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Non-Viral Based miR Delivery and Recent Developments

Annalise Elizabeth Labatut and George Mattheolabakis*

Department of Basic Pharmaceutical Sciences, School of Pharmacy, University of Louisiana at Monroe

Abstract

miRNAs are promising therapeutic targets or tools for the treatment of numerous diseases, with most prominently, cancer. The inherent capacity of these short nucleic acids to regulate multiple cancer-related pathways simultaneously has prompted strong research on understanding miR functions and their potential use for therapeutic purposes. A key determinant of miR therapeutics' potential for treatment is their delivery. Viral and non-viral vectors attempt to address the major limitations associated with miR delivery, but several hurdles have been identified. Here, we present an overview on the general limitations of miR delivery, and the delivery strategies exploited to overcome them. We provide an introduction on the advantages and disadvantages of viral and non-viral vectors, and we go into detail to analyze the most prominently used non-viral systems. We provide with an update on the most recent research on this topic and we describe the mechanism and limitations of the lipid-, polymer- and inorganic material- based miR delivery systems.

Graphical abstract

*Corresponding author, Department of Basic Pharmaceutical Sciences, Room 380, School of Pharmacy, University of Louisiana at Monroe, 71201, Phone: (318) 342-7930, matthaiolampakis@ulm.edu.

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Keywords

Nanoparticles; non-viral vectors; miRNA therapeutics; delivery

1. Overview

During the recent years, microRNAs (miRs) have emerged as an attractive tool for regulating gene expression. miRs are single-stranded RNAs of ~20–22 nucleotides, and are natural endogenous products of the gene transcription process [1], similar in structure with the exogenous siRNAs. miRs, whose biogenesis has been described in detail elsewhere [2], are non-coding RNAs that utilize the cell's RNAi mechanism to target mRNAs post-transcriptionally, and downregulate their final expression [1] (Figure 1).

The RNAi mechanism was discovered in parallel with miRs, originally in the *Caenorhabditis elegans* genome. The Argonaute and Dicer proteins have a central and prominent role to the RNAi mechanism, being at the core of the RNA Induced Silencing Complex (RISC), a complex system of proteins that performs the gene silencing, by incorporating a miR strand into the RISC complex and targeting mRNAs with complementary sequences [3, 4]. To appreciate the importance and mechanistic potential of the miRs, one can simply realize that in order for the miRs to exert their action, either partial or full complementarity between the seed region of the miR and (primarily) the 3' untranslated region (3' UTR) of a messenger RNA is required [5, 6], although additional mechanisms of interactions have been reported [7]. This allows for a single miR to have multiple mRNAs as potential targets, thus being capable to influence different pathways. Acknowledging that there have been more than 5,000 miRs currently identified, with more than 3,500 just recently been added to our knowledge in 2015 [7], it is not surprising that miRs arise as critical post-transcriptional regulators of gene expression, and consequently of cell proliferation, apoptosis and differentiation, among others [1]. In fact, it is estimated that the miRs regulate >30% of the cell's genes [3].

Recurring reports indicate that the onset and progress of many diseases are associated by misexpression or dysfunction of miRs, with among such diseases being cancer biogenesis, progression and metastasis [8–11]. Profiling of miR expression between normal and cancer tissue and subsequent evaluation of the activity of miRs led to the development of two major classifications for the miRs, oncogenic or tumor suppressors [12]. As their name indicates, the former class of miRs is associated with being upregulated in tumor samples and promoting the development, growth or metastatic potential of tumors, while the latter is associated with being downregulated in tumor samples, having the capacity to inhibit tumor growth, proliferation, angiogenesis, metastasis, and inducing apoptosis or other mechanisms that impede cancer progression [12]. Indicatively, miR-155 is recognized as an oncogene, associated with tumorigenesis and metastasis and is consistently upregulated in tumors samples compared to normal tissues [13], while has demonstrated oncogenic capacity in breast [14, 15], lung [16], colorectal [17], prostate [18], and skin cancers [19], among others [13, 20]. In contrast, miR-34a is regarded as a master of tumor suppressor [21], having potential mRNA targets in critical pathways, such as Wnt1, Notch1, Wnt3, MTA2, CD44, MYC among others [22–27]. Not surprisingly, ectopic expression of miR-34a results in reduction to cancer cell proliferation [28], migration, invasion [29], and induces apoptosis, in colon [30], lung [31], pancreatic [32], liver [33], and breast cancer [34], among others.

Understandably, miR therapeutics have become of great interest as therapeutic targets or tools in the recent years. Unfortunately, their utilization as cancer therapeutics has been impeded by inherent hurdles regarding their efficient and safe systemic delivery *in vivo*. Among the major limitations are the instability and relatively short half-life of miRs in circulation, the limited cellular uptake, instability within the endosomal compartments and limited presence in the cytosol [35].

Although several modifications of the miR structure have been studied to improve miR stability, primarily originating from prior research on siRNAs, issues with the circulation time, parenteral administration and cell uptake have not been adequately resolved [36, 37]. To this end, nanovectors were the obvious next step. There have been two primary categories of nucleic acid delivery approaches, viral and non-viral vectors, both with advantages and disadvantages. Briefly, the former, as the name states, are based on genetically modified, non-pathogenic viral vectors, such as adenoviruses, capable to carry and deliver genetic material to the cells [38]. Their major limitation of the viral systems has been their immunogenic potential, creating significant side effects from the treatment, such as inflammatory responses, toxin production, even mortality [39].

In this review, we are focusing on delivery approaches based on non-viral methodologies, and we will provide an overview of current research on some of the major miR delivery systems, recognizing the existing limitations and barriers in delivering miRs.

2. Therapeutic action and delivery of miRs. Major limitations

miRs, similarly to the siRNAs, are water-soluble, making them appropriate for parenteral administration. Unfortunately, following i.v. injection, naked miRs are rapidly degraded by the abundant nucleases present in the extracellular and plasma environment, such as RNase

A-type nucleases [40]. Furthermore, the miRs tend to accumulate and be removed from the circulation to the liver and kidneys [41]. This behaviour results into a rapid drop in plasma levels within minutes post administration.

The duration of miR presence in the tissues primarily depends on whether the miRs will be uptaken by the cells and any structural modifications of the miRs that have taken place. Overall, chemical modification of the miR's structure attempts to enhance the stability, making them more resistant to degradation and hence improve their systemic presence. For example, 2' OH group modification of the ribose ring towards 2'-O-methoxyethyl, 2'-O-methyl or 2'-fluoro modification can improve stability and binding affinity for anti-miRNA nucleic acids [42]. Wang et al. [43], administered intravenously three 2'-methoxyphosphorothioate-miRs in mice at 7.5 mg/kg, and determined that the three miRs had a plasma half-life slightly improved compared to unmodified miRs, and tissue presence, detectable up to 24 h post injection. Alternatively, Cantafio et al. [44] indicated that locked nucleic acid LNA-miR-221 inhibitor, given i.p. 25 mg/kg to mice, was detected in tumor and tissue samples up to 7 days post administration. The LNA conformation of a miR, in which a methylene bridge "locks" the ribose ring connecting the 2'-O atom with the 4'-C atom, additionally enhances the affinity and efficiency of an antagomir to inhibit the action of a target miR [45].

Among the different parameters that contribute in the duration of the presence or action of miRs in the cells include the stage of the cell cycle, the expression of growth factors or other miRs, the plurality or extend of complementary targets, and the miR itself. It is important to note that the natural miR turn-over present in the cells can further affect the levels of an exogenous miR. The reader is encouraged to read the review paper by Rugger and Grosshans [46], who present these in detail. Furthermore, there is an inherent lag between the levels of miR in a cell, the suppression of a targeted mRNA translation, the destabilization and degradation of targeted mRNA, and eventually the downregulation (reduction) at the protein level. As it was demonstrated by Bazzini et al. [47], increased levels of miR-430 do not immediately cause the downregulation of the targeted mRNA, but a lag of approximately 2 h takes place, with prior slow translational repression for the targeted mRNA. Similarly, Subtelny et al. [48], injected one-cell zebrafish embryos with miR-155 and demonstrated that there was a lag between the induced high levels of miR-155 and the destabilization of targeted mRNA. These results indicate that there will be an inherent lag between the upregulation of a miR and an observed downregulation of a specific gene.

The pharmacokinetic properties of the miRs suggest that a sufficient concentration of the miR in tumor areas will be challenging to achieve, and often result in limited presence of the miR in the tissue. Furthermore, the abnormal tumor vasculature and the higher interstitial fluid pressure in the tissue further inhibits the delivery of miRs to the targeted tumor tissue [49, 50]. Finally, the tumor microenvironment has numerous cells of different types besides the malignant cells, such as macrophages, lymphocytes, adipocytes, T cells, among others, which in correlation with the complex extracellular matrix, poses an additional burden for any active compound to reach the tumor cells in sufficient quantities, including miRs [51]. The complex extracellular matrix makes it difficult for any compound to travel towards the

tumor cells from the already impaired vascular system, and the non-malignant cells will uptake miRs present in the tumor area. These parameters overall will result into significantly reduced presence of the miR in the tumor environment and low availability for the tumor cells to uptake them, without potentially reaching therapeutic concentrations intracellularly. In our analysis above, we describe the case for the tumor tissue to be located in areas outside the central nervous system. In the opposite scenario, the blood brain barrier (BBB), a diffusion barrier preventing the entry of most compounds from the blood to the brain [52], poses an additional challenge for the delivery of miRs [53, 54]. All these limitations, starting from the instability of the miRs in the circulation to the complex and multiple barriers for them to reach the tumor cells constitutes miR delivery a complex and challenging proposition for cancer treatment (Figure 2).

Assuming that naked miRs have reached the tumor cell area, by overcoming the barriers described above, there are additional barriers for the miR to reach the cytoplasm of the tumor cells, the cellular compartment that exerts its activity. The water soluble and negatively charged miRs are naturally repelled by the negatively charged phospholipids of the cell membrane. Furthermore, the cellular membrane consists of a bilayer of the phospholipids, with a highly lipophilic environment between the two layers, hence, the transition or diffusion of the miRs through the cellular membrane is not favorable. The main mechanism for cellular uptake is through endocytosis, with the formed endosomes eventually evolving towards the development of late endosomes/lysosomes, with gradual pH drop and the accumulation of digestive peptides, causing the degradation of the miRs [55]. The most important challenge for the miR delivery is the endosomal escape to the cytoplasm [56, 57]. Endosomal escape should take place during the early endosomal stage, and different approaches have been utilized to achieve it, some of which we will describe later.

Finally, one critical factor for regulating gene expression with miRs is the expression levels of the RISC complex. As we mentioned above, the RISC complex performs the gene silencing, by interacting the mature miR with the 3'-UTR region of a target mRNA. Although this is not a topic associated with the delivery of miRs, it is important to note that the activity of miRs is directly associated with the presence of the RISC complex and the expression of the RISC enzymes in the cell needs to be sufficient. Under certain conditions, it has been reported that the expression of the RISC proteins is downregulated, such as in hypoxic conditions [58]. Sufficient expression of the RISC complex in cells should be taken into consideration, during the evaluation of exogenous miR treatment, especially recognizing that limited RISC expression will result in a competition of the exogenous miR with the endogenous miR [41], overall diminishing the effectiveness of the treatment.

It is important to note, the miR delivery limitations and methodologies presented in this review have significant similarities or can expand to the delivery of siRNA, shRNA and other nucleic acid constructs. Although the mode of action of the different RNA-based molecules may differ to a certain extent, all three siRNA, shRNA and miRs are nucleic acids that need to be exogenously and systemically administered, thus the same limitations described here may apply [59, 60].

3. miR delivery approaches

The major obstacles for gene therapy is the efficient delivery of the nucleic acids to the targeted tissues, the efficient uptake by the cells, and the final localization of the nucleic acids to the cytoplasm. As we mentioned above, there have been two major categories of carriers that researchers use, viral and non-viral carriers. The authors do not suggest that either type of carriers is the most promising solution for gene therapy, because both systems have significant advantages and disadvantages, with arguments towards the optimal approach to be possibly made for both. The focus of this review is on non-viral vectors.

Ideally, the miRs should be protected in the circulation from degradation, and be directed to the desired tissue. Viral vectors were quickly recognized for their capacity to efficiently carry nucleic acids and deliver their genetic material into the cell [39].

The viral vectors demonstrate great capacity in transfecting cells *in vitro* and *in vivo*, easily entering cells, producing high expression of the introduced gene, and are able to induce stable transfections. Unfortunately, the associated high cost and difficulty in production, potential mutagenesis, and immunogenic behavior have prompted for alternative approaches [61, 62].

In contrast, non-viral vectors were utilized to address some of the safety concerns associated with the viral vectors, primarily the cost, immunogenicity and toxicity. Although the non-viral vectors are less efficient to transfect cells, significant progress in their development has steadily introduced them to miR delivery with encouraging results. Primarily, this is attributed to the overall progress of nanotechnology in the field of drug delivery, and the prior, existing knowledge was quickly adjusted to meet the needs of miR delivery.

Nanotechnology has provided significant improvements on the delivery of molecules with low aqueous solubility, short circulation life, or unfavorable pharmacokinetics [63]. Similarly, nanotechnology methods have been utilized to overcome some of the limitations described above associated with the delivery of miRs.

3.1 Lipid based drug carriers

Since the development of the first liposomes, lipids have had a prominent role on the development of novel drug delivery approaches, with significant existing research on liposomal drug delivery, and most prominently the development of Doxil, the first liposomal formulation used to treat cancer patients [63]. Liposomes are composed of a lipid bilayer and an internal aqueous phase, which allows them to easily carry water-soluble active compounds, while they are easily uptaken by cells through endocytosis [63]. Through surface modification of the liposomes with neutral polymers, such as PEG, prolonged circulation times can be achieved *in vivo*, in order to take advantage of the EPR effect in cancers [63], and with the attachment of lipopeptides, fusion of the liposomes with the cell membrane can be enhanced for direct delivery of the load to the cytoplasm [64, 65].

Similarly, lipids/liposomes are utilized to entrap nucleic acids for delivering to the cells. Positively charged lipids are predominately used for miR delivery to create lipoplexes, a

complex occurring by the binding of the negatively charged miRs on the positively charged lipids [66]. Furthermore, the positively charged lipids enhance interaction between the formed liposomes and the negatively charged cell membranes, enhancing cellular uptake [65]. Unfortunately, the cationic lipids demonstrate a dose-dependent toxicity against all types of cells, with higher toxicity identified against macrophages, a phenomenon attributed to the higher phagocytic activity of these cells [67–69]. In fact, high levels of lipoplexes can lead to cell lysis and necrosis [70].

Furthermore, cationic lipoplexes induce immune activation, associated with the systemic release of IL-6, IL-12 and IFN- γ , indicating a pro-inflammatory response [71]. Possible toxicity from the cationic lipids can be abrogated to a certain extent during the liposomal formulation, by using a mixture of cationic and anionic or neutral lipids, in addition to the inclusion of PEG polymers on the surface of liposomes [72]. Although PEGylation assists in the stabilization of the formed lipoplexes and promotes increased circulation times *in vivo* [73–75], it negatively affects the efficiency of the lipoplexes to be uptaken by and transfect cells [76], sterically inhibiting interaction between the nanocarriers and the cells [77]. Nonetheless, the liposomes efficiently protect the miRs while in circulation and can enhance endosomal uptake or directly deliver the miRs to the cytoplasm by fusion with the cell membrane [72].

Lipoplexes have been used in several applications for the delivery of miRs. Indicatively, Wu et al. [78] developed lipoplexes with miR-29b for the treatment of lung cancer *in vitro* and *in vivo*. miR-29b downregulation has been reported in lung cancer samples, and upregulation of the miR-29b in lung cancer cells can inhibit migration and invasion. The authors developed lipoplexes by the prior preparation of empty liposomes using the cationic 1,2-Di-O-octadecenyl-3-trimethylammonium propane, cholesterol and TPGS, and subsequent incubation of the liposomes in the presence of miR-29b mimics [78]. The lipoplexes demonstrated strong cellular uptake, upregulation of miR-29b *in vitro* and *in vivo*, and strong tumor growth inhibition. Similarly, Sharma et al. [79] studied the development of cationic liposomes for the delivery of anti-miR-191 for the treatment of breast cancer. They developed lipoplexes using the cationic stearylamine, along with phosphatidylcholine and cholesterol, with the complexation with the anti-miR taking place during the preparation of the liposomes. The lipoplexes were efficiently uptaken to the cells *in vitro*, and induced strong cytotoxic and apoptotic activity, suppressed the migration of the cancer cells and increased chemosensitivity of the cancer cells to chemotherapeutics drugs *in vitro*.

3.2 Polymer based nanocarriers

Polymeric nanocarriers have a long and promising presence in drug delivery in general. A plethora of studies have demonstrated the capacity of polymer-based nanocarriers to entrap active ingredients, improve their aqueous solubility, protect them from degradation and deliver them *in vivo* to the desired tissue [63]. Similarly, polymers, such as the biodegradable and biocompatible poly(lactic-co-glycolic acid) (PLGA) polymer, and the polyethyleneimine (PEI) polymer, have been commonly utilized for nucleic acid delivery.

In particular, PEI has attracted significant attention due to its unique capacity to transfect cells *in vitro* and *in vivo*, and to deliver nucleic acids, including miRs, to the cytoplasm. This

is due to the inherent properties of the polymer. The PEI, similar in structure with PEG but with the replacement of the oxygen groups with nitrogen, consists of multiple positively charged amines (primary, secondary and tertiary amino groups, depending on the linear or branched structure of the polymer). The positive charge of the polymer is suitable for condensing with nucleic acids, which are negatively charged, forming polyplexes [96]. The polyplexes can enter the cells through endocytosis [97, 98], with the primary cause for the endosomal escape to be a proposed mechanism called “proton-sponge” [99]. Briefly, due to the nitrogen-based structure of the polymer, PEI appears to have considerable buffering capacity [100], with the unprotonated amines to be able to absorb protons entering into the lysosome. This overall induces an increased influx of chloride anions and water, due to osmotic pressure, and the resulting swelling of the endosomes eventually leads to rupture of the endosomal membrane and subsequent release of its contents directly into the cytoplasm [99]. Overall, the PEI is recognized for its excellent capacity to complex with miRs and transfect cells.

Nonetheless, it is important to note that the PEI has been associated with significant toxicity. Initially, PEI polymers of different structure exhibit different cytotoxicity, as was demonstrated by Kafil et al. [101], who compared linear and branched PEI polymers (25kD) and determined that the branched PEI exhibited higher cytotoxicity compared to linear, although the authors also mention that the branched PEI exhibited higher cell uptake and transfection efficiency compared to its linear counterpart. Furthermore, the molecular weight of the PEI polymer has significant effect on its toxicity, with lower molecular polymers exhibiting lower cytotoxicity (i.e., 1,800) compared to larger (>10,000) [102]. Overall, it is mentioned that the transfection efficiency of the polymer also diminishes with the reduction of its molecular weight, although contradicting research exists [102, 103]. Thus, a balance between transfection efficiency and cytotoxicity should be met, with the molecular weights between 1,800 and 10,000 being the most promising on this aspect.

Alternatively, the PLGA polymers are also commonly used in miR and drug delivery in general, due to their versatility (different molecular weights with different compositions endow different properties to the final formulation), biocompatible and biodegradable properties, and the development of stable nanoformulations for sustained drug release, while protecting the active compound from degradation *in vivo* [63]. Furthermore, the PLGA polymers can further be modified, for example with PEG, to achieve pro-longed circulation *in vivo*, with peptides or antibodies, to achieve active targeting to cells, or other positively charged polymers (e.g. poly-lysine) to induce complexation with the negatively charged nucleic acids [104]. Interestingly, PLGA nanoparticles efficiently escape the endo-/lysosomal compartment to the cytosol, due to selective reversal of the surface charge of the polymeric nanoparticles in the increasingly acidic environment of the endosomes, changing from anionic to cationic. This behavior promotes interaction between the nanoparticles and the endosomal membrane, and their escape to the cytosol [105]. On the other hand, pegylation can have detrimental effect on the endosomal escape of the nanoparticles, despite the overall improvement on the pharmacokinetics of the nanoparticles and the tumor area accumulation through the EPR effect [106]. Unlike the PEI polymer, the PLGA polymers are considered non-toxic and there are currently several FDA-approved products based on these polymers [107].

Not surprisingly, the PLGA polymers are extensively studied for miR delivery. Indicatively, Bhargava-Shah et al. [108] developed nanoparticles with PLGA-PEG polymer for the delivery of anti-miR-21 for the treatment of triple negative breast cancer, and determined that the treatment sensitized the cells to Orlistat treatment, which was proposed as a novel repurposing drug for breast cancer treatment. In another study, Cai et al. [104] developed a nanoparticle formulation with monomethoxy (polyethylene glycol)-poly(D,L-lactide-co-glycolide)-poly(L-lysine)-lactobionic acid- anti-vascular endothelial growth factor antibody (mPEG-PLGA-PLL-LA/VEGFab) polymer for the delivery of miR-99a for the treatment of hepatic carcinoma. In their study, they utilized active targeting against VEGFab for the delivery of the miR, and observed a downregulation of the targeted genes, resulting to reduced proliferation, migration and invasion of HepG2 cells. Furthermore, the proposed therapy inhibited tumor growth in a subcutaneous xenograft mouse model of the disease.

Another class of polymeric structures that find increasing interest in nucleic acid delivery are dendrimers. Dendrimers are highly branched polymer-based molecules, with modifiable surfaces [63]. Some of the polymers used for dendrimer-based miR delivery are polyamidoamine (PAMAM), Poly(propylene imine) (PPI), and Poly-L-lysine (PLL), which are cationic and allow the complexation with the nucleic acids, and the large number of tertiary amines allow them to escape the endosomal compartments through the “proton sponge” effect [109], similar to PEI. Polymeric carriers for the delivery of miRs *in vitro* and *in vivo* are very promising. The versatility of the polymeric structures, with the capacity to combine cationic, anionic or neutral polymers in the same structure, the ability to control drug release, by controlling the polymer composition, and the capacity to easily modify the surface of the resulting nanocarriers provide the polymeric constructs significant benefits for nucleic acid delivery. In Table 2, we illustrate representative examples of polymer based research for the years 2015 to 2017.

3.3 Inorganic material based carriers

Nanocarriers developed with inorganic materials are particularly unique transport vehicles for non-viral gene delivery because they can potentially be biocompatible, non-immunogenic, nontoxic, and can be easily manufactured on a large scale. Among the different types of inorganic nanoparticles, gold nanoparticles (AuNPs) have shown great promise due to their advantageous physico-chemical properties, specifically biocompatibility, amphiphilicity, large surface area, and ease of surface functionalization. Gold-based nanocarriers also have undesirable attributes, such as low encapsulation efficiency, poor storage stability, and a slow endosomal escape [132].

AuNPs have been used to effectively deliver miRs to prostate and breast cancer cells *in vitro*. Ekin et al. [133] hybridized the tumor suppressor miR-145 to RNAs that had been chemically attached to 13-nm AuNPs via thiol oligonucleotide conjugation. This conjugation gives AuNPs a high affinity for biomolecules. These AuNPs were successfully transfected into the PC3 line of human prostate cancer cells and the MCF7 line of human breast cancer cells.

Silica-based vehicles have also had success as inorganic delivery systems [41]. Zheng *et al.* [134] designed a silica-based nanocarrier that co-delivered sorafenib and a vascular

endothelial growth factor (VEGF) targeted siRNA (siVEGF) for the treatment of hepatocellular carcinoma. They identified that the nanoparticles with the combinatorial treatment were successfully uptaken by the cells, with minimal toxicity, induced strong cell cycle arrest at the S phase, enhanced the cytotoxicity of the drug and downregulated the expression of VEGF.

Although inorganic materials for the delivery of miRs is promising, limited understanding exists on their capacity to protect their payload *in vivo* from degradation, and their capacity to successfully escape the endosomal compartment [41].

3.4 Clinical applications of miR therapeutics

Despite the recent discovery of miRs and the significant limitations associated with their delivery, their clinical significance has been recognized and efforts take place to translate miR-based therapeutics to patient care.

In Table 4, we present some of the miR-based therapeutics currently in clinical trials [142, 143].

4. Conclusion and Future Perspectives

miR therapeutics are a promising approach for the treatment of numerous diseases, among them, cancer. The recent discovery of the miRs indicates that there is significant research remaining that needs to take place, to fully understand their function and capabilities, and intensified efforts are required. Non-viral vectors are contributing to this purpose, and their rapid development and plurality of capabilities, allow researchers to progress in further understanding the potential of miR therapeutics. However, in order to translate any work on the non-viral vectors to clinical applications, some issues need to be addressed, such as their potential toxicity. Furthermore, non-viral vectors need to efficiently target specific tissues, to maximize therapeutic efficacy and minimize side-effects. Targeted delivery, through active targeting, is an approach that researchers have made great efforts to address and is associated not only with miR delivery, but drug delivery in general. Thus, significant developments on the miR-delivery utilizing non-viral vectors should be anticipated. Nonetheless, the overall progress for the development of miR therapeutics in the short period since their discovery is noteworthy.

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List of Abbreviations

EPR	Enhanced permeability and retention
PEG	poly(ethylene glycol)
FDA	Food and Drug administration

PLGA	poly(lactide-co-glycolide)
PLL	poly(L-lysine)
PAMAM	polyamidoamine
PPI	Poly (propylene imine)
DOTAP	1, 2-Dioleoyl-3-Trimethylammonium-Propane
DOPC	1,2-dioleoyl-sn-glycero-3-phosphatidylcholine
HSPC	hydrogenated soybean phospholipid
DODAP	1,2-dioleoyl-3-dimethylammonium-propane

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Figure 1. Biogenesis and mode of action for miRNAs

miR biogenesis includes the transcription of the pri-miRNA from the respective gene and its cleavage by the drosha enzyme, followed by the transfer of the produced pre-miRNA by the exportin 5 proteins to the cytoplasm and the creation of the mature duplex miRNA, with the assistance of the Dicer protein. (Reproduced from [144], authored by B. Ryan, G. Joilin and J. Williams. Copyright 2015 Ryan, Joilin and Williams)

Figure 2. Tumor microenvironment and the plethora of cells and structures present
The complexity of the tumor microenvironment includes numerous cells of different types, such as lymphocytes, fibroblasts, endothelial cells and other, along with a complex vascular and extracellular matrix, adding to the difficulties associated with cancer drug delivery in general. (Reproduced with permission from [51]. Copyright the Company of Biologists Ltd.)

Figure 3. Illustration of *in vivo* miR-delivery barriers in cancer therapy
Several hurdles are associated with systemic miR delivery, such as the RES clearance, complex extracellular matrix and environment around the tumor cells, off-targeting and poor cellular uptake. (Reproduced with permission from [41]. Copyright Elsevier 2015.)

Table 1

Representative research in recent years on miR therapeutics based on lipoplexes

miRNA	Disease	Carrier	Reference
Anti-miR-124	Planarian brain regeneration	Liposomes with cationic lipids with symmetric (di-octadecyl, Sym-Lip) and asymmetric (octadecyl and oleyl, Asym-Lip) hydrophobic chains	[80]
miR-148a and miR-152	Pregnancy prevention as contraceptive	Cationic liposomes	[81]
miR-101	Hepatocellular carcinoma	Liposomes with DOTAP	[82]
miR-603	Breast Cancer	Liposomes with dimyristoyl-sn-glycero-3-phosphocholine (DMPC)	[83]
miR-215	Colorectal Carcinoma	MaxSuppressor™ In Vivo RNA-LANCer II	[84]
miR-145	Formulation optimization	Liposomes with DOTAP	[85]
Anti-miR-21	Lung Cancer	QTsome with DODMA/DOTAP/DOPC/CHOL/mPEG-DPPE	[86]
miR-630	Ovarian Cancer	Liposomes with DOPC	[87]
miR-375	Hepatocellular Carcinoma	Liposomes with DOTAP	[88, 89]
miR-34	Lung Cancer		[90]
miR-145	Myocardial Infarction	Lipofectamine	[91]
Anti-miR-221	Hepatocellular Carcinoma	Liposomes with HSPC	[92]
Anti-miR-145	Pulmonary hypertension	Liposomes with Staramine	[93]
Anti-miR-21	Glioblastoma	Liposomes with DODAP	[94]
Anti-miR-191	Breast Cancer	Liposomes with Stearlyamine	[79]
Anti-miR-10b	Inhibition of Breast Cancer metastasis	Liposomes with DOTAP	[95]

Table 2

Representative research in recent years on miR therapeutics based on polyplexes

miRNA	Disease	Polymeric system	Reference
miR-99a	Hepatic Carcinoma	mPEG-PLGA-PLL-LA/VEGFab	[104]
miR-26a	Bone Defects regeneration	PLGA	[110]
miR-199a	Osteosarcoma	Dextran acrylate	[111]
miR-223	Inhibition of macrophage fusion	PLGA	[112]
miR-145	Breast Cancer	Chitosan	[113]
Anti-miR-21	Hepatocellular Carcinoma	PLGA-PEG	[114]
miR-146a/b	Inflammation Response and Tissue Regeneration	PEG-PEI	[115]
miR-34a	Multiple Myeloma	Chitosan/PLGA	[116]
Anti-miR-221		PLGA/acetylated PEI/hyaluronic acid	[117]
Anti-miR-10b/21	Breast Cancer	PLGA-PEG	[118]
miR-125b	Lung Cancer	HA-PEG/PEI	[119]
miR-122	Colon Cancer	PLGA-PEG	[120]
miR-34a	Breast cancer	Ethylenediamine modified BSA	[121]
miR-34a	Prostate Cancer	Chitosan	[122]
Anti-miR-126	Acute Myeloid Leukemia	PEI/liposomes	[123]
miR-708	Lung Cancer	PEI	[124]
miR-146a	Renal Fibrosis	PEI	[125]
miR-223	Inflammatory Regulation	HA-PEI/PEG	[126]
Anti-miR-21	Gastric Cancer	poly(ϵ -caprolactone) (PCL)-poly (ethylene glycol) (PEG)	[127]
miR-34a	Lung Cancer	PAMAM dendrimers/PEG	[128]
Anti-miR-21	Breast Cancer	PLGA-PEG	[129]
miR-221/222	Nerve Regeneration	2,6-pyridinedicarboxaldehyde-PEI	[130]
Anti-miR-214	Osteoporosis	Polyurethane	[131]

Table 3

Representative research in recent years on miR therapeutics based on inorganic material

miRNA	Disease	Carrier	Reference
anti-miR-221	Drug-resistant glioma	Silica Nanoparticles	[135]
miR-29b	Tissue engineering and regeneration medicine	Gold-PEI nanoparticles	[136]
Anti-miR-21	Breast cancer	PAMAM modified gold nanoparticles	[137]
miR-122	Liver Cancer	Gold nanoparticles on graphene nanocomposites	[138]
Anti-miR-155	Macrophage delivery	Gold nanoparticles	[139]
miR-375	Hepatocellular Carcinoma	Gold nanoparticles	[140]
Anti-miR-21	Pancreatic Cancer	Polyethylenimine-magnetic iron oxide	[141]

Table 4

miRNA-based therapeutics that have advanced to the clinical trials.

Therapeutic	miR	Disease	Stage	Agency/Company
MRX34	miR-34	Cancer treatment	Phase I	Mirna Therapeutics
LNA miravirsen	AntimiR-122	Hepatitis C virus infection	Phase II	Santaris Pharma
RG-101	AntimiR-122	Hepatitis C virus infection	Phase II	Regulus Therapeutics
RG-012	AntimiR-21	Alport Syndrome	Phase I	Regulus Therapeutics
MesomiR-1	miR-16	Malignant pleural mesothelioma or NSCLC	Phase I	EnGeneIC/Asbestos Diseases Research Institute
MRG-201	miR-29	Scleroderma	Phase I	miRagen Therapeutics
MRG-106	antimiR-155	Cutaneous T cell lymphoma	Phase I	miRagen Therapeutics
RG-125	antimiR -103/107	Non-alcoholic steatohepatitis	Phase I	Regulus Therapeutics