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Engineered polymeric nanoparticles to guide the cellular internalization and trafficking of small interfering ribonucleic acids

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ABSTRACT

Ribonucleic acid interference therapy is a promising cancer treatment, which uses small interfering RNAs (siRNAs) to target and degrade messenger RNAs. Due to endogenous nuclease activity, siRNA is degraded rapidly, resulting in poor cell uptake and hence specificity. Moreover, it will not readily cross the cell membrane by passive diffusion. In order to take advantage of the therapeutic power of siRNA for the treatment of cancer, specialized delivery vehicles have been designed. In this review, we highlight advances in optimizing nanoparticle functionalization for guided siRNA delivery at the cellular level – that is, promoting cell uptake, escaping the endosome, and releasing siRNA from the delivery vehicle.

Contents

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1. Introduction

Ribonucleic acid interference (RNAi) is a powerful tool for the regulation of gene expression, making it an ideal therapeutic for diseases caused by genetic mutations, such as cancer. Often, the cancer cells overexpress oncogenic genes, providing potential targets for gene knockdown [1,2]. Small interfering ribonucleic acids (siRNAs) are short strands of ribonucleic acid typically composed of 21–30 base pairs with overhanging 3′ ends that can induce sequence-specific gene silencing at low (picomolar) concentrations when transfected into cells [3]. Although other silencing technologies are available, including the CRISPR/Cas9 system and antisense oligonucleotides, among others, siRNAs are advantageous due to their high potency and small size. Additionally, delivery of CRISPR/Cas9 components *in vivo* is still a major challenge, whereas there are a plethora of siRNA delivery strategies, as described herein. Naturally occurring small interfering ribonucleic acids (siRNAs) were first reported in 1999 in plants [4], and synthetic siRNAs were used to effect gene knockdown in mammalian cells two years later [5]. "Naked" siRNA therapeutics have been successful in clinical trials for ocular diseases when locally delivered at high concentrations, despite limitations including inflammation and increased ocular pressure [6]. The systemic delivery of these therapeutics presents additional challenges following intravenous injection in order to reach cancerous tissue. When injected, siRNA formulations must (1) evade the immune system, (2) avoid interactions with non-target cells, (3) avoid premature renal clearance, and (4) reach target tissues. These requirements have been reviewed extensively [7–9], thus here we will focus on overcoming further roadblocks once siRNA formulations reach their target tissues, including degradation by extracellular nucleases, poor cell uptake, and trafficking into the lysosomal compartments where the RNA strands are quickly degraded [8,10]. These challenges often require that siRNA therapeutics are combined with specialized delivery materials in order to be effective.

Nanotechnologies, encompassing a wide variety of formulations including metallic nanoparticles, micelles, liposomes, nanocrystals, nanogels/capsules, among others, are important delivery vehicles for a wide range of therapeutics including small molecule drugs, proteins, and siRNAs [11–13]. Advantages of nanoformulations include improved biodistribution and pharmacokinetics, stabilization of therapeutics, solubilization of hydrophobic drugs, and attenuating toxicity to off-target tissues [14–16]. The size, surface charge, and morphology of the delivery vehicle must be considered as they have a significant impact on pharmacokinetics and biodistribution [17–21]. The morphology of the vehicle can also have a significant impact on cellular internalization rates [22]. Moreover, in order for the nanoparticles to respond to both stimuli on the surface of the cell and within intracellular trafficking pathways, they must be flexible in terms of structure, functionalization, and resultant properties [23–25]. Although lipid-based nanoparticles have played an important role in the development of siRNA delivery strategies, lipid-based formulations are limited to a smaller number of well-established lipid components [26], whereas there are numerous monomers for polymer synthesis [27–30]. Therefore, while lipid-based strategies have been extensively studied and reviewed [31–33], this review will focus on fundamental and novel research in polymeric micelles, nanoparticles, and polyplexes for siRNA therapeutic delivery.

We begin with a brief discussion of stability of siRNA in the extracellular environment, and then examine some of the key challenges of siRNA delivery and trafficking in the target tissues using polymeric delivery vehicles, including: enabling cellular uptake, avoiding degradation within the cell, and successfully releasing the therapeutic payload. While there are many parameters that influence the success of an siRNA nanoparticle delivery system, including uptake specificity, rate of clearance and degradation, the key parameter is efficiency of knockdown and it is this parameter on which we have based our review.

2. Stability of siRNA in the extracellular environment

In order to increase the delivery efficiency of siRNA payloads, siRNAs are often conjugated or complexed to nanoparticles that protect them from nucleases and rapid clearance (Fig. 1). Within 15 min of injection in mice, >90% of standard 21-mer siRNAs are degraded by serum nucleases or lost *via* renal or lymphatic clearance [34], underlining the importance of the delivery vehicle. Polymeric nanoparticles can increase the stability of siRNAs against degradation: Raja et al. demonstrated that crosslinked chitosan nanoparticles increased the stability of siRNAs against serum during a 15 day storage at 4 °C [35] while Zhu et al. increased the half-life of siRNA in the blood to approximately 8 h by encapsulating it within a PLGA-based delivery vehicle, resulting in better tumor accumulation [36]. It is hypothesized that the nuclease resistance conferred by nanoparticle formulations is due to the steric bulk of the polymeric corona, preventing nucleases from reaching the siRNA. Therefore, with increasing density of the polymeric corona, the stability of siRNA in biologically relevant conditions is increased [37,38].

SiRNAs can also be chemically modified in order to increase their stability against nucleases. These modifications include any change to the native siRNA structure, typically employed on the phosphodiester bond or sugar ring (Fig. 2). These modifications enhance siRNA stability and potency, provide longer knockdown duration, reduced off-target effects, and lower immunostimulatory effects [39–42]. Modified siRNAs are now commonly used in research [43–45]. As shown in Fig. 2, some of the most common modifications of oligonucleotides include modifications to the backbone or nucleosides. For example, backbone modifications include phosphorothioate [46] and boranophosphonate [47] linkages, which increase nuclease resistance, while nucleoside modifications include 2′-*O*-methyl [48,49], 2′-deoxy-2′-fluoro [50], and locked nucleic acids [51], which increase stability and target binding affinity. Chemical modification of oligonucleotides and the effect on potency have been extensively reviewed by Deleavey et al. [40].

3. Cellular internalization

Most clinically relevant hydrophobic small molecule drugs can passively diffuse through the cell membrane. SiRNAs are large, hydrophilic, and negatively charged, so their passage across the cell membrane in the absence of a specialized carrier is hindered or blocked entirely. In a nanoparticle formulation, several different internalization pathways

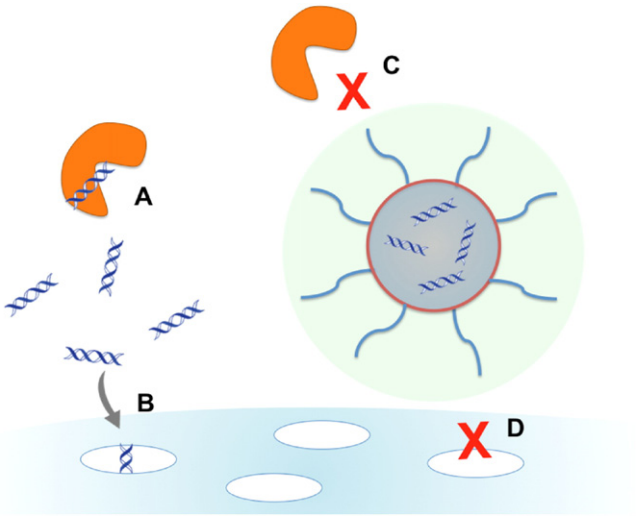

Fig. 1. SiRNA nanocarriers protect it from nuclease degradation. (A) Free siRNA (blue double helix) is rapidly degraded by nucleases (orange semi-circle) and (B) cleared by lymphatic drainage (pale blue ovals). (C) Nanoparticles may protect siRNA from nucleases and (D) reduce clearance.

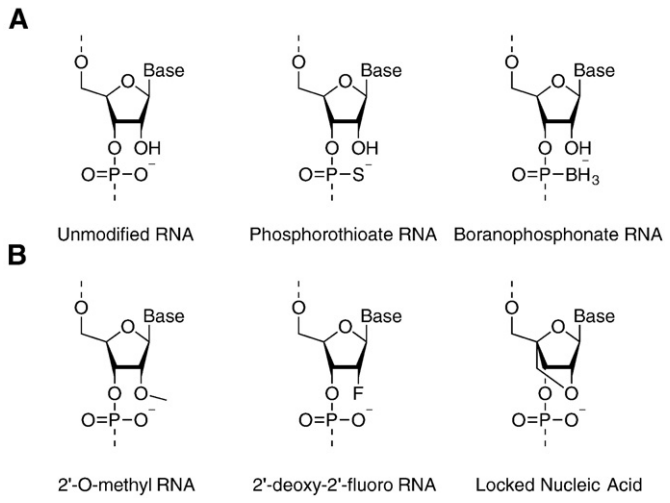

Fig. 2. Common modifications to siRNA include modifications to both: (A) the phosphodiester linkage and (B) the 2′ sugar.

are possible - clathrin/caveolar-mediated endocytosis, phagocytosis, macropinocytosis, and pinocytosis - but each ultimately leads to the endolysosomal pathway [52]. Although not directly addressed in the examples that we highlight herein, it has been shown that subsequent endocytic recycling pathways [53] or autophagy [54] may also play a role in limiting the dose of oligonucleotides delivered and should be more extensively studied for polymeric siRNA delivery systems. There are several different interactions that a polymeric vehicle can use to trigger one of these internalization pathways and carry siRNA across the cell membrane, including cationic charge, cell penetrating peptides (CPPs), antibodies, and aptamers (Fig. 3). Non-specific uptake, *via* cationic charge or CPPs, is often efficient *in vitro*, but a more selective strategy requires ligands (antibodies or aptamers) to be taken up by specific cells. Selective strategies may be more relevant in an *in vivo* setting in order to reduce off-target effects. Here, we present a variety of strategies used to cross the cell membrane from low to high specificity.

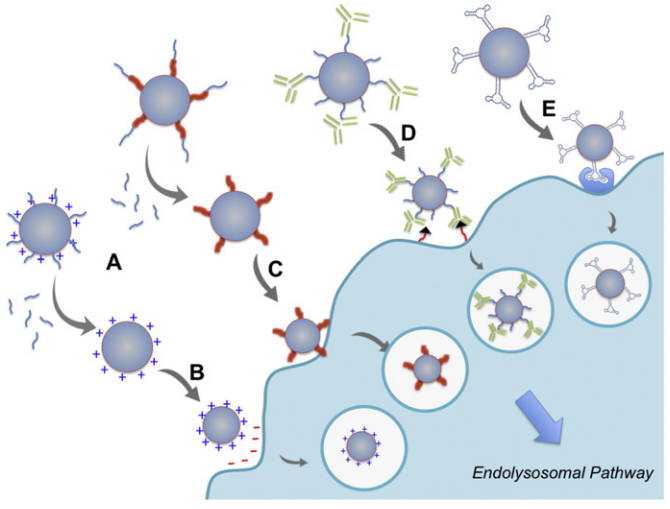

Fig. 3. Nanoparticle formulations can cross the cell membrane by multiple pathways: (A) deshielding of cationic charges triggered by cues in the tumor microenvironment, yields (B) positively charged nanoparticles that interact with the anionic cell membrane; (C) deprotection of cell penetrating peptides (CPPs) yields nanoparticles that cross cell membranes non-specifically; (D) Antibody-modified nanoparticles target specific receptors on the cell surface for internalization; (E) Nanoparticles modified with aptamers, selected from phage-display libraries that bind specific cell membrane targets, trigger internalization.

3.1. Cationic polymers enhance penetration of the cell membrane

Cationic polymers are often used to facilitate siRNA penetration of the cell [55] because they interact with the anionic proteoglycans of the cell membrane, facilitating endocytosis [56,57]. One classically used cationic polymer for siRNA delivery is polyethyleneimine (PEI) [58]. Highly branched and high molecular weight PEI (>20 kDa) is toxic, so low molecular weight PEI (<2 kDa) is often used [59]. In one example, Lee et al. used low molecular weight PEI for the delivery of 'polymerized' siRNA - that is, chains of repeating siRNA segments connected by disulfide bonds. By using this PEI delivery system, they were able to achieve 70% knockdown *in vitro* of red fluorescent protein (RFP) in RFP + melanoma cells [60]. Despite efficient transfection, any cell will non-specifically take up PEI and other positively charged polymers. Since most nano-scale formulations naturally accumulate in the liver [61,62], many strategies deliver therapeutics against diseases of the liver [62,63]. In order to target other tissues, the positively charged polymer must be shielded until it reaches the tumor site. To temporarily shield their positive surface charge, cationic nanoparticles are modified with sheddable poly(ethylene glycol) (PEG) coronas using various stimulus-responsive coupling strategies. For example, Li et al. developed a polymeric nanoparticle that is responsive to matrix metalloproteinase 7 (MMP-7), an enzyme that is overexpressed by breast cancer cells and found at high concentrations in the tumor microenvironment [64]. The nanoparticle corona is composed of a PEG block linked by an MMP-7 cleavable peptide to a cationic block. When the nanoparticle reaches the tumor microenvironment, extracellular MMPs cleave the peptide, shedding the PEG layer and exposing the cationic layer, raising the zeta-potential of the nanoparticle from +5.8 to +14.4 mV and increasing cellular internalization 2.5-fold. Nanoparticles pre-treated with MMP-7 resulted in knockdown efficiency of approximately 50% *in vitro*; however, this system was not studied *in vivo*, so it is still unclear whether this strategy will result in improved biodistribution [64]. Despite the shielded cationic charge, significant toxicity was observed at high nanoparticle: siRNA ratios, underlining the importance of nanoparticle safety to their utility. Perche et al. took advantage of the hypoxic tumor microenvironment to develop a nanoparticle shielded by PEG conjugated through an azobenzene moiety that undergoes reduction-mediated cleavage under hypoxic conditions [65]. Once the PEG layer has been shed, an siRNA-PEI complex, conjugated to a hydrophobic anchor, is revealed that readily enters the surrounding cells. Using green fluorescent protein (GFP) as a model target, knockdown of ~30% was achieved *in vitro*. In an *in vivo* mouse model, the nanoparticle formulation significantly reduced tumor GFP expression compared to a scrambled control [65]. In this case, no significant cytotoxicity was observed.

3.2. Cell penetrating peptides for cellular uptake

CPPs have been exploited to bring "cargo" into cells. The CPPs are typically <40 amino acids, cationic, and viral-derived [66]. There have been many reviews focused on the characteristics and mechanisms of CPPs [66–68] and while there are numerous CPP sequences (Table 1), they usually lack specificity as they will cross any cell membrane. While the internalization pathways of most CPPs are not well-defined, internalization is initialized *via* interactions of the cationic CPPs with the phospholipids of the cell membrane [69].

To achieve greater specificity of CPPs, one of three strategies is typically employed: (1) triggering CPP deprotection at the tumor site; (2) local delivery of the CPP to the tumor site; or (3) conjugation to cell-targeting ligands.

Using the first strategy, Sun et al. synthesized a polyarginine CPP, used for siRNA complexation and siRNA release, sandwiched between a hydrophobic poly(caprolactone) (PCL) block and a hydrophilic PEG block [79]. The PEG corona was conjugated to the CPP through 2-propionic-3-methylmaleic anhydride linkers, which are cleavable under the acidic environment of the tumor site, deshielding the CPP

Table 1
Common cell-penetrating peptides.

Name	Sequence	Origin	Charge[a]
TAT (48–60) [70,71]	GRKKRRQRRRPPQ	Derived from HIV type 1	+8
Penetratin [72]	RQIKIWFQNRRMKWKK	Antennapedia homeodomain	+7
Transportan/TP10 [73,74]	GWTLNS/AGYLLGKINLKALAALAKKIL[b]	Neuropeptide galanin-mastoparan fusion	+4
VP22 [75]	NAKTRRHERRRKLAIER	Herpes simplex virus	+7
Polyarginine [76]	R_n[b], n = 8–9	Engineered for positive charge	+8 or +9
Pep-1[77]	KETWWETWWTEWSQPKKKRKV[c]	Fusion of NLS from simian Virus 40 and reverse transcriptase of HIV-1	+3
CADY [78]	GLWRALWRLLRSLWRLLWRA[c]	Derived from PPTG1 peptide, addition of W and charged amino acids	+5

[a] pH 7.4.
[b] *C*-terminal amide.
[c] *C*-terminal cysteamide.

and facilitating cellular uptake. *In vitro* experiments revealed 60% PEG cleavage under acidic conditions and ~70% knockdown of cyclin-dependent kinase 4 (CDK4) in adenocarcinoma cells. *In vivo* experiments in a mouse model of adenocarcinoma resulted in significantly delayed tumor growth compared to scrambled controls over 21 days and 50% knockdown of CDK4.

For the second strategy of local CPP delivery, Kanazawa et al. used an intranasal delivery route to carry siRNA directly to the brain in a mouse model of brain cancer using a CPP-nanoparticle formulation [80]. PCL nanoparticles were conjugated to a TAT CPP and hydrophilic PEG. The authors were able to use the TAT peptide for siRNA complexation and delivery. *In vitro* experiments demonstrated significant nanoparticle uptake, minimal cytotoxicity, and 70% knockdown of Raf-1, a gene associated with cell proliferation and apoptosis, and ultimately resulted in significantly lengthened survival in an *in vivo* rat model of malignant glioma.

For the third strategy, conjugating CPPs to cell targeting peptides can increase their specificity. Fang et al. conjugated the TAT CPP to A1, a peptide with high affinity for vascular endothelial growth factor receptor-1 (VEGFR1) and demonstrated selective delivery to tumor cells overexpressing VEGFR [81]. Similarly, R9 can be fused to a cyclic arginine-glycine-aspartic acid (cRGD) peptide for targeting [82]. However, this hybrid strategy has not yet been reported for delivery of a synthetic polymeric formulation of siRNA.

Although stimuli-responsive nanoparticles or local delivery routes can offer improvements in activity, cationic peptides and polymers may be limited by non-selectivity and cytotoxicity. Therefore, strategies that avoid reliance on cationic charges should be considered such as antibodies or ligands for receptor-mediated endocytosis or aptamer-mediated uptake. While in these strategies protein corona formation [83,84] may hinder cell uptake, avoiding the toxicity and non-specificity of cationic charges offers a significant advantage. The protein corona can be at least partially overcome by, for example, functionalizing the polymeric nanoparticles with a PEG corona to prevent opsonisation [85,86].

3.3. Receptor-mediated cell uptake via small molecule ligands or antibodies

Targeting ligands can be attached to polymeric delivery vehicles to increase the specificity of cellular uptake. These specifically bind receptors overexpressed on cancer cell membranes, facilitating receptor-mediated endocytosis of the nanoparticle [87]. Interestingly, the MMP-7 responsive nanoparticle, previously discussed [64], was conjugated to folate ligands [88]. In this case, PEG cleavage was triggered by MMP-7 at the tumor site, exposing folate-conjugated nanoparticles for receptor-mediated endocytosis. *In vitro* experiments, including MMP-7 pretreatment and folate ligand competition assays, revealed that knockdown was dependent on both MMP-7 activity and folate receptor binding. Under optimal conditions, the formulation achieved >50% luciferase protein knockdown with no detectible cytotoxicity in a luciferase positive breast cancer cell line [88].

Antibodies can also be conjugated to nanoparticle formulations for targeted siRNA delivery, triggering internalization *via* a receptor-mediated endocytosis pathway [89]. Palanca-Wessels et al. synthesized a nanoparticle in which siRNA was encapsulated and to which anti-human epidermal growth factor receptor 2 (HER2) antibodies were conjugated for cellular internalization [90]. Delivery of siRNAs against a variety of chemotherapy resistance-associated mRNAs resulted in 80% knockdown *in vitro* and 70% knockdown of a target gene *in vivo* in a mouse model of ovarian cancer. However, the authors noted a slight immune response in some of the streptavidin-containing control groups [90,91].

3.4. Aptamers for highly specific cellular uptake

Nucleic acid aptamers are relatively short strands of DNA or RNA that are identified by screening from a large random sequence pool that tightly bind to specific receptors [92]. Preclinical studies using soluble aptamer-siRNA chimeras have been very successful in mouse models of cancer [92]. Building on this strategy for use in a nanoparticle formulation, Subramanian et al. synthesized an aptamer-PEI-siRNA polyplex stabilized with sodium citrate for targeting and knockdown of epithelial cell adhesion molecule (EpCAM) [93]. Using an anti-EpCAM aptamer to target breast cancer and retinoblastoma cell lines, the delivery of anti-EpCAM siRNA resulted in an approximately 50% reduction in EpCAM expression leading to 80–90% reduction in cell proliferation *in vitro* in both cell lines (Fig. 4) [93]. Aptamers are advantageous as targeting ligands for several reasons, including their low molar mass, low immunogenicity, and high specificity for cellular antigens [94]. The use of aptamers is a promising strategy for cell binding and internalization of polymeric nanoparticles.

4. Escaping the endolysosomal pathway

Regardless of the cell surface target, the majority of nanoparticle formulations enter the cell through the endolysosomal pathway where rapid acidification of the endolysosomes results in pH ranges from pH 6.5 to 5.5 in the endosomes and pH 5.5 to 4.0 in the lysosomes [96]. SiRNAs will eventually degrade under the acidic conditions and enzymatic activity in the lysosomes [97], and thus endosomal escape agents must be used. These agents function by the "proton sponge" effect, pore formation or membrane destabilization [70,98–100].

4.1. Amines for endolysosome escape

To achieve the "proton sponge" effect (Fig. 5), nanoparticle delivery systems are functionalized with groups that are protonated at acidic pH, causing an influx of chloride anions, followed by osmotic swelling and endosomal lysis [101], enabling contents within the endosome to be expelled into the cytosol. Functional groups that are commonly used for endosomal escape include primary or secondary amines, such as linear or branched PEI [102–104], guanidines [105–107], lysines [108–110], and imidazoles, such as histidine [111]. Polymer synthesis can be designed for facile incorporation of each of these functionalities through monomer modification or post-polymerization modification.

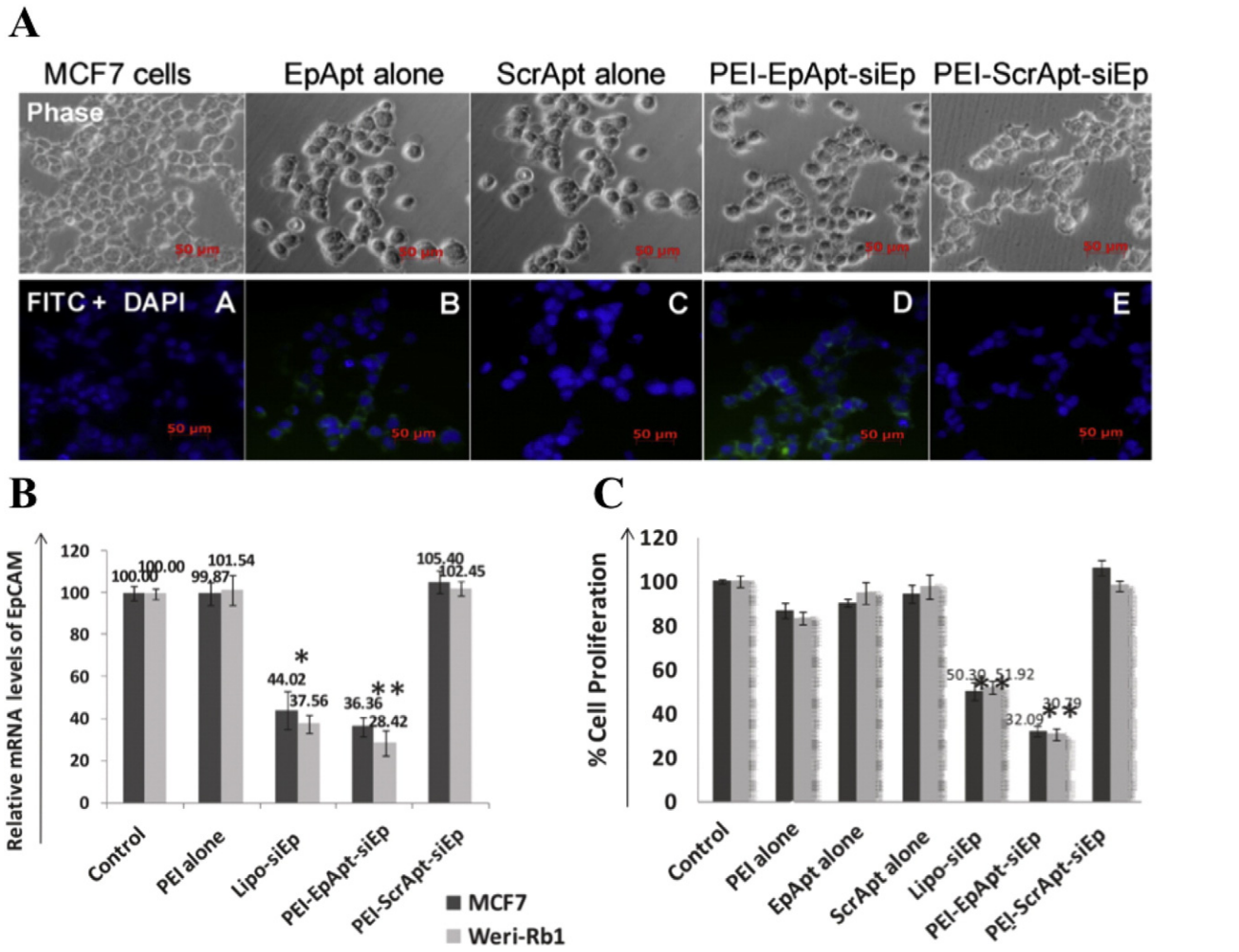

Fig. 4. A) Uptake of EpCAM aptamer-conjugated complexes. The scrambled aptamers (EpApt) and the corresponding conjugates (PEI-ScrApt-siEp) show no uptake whereas the active aptamer (EpApt) shows uptake and the polymer conjugate (PEI-EpApt-siEp) shows enhanced uptake compared to the aptamer alone. B) mRNA levels following treatment. Significant EpCAM mRNA decrease is seen in two cell lines when the anti-EpCAM siRNA is transfected (Lipo-siEp) or delivered by the polymer conjugate (PEI-EpApt-siEp). C) mRNA knockdown leads to a decrease in cell proliferation. Figure reproduced from Subramanian et al. [95] with open access permissions.

Many of the cationic polymers and peptides already discussed contain amine moieties that, in addition to crossing the cellular membrane, will lead to the "proton sponge" effect and endosomal escape. For example, Cheng et al. synthesized mPEG-poly(ε-caprolactone)-*graft*-poly(2-(dimethylamino) ethyl methacrylate by ring opening polymerization of the caprolactone, followed by atom-transfer radical-polymerization of 2-(dimethylamino) ethyl methacrylate (pDMAEMA) [112]. DMAEMA contains a tertiary amine that aids endosomal escape through proton sponge effects [113]. The authors hypothesize that the nanoparticles showed significant uptake and efficacy in MDA-MB-231 cells *in vitro* and *in vivo* due, in part, to endosomal escape caused by protonation of the pDMAEMA. However, the presence of the cationic pDMAEMA caused significant cytotoxicity when treating cells with blank nanoparticles at increasing concentrations, demonstrating that the vehicle itself needs to be safe for efficacy [112].

Oligoarginines are known as cell-penetrating peptides, but are also active in the endosome for endosomal escape due to the bidentate hydrogen binding of the guanidine side group of arginine to the negatively charged endosomal membrane [114]. Zhao et al. synthesized a monomethoxy poly(ethylene glycol)-*block*-poly(D,L-lactide)-*block*-poly(arginine) ($mPEG_{3000}$-PLA_{2000}-R_{15}) for the delivery of siRNA targeting epidermal growth factor receptor (EGFR) [115]. The addition of the polyarginine peptide led to a significant increase in the zeta potential, and delivery of anti-EGFR siRNA, resulting in 60% protein reduction *in vitro* in MCF7 cells. *In vivo* data revealed significant volume reduction in xenografted MCF-7 tumors following nanoparticle-siRNA treatment (Fig. 6A). More recently, Nam et al. incorporated an arginine grafted bio-reducible poly(cystamine bisacrylamide-diaminohexane) onto a poly(amido amine) to deliver VEGF siRNA to three cancer cell lines [116]. The authors reported 70–80% knockdown of VEGF with minimal cytotoxicity using this system *in vitro*.

A major concern when using primary or secondary amines with pK_as greater than physiological pH is the off-target effects and cytotoxicity of cationic delivery vehicles. Imidazole groups are attractive functionalities to incorporate into polymers because they have pK_as of ~6.0 [117]. Therefore, these groups will be neutral outside of the cell, and only become protonated once inside of the endosomes, reducing off-target effects and cytotoxicity. Ghosn et al. used imidazole acetic acid to modify 20–30% of the amino groups of chitosan [118]. The authors reported the delivery of siRNA targeting glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and demonstrated 90% protein knockdown of lung A549 cells *in vitro* using the 30% imidazole-substituted chitosan. This system did not demonstrate significant cytotoxicity *in vitro*. Furthermore, Han et al. formed nanoparticles from imidazole-modified urocanic acid-modified galactosylated trimethyl chitosan [119]. While the non-imidazole modified nanoparticles delivered siRNA with strong uptake in hepatocellular carcinoma QGY-7703 cells, they were mainly localized and trapped in the lysosomes after 4 h *in vitro* (Fig. 6C). The imidazole-modified nanoparticles exhibited a diffuse fluorescence in the cytosol, indicative of endosomal escape. Furthermore, the imidazole

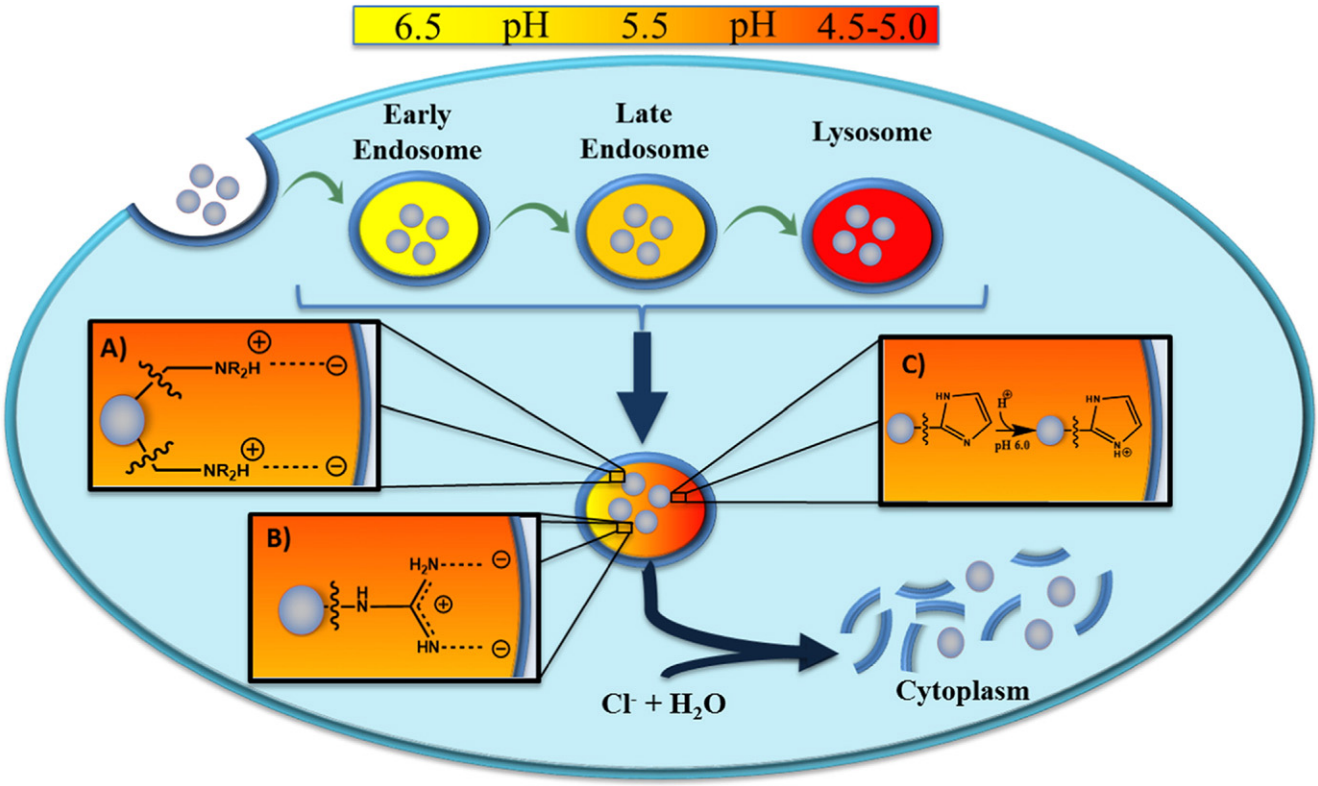

Fig. 5. Nanoparticle escape from the endolysosomal pathway *via* the "proton sponge" effect. Nanoparticles enter the early endosome, which matures into late endosome and then lysosome. Nanoparticles bearing: (A) primary amines, such as lysine, (B) guanidines, such as in arginine, or (C) imidazoles, such as in histidine, promote the "proton sponge" effect where an influx of chloride ions along with water leads to an eventual rupture of the endosome. The contents of the endosome are then freed into the cytosol for intracellular delivery of siRNA to RNAi machinery.

modified nanoparticles outperformed Lipofectamine 2000, a commercially available transfection reagent, *in vitro*, with negligible cytotoxicity. Using this formulation, imidazole-modified nanoparticles demonstrated significantly more tumor inhibition *in vivo* compared to non-imidazole nanoparticles due to their enhanced endosomal escape. Thus, incorporation of imidazoles in nanoparticle formulations is promising as it results in a stimulus-responsive nanoparticle where endosomal escape is only activated in the acidic organelles.

4.2. Pore-forming peptides for endosomal escape

An emerging alternative to cationic charges for endosomal escape is the use of pH-switchable groups or peptides, such as endosomolytic peptides [120–122]. Endosomolytic peptides destabilize membranes when a critical concentration of the peptide is located near a membrane, followed by interaction with the negatively charged phospholipid bilayer and pore formation. The resultant pore then destabilizes and disrupts the membrane [123]. Examples of this strategy for siRNA delivery using polymeric delivery vehicles are still rare, but there is some strong proof-of-concept work in the field of gene delivery. For example, Cheng et al. synthesized virus-inspired polymers for endosomal release (VIPER) by grafting 'caged' melittin, a membranolytic peptide, through a disulfide bridge to a polymer containing a hydrophilic cationic block for therapeutic loading and a pH-sensitive block for the triggered display of the melittin peptide [124]. The authors demonstrated nanoparticle disassembly at pH 5.7, enhanced hemolytic activity at acidic pH compared to neutral pH, and greater transfection *in vitro* and *in vivo* relative to commercially available reagents (Fig. 6B). Although this system was used to deliver DNA, the same strategy could be used for the delivery of siRNA.

Melittin is a non-specifically membranolytic peptide, and thus it needed to be shielded by additional chemical modifications until reaching the acidic pH of the endosomes. An alternative that has not yet been explored for polymeric nanoparticle systems, but has been successful for siRNA delivery in other forms, is a class of peptides called 'fusogenic' peptides (Table 2) [125]. These peptides do not interact with cells until they reach the endosomal pH, where they adopt a pore-forming α-helical structure. Although these peptides have not yet been used with synthetic polymer systems, they have shown efficacy in cationic lipid [126] and oligo(amino acid)-based systems [127] and should be promising for use with polymeric delivery vehicles.

5. Releasing the siRNA

One of the most common methods of delivering siRNA is by cationic vehicles that complex the anionic siRNA, and then the siRNA is released *via* spontaneous dissociation [132]. In order to increase extracellular stability, many vehicles incorporate covalently conjugated siRNA. The siRNA must be released from the polymer vehicle in the cytosol in order to be effective [133,134], and release of the siRNA from covalent conjugation requires a specific mechanism. As shown in Fig. 7, there are many cue available for the release of siRNA that can be exploited including: acidic pH, enzymatic activity, reducing conditions, and the presence of specific bioactive molecules.

5.1. Acid-triggered siRNA release

The acidification in the endolysosomal pathway can be exploited to trigger the cleavage of the siRNA from the polymeric vehicle in conjunction with endosomal escape strategies previously discussed. Takemoto et al. developed an siRNA conjugate nanoparticle designed to be destabilized under acidic conditions to promote endosomal escape and siRNA cleavage, all based on one acid-sensitive maleic acid amide (MAA) linkage [135]. Under acidic conditions, the MAA groups of the

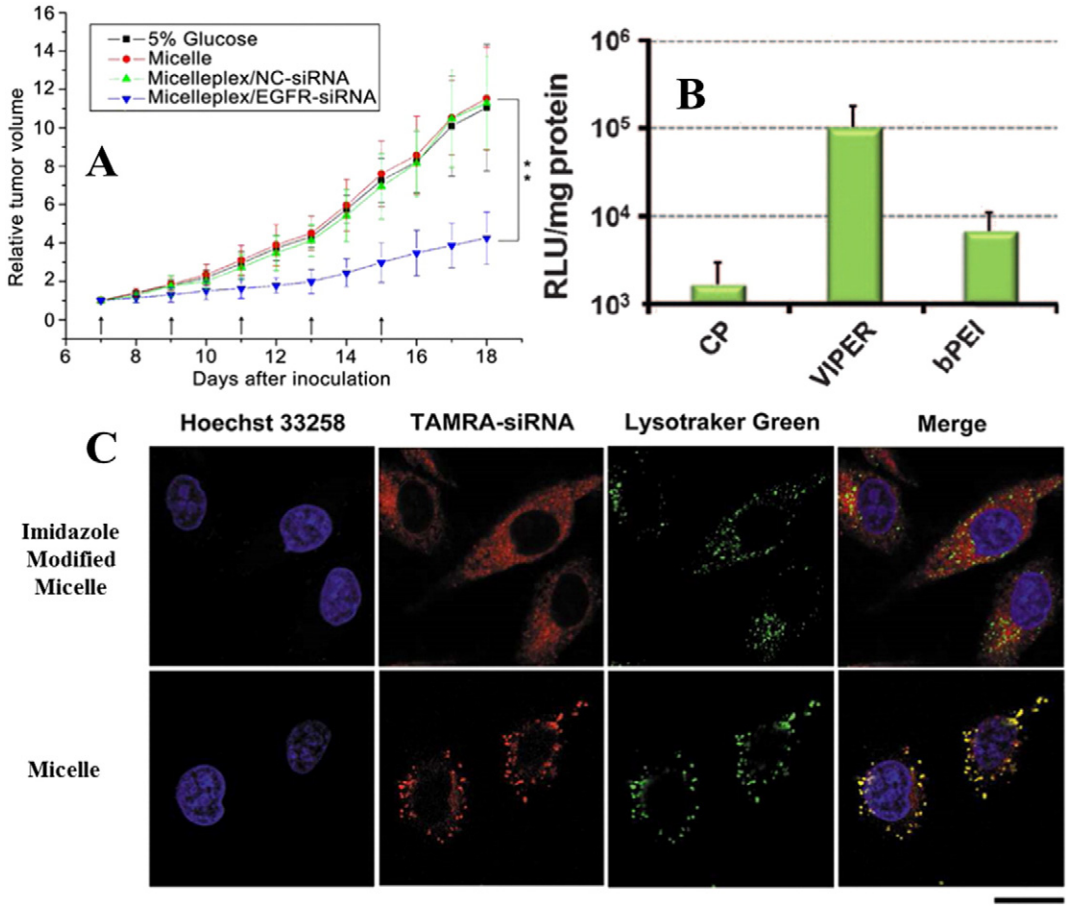

Fig. 6. A) Relative volume changes of xenografted MCF- 7 tumors when injected intravenously with micelle/siRNA complexes targeting EGFR at 1 mg/kg for 5 injections. Micelleplexes contained an R_{15} peptide for cancer cell uptake, and endosomal escape. Reprinted from Zhao et al. [115] with permission from Elsevier. B) Luciferase activity from excised A549 tumor tissues of mice treated with polyplexes containing no membranolytic peptide, mellitin, (CP), polymer grafted with melittin (VIPER), and branced PEI (bPEI). Data are shown as mean ± SD (n = 4; student's *t*-test, *p < 0.05,**p < 0.01). Reproduced from Cheng et al. [124] with permissions. C) Confocal laser scanning microscopy images of QGY-7703 cells treated with TAMRA-siRNA loaded micelles (red) for 4 h and stained with Lysotracker (green) and Hoechst 33258 (blue). Bar represents 20 μm. Adapted from Han et al. [119] with permission from Elsevier.

polyplex are cleaved, releasing the siRNA and revealing primary amines that destabilize the endosomes. Using this system, delivery of various siRNAs to ovarian cancer and adenocarcinoma cells resulted in 50–70% gene knockdown *in vitro*; however, no *in vivo* experiments have been reported using this system.

Another strategy is to encapsulate the siRNA within an acid-degradable polymer shell. Hong et al. used this strategy to deliver siRNA in a core-shell nanoparticle platform, with acid-cleavable diamines crosslinking the PCL shell [136]. The shell degrades within the acidic lysosomes, releasing the siRNA. This system achieved 40% knockdown of GFP *in vitro* using GFP + breast cancer cells, while demonstrating significantly less cytotoxicity than traditionally used transfection reagents. However, it is unclear how the siRNA escapes from the endolysosomal pathway, so it is possible that incorporating a specific endosomal escape strategy would further increase potency. A better strategy would be to use a sequential siRNA release mechanism that occurs after endosomal escape of the nanoparticle, taking advantage of bioactive molecules and enzymes in the cytosol.

5.2. Glutathione-triggered siRNA release

Once inside the cytosol, a diverse array of enzymes and bioactive molecules are available for triggered siRNA release. One of the most commonly exploited biomolecules for siRNA release is glutathione (GSH), which reduces disulfide bonds often used to immobilize siRNA to a polymeric carrier [137–139]. GSH is found at high concentrations (5–10 mM) in the cytosol of most mammalian cells, which is significantly higher than concentrations typically found in the blood (0.05 mM) [140]. Namgung et al. took a unique approach and conjugated the sense and antisense strands of the siRNA to separate polymer backbones through disulfide bonds, and then annealed them together, yielding a system where the siRNA is tethered on both ends within the core of the nanoparticle and effectively acts as a crosslinker [141]. The backbone consists of chitosan polymers, and together the chitosan and crosslinked siRNAs form the core of the nanoparticle, while peptide aptamers targeting prostate cancer cells form the corona. Although

Table 2
Commonly used fusogenic peptides.

Peptide	Origin	Sequence	Charge (pH 7.4)
HA2 [128,129]	Derived from influenza hemagglutinin (HA) proteins of influenza viral capsid	GLF GAI AGF IEN GWE GMI DGW YG	−3
INF-7 [130]	Derivative of HA2; glutamic-acid enriched for improved endosomal escape	GLF EAI EGF IEN GWE GMI DGW YGC	−5
GALA [131]	Synthetic peptide with EALA repeats; E for pH-sensitivity and ALA for hydrophobicity	WEA ALA EAL AEA LAE HLA EAL AEA LEA LAA	−6

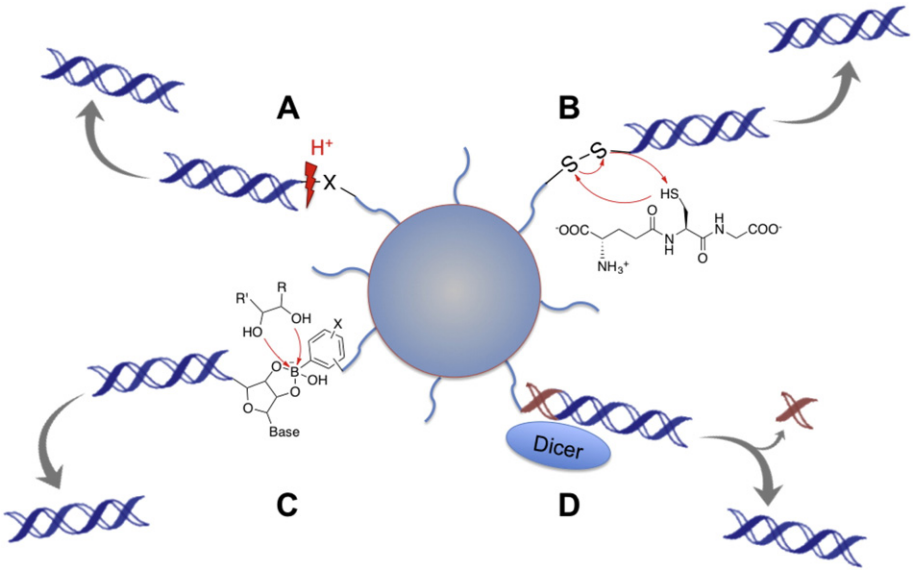

Fig. 7. Cleavage strategies for siRNA release from a nanoparticle system. (A) Acid sensitive groups (ie. maleic acid anhydride, hydrazone, thiolmaleiamide, *etc.*) are cleaved under the acidic endosomal conditions. (B) Disulfide bonds are cleaved by high glutathione levels in the cytosol. (C) Phenylborate associates strongly with the terminal diols of siRNA but is displaced by the high diol (ATP) concentration in the cytosol. (D) The Dicer enzyme cleaves Dicer-substrate siRNA.

this system was able to affect gene knockdown to 50% *in vitro*, the concentration of siRNA required was significantly higher (200–400 nM) than typical siRNA concentrations of <50 nM. The high doses required may reflect the slow kinetics of siRNA release when it is tethered on both ends. This system requires further optimization before it will be a useful *in vivo* delivery strategy [141].

Instead of attaching the siRNA *via* a disulfide bond, it can be complexed to a positively charged pendant group that is cleaved from the polymer backbone in the cytosol. For example, Li et al. designed a copolymer of PEG and poly(L-lysine) that was grafted to polyethyleneimine through reducible disulfide bonds and to which siRNA was complexed non-covalently [142]. Anti-HER2 was conjugated to this nanoparticle for selective uptake by HER2 overexpressing cells while the proton buffering capacity of PEI enhanced endosomal escape. Once in the reducing conditions of the cytosol, the disulfide bonds were cleaved, releasing PEI and its siRNA cargo. This formulation led to an 80% knockdown of XIAP, a gene associated with apoptosis, *in vitro* in a HER2 + ovarian cancer cell line. *In vivo* studies in a subcutaneous model of ovarian cancer revealed an 80% increase in apoptosis following treatment, as well as significantly delayed tumor growth and longer survival. Notably, 80% of the animals in the targeted siRNA formulation group were alive after 45 days whereas none remained alive in the control groups [142].

Another strategy is to 'cage' the siRNA within disulfide crosslinked polymer constructs, which will degrade and release the siRNA within the cytosol. Yoon et al. used a hyaluronic acid scaffold conjugated to both pDMAEMA and a crosslinker which, with the addition of siRNA and a redox reagent, formed a crosslinked hyaluronic acid nanoparticle encapsulating siRNA with cationic pDMAEMA for siRNA complexation [143]. Overexpressed CD44 receptors for hyaluronic acid on the cell surface promoted internalization of the nanoparticle, and once inside the cytosol the disulfide bonds were cleaved by GSH, releasing siRNA. This system demonstrated efficient cell uptake and RFP knockdown *in vitro* in an RFP + melanoma cell line, although significant cytotoxicity in CD44 + cells was also observed, potentially due to the toxicity of pDMAEMA. *In vivo* studies demonstrated reduced RFP levels in melanoma tumors [143].

5.3. ATP-triggered siRNA release

Adenosine triphosphate (ATP) can be used to trigger release in the cytosol as well. This strategy was employed by Naito et al., who synthesized a polyion complex micelle for ATP-triggered release of siRNA [144]. In this study, some of the lysine residues of PEG-b-poly(lysine) were modified with phenylboronic acid, which binds strongly to siRNA but can be displaced by an excess of other diols (ie. ATP) in solution. Although the authors were able to demonstrate ATP-triggered siRNA release, the *in vitro* studies are limited, with the supporting information showing 30% gene knockdown against polo-like kinase 1 (PLK1) in renal carcinoma cells at 500 nM. The concentration used is very high for siRNA where typically sub-50 nM concentrations are used [144]. Therefore, although this approach is interesting, more work is required to prove its utility *in vitro* and *in vivo*.

5.4. Dicer-mediated cleavage of siRNA

One key enzyme particularly important in siRNA trafficking is Dicer, which cuts longer siRNAs (27–30 base pairs) into 21 base pair siRNAs and traffics them into the RNA-induced silencing complex (RISC) [145]. In addition to providing a mechanism for cleavage from a polymer vehicle, Dicer-substrate siRNAs have been reported to be 10–100 fold more potent than non-Dicer substrates [9]. Thus, Dicer-substrate siRNAs are one of the most promising strategies for siRNA release. However, some cancer tissues have been shown to have less Dicer expression than normal tissues [146,147], so the target tissue must be carefully considered when choosing to use Dicer-substrate siRNAs.

Chan et al. developed a polymeric micellar system composed of a poly(lactide-*co*-2-methyl, 2-carboxytrimethylene carbonate) backbone with grafted PEG to deliver both Dicer-substrate siRNAs as well as targeting antibodies [148]. SiRNA-modified with DBCO and anti-HER2 antibody-modified with maleimide were conjugated to the terminal ends of PEG-azide and PEG-furan, respectively, through click conjugation reactions. The nanoparticles carrying both Dicer-substrate siRNA and anti-HER2 antibodies effectively knocked down luciferase expression by approximately 80% *in vitro* in luciferin-positive ovarian cancer cells [148]. This study demonstrates the proof-of-concept of covalently bound siRNA for effective knockdown, taking advantage of the siRNA duplex, with stable siRNAs and the sense strand covalently immobilized, leaving the anti-sense available to Dicer for facilitated processing.

Dicer-substrate siRNA has also shown promise *in vivo*. Liu et al. synthesized a dendrimer platform for siRNA delivery comprising poly(amidoamine) dendrimers which are capable of complexing both siRNA and anionic targeting peptides [149]. Interestingly, when using

this system to deliver siRNA against heat shock protein 27 in human prostate cells *in vitro*, no significant gene knockdown was observed using a conventional 21-mer siRNA. However, when using Dicer-substrate siRNA, significant gene silencing (50%) was observed. Delivery of this Dicer-substrate formulation with a targeting peptide to a prostate cancer xenograft model resulted in significantly slower tumor growth compared to controls [149].

Overall, although there are many effective ways to release siRNA from the polymer delivery vehicle, one of the most promising strategies is Dicer cleavage. Not only does it provide a specific enzymatic mechanism for siRNA release, it also increases the potency of siRNA. Examples of Dicer-siRNA in the literature are still rare, and should be considered for future work in the field.

6. Combination therapies

Importantly, the combination of sequence-specific siRNA with cytotoxic chemotherapeutics offers interesting advantages, and there are multiple examples of combination therapies already in the literature. For example, Sun et al. used biodegradable triblock poly(ethylene glycol)-*b*-poly(ε-caprolactone)-*b*-poly(2-aminoethylethylene phosphate) micelles to deliver both paclitaxel and siRNA targeting polo-like kinase 1 (Plk1) to MDA-MB-435 breast cancer cells [150]. Delivering siRNA with this system resulted in ~70% reduction of Plk1 protein levels, and provided a synergistic reduction in cell proliferation when delivered with paclitaxel relative to each therapeutic alone. Interestingly, when the authors delivered paclitaxel along with control siRNA *in vivo*, they required a thousand-fold higher paclitaxel concentration to observe similar tumor reduction relative to delivering paclitaxel with siRNA targeting Plk1 (Fig. 8A). Although siRNA targets can be chosen to act synergistically with chemotherapeutics when delivered simultaneously, sequential delivery can sensitize the cells to the chemotherapeutic. For example, Zhang et al. first delivered siRNA targeting Bcl-2, an anti-apoptotic siRNA, to multi-drug resistant (MDR) cells, which then sensitized the cells to doxorubicin treatment, resulting in reduced MDR cells [151]. This siRNA delivery system reduced Bcl-2 protein levels to ~30% while reducing the IC_{50} of doxorubicin by 2.5-fold when compared to delivering scrambled siRNA (Fig. 8B). The combination of chemotherapeutics and siRNA adds more complexity; however, the combination may require lower doses of both therapeutics, thereby reducing toxicity from the vehicle or drugs.

7. Current clinical status, outlook, and conclusions

As demonstrated by this review, many siRNA-nanoparticle formulations have shown *in vitro* success, but have not been translated *in vivo* or to the clinical setting. There are, however, some siRNA-nanoparticle formulations that are being tested in clinical trials, as summarized in Table 3. More complete summaries of the current clinical status of siRNA therapeutics can be found in recent reviews published by Barata et al. [152] and Kim et al. [153]. The majority of delivery systems in current clinical trials are lipid-based or composed of cationic polymers.

Although cationic vehicles work well for siRNA delivery *in vitro*, *in vivo* they result in non-specific uptake, cellular toxicity and elicit an immune response. With a neutral or negatively charged polymer system, these off-target effects are avoided; however, a cell uptake strategy, such as an antibody or aptamer, is now required. An interesting strategy would have a nanoparticle that responds to the tumor microenvironment by shielding the antibody or aptamer with a polymer that is cleaved by specific cues such as pH or MMPs. To achieve maximum potency, we suggest incorporating a chemically stabilized Dicer-substrate siRNA and a fusogenic peptide for greatest siRNA release. This strategy should minimize toxicity while taking advantage of the tumor microenvironment and maximizing cellular uptake, endosomal escape, and potency.

Overall, polymeric vehicles offer a multitude of functionalities for guided siRNA delivery. While the biggest challenge to the field remains localizing the nanoparticles at the tumor site, once there, siRNA stability, cell uptake, and endosomal escape are the key issues. To increase clinical efficacy, we suggest the following: modified siRNA for greater stability and potency, highly specific cell uptake mechanisms, improved endosomal escape agents, and sophisticated siRNA release mechanisms. Recent advances in these strategies demonstrate the promise of polymeric vehicles for the delivery of potent biomolecules, such as siRNA, and their translation to the clinic.

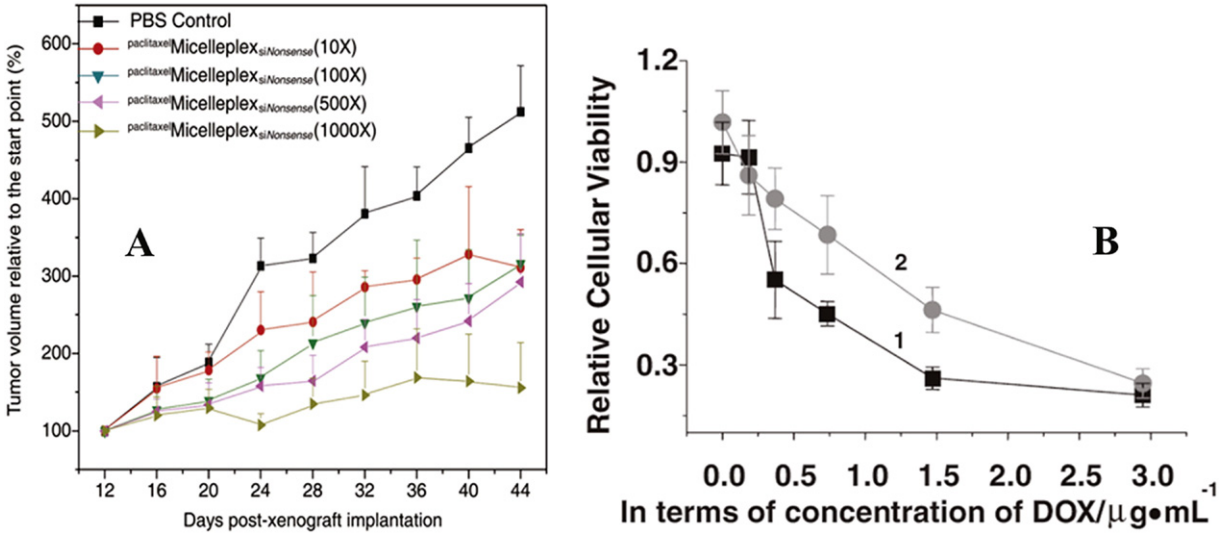

Fig. 8. A) Dose-response study of paclitaxel delivered by $^{paclitaxel}micelleplex_{siNonsense}$ (control siRNA) on inhibition of MDA-MB-435s xenograft tumor growth. Paclitaxel doses were 10 to 1000-fold increase (10× to 1000×) compared to those used in $^{paclitaxel}micelleplex_{siPlk1}$ (siRNA targeting PLK-1). Comparable results to $^{paclitaxel}micelleplex_{siPlk}$ were only achieved with a 1000-fold more paclitaxel dose when using a control siRNA. Reprinted with permission from Sun et al. [150]. Copyright 2011 American Chemical Society. B) Using a PEI-graphene oxide NPs, relative viability of HeLa cells after being treated with either (1) Bcl-2 siRNA or (2) scrambled siRNA for 48 h followed by incubation with PEI-graphene oxide NPs loaded with doxorubicin for 24 h. The Bcl-2 knockdown sensitized the cells to doxorubicin treatment. Reproduced from Zhang et al. [151] with permissions.

Table 3
Selected siRNA-nanoparticle formulations in clinical trials.

Name	Formulation type	Cancer type	Size (zeta potential)	siRNA target	Clinical trial phase
CALAA-01	Cyclodextrin-based polymer	Solid	70 nm (+10 mV) [154]	RRM2 [155]	Phase I (NCT00689065)
Atu027	Liposome	Solid	102 nm (+38.9 mV) [156]	PKN3 [156]	Phase I (NCT00938574)
TKM080301	SNALP (stable nucleic acid lipid particle)	Liver	N/A	PLK1 [157]	Phase I (NCT01437007)
ALN-VSP	Lipid nanoparticle	Liver	80–100 nm (+6 mV) [158]	KSP, VEGF [158]	Phase I (NCT00882180)

RRM2 - ribonucleotide reductase M2, PKN3 - protein kinase N3, PLK1 – polo-like kinase 1, KSP – Kinesin spindle protein, VEGF – Vascular Endothelial Factor.

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