	Anti-miR	Hepatocellular carcinoma	[60]
Liposome-polycation-hyaluronic acid	scFV targeting	34a	Mimic	Melanoma	[61]
Neutral lipid		34a	Mimic	Lymphoma	[64]
		34a;let7	Mimic	Lung	[65, 66]
		34a	Mimic	Prostate	[67]
Cationic polymers					
PU-PEI		145	Mimic	Lung	[71]
CD-PEI	RGD-targeting peptide (CC9)	34a	Mimic	Pancreas	[74]
Dendrimers					
PAMAM	5-FU	21	Anti-miR	Glioma	[76]
Nanoparticles					
Gold nanoparticles		31; 1323	Mimic; Anti-miR	Neuroblastoma; ovarian	[81]
Encapsulation					
PLGA nanoparticle	Cell-penetrating peptide	155	Anti-miR	Lymphoma	[82]
PLGA nanoparticle		155	Anti-miR	Keratin-forming tumor cell line HeLa	[83]
Silica nanoparticles	Cell surface antigen GD2 coating	34a	Mimic	Neuroblastoma	[85]
Conjugation					
Gold nanoparticles		29	Anti-miR	HeLa cells	[86]
Magnetic nanoparticle	Fluorescence and AS1411 aptamer targeting nucleolin	221	Anti-miR	Astrocytoma	[87]

Lipid vesicles

Liposomes are lipid vesicles composed of bilayer phospholipid membrane encasing a water compartment. Lipid composition can be varied greatly to create liposomes with different surface charges. Lipids can be chemically attached to targeting moieties and fluorescent molecules. Often, liposomes are PEGylated, i.e., coated with PEG to avoid immune system recognition and RES uptake and to reduce immunogenicity. Other options for liposomal coatings are polysaccharides such as hyaluronan (HA), which result in long circulation and high affinity to recognition sites that are overexpressed in tumors [50, 51]. However, polysaccharides-coated liposomes have not been exploited yet for miRNA delivery. For nucleic acid delivery, liposomes composed of cationic lipids are usually used to achieve complexation with the negatively charged miRNA, rather than encapsulation. Wu et al. [52, 53] used cationic lipoplex-based delivery system to deliver miRNA for the treatment of lung cancer. Empty positively charged liposomes were prepared and complexed with miRNA. The formulation was used to deliver miR-29b or miR-133b, potential tumor suppressors in nonsmall cell lung cancer (NSCLC), that reduce the expression of key targets CDK6, DNMT3B, and myeloid cell leukemia sequence 1 (MCL1). They demonstrated that cationic lipid formulations were more effective in delivering the miRNAs, both in vitro and in vivo, compared to standard transfection reagent (siPORT NeoFX transfection agent, Ambion).

Similar system based on cationic empty liposomes was used by Pramanik et al. to deliver miR-34a or miR-143/145 cluster for the treatment of pancreatic cancer. miR-143 and miR-145 target the oncogene KRAS. Constitutively active KRAS activates RAS-responsive element binding protein 1 (RREB1), which directly inhibits the transcription of the miRNA cluster encoding miR-143 and miR-145. Loss of this cluster, as occurs in pancreatic, bladder, lung, and colorectal carcinomas, or elevations in KRAS expression can perturb this balance, thus leading to carcinogenesis. Overexpression of miR-143 and miR-145 through this lipid-based nanovector inhibited tumor growth in an orthotopic xenograft mouse model of pancreatic cancer and downregulated both KRAS and RREB1 [54]. This nanovector represents an electrostatic complex of positively charged liposomal nanoparticle and negatively charged plasmid DNA and was prepared by mixing pMSCV-puro vectors expressing corresponding miRNAs and liposome on a charge ratio basis. The plasmid DNA-complexed lipid-based nanovector was approximately 100 nm in diameter and showed no apparent histopathological or biochemical evidence of toxicity upon intravenous injection [54].

miR-34a, a transcriptional target of p53, is a master tumor suppressor miRNA, which is downregulated in most human cancers. miR-34a inhibits malignant growth by repressing genes involved in various oncogenic signaling pathways, including cellular proliferation and apoptosis. These effects are achieved through inhibiting the expression of MYCN, BCL2, SIRT1, SFRP1, CAMTA1, NOTCH1, JAG1, CCND1, CDK6, and E2F3 [55]. Consequently, miR-34a antagonizes processes that are necessary for basic cancer cell viability, as well as cancer stemness, metastasis, and chemoresistance [56].

A phase I study of miR-34 (MRX34), given intravenously to patients with unresectable primary liver cancer or metastatic cancer with liver involvement has recently begun, sponsored by Mirna Therapeutics, Inc. [57]. The delivery technology for MRX34 is the NOV340 SMARTICLE technology owned by Marina Biotech. NOV340 SMARTICLE consists of an ionizable liposome that forms a particle with a diameter of ~120 nm. The liposome contains amphoteric lipids that are cationic at low pH and neutral or anionic at neutral and higher pH. The lipids and miRNA mimics are mixed under acidic conditions to facilitate efficient miRNA encapsulation and liposome formation. In biofluids with a pH of 7–7.5, the nanoparticles assume a slightly anionic character that may prevent unwanted interactions with the negative charge of cellular membranes in the endothelium and other tissues. Since the pH tends to be lower in tumor areas, the NOV340 particles may become cationic in these areas and adhere to tumor cells [58]. In short, this liposomal delivery technology employs ionizable amphoteric lipids, lipids that can take on both positive and negative charge depending on pH, and not ionizable cationic lipids, lipids that merely become positively charged at acidic pH.

Delivery efficiency can be enhanced by modifying the liposomes with a targeting moiety. Anand et al. [59] achieved selective delivery of anti-miR-132 to the tumor endothelium of mice, using liposomes with a targeting moiety directed to $\alpha_v\beta_3$, an integrin overexpressed on tumor vasculature. miR-132 acts as an angiogenic switch by targeting p120RasGAP in the endothelium and thereby inducing neovascularization. miR-132 was found to be highly expressed in the endothelium of human tumors and hemangiomas but was undetectable in normal endothelium. $\alpha_v\beta_3$-targeted delivery of anti-miR-132 restored p120RasGAP expression in the tumor endothelium, suppressed angiogenesis and decreased tumor burden in an orthotopic xenograft mouse model of human breast carcinoma [59]. In a recent study, novel hepatocellular carcinoma (HCC)-targeted lipid nanoparticles (LN) with the capability of overcoming in vivo delivery barriers were designed and synthesized, and the delivery efficiency of anti-miR-155 was evaluated in both HCC cells and in mice. miR-155 is an oncogenic microRNA that regulates several pathways involved in cell division and immunoregulation. It is overexpressed in numerous cancers, including HCC, and is often correlated with poor prognosis, thus being a key target for future therapies. These lipid nanoparticles contained *N*-lactobionyl-dioleoyl phosphatidylethanolamine (Lac-DOPE), which is a ligand for the asialoglycoprotein receptor expressed on HCC cells, and an antibiotic peptide gramicidin A, a hydrophobic peptide that acts as an ionophore, which was incorporated to facilitate endosomal release of the anti-miRNA following endocytosis [60]. The nanoparticles contained a cationic lipid that complexes miRNA; however, the use of lactobionic acid partially reduced the positive surface charge of the liposomes, potentially reducing toxicity. This formulation was named lactosylated gramicidin-based LN (Lac-GLN). When Lac-GLNs were delivered in vivo, they favored localization to the liver and diminished the off-target uptake from other tissues to a great extent [60].

Chen et al. developed liposome-polycation-hyaluronic acid (LPH) nanoparticles that are self-assembled by charge–charge interaction. A slight excess amount of HA and siRNA or miRNA was first complexed with protamine in a way that the condensed cores were negatively charged. The complex was then encapsulated by cationic liposomes composed of DOTAP/cholesterol (1:1 mol/mol) via charge interaction. The nanoparticles were further PEGylated and modified with the tumor-targeting GC4 single-chain antibody fragment scFv to selectively deliver the cargo into the tumor cells. These GC4-targeted nanoparticles effectively delivered miR-34a systemically and reduced tumor load in the lung metastasis of murine B16F10 melanoma [61]. Similar system was presented by Liu et al, who developed PEGylated LPH nanoparticle formulation modified with cyclic RGD peptide (cRGD) for specific and efficient delivery of anti-miR-296 antisense oligonucleotides into $\alpha_v\beta_3$ integrin-positive angiogenic endothelial cells. miR-

296 belongs to the family of angio-miRs and contributes significantly to angiogenesis by downregulating the hepatocyte growth factor-regulated tyrosine kinase substrate mRNA, leading to degradation of the growth factor receptors VEGFR2 and platelet-derived growth factor receptor. This delivery system significantly increased cellular uptake of anti-miRNAs, leading to profound downregulation of miR-296 and inhibition of angiogenesis [62].

Another promising delivery system is a commercially available neutral lipid emulsion (MaxSuppressor™ In vivo RNA-LANCEr II, BIOO Scientific). It is a patent-pending, proprietary formulation composed of neutral lipid, nonionic detergent, oil, and small molecules [63]. It was recently used in several studies to systemically deliver the tumor suppressor miRNAs let-7 and miR-34a [64–67]. let-7 is one of the most studied miRNAs, and it is downregulated in multiple cancers [68]. let-7 was originally identified as a switch gene required for proper development in *Caenorhabditis elegans* [16] and was subsequently found as the first known human miRNA. let-7 and its family members are highly conserved across species in sequence and function, and have several known targets genes, including KRAS, HMGA2, MYC, LIN28, CDK6, CDC25A, and CCND2 [66]. Systemic treatment of a KRAS-activated autochthonous mouse model of NSCLC led to a significant decrease in tumor burden. Specifically, mice treated with miR-34a displayed a 60 % reduction in tumor area compared to mice treated with a control miRNA [66]. Similar results were obtained with the let-7 mimic [66].

The therapeutic benefit of systemically delivered miR-34a mimic was also examined in a xenograft model of diffuse large B-cell lymphoma. A strong reduction in tumor growth by 76 % on average was observed in mice receiving neutral lipid emulsion-formulated miR-34a mimic compared to the control group. miR-34a-treated tumors expressed elevated levels of miR-34a and exhibited significantly higher apoptosis rates than the control tumors [64]. In another study, delivery of miR-34a using the RNA-LANCEr II reagent, inhibited prostate cancer metastasis and extended survival of tumor-bearing mice [67]. Intravenous delivery of formulated miR-34a did not induce an elevation of cytokines or liver and kidney enzymes in serum, suggesting that the formulation is well tolerated and does not induce an immune response [65].

Polymer carriers

A polymeric backbone represents a versatile platform for delivery of genetic material. Polymers, from natural or synthetic sources, can be tailored in size and charge to maximize the payload of RNAi and to obtain nanoparticles in the nanosized scale with specific pharmacokinetic and biodistribution profile. Polyethylenimine (PEI) is a synthetic cationic polymer often used for nucleic acid delivery. PEI's high charge density enables the formation of stable complexes with miRNA and facilitates endosomal escape via the proton sponge effect [43]. However, this polymer is not biodegradable and is known to interact with blood components and cause cytotoxicity [69, 70]. A recent study assessed the utility of polyurethane-short branch-polyethylenimine (PU-PEI) as a vehicle for miR-145 delivery and evaluated its therapeutic efficacy on lung adenocarcinoma tumorigenesis and metastasis. PU-PEI-mediated miR-145 overexpression inhibited the cancer stem cells (CSCs)-like properties and sensitized highly tumorigenic CSC-like cells to chemo-radio treatment by repressing its downstream targets, Oct4, Sox2, and Fascin1. However, the toxicity of this formulation was not examined at that time [71]. Cyclodextrins (CD) are natural cyclic oligosaccharides, which are nontoxic and nonimmunogenic. Conjugation of polymers, such as PEI, to CDs is often practiced in order to reduce their toxicity [72]. β-cyclodextrin-PEI (PEI-CD) carrier was developed for delivery of tumor-suppressor miR-34a mimic to pancreatic cancer cells. The PEI-CD nanoparticles were conjugated with CC9, a specific tumor-homing and tumor-penetrating bifunctional peptide via its CRGDK motif, which binds to neuropilin-1 (NRP-1) [73]. This delivery system could greatly upregulate the miR-34a level in PANC-1 cell line and substantially inhibit the target gene expressions such as E2F3, Bcl-2, c-myc, and cyclin D1, inducing cell cycle arrest, apoptosis, and suppressing migration. More importantly, the in vivo evaluation of the antitumor activity indicated that the delivery of miR-34a significantly inhibited tumor growth and induced cancer cell apoptosis [74].

Other potential novel carriers for the delivery of miRNA are dendrimers. Dendrimers are synthetic polymers with a unique structure. They are repetitively branched spherical macromolecules with multiple functional end groups at the surface. The formulations used for nucleic acid delivery usually bear cationic amine groups for complexation with the negatively charged miRNA. Polycationic dendrimers offer high transfection efficacy attributed to their high buffering capacity, which facilitates endosomal escape [75]. Ren et al. developed poly(amidoamine) (PAMAM) dendrimers that were simultaneously loaded with the antimetabolite 5-fluorouracil (5-FU), a chemotherapeutic drug, and anti-miR-21 in order to achieve in vitro co-delivery to human glioblastoma cells. miR-21 is an onco-miR that is overexpressed virtually in all human cancers. The co-delivery of anti-miR-21 significantly improved the cytotoxicity of 5-FU and dramatically increased the apoptosis of glioblastoma cells, while the migration ability of the tumor cells was decreased [76].

Gold nanoparticles

Gold nanoparticles have received attention as a gene delivery vector due to advantageous surface characteristics that allow easy functionalization with chemical and biological molecules and also due to their apparently low toxicity [77].

Gold nanoparticles were found to be noncytotoxic and nonimmunogenic [78], and their surface can be modified with functional groups, such as thiol and amino groups, enabling conjugation or complexation with different active and targeting moieties [79]. However, in most cases, oligonucleotides have been modified to facilitate the delivery [80]. Amino-functionalized gold nanoparticles complexed with miRNA, coated with PEG were developed by Ghosh et al. These nanoparticles were noncytotoxic, easily taken up by cells through endocytosis, and efficiently released the tumor suppressor miRNA, miR-31 and an oncogenic miRNA, miR-1323, to the target ovarian cancer and neuroblastoma cell lines, respectively [81].

Encapsulation

Another approach for the delivery of miRNA is encapsulation inside biodegradable nanoparticles. The cargo is released following intracellular dissolution of the particle. This method is advantageous since it does not require the use of potentially toxic cationic materials. However, the preparation of such carriers is more challenging compared to simple "mix and use" approach of complexation, and care needs to be taken not to damage the sensitive miRNA molecules in the process.

PLGA nanoparticles

Nanoparticles composed of poly(lactic-co-glycolic acid) (PLGA) are often used for enhancing the delivery of various therapeutic agents. PLGA is a biocompatible and biodegradable Food and Drug Administration-approved polymer, synthesized by co-polymerization of lactic acid and glycolic acid. Babar et al. [82] developed polymer nanoparticles composed of PLGA encapsulating polylysine-conjugated peptide nucleic acids (PNAs), which are chemically modified anti-miRNA molecules. Unlike most nucleic acids, PNAs have a charge-neutral backbone, which makes their encapsulation into polymer nanoparticles uniquely independent of electrostatic interactions between cargo and delivery vehicle. In another study, the PLGA nanoparticles were decorated with a cell-penetrating peptide, penetratin, to further enhance intracellular delivery. Systemic delivery of anti-miRNA PNAs encapsulated in those PLGA nanoparticles, inhibited miR-155 and slowed the growth of pre-B-cell lymphoma tumors in vivo [83].

Silica nanoparticles

Silica nanoparticles are stable and inert delivery vehicles with biodegradable and nontoxic properties both in vitro and in vivo [84]. Encapsulation of miR-34a in porous silica nanoparticles resulted in significantly decreased tumor growth, increased apoptosis, and reduced vascularization. The proposed mechanism of drug delivery was dissolution of the nanoparticle and release of the cargo under physiological conditions via hydrolysis of the silica network. The particles were conjugated to GD2 antibody to facilitate tumor-specific delivery following systemic administration into tumor-bearing mice [85].

Conjugation

Conjugation is an innovative method for the delivery of miRNA, where the nucleic acid is covalently bound to its carrier. Conjugation results in a highly stable delivery system that can effectively protect miRNA in the bloodstream. Conjugation can be achieved using different linkers that will release the cargo specifically at the target site either by hydrolysis or reduction. On the other hand, chemical conjugation is a complex procedure that also requires alteration of the miRNA molecule and can therefore affect its function. Kim et al. developed functionalized gold nanoparticles (AuNPs) coated with cargo DNA, which was then coupled to complementary DNA linked to an antisense miR-29b sequence. It was demonstrated that this convenient AMO delivery system successfully blocked miRNAs, resulting in the upregulation of its target protein, MCL-1 [86]. In another study, an aptamer- and miRNA-based cancer-targeting theranostic (*thera*py and diag*nostic*) fluorescent-magnetic nanoparticles were designed, in which miRNA was covalently conjugated. For targeted delivery, the authors used AS1411 aptamer targeting nucleolin protein, which is highly expressed on cancer cells. In order to detect and inhibit miR-221, which is highly expressed in papillary thyroid carcinoma, DNA molecular beacon (MB) containing perfectly complimentary oligonucleotides against miR-221 were used. miR-221 MB-conjugated magnetic fluorescence (MF) nanoparticles (MFAS miR-221 MB) were able to simultaneously target cancer tissue, image intracellularly expressed miR-221 and treat miR-221-involved carcinogenesis [87].

Conclusions and future prospective

miRNAs are key regulators in cellular pathways, and their dysregulation is linked to many pathologies, such as cancer. They contribute to cell proliferation, apoptosis, tumor progression, invasion, metastasis, and drug resistance. An important mission of translational medicine is to transform the novel research of the oncogenic and tumor-suppressive functions of miRNA into clinical applications. Two approaches can be used for the therapeutic application of miRNAs. The first approach is directed towards inhibiting miRNAs with oncogenic properties using miRNA antagonists, such as anti-miRNAs or LNAs. These oligonucleotides have sequences complementary to the endogenous miRNA. Enhancing their

affinity to the target miRNA is done by chemical modifications, which also help trapping the miRNA in a configuration that cannot be processed by the RISC. Although still controversial, these antagonists might lead to the degradation of the endogenous miRNA. The second approach uses miRNA replacement to restore a loss of function of a tumor suppressive miRNA. This approach represents a new strategy for miRNA therapeutic. Various delivery systems have been developed for the delivery of miRNA for cancer therapy. As miRNA therapeutics is a relatively new field, the variety of the carriers tested is limited and many of them have only been evaluated in vitro so far. However, since the chemical development is similar to siRNA, we expect to witness an exponential growth of new preclinical and clinical studies of miRNA delivery for cancer in the near future.

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