**Lipid Nanocarriers for microRNA Delivery**

ABSTRACT: Nanomedicine and microRNA (miRNA) research are innovative and young scientific fields that offer interesting perspectives for future diagnostic and therapeutic directions in precision medicine. In this article, we describe the progress in the development of therapeutic lipid-based nanoparticles for miRNA delivery, outline challenges and opportunities for advanced miRNA-based therapies, and discuss the complexities associated with the delivery of functional miRNAs. At present, toxicity issues, specific targeting of diseased sites, proper cellular uptake and endosomal escape of miRNA are among the most critical challenges facing miRNA delivery. Novel strategies how to deal with these limitations are addressed in this review. We will also discuss current fields of application and provide an overview of preclinical settings involving miRNA therapeutics and give an outlook to the future of such therapeutic approaches with special focus on lipid based delivery systems. Following the current trends and technological developments in nanomedicine exciting new delivery systems for oligonucleotide based therapeutics can be expected in upcoming years.

**1. Introduction**

MicroRNAs (miRNAs) are non-coding RNA molecules that play a central role as regulators of gene expression. MiRNAs are single-stranded RNAs with 19- to 25 nucleotides that can prevent messenger RNA (mRNA) translation. Unlike double-stranded short interfering RNAs (siRNAs) that only target a specific single gene, miRNAs may target up to 500 different mRNAs (Mack, 2007). Accordingly, miRNAs are deeply involved in different cellular processes such as differentiation, proliferation, apoptosis or autophagy. Yet, a dysregulation of miRNAs can cause numerous pathological disorders like cancer, atherosclerosis or cardiovascular diseases (Feinberg and Moore, 2016; Iorio and Croce, 2012; Rupaimoole et al., 2016a; Rupaimoole and Slack, 2017). Especially, the capacity of miRNAs to target multiple mRNAs that are altered in disease conditions makes these molecules highly interesting candidates either as therapeutics or as therapeutic targets. MiRNA based therapeutics are applied either as synthetic double stranded small RNA molecules, so called miRNA mimics or as miRNA antagonists. The function of miRNA mimics used in miRNA replacement therapy is to bind to the target gene, induce the expression of silenced genes and restore miRNA function (Hosseinahli et al., 2018; Rothschild, 2014; Wang et al., 2016). In contrast to miRNA mimics, miRNA antagonists are single-stranded antisense oligonucleotides (ODN), which have a complementary sequence to the miRNA to be targeted and silenced (Rupaimoole and Slack, 2017).

To be therapeutically applicable miRNAs typically require a delivery system to improve their efficiency *in vivo* and to enhance their therapeutic index. Thus, miRNAs are encapsulated into various nanocarriers, which have the ability to provide a higher molecular stability and efficient protection from nuclease digest by nucleases abundantly present in serum (Chen et al., 2015). In principle, the nanoparticles have to be specifically designed to permit an effective transport of high concentrations of active miRNAs to the target organs and enable cellular entry by endocytosis followed by endosomal escape and cytosolic release. Among non-viral delivery systems lipid-based vectors are the most investigated carrier systems. Some of the lipid-based formulations that are developed for the delivery of DNA/RNA or non-coding RNA (ncRNA) are already in clinical practice, predominantly for cancer therapy (for a recent review see (Campani et al., 2016)). A great advantage of lipid nanocarriers is the biodegradability and biocompatibility of their individual components. Likewise, most lipid nanoparticles show tolerable toxicity and low immunogenicity. Moreover, nanoparticles assembled from lipids are versatile in their physicochemical characteristics attributable to differences in chemical composition, size variability and particle surface properties. All these parameter can be easily tuned and adjusted. Usually, lipid particles are also easy to prepare and scale-up procedures for large-scale manufacturing are readily available. The most used preparation methods for lipid based nanoparticles were recently reviewed by Patil (Patil and Jadhav, 2014) and Pattni (Pattni et al., 2015). Among them the most prominent techniques are conventional thin lipid film rehydration techniques followed by size extrusion procedures, ethanol-lipid dilution techniques or more recently microfluidics-based rapid mixing procedures. An exemplary scheme for the preparation of miRNA loaded liposomes using microfluidic devices is presented in Figure 1. Notably, the behavior of the nanoparticles might be influenced by the manufacturing technique. For some cases, it was shown that the same substances formulated into nanoparticles by microfluidics performed much better than those formulated by extrusion techniques (Chen et al., 2012; Valencia et al., 2012; Wan et al., 2014).

Most commonly, the lipid nanoparticles are coated with polymers offering the opportunity to increase particle stability in plasma leading to prolonged half-life time in circulation (Barenholz, 2001). In many studies, polyethylene glycol (PEG) with a mean molecular weight of 2000 is used as polymer chain conjugated to phosphatidylethanolamine (PE-) lipids. When the PEGylated lipid is used in a defined concentration range between 3-5 mol % PEG2000-PE of total lipids, a polymer coat is formed that tightly surrounds the lipid nanoparticle and confers sterically stabilization to the particles without inducing particle deformation (Marsh et al., 2003). The inherent hydrophilicity and slightly negative charge of the PEG shell diminishes particle uptake by the reticuloendothelial system (RES) and significantly prolongs particle´s life-time in blood circulation (Zylberberg and Matosevic, 2016).

Another attractive approach to improve drug delivery efficacy is to conjugate targeting sequences onto the surface of the nanoparticles to achieve a more specific recognition at diseased sites or target organs. Such ligands include peptide sequences, proteins, specific antibodies or aptamers (Belfiore et al., 2018; Torchilin, 2005). Ligand targeted liposomes enable an active targeting of cell receptors or specifically surface exposed biomarkers. Alternatively, passive accumulation of nanoparticles in tumor tissues can be attained by the so called enhanced permeability and retention (EPR-) effect. Owing to the overall leaky structure of the tumor vasculature, which is induced by gaps of varying size between the endothelial cells and widespread fenestrations of blood vessels, an enhanced extravasation of nanoparticles in the tumor microenvironment is gained (Miao and Huang, 2015). The longer retention time for nanoparticles in tumor tissues is attributed to the lack of efficient lymphatic drainage (Bazak et al., 2014).

Particle size is a key factor for determining the *in vivo* fate of the particle involving cellular uptake mechanisms, biodistribution profiles and clearance rates. Following these lines, therapeutic nanoparticles for intravenous applications need to have an optimal size that typically ranges between 30-200 nm. Bigger particles are mostly taken up by the RES for phagocytotic clearance, while smaller nanoparticles with the size of only a few nanometers are rapidly cleared by renal excretion (Choi et al., 2007). Apart from nanoparticle size other physicochemical parameter like chemical nature of lipids, charge or particle shape are relevant features for delivery purposes. For example, charged nanoparticles might be opsonized and removed more rapidly from circulation than their neutral counterparts (Carrstensen et al., 1992). Yet, different clearance rates are reported for negatively and positively charged liposomes depending on their propensity to interact nonspecifically with oppositely charged plasma components and their intrinsic tendency for particle aggregation (Deshpande et al., 2013; Miller et al., 1998). Once in circulation, nanoparticles adsorb plasma proteins to become surrounded by the so-called protein corona, which might readily affect their biodistribution and pharmacokinetics (Bertrand et al., 2017). Often the protein corona impairs targeting specificity and cellular uptake. On the other hand, for some lipid-based delivery systems it is essential to interact with plasma components like lipoproteins after their entry in the bloodstream to be directed to hepatocytes in the liver for the active treatment of liver diseases (Akinc et al., 2010; Wolfrum et al., 2007). This exemplifies the fact that nanocarriers have to be flexibly adopted to meet individual treatment requirements.

In any case, nanocarriers for the successful delivery of miRNAs need to be carefully designed and optimized in terms of lipid composition, particle size, charge distribution, miRNA to lipid ratio and encapsulation efficiency. Additionally, surface coatings and coupling of targeting moieties are important assets to improve particle stability, circulation lifetime and tissue specificity. Particularly for miRNA therapy the biggest challenges to be met are acceptable toxicity, tissue specific targeting and proper cellular uptake as well as cytosolic release of the miRNA cargo.

In this review, we will focus on the design and applicability of the lipid-based delivery systems for miRNA delivery rather than on specific miRNAs to be transported, as we expect the delivery vehicles to be similar effective irrespective of which miRNA molecule is encapsulated. Rather, we proceed from the assumption that the cellular species and the target organ to be addressed determine which kind of nanoparticle assembly is most appropriate. In this respect, lipid-based assemblies and liposomes serve as flexible nanoparticle platform for the delivery of drugs including nucleic acid derived therapeutics. To date most studies are reported on the delivery of siRNA, however, it can be expected that the physicochemical characteristics of the nanoparticle will not significantly change using different sequences of nucleic acids. Hence, the delivery systems appear to be adequate for both siRNA and miRNA molecules as well, making lipid-based nanoparticles to a universal delivery vehicle for oligonucleotide based drugs. Moreover, all systemically administered oligonucleotides face similar physiological hurdles that might impede their therapeutic applicability. In circulation, they have to escape enzymatic degradation and rapid clearance by the immune system. They have to reach their target cells, accomplish cellular uptake to be released in the cytosol (Kanasty et al., 2013; Tibbitt et al., 2016; Whitehead et al., 2009).

**2. Lipid platform for nucleic acid delivery with specific focus on miRNA delivery**

At present, various different lipid systems have been explored for gene transfection or ODN delivery. In the following, some of them will be discussed in more detail, supported by representative examples of their potential therapeutic applications with special focus on miRNA deliver. However, this review does not take into account approaches using commercially available lipid-based transfection reagents as such reagents are primarily intended for *in vitro* cell culture experiments. Although currently significant efforts are being made to develop new transfection reagents that can be used for *in vivo* gene delivery in preclinical settings.

*2.1. Cationic lipoplexes*

Among lipid-based nanoparticles cationic liposomes, often termed lipoplexes, are the most prominent and widely explored delivery vehicles for DNA (Felgner et al., 1987; Zuidam et al., 1999), mRNA (Islam et al., 2015), siRNA (Xia et al., 2016) or ODN (Garbuzenko et al., 2009). For the development of lipoplexes a great variety of different cationic lipid molecules are available which are mostly used in combination with a zwitterionic “helper” lipid, such as 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). In general, the cationic lipid molecules spontaneously interact with the negatively charged nucleic acid molecules to form a stable complex via electrostatic interactions. Among such cationic lipids, 1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP) is the most prominent one that was synthesized first about 30 years ago (Leventis and Silvius, 1990). DOTAP has a quaternary amino headgroup linked to a glycerol backbone with two C18:1 chains. At physiological pH DOTAP is fully protonated and due to its biodegradable ester bond DOTAP shows a relatively low toxicity. Upon particle formation due to self-assembly of DOTAP molecules the nucleic acid molecules become sandwiched between single lipid bilayers. The inter-lamellar spacing of DOTAP/oligonucleotide lipoplexes was determined by small angle X-ray scattering (SAXS) and transmission electron microscopy (TEM) to be about 4.9 nm, and the DOTAP bilayer thickness as 3.72 nm. The interbilayer aqueous layer thickness of about 1.2 nm is appropriate to accommodate sufficient amounts of nucleic acid molecules (Weisman et al., 2004). The positive surface charge of the nanoparticles further promotes the association with the negatively charged cell surface. N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) is structurally similar to DOTAP but has an ether bond to link the headgroup to the acyl chains instead of an ester bond. While DOTAP and DOTMA are monovalent cationic lipids, 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-l-propanaminium trifluoroacetate (DOSPA) and dioctadecylamidoglycylspermine (DOGS) are multivalent with different ammonium groups. In DOSPA a multivalent spermine group is linked via a peptide bond to a quaternary amine conjugated to the hydrophobic alkyl chains, which are monounsaturated. DOGS is structurally very similar to DOSPA but lacks the quaternary amino group and has two saturated 18-carbon alkyl chains. Due to the high affinity of the spermine head group to nucleic acids, the oligonucleotides can be more efficiently bound. DOSPA and DOGS are the major components of the commercially available transfection reagents Lipofectamine and Transfectam, respectively. Another cationic lipid that is frequently used for transfection is DC-Chol (3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol hydrochloride) that contains a tertiary amine in the dimethylethylenediamine headgroup that is conjugated via an ester bond to a cholesterol moiety (Balazs and Godbey, 2011). A big advantage of DC-Chol is its very low toxicity confirmed by measurements on various mammalian cell lines (Gao and Huang, 1991). A list of the most largely used cationic lipids for lipoplex formation is provided in Figure 2.

The aim of helper lipids incorporated into lipoplexes is to promote a pH-dependent conversion of a lamellar structure to a non-lamellar phase due to the geometry of the helper lipid. DOPE, for example, has a conical shape with a cross-sectional area of the headgroup that is much smaller than the hydrophobic tail region and thus the packing parameter exceeds 1 (Israelachvili and Mitchell, 1975). Thus, DOPE tends to induce a lamellar to inverted hexagonal phases transition at low pH through hydrogen bond formation, and so exerting a destabilizing effect on endosomal membranes to facilitate fusion and cytosolic release of nucleic acids. The effects of fusogenic neutral helper lipids on lipoplex assembly and structural features relevant for endosomal uptake and release are reviewed elsewhere in more detail (Balazs and Godbey, 2011; Wasungu and Hoekstra, 2006). Cholesterol can also be useful as helper lipid that resides in the hydrophobic acyl chain region in the lipid bilayer. Higher concentrations of cholesterol (about 30-40%) incorporated in the lipid bilayer significantly decrease the permeability of the lipid membrane making it more rigid. Through the addition of cholesterol the liposomes become stabilized *in vivo* and the exchange of lipid molecules occurring between liposomes and lipoproteins or circulating cells becomes significantly reduced. In addition, cholesterol shields the oligonucleotides from nuclease degradation increasing *in vivo* transfection efficiency of lipoplexes (Sercombe et al., 2015).

Another lipid formulation was created to display a highly ordered bicontinuous cubic phase as internal structural motif revealing a high colloidal stability combined with steric stabilization. Such nanoparticles called PEGylated cuboplexes are primarily composed of glycerol monooleate (GMO), DOTAP and a GMO lipid conjugated to polyethyleneglycol. Due to the cubic internal structure of the particles they can be loaded with large amounts of oligonucleotides. These systems are special in terms of particle-endosomal membrane interactions that are controlled by elasticity energetics and topologically activated mechanisms rather than by electrostatics (Kim and Leal, 2015).

In the following we provide some representative examples for lipoplex based delivery systems with specific focus on miRNA delivery. Most of the lipoplex formulations for miRNA are intended for cancer therapy. E.g. lipoplexes loaded with pre-miRNA-133b were examined for the treatment of lung cancer (Wu et al., 2011). The samples were prepared by ethanol injection technique using a lipid mixture of DOTMA/cholesterol/ D-α-tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS). The final ethanol concentration in the solution was 10%. Vitamin E TPGS was added to increase particle stability and circulation time in blood. The empty liposomes were incubated with the pre-miRNA-133b and used immediately after complexation (Jin et al., 2010). The efficacy of the lipoplexes was evaluated in A549 non-small cell lung cancer (NSCLC) cells overexpressing MCL-1 protein that could be efficiently downregulated by the use of lipoplex encapsulated pre-miRNA-133b. The *in vivo* biodistribution study in mouse showed a 30% accumulation of the lipoplex nanopartices in lung tissues (Wu et al., 2011). A very similar lipoplex formulation was loaded with miRNA-29b to reduce the expression of the oncogene cyclin-dependent protein kinase 6 (CDK6) as a direct target of miRNA-29b in lung cancer (Wu et al., 2013). In this study, the mRNA expression of CDK6 was downregulated by ~ 57% and the tumor growth was inhibited by ~ 60% compared to negative control. A comparable cationic formulation prepared with dimethyldioctadecyl ammonium bromide (DDAB), cholesterol and vitamin E TPGS was used to transport pre-miR-107 to head and neck squamous cell carcinoma (HNSCC) cells (Piao et al., 2012). This formulation significantly suppressed the tumorgenesis of HNSCC *in vitro* and *in vivo* experiments. In another example ephrin-A1 was conjugated to the surface of the DOTAP/cholesterol/DSPE-PEG-cyanur lipoplexes to target ephrin type-A receptor 2 (EphA2) that are overexpressed in aggressive malignancies including NSCLC cells (Lee et al., 2013). Ephrin-A1 inhibits proliferation and migration of cancer cells by downregulation of EphA2 expression via binding to EphA2 receptors on the cell membrane.In addition, the tumor-suppressive properties of ephrin-A1 are due to the expression of let-7a miRNA. Thus, the combination therapy using ephrin-A1 targeted lipoplexes delivering let-7a miRNA to NSCLC cells led to a significantly reduced proliferation rate, migration and clonogenic expansion of lung cancer cells by downregulating Ras mRNA as target gene (Lee et al., 2013). Only recently, the co-delivery of doxorubicin and miR-101, a tumor suppressor micro-RNA, was achieved using cationic DOTAP-liposomes to target hepatocellular carcinoma (HCC) cells. Synergistic anti-tumor effects of this combination therapy could be validated *in vitro* and *in vivo* (Xu et al., 2017).

Apart from tumor treatment, cationic lipoparticles containing anti-miR-712 were synthesized to target inflamed endothelial cells to prevent atheroma formation in atherosclerosis (Kheirolomoom et al., 2015). To prepare this formulation the hydrophilic anti-miR-712 in water and the hydrophobic DOTAP in chloroform were reacted by addition of methanol to induce the Bligh Dyer monophase for creating a hydrophobic DOTAP-anti-miR-712 complex. The complex was extracted in the organic phase and added to a mixture of neutral and PEGylated lipids. Upon addition of water a stable water-in-oil microemulsion was created. Gradual removal of chloroform resulted in the formation of cationic lipoparticles with asymmetric lipid bilayers entrapping anti-miR-712 within the core. The particles were about 150 nm in size and slightly negatively charged. To selectively target vascular cell adhesion molecule 1 (VCAM1) the particles were decorated with a targeting peptide sequence (VHPK) by post insertion into the outer leaflet of the preformed miRNA loaded liposomes. Optical imaging validated disease-specific accumulation of anti-miR-712 that was efficiently delivered to inflamed mouse aortic endothelial cells *in vitro* and *in vivo* in an Apo E-/- mouse model of atherosclerosis (Kheirolomoom et al., 2015).

All these studies amply demonstrate that cationic liposomes – lipoplexes - could serve as potent delivery systems for miRNAs, however, problems associated with potential cytotoxicity and immunogenicity of cationic lipids or intolerable side effects might pose potential limitations and hamper their successful translation into the clinics (Pecot et al., 2011).

*2.2. Neutral or negatively charged lipoplexes*

Except for cationic lipoplexes an interesting approach concerns actively targeted neutral or negatively charged miRNA loaded lipoplexes (Huang et al., 2013). Thereby, transferrin (Tf) an 80 kDa glycoprotein is used as active ligand to target transferrin receptors overexpressed on certain cancer or leukemia cells. Potential benefits of neutral or anionic formulations are their lower propensity to cause non-specific immune responses and pro-inflammatory reactions (Lonez et al., 2012). Additionally, the overall neutral surface charge of the lipoplexes is less prone to opsonization, plasma protein binding and unspecific cellular uptake. The anionic lipoplexes are formed by ethanol injection technique using a lipid mixture of DOPE, linoleic acid and 1,2-dimyristoyl-rac-glycero-3-methylpolyoxyethylene (DMG-PEG). The negatively charged miRNA molecules are complexed and condensed with low molecular weight polyethylenimine (PEI) before mixing with empty lipoplexes. Through sonication the miRNA-PEI core complex is loaded into the lipoplexes. The particles are about 130 nm in size and slightly negatively charged. The targeting ligand is incorporated by post insertion technique upon incubation of miRNA-lipoplexes with micelles of DSPE-PEG conjugated with Tf . The Tf-targeted miR-29b loaded lipoplexes showed anti-leukemic activity by improving the survival rate in an acute myeloid leukemia (AML) mouse model (Huang et al., 2013). Lipid nanoparticles with the same composition targeted either with Tf or anti-CD45.2 antibodies conjugated to DSPE-PEG2000 maleimide are used to deliver antagomiR-126 to leukemia stem cell (LSC) enriched cell subpopulations in AML (Dorrance et al., 2015). The results show a reduction of LSCs *in vivo* in mice transplanted with human AML primary blasts taken from AML patients, most likely by depletion of the quiescent cell subpopulation. The same group was able to show that Tf-targeted lipoplexes delivering miR-181a mimics can target the RAS-MAPK-pathways presenting a novel promising therapeutic approach for the treatment of AML and possibly also for other RAS-driven cancers (Huang et al., 2016). In another study Tf-targeted lipoplexes are formulated with anti-miR-221 to target HepG2 cells overexpressing Tf-receptors. The anionic liposomes successfully accumulate at tumor sites promising a potential therapy of human hepatocellular carcinoma (Zhang et al., 2015). Finally, Tf-targeted lipoplexes are also successfully applied to restore miR-1 levels in patient derived glioblastoma stem cells (Wang et al., 2014).

Nanoparticles based on 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) have been successfully developed for siRNA delivery. In principle, DOPC and siRNA are complexed in the presence of excess tertiary butanol (Landen et al., 2005). The neutral DOPC particles have certain advantages over charged particles. For instance, they are less prone to aggregation in biological fluids, they do not preferentially adhere to the endothelium, and are less frequently taken up by scavenger macrophages (Landen et al., 2005). The authors could show that siRNA-DOPC nanoparticles targeted with the oncoprotein EphA2 and combined with paclitaxel dramatically reduce tumor growth compared to treatment with paclitaxel and siRNA separately. The neutral DOPC-based nanoparticles are equally efficient for the delivery of miRNA mimics as shown in preclinical testing for tumor targeting (Joshi et al., 2014; Nishimura et al., 2013; Pecot et al., 2013; Rupaimoole et al., 2016b). DOPC liposomes formulated with miRNA-506 mimics or miRNA-520 applied in an ovarian cancer orthotopic mouse model resulted in a decreased expression of the respective mRNA targets and significant tumor regression (Nishimura et al., 2013; Yang et al., 2013). Similarly effective was the delivery of miR-2000 complexed with DOPC liposomes in orthotopic lung cancer models resulting in a significant decrease in the primary tumor mass (Pecot et al., 2013).

Another promising study is based on conventional liposomes composed for egg-phosphatidylcholine (EPC)/CHOL/DSPE-PEG-mal conjugated to anti-cardiac troponin1 antibody to deliver anti-miR-1 ODN (AMO-1) to ischemic myocardium tissues. By silencing of miR-1 in ischemic myocardium using AMO- 1 loaded liposomes ischemic arrhythmogenesis could be efficiently relieved in a rat model after myocardial infarction (Liu et al., 2014).

*2.3. Novel ionizable lipids for cationic liposomes: aminolipids*

A new generation of ionizable cationic lipids with low pKa-values has been developed aiming to achieve a high encapsulation efficiency for nucleic acids at low pH values when the lipids are positively charged. Since ionizable lipids are neutrally charged at physiological pH they have a lower toxicity than cationic lipids. Among the first ionizable aminolipids reported in literature was 1,2-dioleoyl-3-dimethylammoniumpropane (DODAP) with an intrinsic pKa value between 6.6 and 7. However, the apparent pKa value of DODAP can be further adjusted to much lower values by increasing the ionic strength of the solution (Maurer et al., 2001). When particles are formed at acidic pH in the presence of up to 40% ethanol a very high encapsulation efficiency can be achieved. At present, a broad range of ionizable cationic lipids with varying chemical structures have been synthesized and assessed for their transfection efficiency. Representative examples are DLinDMA (1,2-dilinoleyloxy-3-dimethylaminopropane), DLinDAP (1,2-dilinoleoyl-3-dimethylaminopropane), DLin-2-DMAP (1-linoleoyl-2-linoeyloxy-3- dimethylamino-propane), DLin-C-DAP (1,2-dilinoleylcarbamoyloxy-3-dimethylaminopropane), DLin-K-DMA (2,2-dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane). The chemical structures of the most prominent aminolipids are presented in Figure 3.

An additional strategy is to add low amounts of poly(ethylene glycol)-lipids during the formulation process to prevent aggregation. These nanoparticles termed stable nucleic acid lipid particles (SNALPs) reveal a longer lifetime in circulation when administered intravenously (Semple et al., 2001). The efficiency of SNAPS for nucleic acid delivery *in vivo* was assessed in a Factor VII mouse model (Semple et al., 2010). The silencing potential of siRNA containing SNALPs with a lipid composition of cationic aminolipid/ 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)/cholesterol/PEG-lipid in a molar ratio of 40/10/40/10 was tested using Factor VII as target in hepatocytes. Thus, Factor VII becomes secreted in the circulation and could be measured directly one day after i.v. administration of SNALPs. Following a screening procedure DLin-KC2-DMA (2,2-dilinoleyl -4-(2-dimethy aminoethyl)-[1,3]-dioxolane) was identified as best performing lipid (Semple et al., 2010). The pKa value of 6.4 indicates that SNALPs based on DLin-KC2-DMA have limited surface charge in circulation, but become positively charged in the acidified endosomes. More recently, studies on the use of SNALPs for miRNA delivery became available. One of such examples is miRNA 119b-5p that was efficiently encapsulated into DODAP based particles composed of DSPC/cholesterol/DODAP/PEG-lipid. The formulation was tested in a range of different tumor cell lines and its delivery efficiency was demonstrated by a significant impairment of the transcription factor HES1 levels and cancer stem cell markers (de Antonellis et al., 2013). The same lipid composition was used to incorporate miR-34a (Di Martino et al., 2014). In this study, the activity was tested against multiple myeloma xenografts in a mouse model in which a significant tumor growth inhibition could be achieved. To address lung cancer a lipid formulation based on 1,2-dioleyloxy-3-dimethylaminopropane (DODMA), which is a lipid with a protonatable tertiary amino head group, EPC, cholesterol and cholesterol conjugated PEG2000 was loaded with miR-122, a liver-specific tumor suppressor microRNA, to restore deregulated gene expression in hepatocellular carcinoma (HCC) cells (Hsu et al., 2013). Upon reaching the liver the nanoparticles could exit the intravascular space to directly access hepatocytes by passive targeting as long as they are smaller than the pore size of fenestrated vasculature of the liver, which is about 100-150 nm in diameter (Wisse et al., 2008). Active targeting of HCC cells was achieved using lactosylated lipid nanoparticles being composed of DODAP, DOPE, DMG-PEG and N-lactobionyl (LAC)-DOPE, as targeting ligand to address asialoglycoprotein receptors on HCC cells. The particles are prepared by ethanol injection method and loaded with anti-miR-155 by post incubation at room temperature (Zhang et al., 2013a). The delivery efficiency could further be improved by the incorporation of gramicidin A, a hydrophobic peptide that is known for its channel forming properties in lipid bilayers. Presumably, gramicidin A could provide additional ion transport and increase membrane permeability, in turn causing the swelling of the endosome and release of the trapped substances into cytoplasm, preventing its lysosomal degradation (Bolkent and Zierold, 2002). A more sophisticated lipid formulation was designed for the delivery of antimiR-21 for lung cancer treatment (Yung et al., 2016). These nanoparticles, termed QTsomes, are composed of a mixture of quaternary and tertiary amino cationic lipids, neutral lipids, cholesterol, and a PEG-lipid (DODMA/DOTAP/DOPC/CHOL/PEG-DPPE). The efficiency was assessed in an A549 xenograft mouse model in terms of tumor regression and a prolonged survival rate (Yung et al., 2016). Ando et al., for example, developed miRNA loaded polycationic liposomes for anti-angiogenesis based cancer therapy (Ando et al., 2013). The liposomes were formulated from dicetyl phosphate-tetraethylenepentamine (DCP-TEPA)/DPPC/DOPE and cholesterol. MiR-92a, one of the miRNAs known to regulate angiogenesis, was grafted to cholesterol and bound to the preformed polycationic liposomes. The authors could show that the miR-92a loaded liposomes were efficiently taken up by human umbilical vein endothelial cells (HUVECs) via micropinocytosis, escaped from the endosomes into the cytoplasm suppressing the expression of several proteins encoded by miR-92a-target genes (Ando et al., 2013).

Apart from tumor targeting, aminolipid based nanoparticles were successfully applied to target the liver for the treatment of metabolic diseases. Such particles were prepared from Dlin-KC2-DMA/DSPC/cholesterol/DMG-PEG at a molar ratio 50/10/38.5/1.5 and loaded with a combination of antagomirs to silence both miR-103 and miR-107 (Trajkovski et al., 2011). Here it was demonstrated that upon miR-103/107 inactivation by antagomir loaded nanoparticles caveolin-1 is upregulated in adipocytes leading to improved glucose homeostasis and insulin sensitivity in obese animals. The results clearly imply that miR-103/107 are promising therapeutic targets to be silenced by antagomir loaded lipid formulations when aiming to treat diabetes (Trajkovski et al., 2011). A novel pH-sensitive lipid, termed YSK05, with a pKa value of 6.40–6.45 was formulated with cholesterol and DMG-PEG at a molar ratio of 70/30/3, and loaded with anti-miR-122 to regulate liver specific miRNA-122. Due to the enhanced escape of antago-miR-122 in the endosome a high activity in liver cancer cells was achieved. Systemic administration of YSK05 containing liposomes induced the knock-down of miR-122 and an increase in target genes in the liver (Hatakeyama et al., 2013).

In another example, miRNA-126 was entrapped in 1,2-distearoyl-3-dimethylammonium-propane (DSDAP), DSPC and PEG2000 containing bubble liposomes to treat hindlimp ischemia (Endo-Takahashi et al., 2014). Bubble liposomes contain an ultrasound contrast gas for diagnostic imaging. In this study, therapeutic miRNA-126 delivery for angiogenic treatment and ultrasound for detection in a hindlimp ischemia model could be combined in a single bubble liposome formulation (Endo-Takahashi et al., 2016).

*2.4. Lipidoid based nanoparticles*

Lipidoids are lipid-like molecules which were synthesized by a high-throughput combinatorial approach (Akinc et al., 2008). So far a library of 1400 degradable lipidoid molecules has been synthesized by applying Michael addition chemistry. To do so, alkyl-amines were reacted with alkyl-acrylates of varying carbon chain tail length, which have ester groups that can be hydrolyzed by enzymes to make them biodegradable (Whitehead et al., 2014). A general scheme for lipidoid synthesis and a representative example are provided in Figure 4.

The efficiency of the lipidoid based particles in siRNA delivery was screened in hepatocytes and immune cell populations after i.v. administration to mice (Love et al., 2010). The nanoparticles were formulated from lipidoid molecules, cholesterol, DSPC and DMG-PEG with a molar ratio of 50:38.5:10:1.5. The particle size varied between 60 and 120 nm in diameter. Based on these screening results, Whitehead et al. identified four criteria, three structural requirements and the pKa-value as important parameter to predict the ability of lipidoid based nanoparticles to mediate greater than 95% protein silencing *in vivo*. More precisely, lipidoids which were synthesized with amide bonds linking lipid tails containing at least one tertiary amine and at least three substitution sites had the highest probability to facilitate potent gene silencing in mice. The pKa-value appeared to be most influential in determining *in vivo* efficacy of the nanoparticles. It was ruled out that the pKa-value should be equal or less than 5.4 (Whitehead et al., 2014). The most promising data achieved so far for lipidoids are summarized in a recent review by Dahlman (Dahlman et al., 2014b). As an example, one lead lipidoidthat is synthesized with alky acrylate tails containing ester groups, termed 304O13, efficiently targeted hepatocytes *in vivo* with very low toxicity becoming hydrolyzed by liver enzymes. Interestingly, the targeting efficiency of the lipidoid nanoparticles to liver cells was shown to be dependent on the binding to serum apolipoprotein E, which is naturally endocytosed by hepatocytes. In case of lipidoids, apolipoprotein E functions as endogenous targeting ligand to deliver siRNA-lipidoid nanoparticles to hepatocytes (Akinc et al., 2010). The same effect could not be observed when using cationic liposomes. Thus, the relationship between nanoparticle features and apolipoprotein E binding propensity remains elusive. In another example, concurrent delivery of miR-34a and siRNA targeting Kirsten rat sarcoma viral oncogene analog (Kras) using polymer based lipid nanoparticles resulted in an improved therapeutic response in combination therapy of lung cancer in a genetically engineered mouse tumor model (Xue et al., 2014). A simultaneous silencing of multiple endothelial genes was achieved. The nanoparticles were made of low molecular weight polyamines and lipids derived from a chemical library (Dahlman et al., 2014a; Dahlman et al., 2014b). Briefly, small polyamines were conjugated to alkyl tails via an epoxide ring-opening reaction. A variation of amine backbone, lipid length, and the molar ratio of lipids and amines generated a structurally diverse nanoparticle library of 600 compounds in ethanol. The compounds were mixed with DMPE-PEG2000 at a molar ratio of 80 to 20 to form multilamellar structured nanoparticles with a size of about 50 nm that are neutral at physiological pH. Applying this formulation in combination therapy of miR-34a and si-Kras a strong impact on solid lung tumor growth was observed. In a recent study, lipidoid nanoparticles were synthesized by mixing lipidoids, cholesterol and DOPE to deliver miR335-5p, which specifically targets the 30-UTR sequence of Dickkopf-1 (DKK1) and regulates its gene expression (Sui et al., 2018). The *in vitro* and *in vivo* effects of miR335-5p transfection on the differentiation of stem cells toward the osteogenic lineage and on enhancing calvarial critical-size defect healing in a murine model were investigated. The authors show that the novel lipidoid-miR335-5p formulation is suitable to promote calvarial bone regeneration (Sui et al., 2018). In another approach lipidoid like nanoparticles have been tested to deliver miRNA to human mesenchymal stem cells (hMSCs). The optimal chemical structures for the lipids were derived from a small lipid library and used to deliver miR-9 to promote neuronal differentiation of stem cells (Takeda, 2016). The nanoparticles were formed by mixing synthetic double-stranded miRNA-mimics with bioreducible lipids in sodium acetate buffer. The bioreducible lipids were synthesized from amines and acrylates of different chain lengths containing a disulfide bond that is degraded intracellularly to release the miRNA.

*2.5. Amphoteric liposomes: Smarticles®*

Amphoteric liposomes are composed of differently charged lipids including pH-sensitive lipids and an additive. In principle, amphoteric liposomes are cationic at low pH and neutral or anionic at neutral and higher pH. Accordingly, they can be efficiently loaded with oligonucleotides at low pH. For example, one typical formulation of amphoteric liposomes includes DOTAP, cholesteryl hemissucinate (CHEMS), α-(3′-O-cholesteryloxycarbonyl)-δ-(N-ethylmorpholine)-succinamide (MoChol), 1,2-dimyristoylglycerol-3-hemisuccinate (DMGSucc) (Siepi et al., 2011). The authors demonstrate that the binding of counterions to charged lipids promotes the formation of lamellar membranes while ion desorption causes membrane fusion. The amphoteric liposome delivery technology NOV340 was marketed as Smarticles® by Marina Biotech, Bothell, WA, and exploited for the delivery of miRNA mimics for tumor treatment. Smarticles® are composed of different mixtures of POPC, DOTAP, DMGSucc and cholesterol. The particles are about 120 nm in diameter. At physiological pH Smarticles® are slightly negatively charged, which might prevent their interactions with negatively charged cellular membranes in the endothelium. Once in the tumor the pH tends to be lower and the Smarticles® become positively charged thus facilitating their interaction with tumor cells. Smarticles® technology was first to enter the clinics delivering a miRNA-mimics, miRNA-34a (MRX34). These studies are outlined in more detail later.

*2.6. Liposome polycationic hybrid particles*

Apart from cationic lipids, other cationic excipients such as polycationic peptides or stearylamine (SA) can be used to formulate lipid carriers for gene delivery. Liposome-polycation-hyaluronic acid (LPH) particles containing hyaluronic acid (HA) and a cationic peptide like protamine are produced by self-assembly based on charge-charge interaction. As an example, HA and miRNA are first complexed with protamine, a polycationic peptide isolated from salmon sperm, such that the condensed core is negatively charged. This complex is then encapsulated in cationic liposomes composed of DOTAP/cholesterol by charge interaction. These nanoparticles are further coated with PEG through post-insertion of PEG-lipids (e.g. DSPE-PEG) and ligand-modified PEG-lipids (e.g. DSPE-PEG-anisamide), which specifically target the sigma receptor over-expressed in B16-F10 melanoma cells. The targeted LPH nanoparticles (PEGylated with anisamide ligand) successfully silenced 80% of luciferase activity in the metastatic B16-F10 tumor in the lung after a single i.v. injection (Chono et al., 2008). In another study, PEGylated LPH nanoparticles were surface functionalized with cyclic-RGD peptides and loaded with antisense inhibitors of miRNA-296 to target αvβ3 integrin positive endothelial cells in tumor neovasculature (Liu et al., 2011). The potential of cyclic-RGD targeted LPH nanoparticles for anti-angiogenesis therapy using antogomirs was shown in a Matrigel plug assay in BALB/c nude mice (Liu et al., 2011). In a separate study, the PEGylated LPH particles were modified with the single-chain variable fragment (scFv) of the N-cadherin antibody (GC4) to target lung metastasis in a syngeneic murine model (Chen et al., 2010). The delivery efficacy to tumor metastasis was tested with both, siRNA und miR-34a. MiR-34a is an important p53 regulated tumor suppressor. Encapsulated in targeted LPH particles miR-34a induced apoptosis, inhibited survivin expression and downregulated MAPK pathways in B16-F10 melanoma cells. Interestingly, when miR-34a and siRNAs are co-formulated in LPH nanoparticles targeting GC4 a pronounced inhibition of tumor growth was found in lung metastasis bearing mice (Chen et al., 2010). These data illustrate for the first time the potential of combined delivery of siRNA and miRNA in a single delivery system to target different oncogenic pathways. This is an important step towards future personalized combination therapies in cancer treatment.

An SA based cationic liposome formulation was designed for the delivery of anti-miR-191 to breast cancer cell lines. A combined treatment of SA liposome loaded with anti-miR-191 and anti-cancer drugs markedly enhanced apoptotic cell death and suppressed the migration of cancer cells as shown in *in vitro* in breast cancer cell lines (Sharma et al., 2017).

*2.7. Neutral lipid emulsions*

Neutral lipid emulsions (NLE) used for drug delivery usually consist of a phosphatidylcholine, mostly DOPC, oils like middle-chain (MCT) or long-chain (LCT) triglycerides, polysorbates and antioxidants. NLEs have some advantages over other lipid formulations. Similar to the behavior of neutral liposomes, NLEs are highly stable in biological fluids and do not tend to form aggregates due to electrostatic repulsion. They show low toxicity, are not filtered by the liver, do not adhere to endothelium, and are not taken up by scavenging macrophages (Trang et al., 2011; Wiggins et al., 2010). NLEs have been successfully applied for the systemic delivery of miRNA mimics to inhibit lung tumor in mice (Trang et al., 2011; Wiggins et al., 2010). The same formulation was successfully applied to deliver synthetic miR-34a mimics to B-cell lymphoma *in vivo* to reduce tumor growth with treatment options for aggressive lymphoma. The tumor-suppressive effects of miR-34a therapy are attributed to its anti-apoptotic properties and to the specific knockdown of FoxP1 (Craig et al., 2012).

*2.8. Solid lipid nanoparticles*

Solid lipid nanoparticles (SLNs) are colloidal particles harboring a solid lipid core matrix emulsified by a surfactant that stabilizes the lipid dispersion. As example, cationic solid lipid nanoparticles composed of DDAB, glyceryl monostearate (GMS), polyoxyethylene 50 stearate (Myrj53), cholesterol, and soy phosphatidylcholine (SPC) were synthesized by film-ultrasonic techniques and loaded with miRNA by incubation for 30 minutes at room temperature (Shi et al., 2013). The particles have proven successful to deliver miR-34a to cancer stem cells (CSC) in B16F10-CD44+ bearing lung tumors *in vitro* inducing cell apoptosis and inhibiting cell migration. In an *in vivo* experiment, an accumulation of miR-34a in lung tumor sites was observed (Shi et al., 2013). The same group has used SLNs to load them with anti-miRNA-21 to suppress miRNA-21 in human lung cancer cells (Shi et al., 2012). In this case, the antagomirs were first complexed with DDAB by a Bligh and Dyer extraction method before formation of SLNs through solvent diffusion (Reimer et al., 1995). In this case, an aqueous poloxamer 188 solution was used to form an oil in water emulsion prior to removal of the organic solvent. Cellular uptake and activation properties of antgomir loaded SLNs was tested in human lung adenocarcinoma A549 cells, including studies on antisense efficiency, cell migration and invasion (Shi et al., 2012).

*2.9. Nonionic surfactant vesicles*

Nonionic surfactant vesicles are formed by self-assembly from nonionic surfactants and excipients, most likely cholesterol, and are often referred to as niosomes (Moghassemi and Hadjizadeh, 2014). Niosomes are promising carriers for drugs as hydrophilic and hydrophobic molecules can be incorporated in the aqueous interior of the vesicles or within the closed surface bilayer, respectively. Cationic niosomes formulated by nonionic surfactants such as sorbitan monooleate (Span 80), polyoxyethylenesorbitan trioleate (Tween 85), monopalmitin glycerol, cholesterol and DDAB have been validated as efficient transporter for siRNA to cancer cells displaying a high loading efficiency combined with low toxicity (Obeid et al., 2017; Sun et al., 2015). Recently, a highly interesting approach describes cationic niosomes as theranostic platform for the delivery of siRNA/miRNA to human mesenchymal stem cells (hMSCs) to promote their differentiation. Concurrently, a dye was encapsulated in the niosomes for *in vivo* tracking of the transfected stem cells (Yang et al., 2018). The niosomes are made of Span 80, TGPS and DOTAP by ethanol injection technique incorporating the amphipilic dye indocyanine green (ICG), which is self-quenched due to close proximity of the dye molecules but can be visualized upon release in the near-infrared (NIR) region. siRNA/miRNA are complexed on the surface by electrostatic interaction with the positive head groups of DOTAP. In this study antimiR-138 is used as gene regulator to promote osteogenic differentiation in hMSCs. The particles were efficiently taken up intracellularly resulting in specific gene silencing and NIR labeling of hMSCs upon niosome decomposition. For *in vivo* stem cell tracking the NIR labeled transfected hMSCs are injected subcutaneously into mice and imaged for seven days following implantation. By this way, simultaneous long-term cell tracking and *in vivo* gene silencing could be achieved within one particle. Following these promising results the authors conclude that theranostic niosomes could present a novel platform for gene regulation in stem cell research and regenerative medicine (Yang et al., 2018).

**3. Structural and cellular aspects of miRNA delivery**

*3.1 Structural organisation of oligonucleotide loaded lipid-based nanoparticles particles*

Currently little information is available about the internal structural organisation of ODN or siRNA loaded in lipid-based nanoparticles lacking specific data on miRNA. However, due to the compositional and structural similarity of oligonucleotides the mechanism of interaction accompanying the self-assembling process of the lipid particles as well as the inner molecular organisation should rather depend on the lipid composition, the molar lipid to nucleic acid ratio and the preparation technique of the lipid nanoparticles than on the specific oligonucleotide to be incorporated. Figure 5 shows a very general presentation of miRNA loaded lipoplexes. For lipid nanoparticles containing ionizable cationic lipids produced by rapid microfluidic mixing processes an electron-dense nanostructured core was identified in the particle´s interior (Leung et al., 2012). The internal structure organisation and the localization of the siRNA payload was deduced from cryo-transmission electron-microscopy, P31-NMR, fluorescent energy resonance transfer (FRET), density gradient centrifugation, and molecular modeling The particles were composed of DLinKC2-DMA, DSPC, cholesterol and a PEG2000-lipid. For microfluidics, the lipids were dissolved in ethanol and mixed with an aqueous acidic solution of oligonucleotides, followed by extensive dialysis to remove ethanol (Belliveau et al., 2012). Depending on the mixing conditions homogenous monodisperse lipid nanoparticle of tunable sizes between 20-100 nm and a very high encapsulation range can be produced (Belliveau et al., 2012). Leung et al. found that the lipid nanoparticles have highly structured electron dense inner core in which the ionizable lipids are arranged as inverted micellar structures complexing siRNA molecules. The outer shell is formed by a monolayer consisting predominantly of neutral lipids and PEGylated lipids. Viger-Gravel et al. (Viger-Gravel et al., 2018) investigated a related lipid nanoparticle system composed of the ionizable cationic lipid (DLin-MC3-DMA), DSPC, cholesterol, and DMPE-PEG2000. Again, the particles were synthesized by microfluidics and measured by dynamic nuclear polarization enhanced NMR spectroscopy. With this technique the internal spatial location of the single components could be detected. The authors found a more homogenous distribution throughout the particles comprising a thin outer shell formed by DSPC and PEG-lipid and an interior being made up by evenly dispersed inverted micelles of ionizable cationic lipid and cholesterol.

*3.2. Targeted active transport*

Active transport across biological barriers is specifically important in cases when passive targeting depending on the EPR effect is not viable. The strategy of passive targeting has been successfully used to reach sites of leaky vasculature typical for tumors, sites of inflammation, infection or angiogenesis. Active targeting is a more specific approach to address particular molecules uniquely present or enriched in cells, tissues or pathological sites of interest (Howard et al., 2014). As targeting ligands for liposomes a broad variety of molecules including proteins, antibodies, antibody fragments, peptides, aptamers or small molecules have been explored. The reader is referred to some representative reviews on active targeting approaches for liposomes (Byrne et al., 2008; Deshpande et al., 2013; Pattni et al., 2015; Petrilli et al., 2014; Sawant and Torchilin, 2012; Torchilin, 2007). In brief, transferrin and folic acid are amongst the most promising ligands in cancer therapy to target receptors overexpressed in many cancer cells. Other ligands for nanoparticle targetomg in cancer therapy are antibodies against VEGF, VCAM, matrix metalloproteases (MMPs) or integrins (Zylberberg and Matosevic, 2016). To some extent these ligands are also promising to target inflamed vascular endothelium or angiogenesis. Endothelial cell adhesion molecules, like VCAM-1, E-selectin, P-selectin are preferentially exposed on the surface of activated endothelium and can be targeted by antibodies covalently conjugated to the delivery system (for a recent review see (Howard et al., 2014)). As example, E-selectin targeted immunoliposomes are readily taken up by receptor-mediated endocytosis by E-selectin expressing interleukin-1 activated endothelial cells (Jubeli et al., 2012; Kessner et al., 2001). Peptide ligands primarily belong to the class of cell penetrating peptides (CPP) that translocate through the membrane. Thus, cyclic-RGD, TAT-peptides or octa-arginine peptides have been shown to be very effective for gene delivery and transfection (Kibria et al., 2011; Levchenko et al., 2003; Zhang et al., 2006). A new approach explores aptamers selected against cell surface receptors as targeting sequences for siRNA loaded SNALPS (Wilner and Levy, 2016).

In general, the targeting ligands are conjugated to the surface of the nanoparticles to be recognized by specific receptors or proteins expressed on the cell surface. The ligands can be coupled covalently by chemical reaction either to head group functionalized phospholipids or the distal end group of functionalized PEG-chains anchored to lipid molecules (Nobs et al., 2004). The active transport across the cell membrane permits tissue accumulation of the nanoparticles in diseased sites before drug release occurs within intracellular compartments.

*3.3. Cellular uptake and intracellular trafficking*

Effective cellular internalization and proper intracellular release are particularly relevant for RNA based therapeutics, which require cytosolic delivery for their bioactivity (Schroeder et al., 2010; Whitehead et al., 2009). In case of cationic liposomes, the particles interact with the negatively charged cell surface preferentially via proteoglycans and sialic acids or by unspecific charge-mediated interaction with cellular receptors. The uptake occurs most likely by endocytosis (for a comprehensive review see (Zuhorn and Hoekstra, 2002)). Following endocytosis, the membrane-like structure of the positively charged liposomes allows the exchange of charged lipids between the endosomal membrane and the liposomes facilitating intracellular release of the nucleic acids (for a review see (Leung et al., 2014)). In particular, fusogenic helper lipids added in the formulation can destabilize the endosomal membrane by conformational lipid transition at the low endosomal pH. For instance, DOPE adopts an inverted hexagonal phase at low pH, which readily causes fusion of DOPE containing liposomes with the endosomal membrane (Hafez and Cullis, 2001; Hafez et al., 2001). The fusion process destabilizes the liposomes and their cargo is directly released into the cytoplasm (Torchilin, 2007). In case of pH-sensitive lipid nanoparticles the escape mechanism follows the so called “proton sponge” effect. As the pH value inside the endosome drops the nanoparticles become protonated, which causes an increased influx of protons and counter ions, such as chloride, increasing the osmotic pressure in the endosomes. The osmotic gradient leads to water influx causing rupture and release of its contents into the cytoplasm (Paliwal et al., 2015). Pure cationic liposomes such as DOTAP liposomes efficiently incorporate miRNA/siRNA, but due to their intracellular stability the release rate of active substance and endosomal escape capacities might be limited. Potential cellular uptake and release scenarios for miRNA-liposomes are depicted in Figure 6 (MISSING).

*3.4. Cytotoxicity*

The use of cationic liposomes *in vivo* is often hampered by their inherent dose-dependent toxicity. Multivalent cationic liposomes are generally more toxic than monovalent cationic lipids. Even the hydrocarbon chain length could influence the cytotoxicity of cationic lipids (Filion and Phillips, 1997). These authors found that the toxicity of liposomes to macrophages decreases in mixtures containing DOPE and cationic lipids based on diacyltrimethylammonium propane from DOPE/DOTAP (dioleoyl-) > DOPE/DMTAP (dimyristoyl-) > DOPE/DPTAP (dipalmitoyl-) > DOPE/DSTAP (disteroyl-) acyl chains. By incorporation of polymer conjugated lipids in the lipid formulation the toxicity can be significantly reduced (Xue et al., 2015). For neutral liposomes no toxicities have been reported so far (Landen et al., 2005). Negatively charged liposomes can induce immunogenic response, and are less likely to cross negatively charged cell membranes (Schroeder et al., 2010). Cationic lipids might also activate the complement system which results in binding of complement components to liposomes. Liposomes are then directed to specific receptors found in the lungs or in Kupffer cells in the liver (Landesman-Milo and Peer, 2012). Treatment with cationic liposomes might also provoke a pro-inflammatory response by inducing Th1 cytokine expression. As liposomes might trigger innate immune system and macrophage clearing mechanisms (Szebeni and Moghimi, 2009), potential toxicities associated with lipid carriers need to be addressed carefully before a translation in clinical practice can be implemented. In particular, a careful selection of lipids and formulation strategies might help to get rid of adverse cytotoxic effects.

**4. Clinical developments: Translation of lipid based miRNA therapeutics to the clinics**

Today, several lipid-based technologies are successfully established as proof of principle for miRNA delivery in cellular and pre-clinical settings. Hence, a considerable number of studies have been conducted over the years and the results of all these studies provide important information regarding the therapeutic potential of the carrier system and its cargo to facilitate the translation into clinical development. But despite this, certain challenges like establishment of scaling-up procedures for cGMP production, molecular stability and robustness of the therapeutic delivery system, quality control mechanisms and higher costs associated with the production of innovative nanopharmaceuticals remain and have to be overcome before clinical applications can be considered (Zhang et al., 2013b). When designing novel carrier systems for miRNA therapeutics researcher should therefore focus on untreatable or poorly treated diseases with limited treatment options. Additionally, miRNA expression profiles have to play a central role in the gene regulation of the disease to be therapeutically manipulated (Rupaimoole and Slack, 2017).

The first and only lipid based miRNA-therapeutics, termed MRX34, has entered a phase 1 clinical trial in patients with multiple cancers including primary liver cancer or metastatic cancer with liver involvement in 2013. The miRNA mimic is intended to restore the lost suppressor function of miR-34 on the p53 and wnt/β-catenin cellular pathways (Baumann and Winkler, 2014). The therapeutic MRX34 directly regulates at least 24 known oncogenes, such as those involved in the cell cycle and proliferation, anti-apoptosis, metastasis, chemoresistance, cancer cell self-renewal and oncogenic transcription (Bader, 2012). The miRNA mimics are delivered by the NOV340 technology (Smarticles®), as described above. The miRNA mimics and the lipids are mixed under acidic conditions, when the amphoteric lipids are cationic. At physiological pH the complexes are slightly anionic to reduce the interaction propensity with negatively charged cell membranes. Since the pH tends to be lower in tumor environment the Smarticles® become cationic and increasingly adhere to tumor cells. The efficacy of miR-34a loaded particles to target the liver and to promote tumor regression was verified in a clinically relevant mouse model of hepatocellular carcinoma (Bader, 2012). Mirna Therapeutics (Mirna Therapeutics, Inc., Austin, TX, USA) has initiated a preclinical development program to support manufacturing of cGMP material of MRX34. A phase 1 clinical study using MRX34 was launched in patients with advanced solid tumors critically evaluating dosage issues, safety aspects, pharmacokinetics and off-target effects (Beg et al., 2017). Forty-seven patients with various solid tumors, including hepatocellular carcinoma (HCC; n=14) were enrolled. MRX34 was given intravenously twice a week for three weeks in four week cycles. The maximal tolerated dose, the optimal dosing regime and occurring adverse side effects were assessed. The results show that MRX34 therapy is feasible and tolerable, but only under adequate dexamethasone pretreatment. It was detected that most of the adverse effects are potentially attributed to the carrier independent of the miRNA mimic. Unfortunately, in 2016 Mirna Therapeutics was forced to halt the clinical study following multiple immune-related adverse events diagnosed in patients dosed with MRX34. Interestingly, the same liposomal carrier used in combination with a single stranded DNA oligonucleotide for Bcl-2 targeted therapy (PTN2258, Sierra Oncology Plymouth, MI; former ProNAi Therapeutics Inc.), which was tested in multiple advanced solid tumor (phase I) and in patients with relapsed refractory non-Hodgkin lymphoma and diffuse large B cell lymphoma (phase II) did not show related severe side effects. PTN2258 has been granted orphan drug destination by the U.S. Food and Drug Administration in 2016. Since the focus of the company has changed from DNA knock down approaches to targeting DNA damage response the development of PNT2258 was terminated in 2017.

Such rather disappointing results from clinical human studies indicate that substantial improvements of the carrier systems for nucleic acid therapeutics are needed and that the molecular mechanisms of nanoparticle interactions with blood components, cellular recognition, uptake, release and metabolic response are still poorly understood.

**5. Concluding Remarks**

Indeed, there is a strong need of site-specific delivery systems that integrate several features as high drug loading capacity, stability, improved circulation time, reduced toxicity and protection of its cargo from rapid degradation. In circulation an enhanced transport to the target tissues, proper cellular uptake and drug release are anticipated. Lipid based nanoparticles are very promising candidates for therapeutic delivery. However, many physicochemical and structural parameter have to be taken into consideration when designing a lipid nanocarrier, especially for sensitive drugs like nucleotides. First, for efficient complexation of the negatively charged nucleic acids with lipids positively charged molecules are very helpful. By electrostatic interactions the particles are loaded during formation via lipid self-assembly. As positively charged nanoparticles are often toxic, pH sensitive particles are developed, which are neutral at physiological pH but are positively charged under low pH conditions such as in endosome or tumor microenvironment. Second, the lipid carriers need to have a certain size, which is in a range between 50 to 200 nm to avoid renal filtration or rapid clearance by the immune system. However, the optimal particle size strongly depends on the application and the target tissue. Third, to enhance circulation time and to reduce carrier uptake by RES, the nanoparticles might be decorated and coated with polymers. Accordingly, pegylated lipids are incorporated into the lipid nanoparticle to form a stable polymer corona surrounding the particle surface. Fourth, targeting ligands can be included in the formulation to improve specific recognition and uptake by target cells and tissues. In this vein, advances in the design of proper lipid-based delivery systems may address a number of shortcomings associated with conventional medicine but the complexity of the innovative systems will certainly pose new challenges for clinical approval (Eifler and Thaxton, 2011). MiRNA based therapeutic approaches, applied either as miRNA mimics in miRNA replacement therapy with the aim to restore the function of miRNAs, or alternatively as miRNA antagonists for specific targeting and silencing of miRNAs are still at the very beginning but it can be expected that increasing amounts of studies will become available in the next few years giving promise for clinical translation.

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FIGURE LEGENDS

Figure 1: Schematic overview of a microfluidic system for the manufacturing of miRNA loaded liposomes. Lipids dissolved in ethanol are mixed with an aqueous solution of miRNA at a constant flow rate within a microfluidic chip device. The liposomes are formed by self-assembly. Excess ethanol and non-encapsulated free miRNA are removed by dialysis.

Figure 2: Chemical structures of representative cationic lipids for lipoplex formation

Figure 3: Chemical structures of aminolipids used for the formation of ionizable lipoplexes

Figure 4: Scheme for lipidoid synthesis and a representative example

Figure 5: Schematic presentation of miRNA loaded lipoplexes. The lipoplex nanoparticles are composed of a mixture of neutral lipids, fusogenic lipids, PEGylated lipids, cationic lipids, cholesterol loaded with miRNA molecules.

Figure 1

Figure 2



Figure 3



Figure 4



Figure 5



Figure 6 – UNDER CONSTRUCTION