Biomedical Microdevices (2018) 20: 59
https://doi.org/10.1007/s10544-018-0298-0

Micelle-like nanoparticles as siRNA and miRNA carriers for cancer therapy

Daniel F. Costa [1,2] · Vladimir P. Torchilin [1]

Published online: 12 July 2018

Abstract
Gene therapy has emerged as an alternative in the treatment of cancer, particularly in cases of resistance to chemo and radiotherapy. Different approaches to deliver genetic material to tumor tissues have been proposed, including the use of small non-coding RNAs due to their multiple mechanisms of action. However, such promise has shown limits in *in vivo* application related to RNA's biological instability and stimulation of immunity, urging the development of systems able to overcome those barriers. In this review, we discuss the use of RNA interference in cancer therapy with special attention to the role of siRNA and miRNA and to the challenges of their delivery *in vivo*. We introduce a promising class of drug delivery system known as micelle-like nanoparticles and explore their synthesis and advantages for gene therapy as well as the recent findings in *in vitro*, *in vivo* and clinical studies.

Keywords siRNA · miRNA · Cancer · Micelle-like nanoparticles · Polymers

1 Introduction

The genome is a complex machinery controlled by several pathways. Over the last decades, scientists have confirmed the influence of small RNA molecules, containing between 20 and 30 nucleotides in length, on post-transcriptional events (Carthew and Sontheimer 2009). This specific regulatory mechanism, so-called RNA interference (RNAi), has been shown to be essential in protecting germline and somatic lineages of eukaryotic cells against a variety of pathogens and diseases (Morrison 2018; Gandhi et al. 2014). The first record of RNAi predates 1984, when Nir and colaborators reported the induction of IFN-α1 gene expression and protein synthesis by human fibroblasts exposed to double-stranded RNA (Nir et al. 1984). Since then, the role of different types of nucleic acids in molecular biology has been investigated. In particular, small non-coding RNAs have emerged as one of the most promising categories for RNAi-based medicine. These RNAs comprise many subtypes of molecules, each one presenting a particular origin, structure, synthesis, and mechanism of action. However, two of them have been highlighted: short interfering RNA (siRNA) and microRNA (miRNA) (Carthew and Sontheimer 2009).

Studies in gene therapy application have expanded from simple to multifactorial diseases, such as cancer (Ginn et al. 2013; Wang et al. 2017). Current techniques, including surgical intervention, radio and chemotherapy have not always proven to be efficient strategies for successful treatment. One of the biggest challenges faced in clinics is the resistance of tumor cells to the most commonly used drugs (Tsouris et al. 2014). Among the possibilities for drug resistance, inhibition of efflux pumps and synthesis of antiapoptotic proteins are the most frequently affected by expression of a variety of genes, such as PLK1, Bcl2, survivin, MDR-1, and P-gp (Nakamura et al. 2011; Salzano et al. 2014; Abbasi et al. 2010; Liu et al. 2009; Yadav et al. 2009; Gibori et al. 2018). Knocking these genes down by RNAi has been seen as a way to transiently increase the sensitivity of cells to the action of antitumor drugs, overcoming resistance (Misso et al. 2014; Garofalo et al. 2012).

Despite the promising theory of a synergistic effect between RNAi and chemotherapeutic drugs, preclinical trials have shown many challenges when it comes to delivery of free nucleic acids to the tumor tissue *in vivo*. Poor serum

✉ Vladimir P. Torchilin
v.torchilin@northeastern.edu

Daniel F. Costa
costa.dan@husky.neu.edu

[1] Center for Pharmaceutical Biotechnology and Nanomedicine, Northeastern University, Boston, MA 02115, USA

[2] CAPES Foundation, Ministry of Education of Brazil, Brasília, DF 70040-020, Brazil

stability, off-target effects, and inefficient cellular entry are among the drawbacks (Aaldering et al. 2015; Conde et al. 2015). Thus, the development of drug delivery systems with the ability to carry and protect such payloads is required to improve the efficiency of RNAi-based medicine. Liposomes, dendrimers, lipid nanoparticles, polymeric micelles, inorganic nanoparticles, and some other materials have been used to deliver small RNAs in a safer and efficient manner. They have reduced dose frequency, facilitated targeting, improved biodistribution, and allowed in-time imaging (Navarro et al. 2015; Sarett et al. 2015). This review focuses specifically in one of these alternatives: the micelle-like nanoparticles, a new delivery system which combines the benefits from polymeric and hydrophobic moieties to create cationic amphiphilic conjugates able to form micellar structures by self-assembly in aqueous surroundings (Navarro et al. 2015). Here we discuss their production and characteristics as well as applications and recent findings in RNAi-based therapy for the treatment of cancer.

2 RNAi-based cancer therapy

GLOBOCAN, the latest worldwide project on cancer incidence, mortality, and prevalence conducted by the International Agency for Research on Cancer (IARC), estimated over 14 million new cases of cancer in 2012 (GLOBOCAN 2012). These are new and remissive cases of what has been considered one of the biggest public health challenges in the modern world. Although mortality has been decreasing in the past years, doctors still face reduction in efficacy of the treatments mainly caused by tumor type-dependent resistance and phenotypic variability between patients (Conde et al. 2015).

Several mechanisms of drug resistance have been reported, including pump-type resistance and apoptosis inhibition, the currently preferred targets for RNAi-based therapy (Tsouris et al. 2014). In the first case, cancer cells overexpress ATP-binding cassete (ABC) transporter proteins, such as P-glycoproteins (P-gp). As a result, increased amounts of drug are pumped out of the cells, reducing its concentration at the intracellular site of action. Overexpression of P-gp can be an intrinsic property of tumor tissues or induced by exposure to chemotherapy (Navarro et al. 2015). In the second case, failure of the treatment is caused by overproduction of antiapoptotic proteins, such as Bcl-2, that are responsible for inhibiting one of the major pathways of tumor cell death (Tsouris et al. 2014).

RNAi has been extensively studied among the strategies to overcome drug resistance. This is a natural well-conserved mechanism of most eukaryotic cells, where RNA components recognize a target by Watson-Crick base pairing and regulate post-transcriptional pathways (Carthew and Sontheimer 2009; Gandhi et al. 2014). Recent research has also shown regulation in chromatin structure levels, chromosome segregation, and transcription of genes. RNAi occurs in a programmable fashion, when endogenous patterns of gene expression are activated by metabolic changes in the diseased tissue. Similar events can happen through exogenous pathways, when gene sequences from foreign organisms are internalized and promote changes in the body response (Carthew and Sontheimer 2009; Gandhi et al. 2014; Li and Rana 2014; Ferracin et al. 2010).

This review focuses on two specific agents of RNAi, siRNA and miRNA, whose action depends on the same families of proteins. Briefly, double-stranded precursors are processed by a Dicer protein in the cytoplasm to form small fragments from 20 to 30 nucleotides long (siRNA or miRNA). One strand of this short sequence is then loaded into the RNA-induced silencing complex (RISC) by binding to its catalytic component, the Argonaute protein. This strand acts as a guide to a target mRNA. A high degree of complementarity to the target induces degradation by enzymatic cleavage of the molecule. However, the major pathway described in humans is imperfect binding with consequent repression of mRNA translation (Misso et al. 2014; Kumar and Mahato 2015; Chitkara et al. 2015; Pillai et al. 2007).

Although the mechanism of modulating mRNA expression is basically the same, variability at the end of the double-strand sequence seems to be a key factor differentiating siRNA and miRNA. It has become clear that siRNAs are fragments excised from fully complementary double-stranded RNAs of foreign nucleic acid and endogenous genomic loci (Tomari and Zamore 2005). In contrast, miRNAs are fragments from stem-loop precursors with an incomplete double-strand intimately related to cancer establishment and progression (Aaldering et al. 2015; Nicoloso et al. 2009). Compared to siRNAs, whose action relies on the degradation of a mRNA after its binding to RISC, miRNAs can also regulate genes involved in DNA damage and repair (Aaldering et al. 2015). Also, their lower sequence complementarity to the target reduces the chances of developing resistance to treatment, which would require mutations in multiple genes (Chitkara et al. 2015). On the other hand, it increases the induction of off-target effects (Kumar and Mahato 2015).

Some families of miRNA have a key role in promoting tumor suppression. Others, called oncomiRs, favor the development of cancer by targeting tumor suppressor genes (Table 1). These opposing classes divide miRNA-based therapy into two strategies: miRNA inhibition and replacement (Aaldering et al. 2015). The first is achieved by using specific molecules, such as anti-miRNA oligonucleotides (AMOs), able to neutralize and degrade oncomiRs (Conde et al. 2015). miR-519a and miR-32 are examples that have been considered relevant triggers to tumor development leading to decrease in patient survival (Shao et al. 2015; Jin et al. 2015;

Table 1 Examples of miRNAs and their action in miRNA-based cancer therapy

miRNA	Action	Type of cancer	REF
miR-519a	OncomiR	Hepatocellular carcinoma	(Shao et al. 2015)
miR-17-5p		Triple negative breast carcinoma	(Jin et al. 2015)
miR-32		Prostate carcinoma	(Liao et al. 2015)
miR-1290		Laryngeal carcinoma	(Janiszewska et al. 2015)
miR-21		Squamous cell carcinoma	(Ge et al. 2016)
miR-34a	Tumor suppressor	Osteosarcoma	(Wu et al. 2013)
let-7b		Lung carcinoma	(Stahlhut and Slack 2015)
miR-145		Colorectal carcinoma	(Drebber et al. 2011)
miR-494		Ovarian carcinoma	(Li et al. 2015a)
miR-212		Gastric carcinoma	(Li et al. 2015b)

Liao et al. 2015; Janiszewska et al. 2015; Ge et al. 2016). On the other hand, downregulation of tumor suppressor miRNAs, such as miR-34a and let-7b, are closely associated with poor prognosis, drug resistance, invasion and metastasis (Wu et al. 2013; Stahlhut and Slack 2015; Drebber et al. 2011; Li et al. 2015a, b). In these cases, exogenous miRNAs (or miRNA mimics) can be delivered to the tumor site to restore normal levels and promote cell homeostasis. However, miRNA mimics are known to be 100 to 1000-fold less potent than their endogenous counterparts (Zhang et al. 2013).

Such diversity in mechanisms of action suggests the use of miRNAs as prognostic, diagnostic, and therapeutic tools to improve treatment of cancer (Chitkara et al. 2015). The utility of these tools will depend on the design of an ideal vector, capable of delivering the payload specifically to the target tissue in sufficient amounts to modulate gene expression (Ferracin et al. 2010; de Planell-Saguer and Rodicio 2011).

3 Challenges for siRNA/miRNA delivery

A therapeutic effect of RNAi is the result of an efficient delivery of small non-coding RNAs into the cytoplasm of a target cell (Navarro et al. 2015). However, before reaching the site of action, many biological barriers must be overcome, including the blood milieu and tumor microenvironment (Chitkara et al. 2015). Technological advancement in transporting the payloads should greatly improve efficacy as well as reduce costs of the treatment (Aaldering et al. 2015).

Despite the promising results with RNAi in cell culture and animal studies, the majority of clinical trials have not advanced to late stages (Chen et al. 2018). Some of the non-conclusive results regarding its efficacy in humans rely partially on intrinsic characteristics of nucleotide-based molecules, such as: (1) serum instability due to rapid degradation by endo- and exonucleases in the bloodstream; (2) inefficient cell entry inherent in the negatively charged nature of siRNA and miRNA molecules; (3) poor pharmacokinetic profile associated with a half-life of about 5 min and rapid renal clearance due to their low molecular mass (≈ 13 kDa); (4) off-target effects, related mainly to non-specific binding of miRNA; (5) stimulation of the innate immune system after induction of interferon responses; (6) inefficient binding due to mutation in the sequence of the target mRNA and (7) short duration of the silencing effect, which requires high and sustained concentrations of payload in the target tissue (Gandhi et al. 2014; Aaldering et al. 2015; Conde et al. 2015; Navarro et al. 2015; Kumar and Mahato 2015).

In order to minimize most of these drawbacks and retain the bioactivity of the molecules, chemical modifications in the backbone or in the sugar moiety as well as the use of new delivery systems have been taken into consideration (Kumar and Mahato 2015). In the first case, *in vivo* instability and off-target effects are minimized by conjugating non-coding RNAs to lipids, peptides and carbohydrates through covalent linkages (Salzano et al. 2016; Moschos et al. 2007; Zimmermann et al. 2017). Conjugation with natural components of the cell membrane promotes miRNA and siRNA interaction with membrane receptors and thus facilitates their uptake (Broderick and Zamore 2011). In the second strategy, the uptake is increased due to characteristics of the designed carrier. Ideally, a successful delivery system should (1) promote long circulation by escaping immune system recognition, (2) maximize accumulation of the nucleic acid inside the target cell, (3) allow endosomal escape of siRNA and miRNA, (4) be easy to produce on a large scale and (5) be safe, biocompatible and cost-effective (Conde et al. 2015).

Many biomaterials have been investigated as candidates for an optimal delivery of nucleic acids, mostly non-viral lipids and protein carriers (Conde et al. 2015; Chen et al. 2018). Among the options, cationic liposomes and polymeric nanoparticles are the two major choices for delivery of oligonucleotides (Zhang et al. 2013; Gao et al. 2011). However, both systems have shown limitations in delivering chemotherapeutic drugs and small non-coding RNAs to the same cell at the same time. If the two delivery events are independent, the

number of cells receiving both payloads is extremely reduced and the efficacy of a combination treatment is poor (Tsouris et al. 2014). Such challenge led to the development of multicomponent conjugates that combine the advantages of lipids and polymers in a single molecule. Polymeric chains able to form nanoparticles mainly by electronic interactions have been hydrophobized with lipid moieties similar to cellular membrane components. The amphiphilic product of this combination can self-assemble in a micellar structure, giving rise to what has been called micelle-like nanoparticles (MNPs) (Navarro et al. 2015).

4 Micelle-like nanoparticles: a new approach for RNAi-based therapy

Since the emerging of RNAi-based therapies, viral vectors have shown the ability to promote sustained gene silencing (Navarro et al. 2015). However, preclinical trials continue to reinforce safety concerns regarding the insertion of viral genome into human chromosomes. In addition, the production of viral vectors is costly and demands complex scaling up processes (Chen et al. 2018).

In face of these challenges, two different lines of research have emerged: one exploring lipid carriers and another focused on polymeric systems. Biocompatibility and similarity to the plasma membrane composition are two characteristics that made lipid carriers front runners in this field. The cationic surface charge of these particles facilitates their uptake by the anionic membrane of eukaryotic cells, which considerably improves transfection efficiency (Chitkara et al. 2015). Similar interaction is reported for the use of cationic polymers as main components of delivery systems. In comparison to lipids, they show particular advantages such as high stability, variety in architecture and molecular weights (MW), and multiple functional groups available for conjugation (Gandhi et al. 2014; Navarro et al. 2015).

Although both systems succeed in protecting the nucleic acid from degradation and metabolism to preserve bioactivity, the balance between efficacy and safety still needs to be considered carefully (Croy and Kwon 2006). Structure-function screenings have shown toxicity of cationic transfection reagents with high MW and charge, despite their higher transfection efficiency compared to low MW components (Grayson et al. 2006; Gebhart and Kabanov 2001). Additionally, they tend to form aggregates and lose activity in the presence of serum or salt-containing environments. A last concern about their behavior according to changes in pH has been addressed. These changes can significantly affect the release kinetics of the loaded siRNA or miRNA (Sarett et al. 2015).

To overcome some of the related drawbacks, the synthesis of environmentally-responsive copolymers has been suggested. The idea is to conjugate polymeric and lipid blocks with specific chemical groups to create amphiphilic molecules with multiple effects, such as: (1) release of the payload in reducible environments, (2) long circulation and escape from the reticuloendothelial system in the bloodstream, (3) incorporation of hydrophobic and hydrophilic drugs and (4) improvement of cell association and transfection (Navarro et al. 2015; Sarett et al. 2015; Salzano et al. 2016).

Amphiphilic multi-block conjugates can disperse as single units in water until they reach a limit called the critical micelle concentration (CMC), when the accumulation of free energy in the system favors the rearrange of the molecules towards a less energetic and more stable micellar structure (Prameela et al. 2015). In this way, hydrophobic moieties of the conjugates are grouped in the core of a micelle, while the hydrophilic moieties face the exterior and form a water-soluble shell in direct contact with water (Navarro et al. 2015). This shell is essential to protect miRNA and siRNA entrapped in the inner layers of the MNPs. The main challenge here is the complexity of a multi-block structure, which requires careful design and engineering to achieve reproducible formulations (Kumari et al. 2010). The characterization of the copolymers includes not only proving the proposed chemical structure, but also testing each functionalization in a biological model.

The incorporation of small non-coding oligonucleotides into MNPs can be done by electronic interaction or covalent binding (Fig. 1). In the first scenario, compact complexes are formed by interaction between positively charged amino groups of the carrier and negatively charged phosphate groups of the nucleic acids. The nanostructure decreases hydrophilicity and charge of the siRNA or miRNA, which facilitates its cellular uptake mainly by non-specific pinocytosis (Ruponen et al. 2001; Teo et al. 2013; Navarro et al. 2014). In the second case, components of the carrier are covalently bound to small non-coding RNAs mostly by disulfide bridge, which is highly sensitive to reductive intracellular environment (Salzano et al. 2015a).

Three synthetic polymers have been widely used for RNAi-based therapy: polyethylenimine (PEI), poly-L-lysine (PLL), and polyamidoamine (PAMAM). Among natural compounds, chitosan has been the first-choice in the majority of the studies (Navarro et al. 2015).

4.1 Polyethylenimine

The efficacy of PEI as a transfection agent has been proven in a broad range of cells, especially when polymeric branches over 25 kDa MW are the main component of the delivery systems (Chitkara et al. 2015). However, the cationic charge of high MW PEI is strong enough to also disrupt the plasma membrane, causing undesired toxicity in most clinical studies (Parmar et al. 2017). In contrast, low MW PEI (< 2 kDa) has shown minimal toxicity but up to 8-

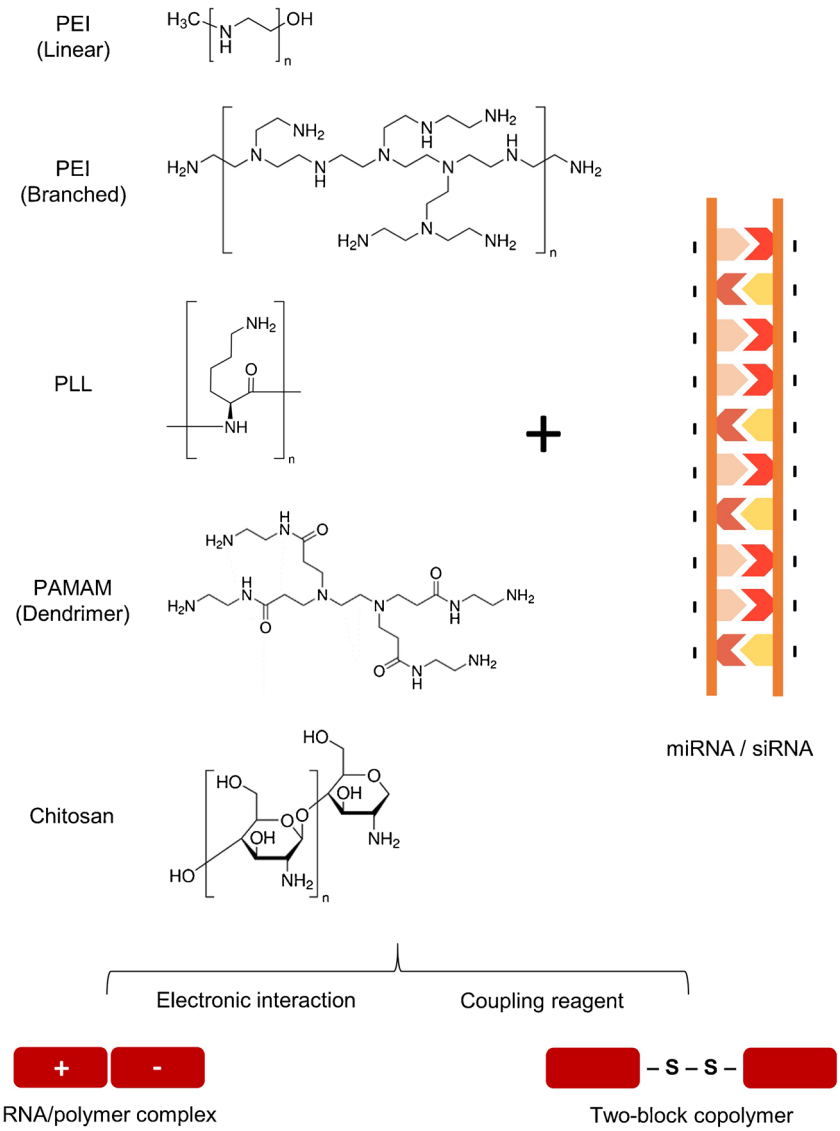

Fig. 1 Chemical structure of the main cationic polymers and schematic representation of their interaction with small non-coding oligonucleotides

fold decrease in gene transfection when compared to bigger branches (Grayson et al. 2006). To overcome such duality, low MW PEI has been attached to lipid moieties able to facilitate its uptake by eukaryotic cells. Copolymers of PEI and saturated fatty acids, such as 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) and caprylic acid (CA), lowered the inherent cytotoxic effects of the delivery system and reduced the amount of polymer necessary to fully complex nucleic acid cargos (Navarro et al. 2014). The conjugation of PEI to unsaturated fatty acids, such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and linoleic acid (LA), could also improve gene silencing by facilitating endosomal escape of the carrier (Navarro et al. 2014; Aliabadi et al. 2011). Different degrees of substitution in the lipid chains causes variation in oligonucleotide binding affinity, surface charge, and cellular uptake (Aliabadi et al. 2011; Alshamsan et al. 2009).

The interaction of oligonucleotides and the majority of cationic polymers occurs through complexation with primary amine ends of the polymeric chains. PEI molecules, in particular, are also formed by secondary and tertiary amines in their chemical structure. These play a key role in the endosomal escape of delivery systems by causing the so-called proton sponge effect (Akinc et al. 2005). During endosomal saturation, ATPase pumps transfer protons from the cytosol into endosomes as an attempt to lower their internal pH. Unsaturated amino groups in the PEI molecule have the ability to sequester these protons, maintaining a continuous influx. The active pumping of protons is followed by passive entry of chloride ions and water. Consequently, there is an increase of osmotic pressure which leads to swelling, rupture of the endosomes and release of the delivery system into the cytoplasm (Nel et al. 2009; Salzano et al. 2015b). It is also suggested that protonated amino groups from the PEI can interact

with the endosomal membrane, which would cause structural destabilization, formation of pores and consequent escape of the entrapped delivery system (Yue et al. 2011; Benjaminsen et al. 2013).

4.2 Poly-L-lysine

One of the first polymers used as carrier for gene delivery, PLL is a linear polypeptide composed of positively charged units of lysine (Toncheva et al. 1998). Although a wide range of polymeric chains is commercially available (0.50 to over 100 kDa), the molecules between 2.4 and 30 kDa have been chosen for RNAi-based therapy (Yogasundaram et al. 2012). The size of the chain is a determinant of the characteristics of PLL assemblies. Low MW (≈ 5 kDa) peptide chains have shown inefficient transfection and high toxicity (Ward et al. 2001).

Despite its ability to form complexes with small non-coding RNAs, PLL shows high affinity for plasma proteins and presents rapid clearance from the circulation (Dash et al. 1999). Its pKa value of around 10.0 lowers the occurrence of proton sponge effect in comparison to other cationic polymers (Sonawane et al. 2003). Instead, particles containing PLL as a backbone are enzymatically digested once transported into lysosomes, which leads to a low transfection efficiency (Patil et al. 2011). Alternatively, conjugation of PLL to fusogenic peptides, fatty acids, histidine, and chloroquine improves endosomal escape. They destabilize the endosomal membrane and allow delivery of the nucleic acids into the cytoplasm (Pouton et al. 1998).

It is important to highlight that many findings related to DNA complexation with cationic polymers differ from what happens in their interaction with siRNA or miRNA. The molecular weight, topology, and charge density of oligonucleotides are some of the factors that directly affect the complexation rates (Zheng et al. 2012). Compared to DNA, flexible and shorter nucleic acid chains form more compact structures with PLL (Shukla et al. 2014).

4.3 Polyamidoamine

In contrast to linear polymers, PAMAM belongs to a class of spherical molecules with multiple branches, the so-called dendrimers. The layers forming a dendrimer (or generations, Gs) are synthesized one at a time, from the core to the periphery, which results in a structure that offers high functionality, defined molecular weight, and low polydispersity (Navarro et al. 2015; Yu et al. 2015). The repeating units of PAMAM provide flexibility to create spaces within the building blocks where small molecules, such as drugs and non-coding RNAs, can be entrapped. Also, the large number of peripheral amino groups allows covalent binding of numerous targets (Singh et al. 2008). Finally, their high stability, non-immunogenicity, and great capability to deliver nucleic acids into different types of cells make PAMAM a promising non-viral delivery system for RNAi-based therapies (Palmerston Mendes et al. 2017).

Increase in the number of generations have been associated with more potent transfection of the cargoes. However, dendrimers bigger than seven generations are known to cause significant toxicity (Boas and Heegaard 2004; Dufès et al. 2005). Alternatively, lipid-modification of non-toxic dendrimers has successfully improved transfection in comparison to their non-modified counterparts (Khopade et al. 2004; Movassaghian et al. 2011).

Since the first applications of PAMAM-based carriers as DNA transfection agents in the early 90s, many variations of this dendrimer have been released in the market (Yu et al. 2015; Haensler and Szoka Jr 1993). A common drawback is reported after the addition of functional groups to PAMAM molecules: it creates heterogeneity in the dendrimer scaffold. Consequently, controlling the number of ligands per particle becomes extremely difficult and could result in uncertain therapeutic effects (van Dongen et al. 2014).

4.4 Chitosan

Among the natural polymers used for drug delivery, chitosan has shown great potential for RNAi-based therapy. It is a derivative of chitin, composed of D-glucosamine and N-acetyl-D-glucosamine blocks, non-cytotoxic, non-immunogenic, biocompatible, and easy to adhere to mammalian cells (Modra et al. 2015). In an acidic environment, the amino groups of the D-glucosamine units (pKa ≈ 6.5) become positively charged and available to complex with siRNA or miRNA through electrostatic interactions (Layek et al. 2015).

Despite its advantages compared to synthetic polymers, unmodified chitosan has limitations in delivering nucleic acids at physiological pH. Experiments with pDNA revealed a very strong binding between the payload and the polymeric carrier, which resulted in inefficient unpacking in the cytoplasm. Furthermore, chitosan is associated with poor endosomal escape due to the lack of buffering amines (Wong et al. 2006; Lavertu et al. 2006). To overcome these drawbacks, the amino (C2 position) and hydroxyl groups of the molecule have been conjugated to hydrophobic moieties, such as lipid chains and bile acids (Hu et al. 2006; Layek and Singh 2012; Mandke and Singh 2012a; Chae et al. 2005). The modification weakens the polymer/nucleic acid interaction and facilitates unpacking inside the cell. Transfection efficiency is even greater when unsaturated lipid chains with one or two double bonds are bound to the polymeric chain (Mandke and Singh 2012b).

4.5 Functionalization of the polymeric blocks

Functionalization of nanoparticles by covering their surface with polyethylene glycol (PEG) was one of the first attempts

used to optimize drug delivery systems. Apart from the advantages of each polymer already mentioned in this review, all of them present a common drawback when assembled in a nanostructure: fast clearance by the reticuloendothelial system (RES) (Brigger et al. 2002). Surface properties appear to be play a more important role in early interactions with components of the RES than size. Attachment of PEG in the surface of nanoparticles has efficiently reduced clearance from the bloodstream, helping to improve the delivery of small non-coding RNAs to sites of action (Yu et al. 2015; Jokerst et al. 2011; Dobrovolskaia and McNeil 2007).

Although the mechanisms of PEG effect are still unclear, recent findings drive attention to the presence of apolipoprotein E (ApoE) in the protein corona formed around nanoparticles after entry in the circulatory system. Lower levels of ApoE in the protein corona favor faster blood clearance. These levels are directly related to PEG density. Polymeric particles with less than 20 PEG chains per 100 nm^2 adsorb higher quantities of ApoE and are more likely to avoid early clearance (Bertrand et al. 2017).

Paradoxically, PEGylated MNPs can escape not only from RES uptake but also from internalization in the target tissue when the shielded cationic core is not available to interact with the negatively charged cell membrane (Choi et al. 1998; Oupicky et al. 2002). Introducing spacers between PEG and the cationic polymer is one of the strategies to overcome this challenge (Fig. 2) (Oumzil et al. 2011). Peptide chains sensitive to proteolytic activity are the linkers of choice when the targeted tissue shows upregulation of extracellular enzymes, such as matrix metalloproteinases (MMPs) and human leukocyte elastase (HLE). The increased enzymatic activity works as a trigger to de-shield the layer of PEG preferentially in the diseased tissue, optimizing the delivery of cargoes (Pak et al. 1999; Mansour et al. 2003).

Some cationic polymers, such as PEI, can be considered linkers themselves. In this case, the acidic environment of inflamed tissues and solid tumors destabilizes the structure of the particles by creating positive charges in the amino groups of the polymeric chain (Salzano et al. 2015b). A similar effect is induced by protonizable imidazole rings when poly-histidine is used as a linker between the core of MNPs and PEG (Midoux et al. 2009; Chang et al. 2010).

Another example of functionalization is the synthesis of bioconjugates sensitive to the reductive intracellular environment of tumor tissues. Elevated concentrations of glutathione (GSH) are commonly detected in the cytoplasm of cancer cells. GSH is capable of breaking disulfide bridges in lipid-modified oligonucleotides, which favors the release of free cargoes into the targeted cells (Salzano et al. 2016; Torchilin 2009). Breast, ovarian and lung tumors showed elevated GSH levels in comparison to healthy or peritumoral tissues, which helps to explain the stability of disulfide bonds in extracellular conditions (Gamcsik et al. 2012).

Functionalization of MNPs and the consequent increase in transfection efficiency of small non-coding RNAs can also be done by attaching cell-penetrating peptides (CPPs) to the surface of the particles (Kumari et al. 2010). Sometimes named "Trojan Horse" peptides, these amino acid sequences, such as the trans-activating transcriptional activator (TAT), improve internalization of the delivery systems through endocytic pathways or by direct translocation of components of the cell membrane (Layek et al. 2015).

Finally, a receptor-specific uptake can be achieved by coupling antibodies, growth factors, peptides, and other small molecules to the surface of the MNPs. The ligand must correspond to receptors overexpressed in the targeted cells, avoiding off-target effects and cytotoxicity to healthy tissues (Jing et al. 2013). Many studies have shown the benefits of attaching folic acid to MNPs. Folate receptor is overexpressed in most cancer cells, so that the ligand induces folate-assisted endocytosis and significantly increases transfection efficiency (Modra et al. 2015). Transferrin has also been used in RNAi-based therapy due to the upregulation of its receptor on the surface of many cancer cell-types (Grabowska et al. 2015; Daniels et al. 2012).

5 Recent achievements in cancer therapy

Many combinations of polymeric blocks and lipid chains have been used to create a broad variety of nanoparticles for the delivery of small non-coding RNAs (Wang et al. 2002; Incani et al. 2009; Abbasi et al. 2008). However, only few of them can be characterized as MNPs.

Low MW (1.8 kDa) and high MW (25 kDa) PEI were linked to DOPE to create amphiphilic conjugates. The resulting products were further functionalized with a peptide sequence sensitive to matrix metalloproteinases (MMPs) and PEG (2 kDa). The triblock conjugates were used to produce MNPs for the co-delivery of anti-GFP siRNA and paclitaxel to A549 cells (lung cancer). Enzymatic cleavage by human MMPs was efficiently proven *in vitro* as well as downregulation of GFP expression. After only a single treatment with low and high MW PEI conjugates, the levels of GFP decreased about 55% compared to untreated cells (Zhu et al. 2014). The same group functionalized low MW PEI-DOPE conjugate with azobenzene-modified PEG (2 kDa). Azobenzene is a linker sensitive to the lower pH of a tumor environment. Cancer cells treated with the formulation carrying anti-GFP siRNA internalized 3.2-fold more MNPs under hypoxia than under normoxia. *In vivo*, 32% of GFP downregulation was detected in A2780/GFP tumors (human ovarian carcinoma) (Perche et al. 2014). Aliabadi et al. (2011) analyzed downregulation of GAPDH and P-gp expression in MDA-MB-435 MDR cells (breast cancer) after treatment with MNPs of PEI attached to caprylic (C8), myristic (C14), palmitic (C16),

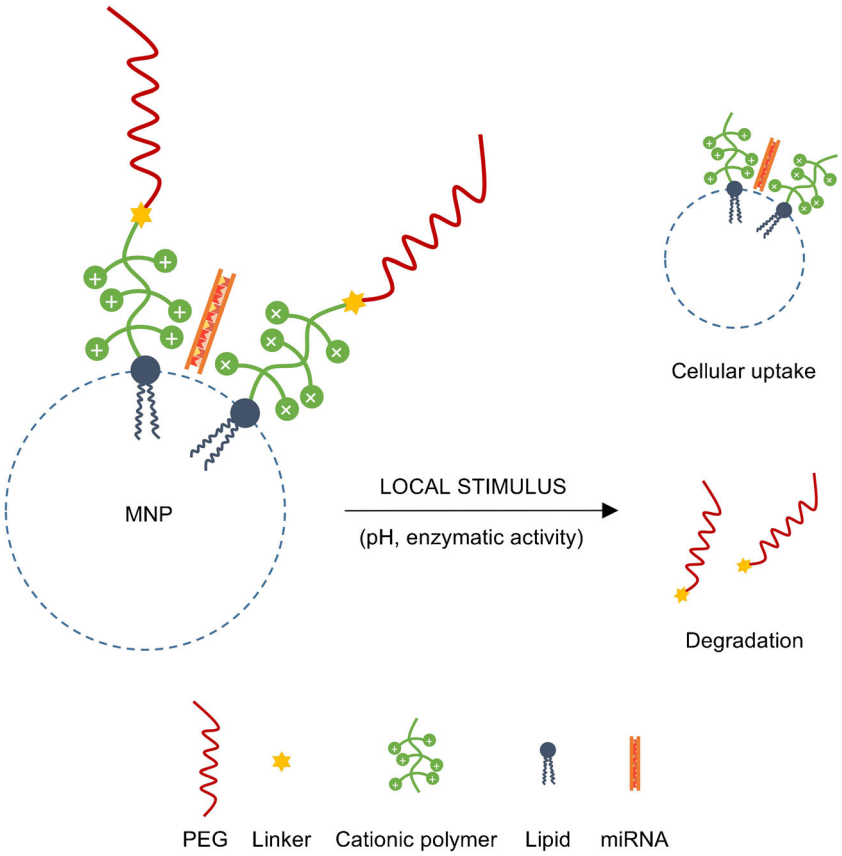

Fig. 2 Schematic representation of a functionalized MNP composed of three blocks: lipid, cationic polymer and PEG moieties. The spacer linking the cationic polymer and the PEG chain is cleaved by local stimuli in the diseased tissue. PEG is thus eliminated in the extracellular environment and the unprotected MNP becomes available to cellular uptake and miRNA transfection

stearic (C18), oleic (C18, one insaturation) and linoleic (C18, two insaturations) acids. The decrease in gene expression was dependent on the nature of the lipid and the level of substitution, but no clear tendency was found (Aliabadi et al. 2011).

PLL has also been lipid-modified to create MNPs. Guo et al. (2012) used cholic acid as modifier of PLL from 15 to 30 kDa. Later on, the amphiphilic chain was coupled to PEG (5 kDa) through a benzoic imine linker, known to be cleaved at the lower pH of extracellular tumor environments. The triblock conjugate formed MNPs able to complex with anti-VEGF siRNA (226–263 nm) and stable in serum for over 48 h. Studies in a mouse model of prostate carcinoma showed approximately 60% of reduction in the target mRNA levels and significant tumor suppression. Final tumor size of the treated group was approximately half of the size in the group control. Also, no marked toxicity or undesirable immune response were noticed (Guo et al. 2012). Zhang et al. (2016) created a triblock copolymer of N-succinyl chitosan, PLL, and palmitic acid (NSC–PLL–PA) to co-deliver Dox and anti-P-gp siRNA. The *in vitro* release of Dox was significantly higher in acidic pH compared to neutral environment. In subcutaneous model of HepG2/ADM tumors (multidrug-resistance hepatocellular carcinoma), the combination therapy with MNPs doubled the tumor growth inhibition reported after injections of free Dox. The antitumor effect was related to efficient downregulation of P-gp (Zhang et al. 2016).

Liu et al. (2015) conjugated the origin of PAMAM (G2) dendrimer to a hydrophobic alkyl chain (C18) and the terminals of the molecule to arginine, a cell-penetrating peptide. MNPs of about 50 nm in diameter protected anti-Hsp27 siRNA from enzymatic degradation *in vitro*. Also, the arginine-decorated particles caused downregulation of the target mRNA in PC-3 cells (prostate cancer) comparable to the commercial transfection vector (Liu et al. 2015). Corroborating with these findings, Márquez-Miranda et al. (2016) used coarse-grained molecular dynamics simulations to compare different combinations of PAMAM (G0, G1, G2) and lipid chains (18C, 15C, or 13C). The most effective siRNA transfection was given by the PAMAM (2G)-C18 alkyl chain conjugate (Márquez-Miranda et al. 2016).

Chitosan, the last example of polymer discussed in this review, was modified with Lipoïd® S75–3 (a soybean lecithin) to form MNPs of about 70 nm in diameter. The system was used to deliver anti-EGFR siRNA to U87MG cells (human glioblastoma). After 96 h of incubation with the treatment, EGFR expression was reduced by 52% (Messaoudi et al. 2014). The same group combined the delivery of anti-Galectin-1 and anti-EGFR siRNAs to decrease Temozolomide (TMZ) resistance in glioblastoma. Survival of the mice treated with combined siRNA therapy and 40 mg/kg of TMZ was significantly higher compared to groups treated with single RNA therapy and the

chemotherapeutic drug. The effect was a result of efficient downregulation of EGFR and Galectin-1 analyzed by immunofluorescence in excised U87MG tumor tissues. Protein levels were, respectively, 24 and 28% lower than control group (Danhier et al. 2015). More recently, El-Sayed et al. (2018) successfully coupled three fatty acyl derivatives of CGKRK homing peptides to chitosan. The conjugates showed over 90% of binding affinity to siRNA targeting kinesin spindle protein and complete protection from early enzymatic degradation. The best cytotoxic effect (35%) on MDA-MB-231 cells (human breast cancer) was caused by the oleoyl derivative at polymer concentration of 10 μg/mL (El-Sayed et al. 2018).

The attachment of hydrophobic groups, such as palmitate, to PEG-PEI might cause false positive siRNA transfection results in luciferase assay. Rheiner et al. (2017) reported changes in the conformation of polymeric nanoassemblies when PEG-PEI was lipid-modified, which may have caused over-ubiquitination and degradation of luciferase in HT29 cells (human colon cancer) (Rheiner et al. 2017).

5.1 Clinical trials

According to the Clinical Trials Database, only eight studies in the United States investigated the use of non-viral drug delivery systems as carriers of siRNA or miRNA for cancer therapy (Table 2) (Clinical Trials Database 2018). These are phase I/IIa studies testing the safety, pharmacokinetics, and dose scaling of the treatments. Seven trials focus on the delivery of siRNA as payload. The only investigation of the therapeutic effect of miRNA was terminated due to occurrence of five serious immune-related adverse events. The majority of the studies on the action of miRNA in humans highlight the use of these oligonucleotides as markers for cancer diagnostic and prognostic, but not treatment.

None of the carriers is classified as MNPs. The most similar to this new class of delivery system is the cyclodextrin nanoparticle, which comprises the idea of linking different chemical blocks that assemble in a nanostructure (Zuckerman et al. 2014). In this case, a cationic cyclodextrin-based polymer forms the core of the particle, which surface is modified by attachment of PEG and human transferrin protein. The siRNA is entrapped in the core of the particle by electronic interaction with the cationic polymer. This was the first trial of RNAi-based cancer therapy in human patients.

6 Conclusion

The combination of chemo- and RNAi-based therapies has been considered a breakthrough in the treatment of cancer and a real alternative to overcome drug resistance. The understanding of the role of small non-coding RNAs in the disease led to the development of a wide range of prognostic, diagnostic, and therapeutic tools. Among them, non-viral carriers have emerged as a promising vector to deliver oligonucleotides specifically to the tumor tissue, which minimizes the off-target effects of gene therapy. In this review, we introduced a new class of non-viral drug delivery systems, the micelle-like nanoparticles. They represent the combination of polymeric and lipid systems in an attempt to have the advantages of both materials in a single carrier. The challenges of finding a perfect backbone for future drug carriers are followed by the idea of functionalization as a strategy to maximize the concentration of therapeutic agents in a targeted tissue. PEGylation, sensitivity to the action of enzymes and reductive microenvironments come along with the inclusion of CPPs and other ligands to the surface of delivery systems. These approaches have improved the therapeutic effect against a variety of cancer cells, including glioblastoma, prostate, lung, and breast carcinomas. However, the complexity of characterizing multicomponent delivery systems and finding reproducible results still hold back the translation from preclinical studies to trials in humans.

Table 2 Registered clinical trials on RNAi-based cancer therapy using non-viral drug delivery systems (Clinical Trials Database 2018)

Payload	Carrier	Type of cancer	Study	Start date (status)
Anti-RMM2 siRNA	Cyclodextrin nanoparticle	Solid tumors	NCT00689065	2008 (terminated)
Anti-PKN3 siRNA	PEGylated lipoplex	Solid tumors	NCT00938574	2009 (completed)
Anti-PLK1 siRNA	Lipid nanoparticle	Solid tumors / Lymphoma	NCT01437007	2011 (completed)
Anti-PKN3 siRNA	PEGylated lipoplex	Pancreatic	NCT01808638	2013 (completed)
Anti-MYC siRNA	Lipid nanoparticle	Solid tumors / Myeloma / Lymphoma	NCT02110563	2014 (terminated)
Anti-MYC siRNA	Lipid nanoparticle	Hepatic	NCT02314052	2014 (terminated)
Anti-EphA2 siRNA	Liposome	Solid tumors	NCT01591356	2015 (recruiting)
miR-RX34	Liposome	Hepatic	NCT01829971	2013 (terminated)

Acknowledgements The authors thank Dr. William Hartner for helpful comments.

References

L.J. Aaldering, H. Tayeb, S. Krishnan, et al., RNA Biol. **12**, 412–425 (2015)
M. Abbasi, H. Uludag, V. Incani, et al., Biomacromolecules **9**, 1618–1630 (2008)
M. Abbasi, A. Lavasanifar, L.G. Berthiaume, et al., Cancer **116**, 5544–5554 (2010)
A. Akinc, M. Thomas, A.M. Klibanov, et al., J. Gene Med. **7**, 657–663 (2005)
H.M. Aliabadi, B. Landry, R.K. Bahadur, et al., Macromol. Biosci. **11**, 662–672 (2011)
A. Alshamsan, A. Haddadi, V. Incani, et al., Mol. Pharm. **6**, 121–133 (2009)
R.V. Benjaminsen, M.A. Mattebjerg, J.R. Henriksen, et al., Mol. Ther. **21**, 149–157 (2013)
N. Bertrand, P. Grenier, M. Mahmoudi, et al., Nat. Commun. **8**, 777 (2017)
U. Boas, P.M. Heegaard, Chem. Soc. Rev. **33**, 43–63 (2004)
I. Brigger, C. Dubernet, P. Couvreur, Adv. Drug Deliv. Rev. **54**, 631–651 (2002)
J.A. Broderick, P.D. Zamore, Gene Ther. **18**, 1104–1110 (2011)
R.W. Carthew, E.J. Sontheimer, Cell **136**, 642–655 (2009)
S.Y. Chae, S. Son, M. Lee, et al., J. Control. Release **109**, 330–344 (2005)
K.L. Chang, Y. Higuchi, S. Kawakami, et al., Bioconjug. Chem. **21**, 1087–1095 (2010)
X. Chen, L.S. Mangala, C. Rodriguez-Aguayo, et al., Cancer Metastasis Rev. **37**, 107–124 (2018)
D. Chitkara, A. Mittal, R.I. Mahato, Adv. Drug Deliv. Rev. **81**, 34–52 (2015)
Y.H. Choi, F. Liu, J.S. Kim, et al., J. Control. Release **54**, 39–48 (1998)
Clinical Trials Database (2018). https://clinicaltrials.gov. Accessed 5 Mar 2018
J. Conde, E.R. Edelman, N. Artzi, Adv. Drug Deliv. Rev. **81**, 169–183 (2015)
S.R. Croy, G.S. Kwon, Curr. Pharm. Des. **12**, 4669–4684 (2006)
F. Danhier, K. Messaoudi, L. Lemaire, et al., Int. J. Pharm. **481**, 154–161 (2015)
T.R. Daniels, E. Bernabeu, J.A. Rodríguez, et al., Biochim. Biophys. Acta **1820**, 291–317 (2012)
P.R. Dash, M.L. Read, L.B. Barrett, et al., Gene Ther. **6**, 643–650 (1999)
M. de Planell-Saguer, M.C. Rodicio, Anal. Chim. Acta **699**, 134–152 (2011)
M.A. Dobrovolskaia, S.E. McNeil, Nat. Nanotechnol. **2**, 469–478 (2007)
U. Drebber, M. Lay, I. Wedemeyer, et al., Int. J. Oncol. **39**, 409–415 (2011)
C. Dufès, I.F. Uchegbu, A.G. Schätzlein, Adv. Drug Deliv. Rev. **57**, 2177–2202 (2005)
N.S. El-Sayed, M. Sharma, H.M. Aliabadi, et al., Int. J. Biol. Macromol. **112**, 694–702 (2018)
M. Ferracin, A. Veronese, M. Negrini, Expert. Rev. Mol. Diagn. **10**, 297–308 (2010)
M.P. Gamcsik, M.S. Kasibhatla, S.D. Teeter, et al., Biomarkers **17**, 671–691 (2012)
N.S. Gandhi, R.K. Tekade, M.B. Chougule, J. Control. Release **194**, 238–256 (2014)
Y. Gao, X.L. Liu, X.R. Li, Int. J. Nanomedicine **6**, 1017–1025 (2011)
M. Garofalo, C. Quintavalle, G. Romano, et al., Curr. Mol. Med. **12**, 27–33 (2012)
Y. Ge, L. Zhang, M. Nikolova, et al., Nat. Cell Biol. **18**, 111–121 (2016)
C.L. Gebhart, A.V. Kabanov, J. Control. Release **73**, 401–416 (2001)
H. Gibori, S. Eliyahu, A. Krivitsky, et al., Nat. Commun. **9**, 16 (2018)
S.L. Ginn, I.E. Alexander, M.L. Edelstein, et al., J. Gene. Med. **15**, 65–77 (2013)
GLOBOCAN (2012). http://gco.iarc.fr. Accessed 13 Mar 2018
A.M. Grabowska, R. Kircheis, R. Kumari, et al., Biomater. Sci. **3**, 1439–1448 (2015)
A.C. Grayson, A.M. Doody, D. Putnam, Pharm. Res. **23**, 1868–1876 (2006)
J. Guo, W.P. Cheng, J. Gu, et al., Eur. J. Pharm. Sci. **45**, 521–532 (2012)
J. Haensler, F.C. Szoka Jr., Bioconjug. Chem. **4**, 372–379 (1993)
F.Q. Hu, M.D. Zhao, H. Yuan, et al., Int. J. Pharm. **315**, 158–166 (2006)
V. Incani, X. Lin, A. Lavasanifar, et al., ACS Appl. Mater. Interfaces **1**, 841–848 (2009)
J. Janiszewska, M. Szaumkessel, M. Kostrzewska-Poczekaj, et al., PLoS One **10**, e0144924 (2015)
Y.Y. Jin, J. Andrade, E. Wickstrom, PLoS One **10**, e0142574 (2015)
F. Jing, J. Li, D. Liu, et al., Pharm. Biol. **51**, 643–649 (2013)
J.V. Jokerst, T. Lobovkina, R.N. Zare, et al., Nanomedicine (London) **6**, 715–728 (2011)
A.J. Khopade, D.B. Shenoy, S.A. Khopade, et al., Langmuir **20**, 7368–7373 (2004)
V. Kumar, R.I. Mahato, Pharm. Res. **32**, 341–361 (2015)
A. Kumari, S.K. Yadav, S.C. Yadav, Colloids Surf. B: Biointerfaces **75**, 1–18 (2010)
M. Lavertu, S. Méthot, N. Tran-Khanh, et al., Biomaterials **27**, 4815–4824 (2006)
B. Layek, J. Singh, Carbohydr. Polym. **89**, 403–410 (2012)
B. Layek, L. Lipp, J. Singh, Int. J. Mol. Sci. **16**, 28912–28930 (2015)
Z. Li, T.M. Rana, Nat. Rev. Drug Discov. **13**, 622–638 (2014)
N. Li, X. Zhao, L. Wang, et al., Tumour Biol. **37**, 7767–7776 (2015a)
D. Li, Z. Li, J. Xiong, et al., Am. J. Cancer Res. **5**, 2980–2997 (2015b)
H. Liao, Y. Xiao, Y. Hu, et al., Oncol. Lett. **10**, 2055–2062 (2015)
C. Liu, G. Zhao, J. Liu, et al., J. Control. Release **140**, 277–283 (2009)
X. Liu, C. Liu, J. Zhou, et al., Nano **7**, 3867–3875 (2015)
R. Mandke, J. Singh, Pharm. Res. **29**, 883–897 (2012a)
R. Mandke, J. Singh, J. Pharm. Sci. **101**, 268–282 (2012b)
A.M. Mansour, J. Drevs, N. Esser, et al., Cancer Res. **63**, 4062–4066 (2003)
V. Márquez-Miranda, I. Araya-Durán, M.B. Camarada, et al., Sci. Rep. **6**, 29436 (2016)
K. Messaoudi, P. Saulnier, K. Boesen, et al., Int. J. Nanomedicine **9**, 1479–1490 (2014)
P. Midoux, C. Pichon, J.J. Yaouanc, et al., Br. J. Pharmacol. **157**, 166–178 (2009)
G. Misso, M.T. Di Martino, G. De Rosa, et al., Mol. Ther. Nucleic Acids. **e194**, 3 (2014)
K. Modra, S. Dai, H. Zhang, et al., Eng. Life Sci. **15**, 489–498 (2015)
C. Morrison, Nat. Rev. Drug Discov. **17**, 156–157 (2018)
S.A. Moschos, S.W. Jones, M.M. Perry, et al., Bioconjug. Chem. **18**, 1450–1459 (2007)
S. Movassaghian, H.R. Moghimi, F.H. Shirazi, J. Drug Target. **19**, 925–932 (2011)
K. Nakamura, A.S. Abu Lila, M. Matsunaga, et al., Mol. Ther. **19**, 2040–2047 (2011)
G. Navarro, S. Essex, R.R. Sawant, et al., Nanomedicine **10**, 411–419 (2014)
G. Navarro, J. Pan, V.P. Torchilin, Mol. Pharm. **12**, 301–313 (2015)
A.E. Nel, L. Mädler, D. Velegol, et al., Nat. Mater. **8**, 543–557 (2009)
M.S. Nicoloso, R. Spizzo, M. Shimizu, et al., Nat. Rev. Cancer **9**, 293–302 (2009)
U. Nir, B. Cohen, L. Chen, et al., Nucleic Acids Res. **12**, 6979–6993 (1984)
K. Oumzil, S. Khiati, M.W. Grinstaff, et al., J. Control. Release **151**, 123–130 (2011)
D. Oupicky, M. Ogris, K.A. Howard, et al., Mol. Ther. **5**, 463–472 (2002)

C.C. Pak, R.K. Erukulla, P.L. Ahl, et al., Biochim. Biophys. Acta **1419**, 111–126 (1999)
L. Palmerston Mendes, J. Pan, V.P. Torchilin, Molecules. **22**, 1401 (2017)
M.B. Parmar, D.N. Meenakshi Sundaram, R.B. K C, et al., Acta Biomater. **66**, 294–309 (2017)
M.L. Patil, M. Zhang, T. Minko, ACS Nano **5**, 1877–1887 (2011)
F. Perche, S. Biswas, T. Wang, et al., Angew. Chem. Int. Ed. Eng. **53**, 3362–3366 (2014)
R.S. Pillai, S.N. Bhattacharyya, W. Filipowicz, Trends Cell Biol. **17**, 118–126 (2007)
C.W. Pouton, P. Lucas, B.J. Thomas, et al., J. Control. Release **53**, 289–299 (1998)
G.K. Prameela, B.V. Phani Kumar, A. Pan, et al., Phys. Chem. Chem. Phys. **17**, 30560–30569 (2015)
S. Rheiner, D. Reichel, P. Rychahou, et al., Int. J. Pharm. **528**, 536–546 (2017)
M. Ruponen, S. Rönkkö, P. Honkakoski, et al., J. Biol. Chem. **276**, 33875–33880 (2001)
G. Salzano, R. Riehle, G. Navarro, et al., Cancer Lett. **343**, 224–231 (2014)
G. Salzano, G. Navarro, M.S. Trivedi, et al., Mol. Cancer Ther. **14**, 1075–1084 (2015a)
G. Salzano, D.F. Costa, V.P. Torchilin, Curr. Pharm. Des. **21**, 4566–4573 (2015b)
G. Salzano, D.F. Costa, C. Sarisozen, et al., Small **12**, 4837–4848 (2016)
S.M. Sarett, C.E. Nelson, C.L. Duvall, J. Control. Release **218**, 94–113 (2015)
J. Shao, J. Cao, Y. Liu, et al., FEBS Open Bio. **5**, 893–899 (2015)
R.S. Shukla, B. Qin, K. Cheng, Mol. Pharm. **11**, 3395–3408 (2014)
I. Singh, A.K. Rehni, R. Kalra, et al., Pharmazie **63**, 491–496 (2008)
N.D. Sonawane, F.C. Szoka Jr., A.S. Verkman, J. Biol. Chem. **278**, 44826–44831 (2003)
C. Stahlhut, F.J. Slack, Cell Cycle **14**, 2171–2180 (2015)
P.Y. Teo, C. Yang, J.L. Hedrick, et al., Biomaterials **34**, 7971–7979 (2013)
Y. Tomari, P.D. Zamore, Genes Dev. **19**, 517–529 (2005)
V. Toncheva, M.A. Wolfert, P.R. Dash, et al., Biochim. Biophys. Acta **1380**, 354–368 (1998)
V. Torchilin, Eur. J. Pharm. Biopharm. **71**, 431–444 (2009)
V. Tsouris, M.K. Joo, S.H. Kim, et al., Biotechnol. Adv. **32**, 1037–1050 (2014)
M.A. van Dongen, C.A. Dougherty, M.M. Banaszak Holl, Biomacromolecules **15**, 3215–3234 (2014)
D.A. Wang, A.S. Narang, M. Kotb, et al., Biomacromolecules **3**, 1197–1207 (2002)
T. Wang, G. Wang, X. Zhang, et al., Sci. Rep. **7**, 14736 (2017)
C.M. Ward, M.L. Read, L.W. Seymour, Blood **97**, 2221–2229 (2001)
K. Wong, G. Sun, X. Zhang, et al., Bioconjug. Chem. **17**, 152–158 (2006)
X. Wu, D. Zhong, Q. Gao, et al., Int. J. Med. Sci. **10**, 676–682 (2013)
S. Yadav, L.E. van Vlerken, S.R. Little, et al., Cancer Chemother. Pharmacol. **63**, 711–722 (2009)
H. Yogasundaram, M.S. Bahniuk, H.D. Singh, et al., Int. J. Biomater. **2012**, 584060 (2012)
M. Yu, X. Jie, L. Xu, et al., Biomacromolecules **16**, 2588–2598 (2015)
Y. Yue, F. Jin, R. Deng, et al., J. Control. Release **152**, 143–151 (2011)
Y. Zhang, Z. Wang, R.A. Gemeinhart, J. Control. Release **172**, 962–974 (2013)
C.G. Zhang, W.J. Zhu, Y. Liu, et al., Sci. Rep. **6**, 23859 (2016)
C. Zheng, L. Niu, J. Yan, et al., Phys. Chem. Chem. Phys. **14**, 7352–7359 (2012)
L. Zhu, F. Perche, T. Wang, et al., Biomaterials **35**, 4213–4222 (2014)
T.S. Zimmermann, V. Karsten, A. Chan, et al., Mol. Ther. **25**, 71–78 (2017)
J.E. Zuckerman, I. Gritli, A. Tolcher, et al., Proc. Natl. Acad. Sci. U. S. A. **111**, 11449–11454 (2014)