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REVIEW

Fundamentals of siRNA and miRNA therapeutics and a review of targeted nanoparticle delivery systems in breast cancer

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Abstract

Gene silencing via RNA interference (RNAi) is rapidly evolving as a personalized approach to cancer treatment. The effector molecules—small interfering RNAs (siRNAs) and microRNAs (miRNAs)—can be used to silence or "switch off" specific cancer genes. Currently, the main barrier to implementing siRNA- and miRNA-based therapies in clinical practice is the lack of an effective delivery system that can protect the RNA molecules from nuclease degradation, deliver to them to tumor tissue, and release them into the cytoplasm of the target cancer cells, all without inducing adverse effects. Here, we review the fundamentals of RNAi, cell membrane transport pathways, and factors that affect intracellular delivery. We discuss the advantages and disadvantages of the various types of nanoparticle delivery systems, with a focus on those that have been investigated in breast cancer in vivo.

Keywords MicroRNA · siRNA · Cellular transport · Delivery · Nanoparticles · Breast cancer

Introduction

Breast cancer represents the leading cause of cancer-related deaths among women worldwide. Globally, breast cancer accounted for the highest number of new cancer cases in 2015 (Jemal et al. 2011; Global Burden of Disease Cancer Collaboration et al. 2017). It has been postulated that nearly 30% of newly diagnosed patients with early stage breast cancer will develop a distant metastasis despite receiving therapy (Redig and McAllister 2013; Morry et al. 2017). In routine clinical practice, breast cancer is traditionally classified as either non-invasive or invasive, and then according to stage and grade. The classification system is based on the histological features of breast tissue, location of abnormal tissues (e.g., milk ducts, lobules), and clinical symptoms presented by the patient (Goljan 2011; Eliyatkın et al. 2015). Non-invasive breast cancers remain localized and do not invade surrounding tissues. The two main types of non-invasive breast cancer are ductal carcinoma in situ and lobular carcinoma in situ. Most breast cancers are invasive and include infiltrating ductal carcinoma, Paget's disease, medullary carcinoma, inflammatory carcinoma, invasive lobular carcinoma, tubular carcinoma, and colloid (mucinous) carcinoma (Goljan 2011). Breast cancers can become metastatic if the cancer cells spread to other parts of the body through the bloodstream and lymph nodes.

Breast cancers are further classified into four molecular subtypes (luminal A, luminal B, HER2-enriched, and basal-like) based on the level of expression of the estrogen receptor (ER), progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) (Mastoraki et al. 2014; Dai et al. 2016). Luminal A cancers overexpress the hormonal receptors only (ER+ and/or PR+ and HER2−), whereas the luminal B type overexpress all three receptors (ER+ and/or PR+ and HER2+). Luminal A and luminal B cancers are defined by the expression of genes in the luminal epithelial layer of the mammary gland. Approximately 70% of breast cancers are hormone receptor-positive and overexpress one or both ER and PR (Bae et al. 2015). Breast cancers that overexpress HER2 only (i.e., HER2+/ER−/PR−) are referred to as HER2-enriched or simply as HER2, and represent around 20% of all cases (Kittaneh et al. 2013). Basal-like breast cancers are based on a distinct gene signature in the basal cells that line the breast ducts. They are mostly triple-negative breast cancers (TNBCs), which do not express any

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of the receptors (i.e., ER−, PR−, and HER−). TNBCs account for about 10% of all breast cancers and are associated with high mortality (Badve et al. 2011; Lü et al. 2017; Peng et al. 2017). Approximately 50–80% of TNBCs are basal-like (Boyle 2012; Li et al. 2017). Further subclassification of breast cancer using more detailed molecular signatures has shown promise, but is yet to reach routine clinical practice (Eliyatkın et al. 2015).

Current therapy options for breast cancer include surgery, hormonal therapy, immunotherapy, chemotherapy, radiation therapy, or a combination of these (Parvani and Jackson 2017). Breast cancers that express any hormone receptors have targeted hormonal therapies available and have a more favorable prognosis than breast cancers that do not show receptor expression (Cho 2016). Hormonal therapies are often prescribed following surgery as an adjuvant treatment. Some block the interaction between receptors and hormones (such as tamoxifen), while others lower the level of hormones (such as aromatase inhibitors). Tamoxifen has been used for more than 30 years to treat hormone receptor-positive breast cancers (ER+ and/or PR+). It works by stopping cells from responding to estrogen (Chang 2012). Similarly, Herceptin (trastuzumab), a humanized anti-HER2 monoclonal antibody, works by binding to HER2 receptors on the surface of breast cancer cells and blocking growth factor signals (Maximiano et al. 2016). Tamoxifen and Herceptin can be administered alone or in conjunction with radiation therapy or chemotherapy drugs such as paclitaxel or doxorubicin (Ranftler and Strasser-Weippl 2017). There are currently no effective targeted treatments available for TNBC patients. The high genetic diversity and absence of ER, PR, and HER2 receptors makes it unresponsive to hormonal therapies. It is also frequently resistant to chemotherapy; the platinum drugs epirubicin and paclitaxel are currently prescribed for TNBC patients, but response rates are poor (Liu et al. 2014; Jing et al. 2016; Parvani and Jackson 2017; Peng et al. 2017).

The heterogeneity of breast cancer remains a key barrier to its accurate molecular classification and individualization of treatment (Eccles et al. 2013). Not all patients who overexpress the hormone receptors (ER+ and/or PR+) respond favorably to tamoxifen. Similarly, not all patients who overexpress HER2 respond to Herceptin alone (Liu et al. 2014; Jiang et al. 2015). The cut-offs to define luminal A and luminal B cancers are currently set in an arbitrary manner rather than from gene expression levels because of substantial ER+ tumor heterogeneity, which can add to the problems in selecting treatment (Eccles et al. 2013; Yersal and Barutca 2014). Several authors suggest that the Ki-67 index can help to differentiate luminal A from luminal B (Dowsett et al. 2011; Eroles et al. 2012; Guiu et al. 2012; Yersal and Barutca 2014; Cho 2016; Dai et al. 2016; Hennigs et al. 2016; Hon et al. 2016; Yip et al. 2016). Ki-67 is a protein that serves as a cellular marker for proliferation and has been used to suggest a fifth molecular subtype, "normal-like" breast cancer, which is similar to luminal A but with the additional characteristic of having low Ki-67, i.e., ER+/PR+/HER2−/Ki-67−. Luminal B can be further subdivided into two groups: ER+/PR+/HER2−/Ki-67+ and ER+/PR+/HER2+/Ki-67+ (Dai et al. 2016). Although the Ki-67 index has gained wide support in the literature as a prognostic and predictive biomarker, it is not yet established for use in the clinical management of breast cancer due to the lack of standardized procedures (Dowsett et al. 2011; Penault-Llorca and Radosevic-Robin 2017).

Several studies have attempted to further subclassify breast cancer based on gene mutations and/or gene expression signatures; however, the prognostic value is not yet clear. Known breast cancer susceptibility genes that make up 25–30% of the heritability include *BRCA1*, *BRCA2*, *CHEK2*, *ATM*, *PALB2*, *BRIP1*, *TP53*, *PTEN*, *CDH1*, and *STK11* (Eccles et al. 2013). *BRCA1* and *BRCA2* are the most commonly mutated genes found in breast cancer, including in TNBC (Liang and Lam 2012; Gasparri et al. 2017). *PLK1* expression has been reported as a potential genetic marker for TNBC (Maire et al. 2013; Morry et al. 2017), and *BIRC5*, *MYBL2*, *IGFBP6*, *TP53*, *GAPDH*, *CCND1*, *HRAS*, and *PCNA* were reported as the most significantly up- or downregulated differentially expressed genes in TNBC compared with normal tissue (Peng et al. 2017). Other studies have reported elevated expression of the AR and EGFR genes in TNBCs (Martin et al. 2012; Pietri et al. 2016).

A better understanding of the genetic mutations and gene expression patterns in breast cancer has the potential to help identify prognostic and predictive biomarkers. In addition, a deeper molecular understanding will also provide new therapeutic targets and candidates for gene silencing. In order to appreciate the potential therapeutic application of gene silencing, it is important to first understand the fundamentals of how this process regulates cell biology.

Gene silencing: history and discovery of microRNA and siRNA

Until recently, the role of non-coding RNAs in our DNA was unknown. The ability of RNA to inhibit gene expression was first observed in plants in the 1990s by a number of independent groups; however, the phenomenon was not fully understood and, so, it was not explained or reported. Then, in 1993, Ambros and coworkers discovered the first microRNA (miRNA) gene, *lin-4*, in the nematode worm *Caenorhabditis elegans* (Lee et al. 1993). They found that lin-4 physically binds to lin-14 RNA and prevents its translation. Their discovery was the first evidence that an miRNA can suppress protein production by inhibiting messenger RNAs (mRNAs). In normal cells, miRNAs play an important role in regulating functions such as proliferation and cell differentiation. In 2002, Calin et al. (2002) found that

miRNAs are involved in cancer by showing that the miR-15a/16-1 locus is deleted or downregulated in 68% of chronic lymphocytic leukemias (Calin et al. 2002). Further research into miRNAs found that they comprise of a broad class of small RNA regulators with important functions in health and disease. Aberrant miRNA expression is frequently observed in various types of cancer, involving global upregulation and downregulation of miRNA expression (Sioud 2015; Mendelsohn et al. 2015).

In 1998, five years after the discovery of the first miRNA, Andrew Fire and Craig Mello discovered the process of RNA interference (RNAi) during their research on gene expressions in the nematode worm *C. elegans*. They found that, when worms were injected with double-stranded RNA coding for specific proteins, genes carrying the same sequence were switched off or silenced (Fire et al. 1998). Their findings resulted in the advent of RNAi as a central tool in modern molecular biology and were recognized by the Nobel Prize in 2006. Soon after the discovery of RNAi, David Baulcombe and Andrew Hamilton discovered naturally occurring small interfering RNAs (siRNAs) in plants (Hamilton and Baulcombe 1999), described as novel small antisense RNAs involved in gene silencing following transcription. Two years later, Thomas Tuschl and colleagues produced the first synthetic siRNAs able to silence genes in mammalian cells (Elbashir et al. 2001; Mattick 2001). This achievement heralded the widespread use of siRNAs to selectively knock down the activity of a specific gene. Currently, miRNAs and siRNAs are the two main classes of RNAs most widely employed for gene silencing. They have similar physicochemical properties but distinct functions and mechanisms of action, making their design requirements and therapeutic applications different.

Process of gene silencing by siRNAs

The first step to employing siRNAs in therapeutic applications is designing an siRNA sequence that is specific to the target mRNA, for which multiple algorithms are available. Once designed, siRNAs are produced by chemical synthesis or through gene expression. The former are completely synthetic RNAs that can be introduced into cells by various means, as will be discussed in the later parts of this review. The latter are transcribed inside the cell from expression constructs (such as plasmids and viral vectors) that express the short-hairpin RNA (shRNA) precursors of siRNAs (Siolas et al. 2005); these lie outside the scope of this review. Chemical synthesis of siRNA allows control over the amount and purity of siRNA and also enables chemical modifications to improve stability, an important aspect required for delivery. Chemically synthesized siRNAs can also be labeled for analyzing siRNA uptake or localization by fluorescence microscopy.

After the siRNA is introduced into the cell, the process of gene silencing is initiated and carried out by the endogenous RNAi machinery of the cell. Inside the cell, the duplex siRNA enters the RNAi pathway. The antisense strand is loaded into a protein complex called the RNA-induced silencing complex (RISC), and serves as the guide for the recognition of complementary mRNAs. After the target sequence is recognized, the mRNA is cleaved by Argonaute 2 of the RISC, resulting in reduced protein expression from the silenced gene (Finlay et al. 2015b; Kim et al. 2016; Parvani and Jackson 2017). Advantages of siRNAs over drug therapies include their high degree of specificity and low toxicity. Nevertheless, off-target effects do occur due to the miRNA-like activity of siRNAs acting through the seed-like sequence at the 5′ end and dsRNA-induced stimulation of the innate immune system, both reviewed recently (Suter et al. 2016; Meng and Lu 2017). A schematic of the siRNA gene silencing process is provided in Fig. 1.

Process of gene silencing by miRNA

There are two ways of employing miRNAs for therapeutic applications: miRNA inhibition and miRNA replacement. miRNA inhibition is used when the target miRNA is overexpressed. It involves introducing synthetic single-stranded RNAs acting as miRNA antagonists that inhibit the action of the target miRNA. In this respect, miRNA inhibition is analogous to antisense inhibition with similar design and delivery considerations, and, as such, is outside the scope of this review. On the other hand, miRNA replacement is employed when the target miRNA is repressed or deactivated, and this tends to be more common in cancer. It involves introducing synthetic double-stranded miRNAs (called miRNA mimics) to mimic the function of the target miRNAs and bind to a target gene to initiate mRNA degradation and produce the gene silencing effect (Rothschild 2014; Wang et al. 2016a). The design of miRNA mimics is more straightforward than that of siRNA, as the sequence should be almost, if not entirely, identical to the endogenous miRNA. Synthetic miRNA mimics and siRNAs enter the same RNAi pathway and both are incorporated into RISC to create the final active complex.

Difference between siRNA and miRNA

While structurally and functionally similar, there are some important differences between siRNAs and miRNAs (Table 1). siRNAs are short double-stranded RNAs typically composed of 21–23 base pairs, often with two nucleotide overhangs at the 3′ ends, but multiple variations in length and overhangs are tolerated (Kim et al. 2016). They consist of an active (guide) strand and a complementary inactive

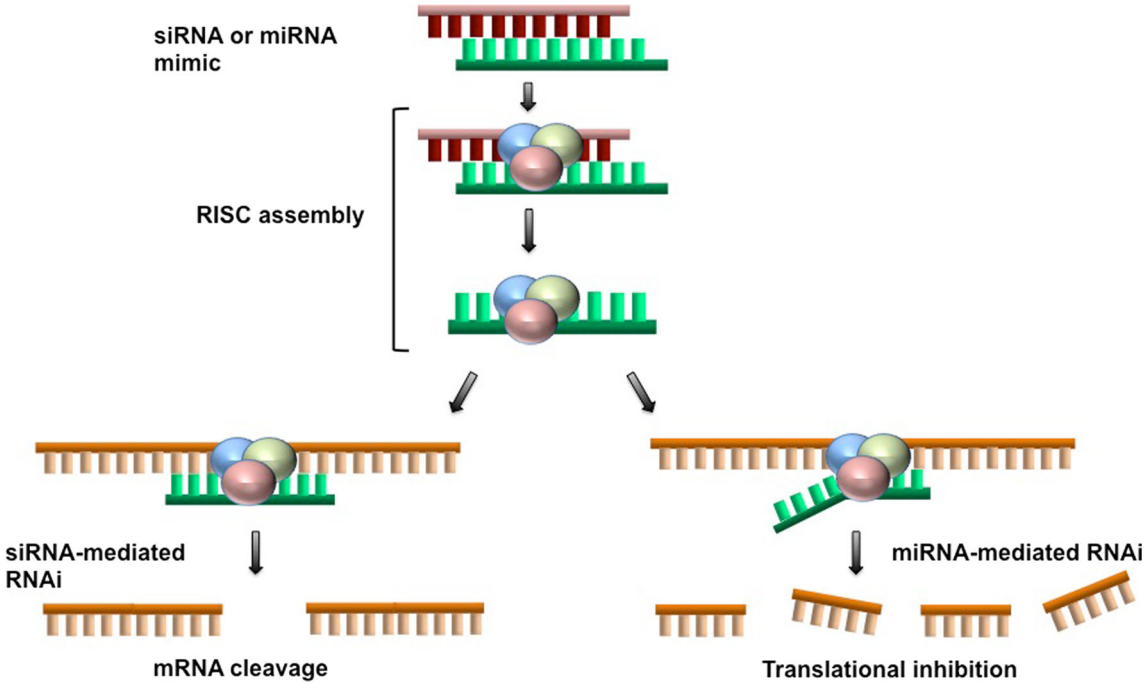

Fig. 1 Gene silencing using siRNA or miRNA mimics. Once the siRNA or miRNA mimic has been introduced into the cytoplasm of the cell, it is unwound and the active antisense strand (green) is incorporated into the RISC. This leads to gene silencing via two distinct mechanisms, depending on the extent of base pairing between the antisense strand and the target mRNA. With siRNA on the left, the complete homology between the antisense and target mRNA (yellow) leads to site-specific cleavage and degradation of the mRNA. In contrast, the partial sequence identity between the active miRNA strand and its mRNA target leads to inhibition of translation, decapping, and subsequent mRNA degradation

(passenger) strand. siRNAs are regarded as exogenous RNAs that enter the endogenous RNAi pathway. miRNAs, on the other hand, are endogenous RNAs produced from within the cells, expressed as long primary miRNA transcripts (pri-miRs) from miRNA genes (Sioud 2015). After partial cleavage by the microprocessor complex in the nucleus, the stem-loop pre-miRNA is exported to the cytoplasm, where it is further processed by Dicer into a double-stranded RNA consisting of the active, or mature, strand and the inactive passenger strand. The mature miRNA is incorporated into the RISC to initiate gene silencing. In contrast to the perfect complementarity between an siRNA and its target mRNA, an miRNA has imperfect complementarity, mostly within the seed sequence at its 5′ end (Carthew and Sontheimer 2009).

In terms of silencing and potential clinical use, a key difference between siRNAs and miRNAs is that an siRNA is specific for a single target site in a single mRNA, and, therefore, inhibits the expression of one target gene, whereas an

Table 1 Differences between siRNA and miRNA

	siRNA	miRNA
Occurrence	Occurs naturally in plants and animals. It is currently unknown whether or not they occur naturally in mammals.	Occurs naturally in plants and animals.
Mean length	Approx. 21–22 nt	Approx. 19–25 nt
Complementarity t o target mRNA	100% perfect match; therefore, siRNAs knock down specific genes, with minor off-target exceptions.	Not exact; therefore, a single miRNA may target up to hundreds of mRNAs.
Biogenesis	Regulate the same genes that express them.	Expressed by genes whose purpose is to make miRNAs, but they regulate genes (mRNAs) other than the ones that expressed them.
Action	Cleave mRNA.	Inhibit or replace translation of mRNA.
Function	Act as gene silencing guardians in plants and animals that do not have antibody- or cell-mediated immunity.	Regulators (inhibitors) of genes (mRNAs)

miRNA can have multiple targets and can regulate multiple genes because mRNA recognition requires binding to the much shorter seed sequence rather than the entire 21-nucleotide sequence of an siRNA. To initiate RNAi, an siRNA must be fully complimentary to its target mRNA, whereas miRNA can be partially complimentary and bind to multiple mRNAs to inhibit their expression, and their mechanisms of action are different: siRNAs cleave mRNA, whereas miRNAs inhibit translation of mRNA (Carthew and Sontheimer 2009). To avoid repression of genes in normal cells, it is important to develop methods that will enable cell specificity, i.e., methods to deliver miRNA and siRNA to tumor cells at the target site (Lam et al. 2015; Takahashi et al. 2015).

In summary, in order to bring RNAi into clinical practice for cancer treatment, three key steps must be taken: (1) the target genes that are directly involved in cancer development must be identified; (2) an siRNA specific to the target gene must be designed and synthesized; (3) the synthetic siRNA must be delivered into the cytoplasm of the target cell. While methods for steps 1 and 2 are already effective, there are several challenges to the delivery of siRNA into the target cells. This last step is the major barrier to implementing siRNA therapy in clinical practice and is the subject of this review.

Current challenges of siRNA and microRNA therapies

The challenges associated with cellular delivery of both siRNA and miRNA are widely reported (Bouclier et al. 2008; Dahlman et al. 2014; Essex et al. 2015; Wang et al. 2016a; Arnold et al. 2017; Tatiparti et al. 2017; Parvani and Jackson 2017). Despite differences in their mechanism of action, siRNAs and miRNA mimics have similar chemical structure and face similar challenges in delivery to target cells. Therefore, the same delivery system can be employed to improve their cellular uptake (Lam et al. 2015). To date, most research on siRNAs and miRNAs as cancer therapies has focused on systemic delivery by intravenous injection. Other routes described include local delivery by intraocular and intratumoral injection, local delivery to the central nervous system (CNS), or intranasal delivery to the airway (Gao and Huang 2009).

When injected systemically, naked siRNAs and miRNAs can be easily degraded by serum nucleases, removed by cells of the immune system, and excreted through renal filtration. At the cell membrane level, naked siRNA and miRNA are negatively charged at normal pH, as is the cell membrane; therefore, they are repelled. They are also water-soluble, which makes it difficult for them to enter cells by passive diffusion (Essex et al. 2015). Other challenges include poor tissue penetration, instability, low efficiency of internalization, and non-specific immune stimulation (Tatiparti et al. 2017). The percentage actually taken up by cells is estimated to be 0.7% of the injected dose (Parvani and Jackson 2017). Naked siRNAs and miRNAs are, therefore, either degraded or entrapped before entering the target cell. There is a need for a delivery system that will not only protect the siRNA and miRNA molecules during systemic delivery, but also maximize delivery to the specific target tissues and promote entry into the target cell.

Delivery methods

Principles of transporting oligonucleotides into cells

There are two key barriers a delivery system must overcome to enable siRNA and miRNA molecules to enter the cell and target mRNA: (1) transport across the cell membrane and (2) escape from the endocytic pathway (Liang and Lam 2012). To determine the ideal characteristics of a delivery system, it is important to first understand the key processes taking place during cellular transport. The cell membrane is a selectively permeable phospholipid bilayer. Each phospholipid molecule consists of a hydrophilic (polar) phosphate head on the outside of the membrane and two hydrophobic (non-polar) fatty acid tails inside the membrane. Generally, the smaller and more hydrophobic a molecule is, the more rapidly it will diffuse across the lipid bilayer (Alberts et al. 2002; Prokop 2011).

Transport across the cell membrane primarily occurs through a process called endocytosis. There are two main categories of endocytosis: phagocytosis (cell eating) and pinocytosis (cell drinking), with particle size being one of the key factors that governs the mode of uptake (Prokop 2011). Phagocytosis involves the cell membrane extending outwards and engulfing the particle into a membrane-bound vesicle called a phagosome, which then fuses with lysosomes for degradation. Phagocytosis is performed by specialized cells such as neutrophils, macrophages, monocytes, and endothelial cells, and plays a role in the clearance of particles that have diameters generally greater than 0.5 μm, including larger particles of up to 10 μm (such as bacteria or cellular debris) (Hirota and Terada 2012). An in vitro study in phagocytic cells showed that macrophages can internalize IgG-coated polystyrene spherical particles that range from 200 nm to 2 μm (Koval et al. 1998). For this reason, delivery systems in this size range are considered undesirable for drug and RNA delivery (Hirota and Terada 2012; Bulbake et al. 2017; Tatiparti et al. 2017). On the other hand, pinocytosis occurs in virtually all cells, including cancer cells, and covers particle sizes ranging from 50 nm up to 5 μm. Particles to be imported by pinocytosis contact the exterior surface of the plasma membrane, triggering the membrane to fold inward, enveloping

them. The enveloped membrane pinches off into a vesicle containing extracellular fluid and the imported particles. There are three main mechanisms of pinocytosis: receptor-mediated endocytosis, receptor-independent endocytosis, and macropinocytosis. Receptor-mediated endocytosis occurs when a particle interacts with a specific receptor and is selectively internalized. There are two main subtypes: clathrin-mediated endocytosis, which is initiated by the protein clathrin on the cell membrane and typically occurs for particles around 120 nm in size, and caveolin-mediated endocytosis, which is initiated by the cholesterol-binding protein caveolin and typically occurs for particles that are around 80 nm in size (Hirota and Terada 2012). Particles of 50 nm in size can be endocytosed without the involvement of clathrin or caveolin receptors (Hirota and Terada 2012). For particle sizes ranging from 100 nm to 5 μm, entry to the cell is via macropinocytosis, a process that involves non-selective uptake of particles by macropinosomes (Prokop 2011; Hirota and Terada 2012; Finlay et al. 2015b; Fan et al. 2016). In addition to controlling size, delivery systems can be designed to be active or passive. For active transport, the surface of the vehicle can be decorated with targeting moieties such as proteins, peptides, small molecules, or antibodies to trigger receptor-mediated endocytosis. Delivery systems for passive transport require the biophysical properties of the delivery vehicle to be manipulated, including its size, shape, and zeta potential (Dahlman et al. 2014).

Once a delivery vehicle passes the cell membrane via endocytosis, it is contained within an intracellular membrane-bound compartment called an endosome, where it enters the endocytic pathway and is recycled or degraded. The pH within the endosome plays an important role as the cell exposes the ingested substance to hydrolytic enzymes in a progressively acidic environment in order to facilitate degradation. The process occurs in stages as follows: (a) early endosomes are sorting organelles and the first compartments of the pathway located in the periphery of the cell, and receive most types of particles coming from the cell surface. They have a mildly acid pH from neutral to around pH 6. From here, many of the molecules are either recycled to the surface by exocytosis or sorted into late endosomes; (b) late endosomes receive the endocytosed materials from the early endosomes through transport carriers or by Rab conversion (Poteryaev et al. 2010). They are rapidly acidified to a pH of around 5.5 by the action of ATPase proton pumps which span the membrane; (c) lysosomes are the last compartment of the endocytic pathway, and have the primary function of breaking down cellular waste products, fats, carbohydrates, proteins, and other macromolecules into their respective subunits (Liang and Lam 2012; Arnold et al. 2017).

Lysosomes contain degradative hydrolytic enzymes and function in an acidic environment (further pH reduction to approximately 4.5). An inability of the delivery system to escape this compartment will lead to degradation of the particle and its cargo. Ideally, the delivery system should release its contents from the acidic compartment of the endosome and avoid exposure to the degrading enzymes contained within the lysosomes. Thus, escaping the late endosomes and being subjected to selective disassembly in cytoplasm are the key challenges to be addressed by successful siRNA and miRNA delivery systems within the cell.

Design considerations of delivery systems

As indicated above, size, shape, and surface charge are the key design considerations for nanoparticle delivery systems (Tatiparti et al. 2017). Delivery systems smaller than the renal filtration cut-off of 50 kDa (or 5–6 nm) are expected to be rapidly removed by renal filtration, whereas delivery systems larger than 100 nm in diameter can be trapped in the mononuclear phagocyte system (Wang et al. 2016a). According to Bedi et al. (2013), the ideal systemic delivery vehicle should be approximately 10–50 nm in size, whereas Wang et al. (2016a) suggest an optimal size range between 10 nm and 100 nm. Other studies suggest that the size should be within the range of 20–100 nm in diameter, with particles less than 100 nm showing higher tissue uptake and transfection efficiency (Young et al. 2016; Tatiparti et al. 2017). There is consensus among several authors that delivery systems should be less than 200 nm for efficient tissue and cellular uptake (Davis 1997; Govindarajan et al. 2012; Draz et al. 2014; Dong et al. 2015; Ngamcherdtrakul et al. 2015), although exceptions exist (Ahmad et al. 2016; Jing et al. 2016). Hirota and Terada (2012) suggested that the optimal size of particles for cellular uptake varies according to cell type, where, for cancer cells, a particle size of around 50 nm is favored (Hirota and Terada 2012).

Shape can also affect particle behavior and cellular uptake. There have been conflicting reports on the effect of particle shape on cellular uptake. Young et al. (2016) suggested that spherical structures have increased rates of endocytosis compared to rod-shaped structures because rods have a larger contact area that can block neighboring receptor sites on cells. Similarly, Li et al. (2015) suggested that spherical particles have the highest uptake and fastest internalization rate because they need to overcome a minimal membrane bending energy barrier compared with non-spherical systems. In contrast, He and Park (2016) showed that shapes with higher aspect ratio and sharper angular features have a higher chance of adhering to the cells and becoming internalized by cancer cells (He et al. 2016). To explain the variations in the cellular internalization of different shapes, Richards and Endres (2016) studied the effects of various shapes on receptor-mediated endocytosis and phagocytosis using 1-dimensional and 2-dimensional physical models. They showed that the orientation plays a critical role in the internalization rate, where non-

spherical shapes can be internalized the fastest when the most highly curved tip is engulfed first (Richards and Endres 2016).

Since positively charged particles are taken up more readily than negatively charged particles, the surface charge is one of the most important properties of a delivery system that influences its uptake. The zeta potential has been used as an indicator of the surface charge (Honary and Zahir 2013). When a material is placed in a liquid, the zeta potential provides a measure of the magnitude of electrostatic repulsive interaction between particles, with a higher zeta potential indicating greater stability. Delivery systems with larger positive zeta potential bind strongly to the cell membrane and show higher cellular uptake, facilitated by the electrostatic interactions between the negatively charged cell membrane and positively charged particle surface (Honary and Zahir 2013; Dong et al. 2015).

Interestingly, negative zeta potentials have also been reported for some delivery systems. Although cell membranes should repel negatively charged particles, it has been reported that cells may adsorb negatively charged particles at the positively charged sites via electrostatic interaction, leading to localized neutralization and subsequent bending of the membrane to initiate endocytosis. Nevertheless, delivery vehicles with positive charge are preferentially taken up by tumors and have higher cellular uptake than negatively charged or neutral particles (Davis 1997; Honary and Zahir 2013; Young et al. 2016). It has been reported that cellular uptake is lowest for particles that have no surface charge as determined by the zeta potential (Hirota and Terada 2012). To improve specificity and cell uptake via receptor-mediated endocytosis, some investigators have suggested attaching cationic polymers such as poloxamers and polyethylene oxide (Draz et al. 2014; Dong et al. 2015; Arnold et al. 2017; Sun et al. 2017b).

Once a delivery system successfully passes the cell membrane and enters the cell, it must escape the endocytic pathway. Nanocarrier delivery systems can be designed to incorporate characteristics that facilitate endosomal–lysosomal escape. A commonly used approach is to make use of the proton sponge effect (Behr 1997), which is based on the hypothesis that pH buffering can facilitate escape from the endocytic pathway by preventing the acidification of the endosomes (Arnold et al. 2017; Dong et al. 2015; Gujrati et al. 2016; Li et al. 2016). When the endosomes struggle to become acidic, it is thought that the cell will continuously pump more protons into the endosomes in an attempt to lower the pH, followed by passive entry of chloride ions and, due to the increase in the ionic concentration, water influx. Eventually, this increase in osmotic pressure causes swelling and rupture of the endosome, releasing its contents into the cytosol (Liang and Lam 2012; Gujrati et al. 2016). The proton sponge effect has been observed with certain cationic polymers that have a high pH buffering capability over a wide pH range, such as polyamidoamine (PAMAM) dendrimers, lipopolyamines, and polyethylenimine (PEI). These polymers usually have secondary or tertiary amine groups with pKa close to the endosomal pH (Liang and Lam 2012). These polymers can be protonated to decrease the acidification of the endosomes. PEI is known for its extensive pH buffering capacity, ability to compact siRNAs into nano-scale complexes, and demonstrated ability to successfully transfect into a range of cells in vitro and in vivo (Dong et al. 2015; Essex et al. 2015). However, the clinical application of PEI has been limited due to reports of its toxicity, which is influenced by its molecular weight. The higher the molecular weight of PEI, the higher its transfection efficiency but also the higher its non-specific toxicity (Essex et al. 2015). Conversely, low molecular weight PEI displays low toxicity and low transfection efficiency (Essex et al. 2015; Li et al. 2016). Essex et al. (2015) found that chemical conjugation of low molecular weight PEI with dioleoylphosphatidylethanolamine (DOPE) improved the intracellular transfection efficiency of siRNA with low cytotoxicity levels.

Examples of polymers that do not have a pH buffering capability include polypeptides such as chitosan and polylysine. To improve the pH buffering capability of these polypeptides, molecules such as histidine can be added as a functional group or incorporated into the peptide sequence, as can pH-sensitive endosome-disruptive peptides such as GALA (Liang and Lam 2012). Bilayer disruption is another escape mechanism that involves destabilization of the cellular membrane via fusion of cationic polymers, peptides, or cationic lipids such as DOPE (Young et al. 2016).

In summary, multiple challenges must be overcome before siRNAs and miRNAs can be implemented as therapeutic agents (Table 2). An effective delivery system must be able to: (a) protect nucleic acid molecules from degradation during systemic delivery; (b) withstand prolonged circulation without being cleared by renal filtration; (c) accumulate into the target tissue from the circulation; (d) enter the target cells via a specific uptake mechanism; (e) initiate cellular uptake via endocytosis; (e) escape from the endosomal compartment to avoid being degraded in the lysosome; and (f) disassemble in the cytoplasm to release its siRNA or miRNA cargo (Prokop 2011; Bedi et al. 2013; Pittella and Kataoka 2013; Gujrati et al. 2016; Arnold et al. 2017). An ideal delivery system should also be biocompatible, biodegradable, non-toxic, and non-immunogenic (Young et al. 2016).

siRNA and miRNA delivery vehicles in breast cancer research

The most common chemical structures explored for gene silencing therapy are cell-penetrating peptides, liposomes, micelles, and polymeric nanoparticles, and all of them have been

Table 2 Barriers to the transport of siRNAs and miRNAs to target cells

Level	Barriers
Circulation	Non-specific interactions with serum proteins, resulting in nanocarrier degradation, dissociation, or aggregation. Clearance by the renal system for particles less than 6–10 nm in size. Nanocarrier toxicity and immune response can be induced.
Tissue permeability	Endothelium penetration. Transport from the bloodstream to a desired tissue.
Extracellular	Extracellular stability and diffusion. Differences in the pH, enzymes, or ions of tissue microenvironment can damage the nanocarrier, causing dissociation before cellular entry.
Internalization	Cell specificity. Cellular uptake via endocytosis.
Intracellular	Escape from endosomal compartment. Dissociation from the nanocarrier in the cytoplasm to release siRNA or miRNA cargo.

applied to experimental models of breast cancer (Fig. 2). To date, there are no reported clinical trials for breast cancer involving siRNA or miRNA therapies using non-viral chemical delivery systems. We have, therefore, focused on in vivo studies in rodent models.

Cell-penetrating peptides

Cell-penetrating peptides are known for their ability to facilitate cellular uptake of various "cargo" into cells, such as nano-size particles, chemical molecules, proteins, and large fragments of DNA (28). They are biologically or artificially manufactured short chains of amino acid monomers (up to 30 amino acids) that are linked by peptide bonds. They are usually positively charged and can bind to negatively charged siRNA to improve its stability in vivo (Jiang et al. 2015; Raucher and Ryu 2015; Jing et al. 2016). From a review of in vivo studies, we observed three design variations of delivery systems involving cell-penetrating peptides: cell-penetrating peptides modified with chitosan and then used to encapsulate the siRNA (Sun et al. 2017a); some studies entrapped or loaded a cell-penetrating peptide onto another primary delivery vehicle (such as liposomes or ultrasound-sensitive nanobubbles) (Jing et al. 2016; Xie et al. 2016); others conjugated a cell-penetrating peptide with other cationic polymers (such as PEG) or micelles to form a nanocomplex for siRNA encapsulation (Wang et al. 2015; Fang et al. 2016; Yang et al. 2016). All in vivo studies demonstrated positive outcomes by showing that tumor growth was inhibited by siRNA.

Cell-penetrating peptides have also demonstrated endosomal escape capabilities through the proton sponge

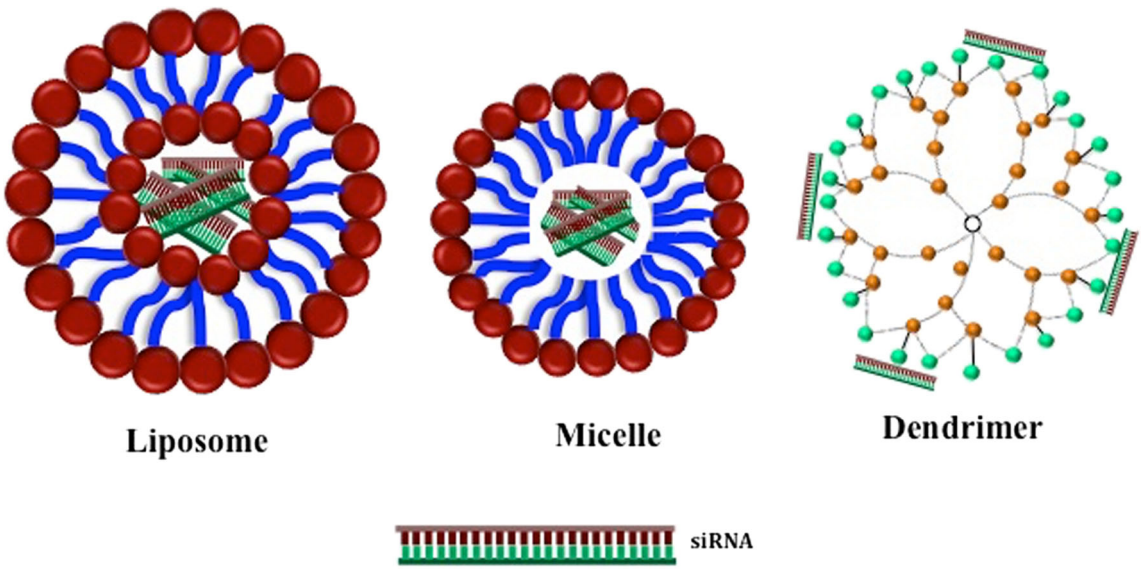

Fig. 2 Schematic representation of delivery vehicles. Liposomes and micelles encapsulate siRNA/miRNA within an aqueous compartment, whereas dendrimers condense RNA via cationic interactions with positive charges on the surface. For simplicity, the major differences between liposomes, micelles, and dendrimers are depicted; as discussed in the text, variations exist in the composition of all three, for example through PEGylation and addition of targeting moieties

effect (Sun et al. 2017a). The main challenge in the use of cell-penetrating peptides are the non-selective interactions of these peptides in vivo and their ability to translocate through any cell membrane, independent of receptors (Jing et al. 2016). Despite promising outcomes from in vivo studies, other challenges facing the use of cell-penetrating peptides are that their intracellular uptake and internalization pathways are not well defined (Raucher and Ryu 2015; Arnold et al. 2017) and they have short blood plasma half-life (Raucher and Ryu 2015). To our knowledge, there are currently no cell-penetrating peptide-based therapeutic delivery systems commercially available.

Liposomes

Liposomes are small artificial vesicles that are made of material similar to the cell membrane. They contain a lipid bilayer with an internal aqueous core and are characterized by their biocompatibility, biodegradability, low toxicity, and ability to trap both hydrophilic and lipophilic drugs. Liposomes fuse with other cell membranes so that their aqueous compartment becomes adjacent to the cytosol of the target cell to deliver the encapsulated material (Mikhaylova et al. 2009). They are commercially used as carriers for delivery of chemotherapy drugs such as doxorubicin and daunorubicin (Bulbake et al. 2017). It is noted that the majority of approved commercial liposome formulations for clinical use are less than 200 nm in size, with the exception of Myocet® (150–250 nm) and Visudyne® (150–300 nm) (Bulbake et al. 2017). Liposomes have been investigated as carriers of siRNAs primarily by encapsulation of siRNA either on its own or co-delivered with a drug. Cationic liposomes currently have the highest encapsulation efficiency (Young et al. 2016). On review of the in vivo studies in Table 3, it is noted that most of the studies used cationic lipid nanoparticles such as DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane), DOPE (dioleoylphosphatidylethanolamine), or DC-Chol (3β[N-(N′,N′-dimethylaminoethane)-carbamoyl]cholesterol). Cationic lipids mimic the chemical and physical attributes of biological lipids and are able to protect siRNAs from degradation; however. their application in gene delivery is limited by variations in their transfection efficiency and systemic toxicity when injected (Piao et al. 2013). Modification of the liposomal surface by coating with polyethylene glycol (PEG), through a process called PEGylation, has been shown to protect the positive charge, reduce immune response, and improve stability in vivo; however, it may compromise cellular uptake, endosomal escape, and resultant silencing efficacy (Ho et al. 2013; Ran et al. 2014; Essex et al. 2015; Gu et al. 2015; Li et al. 2016).

All studies reported positive outcomes for their liposomal delivery system and observed downregulation of the target gene. Nourbakhsh et al. (2015) reported more than 80% downregulation of the drug resistance gene *MDR1* in MCF-7/ADR breast cancer cells using a PEGylated DSPE/DOTAP/DOPE liposome delivery system. It is important to note that liposomes can present with high surface charge, which may induce toxic effects (Li et al. 2016). Other challenges reported for liposomes include their low solubility, high production costs, challenges with manufacturing scalability, short half-life (unless PEGylated), potential hypersensitivity reactions in vivo, and potential to form aggregates with negatively charged serum proteins that can accumulate in the lungs, liver, and spleen (Balazs and Godbey 2011; Bedi et al. 2013; Akbarzadeh et al. 2013; Sioud 2015). Whitehead et al. (2014) studied PEGylated lipid nanoparticles for siRNA delivery in mice. They established four design criteria to predict whether the delivery system will silence at least 95% of protein expression, which are that the scaffold has: (1) a $COOC_{13}H_{27}$ tail, (2) three or more tails, (3) is synthesized from an alkyl-amine precursor containing one or more tertiary amides, and (4) a surface pKa of 5.5 or higher (Whitehead et al. 2014).

Micelles

Micelles are closed spherical monolayers of phospholipids. They differ from liposomes in that they do not have an aqueous core. Polymeric micelles (having diameters ranging from 10 to 100 nm) have attracted attention for their versatile properties and ease of preparation (Yousefpour Marzbali and Yari Khosroushahi 2017). Reported benefits of micelles include simple preparation, low toxicity, long half-life, and good tissue penetration capabilities (Yousefpour Marzbali and Yari Khosroushahi 2017). However, similar to liposomes, they are subject to dilution following intravenous administration. Micelles are either made to encapsulate siRNA and drugs or chemically altered to form a nanocomplex (Falamarzian et al. 2012). They are typically modified at their core to improve encapsulation efficiency and modified at their shell to improve in vivo stability. For example, micelles have been coated with PEG by direct conjugation of PEG to siRNA and condensation of PEG-siRNA to a micelle structure (Wakaskar 2017).

Examples of micelles containing complexed siRNA include PEGPnBA–PDMAEMA (Draz et al. 2014) and PEG–PEI (Falamarzian et al. 2012). Yu et al. (2016) investigated a triple-layered pH-responsive micelleplex formed from PEG-b-PAGA-b-PDPA triblock copolymers. The micelleplex encapsulated a drug at its core and had siRNA loaded at the interlayer. The micelleplexes were delivered in vivo to 4T1 xenografts and were able to inhibit tumor growth. Polymeric micelles are advantageous in that their structure and the physiochemical and biological properties can be changed and multiple molecules can be integrated onto one micelle platform; however. the structure needs to be optimized to obtain a

Table 3 Nanoparticle delivery systems investigated in vivo in breast cancer cells

Materials and design	Properties: size/zeta potential	Carrier/therapeutic agent	Cell line	Reported outcomes	References
Human serum albumin-coated lipid nanoparticles with cationic lipid (DDAB), hydrophilic surfactant, and cholesterol	67.8 ± 3.5 nm/+22.1 mV	phrGFP siRNA	MCF7	Tumor significantly decreased	Piao et al. (2013)
Dexamethasone (dex)-associated liposome	272.1 ± 11.9 nm/23.6 ± 5.7 mV	Anti-cancer drug, ESC8, co-delivered with NRP-1 shRNA-encoded plasmid	ANV-1	~ 4-fold smaller tumor volume	Ahmad et al. (2016)
PEGylated lipid-based nanoparticles mPEG-DSPE/-DOTAP/DOPE	80 to 90 nm/close to neutral surface charge	MDR1	MCF7/ADR cells	Improved chemosensitivity to doxorubicin Downregulated P-glycoprotein Downregulated MDR1 expression by more than 80%	Nourbakhsh et al. (2015
PEGylated liposomal complex PEG covalently linked to hyaluronic acid and coated onto liposome complex (DOTAP and cholesterol)	188.6 ± 10.8 nm and a dramatically declined zeta potential from + 34.9 ± 4.0 mV to − 18.2 ± 2.2 mV	Anti-P-glycoprotein siRNA	MCF-7/ADR cells	Downregulation of P-glycoprotein by 34%	Ran et al. (2014)
Cationic lipid liposomal nanoparticle formulation (DC-Chol, DSPC) containing two PEGylation steps: pre- and post-siRNA insertion	111.3 ± 12.2 to 117.7 ± 10.3/average ~ 5 mV	Luciferase mRNA	MDA-MB-435/LCC6 W-GFP/luciferase cells	Downregulate luciferase mRNA expression by more than 50%	Ho et al. (2013)
Nanosized immunoliposome-based delivery complex (DOTAP:DOPE)	~ 100 nm	Anti-HER-2 siRNA molecule	MDA-MB-435	Silenced target gene Increased response to chemotherapy Tumor growth inhibition	Pirollo et al. (2007)
Liposomal nanoplex (DOTAP:DOPE)	Not specified	Chemically modified short interfering hybrids (siHybrids) HER-2 expression	MDA-MB-435	Inhibited HER-2 expression	Hogrefe et al. (2006)
Cationic liposome, synthetic cationic cardiolipin analogue (CCLA)	110–120 nm	siRNA	MDA-MB-231 cells	73% of tumor growth suppression Transfection efficiency in mice was 7-fold higher than the commercially available DOTAP-based liposome	Chien et al. (2005)
Polypeptide micelle complex, LA-CLss/siATG7 Lipoic acid (LA) conjugated with a cytosol localization and internalization peptide 6, cross-linked into cross-lined micelles (LA-CLss)	< 200 nm/zeta potential ranging from 10 to 30 mV	ATG7 siRNA and docetaxel co-delivery	MCF-7	At least a 1.84-fold greater tumor inhibition compared to that of DTX-loaded micelles in vivo	Gong et al. (2017)
Low-density lipoprotein-N-succinyl chitosan--cystamine-urocanic acid	Avg. 167.90 ± 1.46 nm/− 21.91 ± 1.25 mV	Co-delivery of siRNA and paclitaxel	MCF-7/taxol	Inhibited tumor growth	Zhu et al. (2017)
Pluronic P123-conjugated polypropylenimine (PPI) dendrimer (anti-CD44-P123-PPI) nanocarrier	Average diameter: 155.9 ± 8.9 nm and 182.3 ± 5.4 nm (with antibody conjugation)	Anti-CD44-P123-PPI/pDNA-iMDR1-shRNA nanocomplexes combined with ADR to improve the effectiveness of chemotherapy	MCF-7/ADR	Tumor growth inhibition Knockdown of MDR1/P-gp	Gu et al. (2015)

Table 3 (continued)

Materials and design	Properties: size/zeta potential	Carrier/therapeutic agent	Cell line	Reported outcomes	References
	Zeta potential: 1.9 ± 0.8 to 28 ± 2.7 mV and 1.1 ± 0.5 to 25.8 ± 2.8 mV (with antibody)				
Modified poly(amidoamine) (PAMAM) dendrimer-siRNA complex	100–200 nm	TWISTI gene	SUM1315 TNBC cells	High concentrating ability in xenograft model	Finlay et al. (2015b)
siRNA-loaded nanoparticle (mPEG-PLGA-PLL copolymer) with ultrasound-targeted microbubble destruction	131.5 ± 6.5 nm	ABCG2-siRNA	MCF-7/S MCF-7/ADR	Observed suppressed expression of the target gene in tumor xenografts ADR at 5 mg/kg was harmful to liver and kidney	Bai et al. (2015)
Aqueous-core nanocapsules (NC) of PEG-co-poly(e-caprolactone-co-dodecyl-b-malate) (PEG-PCL/MA)	~ 120 nm	siRNA-loaded nanoparticles targeting ER(alpha)	MCF-7	Significant decrease in tumour growth and a decrease in ER(alpha) expression in tumour cell xenografts.	Bouclier et al. (2008)
Cholesterol-conjugated siRNA loaded onto reconstituted high-density lipoprotein (rHDL)	100 nm Zeta potential ranging from − 16.85 mV to − 4.2 mV	Cholesterol-conjugated siRNA targeting VEGF expression	MCF-7 tumor tissue	Tumor volume reduced by up to 54.5%	Ding et al. (2014)
PHD/PPF/siVEGF nanocomplex via a self-assembly process utilizing the pre-functionalized polymers: (1) a conjugate of polyethylenimine (PEI) with doxorubicin (DOX) via a pH-responsive hydrazone linkage (PEI–Hz–DOX, PHD) and (2) a tumor-targeting folate ligand conjugated to PEI using polyethylene glycol (PEG) as a linker (PEI–PEG–Folate, PPF) in tandem with VEGF siRNA	120 nm/+ 3 mV	Co-delivery of siRNA and low-dose doxorubicin	MCF-7 cells	Up to 55% downregulation of VEGF mRNA expressed in vivo; 1.44-fold lower tumor volume	Dong et al. (2015)
Fusion protein of an anti-HER2 single-chain antibody fragment with a positively charged protamine, F5-P, complexed with siRNA	Not specified	siDNMT (siRNA targeting DNA methyltransferases 1 and/or 3b genes expressing HER2)	HER2-expressing BT474 breast cancer cells	Inhibited tumor growth and restored tumor suppressor gene expression	Dou et al. (2012)
Thioaptamer-conjugated CD44 Thioaptamer (TA)-modified nanoparticles consisting of ligand TA and dendritic polyamidoamine (PAMAM)	~ 105-400 nm/~ − 5 mV to + 20 mV	TA-PEG-PAMAM/miRNA	CD44, a membrane protein that is overexpressed on the plasma membrane of metastatic breast cancer cells (MDA-MB-231 or LM2-4142)	Significant reduction in tumor weight	Fan et al. (2016)
Reduction-sensitive linear cationic click polymer/iMDR1–pDNA complex nanoparticles (RCPN)	150 nm on average/0 mV to ~ 20 mV	To silence the expression of P-glycoprotein (P-gp) to reverse multidrug resistance	MCF-7/ADR cells	RCPNs inhibted expression of P-gp. RCPNs treatment did not show any inhibition effect on the growth of the tumor	Gao et al. (2011)
Cell-penetrating peptide, TAT-MU (TM), and a targeting ligand, HER2 antibody mimetic-affibody (AF)-TMAF	~ 185 nm	HER2	MDA-MB-453	Observed specific and significant reduction of tumor volume when injected intratumorally.	Govindarajan et al. (2012)

Table 3 (continued)

Materials and design	Properties: size/zeta potential	Carrier/therapeutic agent	Cell line	Reported outcomes	References
Chitosan hydrogel (Ch-hG)	Not specified	Alexa555 siRNa/Ch-hG into a375sM-bearing mice	MDa-MB231	Higher localization into tumor cells 92% reduction in tumor growth	Han et al. (2011)
HER2-scFv-arginine nonamer peptide fusion protein (e23sFv-9R) carrier	Not specified	CXCR4-siRNA for treatment of HER2+ breast cancers	MDA-MB-231	Inhibited CXCR4 gene expression, suppressed tumor growth, reduced metastasis, and prolonged survival	Jiang et al. (2015)
Cell-penetrating peptide-loaded nanobubbles with ultrasound radiation	Average 582.80 ± 19.57 nm/+ 23.51 ± 0.32 mV	EGFR siRNA delivery (siEGFR) to TNBC cells	MDA-MB-231	Downregulated expression of EGFR and inhibited growth of TNBC	Jing et al. (2016)
Magnetic nanoparticles (MN for magnetic resonance imaging), labeled with near-infrared dye Cy5.5 (for optical imaging) and conjugated to a peptide (EPPT)	Not specified	Tumor-specific antigen uMUC-1 and siRNA against gene birc5 (survivin) (siBIRC5)	BT-20	Nearly 2-fold decrease in tumor growth rate	Kumar et al. (2010)
Cationic lipid-assisted PEG-PLA nanoparticles	Average diameter of 146.4 ± 5.9 nm	Cyclin-dependent kinase 1 (CDK1), siCDK1	SUM149 and BT549 for c-Myc overexpressed TNBC	CDK1 expression inhibition	Liu et al. (2014)
PEG–PEI polyplexes	~ 30–80 nm ~ − 10 mV to 15 mV	STAT3 siRNA on breast cancer bone metastasis, targeting overexpressed E-selectin	MDA-MB-231 and MCF-7	Knockdown of STAT3 expression in 48.7% of cancer cells inside the bone marrow Tumor growth inhibition	Mai et al. (2014)
Micelleplex system based on an amphiphilic and cationic triblock copolymer, mPEG-b-PCL-b-PPEEA	47.7 ± 0.4 nm/− 20.8 ± 2.8 mV	Targeting the acid ceramidase (AC) gene	BT474-luciferase cells	Significant gene knockdown Suppression of AC expression Inhibited tumor growth	Mao et al. (2011)
Co-delivery of drug (doxorubicin) and siRNA in mesoporous silica nanoparticle functionalized by PEI–PEG copolymer	50 nm	P-glycoprotein to overcome multidrug resistance	MCF-7/MDR	Significant Pgp knockdown Statistically lower Pgp expression (~ 50%) in the tumor from Pgp	
siRNA-Dox-MSNP-treated animals compared to saline controls	Meng et al. (2013)				
Treatment with Pgp siRNA-Dox-MSNP led to ~ 70% reduction in mRNA expression compared to other treatment groups					
Mesoporous silica nanoparticle core coated layer-by-layer with bioreducible cross-linked PEI and PEG polymers, conjugated with an antibody for selective uptake into cancer cells	50 nm/8.10 − 0.25 mV	Polo-like kinase 1 siRNA (siPLK1)	TNBC cells BT549 and MDA-MB-231	Inhibited cancer migration and invasion in TNBC cells Knocked down about 80% of human PLK1 mRNA expression in metastatic breast cancer cells	Morry et al. (2017)
Jet-PEI and ultrasound	Average diameter is 2.5 μm	ANT2 shRNA	MDA-MB-231	Tumor regression and increased survival rate	Park et al. (2015)

Table 3 (continued)

Materials and design	Properties: size/zeta potential	Carrier/therapeutic agent	Cell line	Reported outcomes	References
				(80%), no significant toxicity	
PEI substituted with linoleic acid and caprylic acid	Not specified	CDC20-1 DsiRNA	MDA-MB-435 MDA-MB-231 MCF7	Reduced tumor growth	Parnar et al. (2015)
Multilamellar gold niosomes: siRNA-Nio-Au-thymoquinone	100–160 nm/1.14 mV	Akt-siRNA and thymoquinone MDM2 expression	BALB/c (nu+/nu+) human breast cancer xenograft model	Decrease in tumor mass and volume	Rajput et al. (2015)
PEG-modified chitosan for siRNA encapsulation	100 nm	Surviving	4T1	Significantly reduced growth of tumor in vivo	Sun et al. (2016)
Layer-by-layer films formed by alternately depositing siRNA and poly-L-arginine	120 nm/− 55 mV	GFP-overexpressing cells in TNBC xenograft models	MDA-MB-468	Reduced the target gene expression in the tumors by almost 80%	Deng et al. (2013)
Amphiphilic alkylated PEI/superparamagnetic iron oxide nanovehicles carrying siRNA on the outside through electrostatic interaction	~ 88–130 nm/~ 37–44 mV	P-glycoprotein (P-gp)	MCF-7/ADR	Downregulated P-gp by 71.4%	Lin et al. (2014)

balance between the siRNA stability, cell uptake, and safety in vivo (Falamarzian et al. 2012).

Nanoparticles

Nanoparticles are generally 10–1000 nm in diameter (Wang et al. 2016a; Parvani and Jackson 2017). They possess several advantages to other systems, including small size, high surface area, stability in physiological media, and immunologically inert surfaces that gives them good in vivo retention (Bannunah et al. 2014; Draz et al. 2014; Young et al. 2016). The two main types of nanoparticles investigated for siRNA therapy in breast cancer are inorganic nanoparticles and polymeric nanoparticles (Young et al. 2016; Li et al. 2016; Wang et al. 2016b). Inorganic nanoparticles that have been used for delivery of siRNA include silica, calcium, gold, magnesium, strontium, metal oxides, and carbon nanotubes. Finlay et al. (2015a) delivered siRNA in PEI-coated mesoporous silica nanoparticles and observed knockdown of *TWIST1* expression in vivo. Metal oxides such as iron oxide nanoparticles have magnetic resonance imaging properties, which enables them to offer combined imaging and delivery of siRNA to tumors (Kumar et al. 2010).

Cationic polymer-based nanoparticles are the dominant material used for delivery, owing to their strong electrostatic interactions with negatively charged siRNA and because they can be synthesized relatively easily compared to other types of nanoparticles (Sun et al. 2017b). The most common cationic polymer used for siRNA delivery systems is low molecular weight PEI due to its superior transfection efficiency (Arnold et al. 2017; Sun et al. 2017b). However, clinical application of unmodified PEI is hampered by its cytotoxic effects. Other common polymers include polylysine (PLL), poly(lactic-co-glycolic acid), and chitosan (Navarro et al. 2015).

Dendrimers are a relatively new class of cationic polymers. Dendrimers are hyper-branched globular structures composed of repeating units. They are typically uniform with a high degree of symmetry. Dendrimers have a large number of cavities as well as a large number of functional groups on the surface. The typical structure of a dendrimer consists of three parts: a central core, which defines the interior size and the quantity and direction of the branches; the repetitive branch units, which determine the molecular size and flexibility; and the terminal groups, which determine the chemical property and ability to interact (Fig. 2) (Wu et al. 2013). Dendrimers have positively charged amine functionalities at the surface that enable condensation of negatively charged siRNA molecules through electrostatic interactions. They also have tertiary amine groups in the interior that can be protonated in acidic endosomes to initiate the proton sponge effect (Liu et al. 2016). The most common dendrimer used for siRNA delivery systems is poly(amidoamine) (PAMAM) (Gu et al. 2015; Sun et al. 2017b), a cationic polymer that has good pH buffering

capability. Limited in vivo studies were found for dendrimer-based delivery systems for breast cancer. Finlay et al. (2015b) investigated PAMAM-siRNA complexes in SUM1315 TNBC cells and found that the dendrimer–siRNA complexes were taken up by the cells and led to knockdown of *TWIST1* expression in vivo. Dendrimers offer flexibility in being able to modify their structure and properties, as with liposomes and micelles. However, there are concerns with non-specific cytotoxicity, rapid clearance in vivo, and poor delivery efficiency (Biswas and Torchilin 2013; Wu et al. 2013; Finlay et al. 2015b).

The following types of designs were observed in nanoparticle delivery systems for breast cancer in vivo: the majority of delivery systems were composed of multiple polymers mixed or conjugated to form a nanocomplex; siRNAs loaded onto the surface of a nanocomplex (Lin et al. 2014); and layer-by-layer nanoparticles (Deng et al. 2013). It is noted that PEG and PEI are incorporated in the majority of the siRNA nanocomplexes, providing strong support for cationic polymeric nanoparticles as part of a delivery system. Furthermore, almost all delivery systems were less than 200 nm. Although to our knowledge none have progressed to clinical trials for breast cancer, some such as the commercially available jet PEI have been trialed for other indications (Walther et al. 2008).

A critical consideration in the design of delivery vehicles for breast cancer is a specific targeting capability. In vivo studies have shown that specific gene silencing of breast tumors can be achieved by combining siRNAs with cell type-specific ligands such as antibody fragments or chemokine receptors, which are known to affect protein kinase pathways that control key signaling steps involved in the invasion and growth of the breast tumor cells. These ligands help the delivery vehicles enter target breast cells through receptor-mediated endocytosis. Examples of ligands that have been shown to facilitate specific targeting of breast tumors in in vivo xenograft models include fusion protein containing single-chain antibody fragment (scFv) (Jiang et al. 2015), stromal cell-derived factor (SDF-1α), CXC chemokine receptor 4 (CXCR4) (Jiang et al. 2015), neuropilin-1 (NRP-1), heat shock protein 90 (Hsp90) (Ahmad et al. 2016), c-raf (Chien et al. 2005), and tissue transglutaminase (TG2) (Han et al. 2011). In general, antibody fragments with well-defined cell-type specificity and favorable pharmacokinetics and biodistributions are desired.

Conclusion

RNA interference (RNAi) using small interfering RNA (siRNA) and microRNA (miRNA) is an evolving field for cancer treatment, particularly for cancers that display highly heterogeneous characteristics such as breast cancer. A better understanding of the genetic basis of the molecular subtypes of breast cancer is required to identify the target genes for silencing. There has been considerable progress in employing siRNA in breast cancer through various delivery systems. On review of delivery systems using studies in vivo, we noted that there has been considerable effort directed towards optimizing the design by modifying the core and surface of the delivery vehicles to address the various challenges encountered in circulation and at the tissue and cellular levels. We observed that the surface of the delivery vehicle is primarily where ligands have been attached for cell-specific recognition in breast tumors. The majority of studies employed PEGylation of the surface to protect the delivery system from nuclease degradation. The core of the delivery system has been mainly modified to enhance its ability to escape the endosome, normally through incorporating polymers with proton-sponge effect capabilities such as polyethylenimine (PEI). We also observed that the size of almost all nanoparticle delivery vehicles was less than 200 nm. Concerning translation to the clinic, large-scale production of nanoparticles can be labor-intensive and, therefore, costly. The delivery system must consider careful selection of materials, solvents, and procedures for nanoparticle development, batch-to-batch consistency, reproducibility, and acceptability of the final product during scale-up. Despite these hurdles, gene silencing through RNAi has significant clinical implications for cancer treatment and continued research into the design of an effective delivery vehicle for siRNAs and miRNAs will accelerate the trend towards precision medicine.

Compliance with ethical standards

Conflict of interest Tamkin Ahmadzada declares that she has no conflict of interest. Glen Reid declares that he has no conflict of interest. David McKenzie declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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