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A Role for Peptides in Overcoming Endosomal Entrapment in siRNA Delivery – A Focus on Melittin

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Abstract

siRNA has the possibility to revolutionize medicine by enabling highly specific and efficient silencing of proteins involved in disease pathogenesis. Despite nearly 20 years of research dedicated to translating siRNA from a research tool into a clinically relevant therapeutic, minimal success has been had to date. Access to RNA interference machinery located in the cytoplasm is often overlooked, but must be considered when designing the next generation of siRNA delivery strategies. Peptide transduction domains (PTD) have demonstrated moderate siRNA transfection, which is primarily limited by endosomal entrapment. Strategies aimed at overcoming endosomal entrapment associated with peptide vectors are reviewed here, including osmotic methods, lipid conjugation, and fusogenic peptides. As an alternative to traditional PTD, the hemolytic peptide melittin exhibits the native capacity for endosomal disruption but causes cytotoxicity. However, appropriate packaging and protection of melittin with activation and release in the endosomal compartment has allowed melittin-based strategies to demonstrate both *in vitro* and *in vivo* safety and efficacy. These data suggest that melittin's membrane disruptive properties can enable safe and effective endosomolysis, building a case for melittin as a key component in a new generation of siRNA therapeutics.

Keywords

Melittin; siRNA delivery; endosomal escape

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1 Introduction

1.1 RNA Interference by siRNA

RNA interference (RNAi) refers to an evolutionary conserved mechanism for post transcriptional control of protein expression in which short double-stranded RNA target specific messenger RNA (mRNA) for degradation, thus decreasing protein translation (Eccleston *et al.* 2004). Tuschl *et al.* were the first to demonstrate that RNAi can be artificially induced by the delivery of exogenous small interfering RNA (siRNA) (Elbashir *et al.* 2001). siRNA are short 21-23 base pair duplex RNA oligonucleotides with 5′-phosphorylated ends and 2-nucleotide 3′ overhangs. The “antisense” strand shares sequence complementarity to a target mRNA, while the “sense” strand serves as a bystander. When delivered into the cytoplasm of a cell, siRNA can co-opt the native RNAi machinery and induce assembly of the RNA induced silencing complex (RISC). The RISC unwinds siRNA, binds the antisense strand, and cleaves the sense strand, allowing the RISC to bind and cleave targeted mRNA based on the sequence of the antisense strand (Sakurai *et al.* 2011). This selective degradation of mRNA provides an avenue to decrease the expression of proteins involved in disease pathogenesis. Indeed, initial experimental siRNA therapeutics appeared just three years after the discovery of siRNA (McCaffrey *et al.* 2002). Recently, clinical trials have been initiated for the use of siRNA in ocular, renal, and hepatic diseases with limited success (Haussecker 2012).

1.1 Cellular Barriers to the Therapeutic Use of siRNA

On a cellular level, efficient and non-toxic siRNA delivery to the cytoplasm remains a major impediment to the use of siRNA as a therapeutic. siRNA are large (∼21kDa), highly charged macromolecules, which can not directly translocate across the hydrophobic core of the cell membrane (Overhoff *et al.* 2005). Moreover, the barrier provided by impermeable membrane bilayers not only applies to direct translocation from the extracellular milieu into the cytoplasm, but also applies to cytosolic access of siRNA enclosed in endocytic vesicles (Detzer *et al.* 2009, Gilleron *et al.* 2013). Entrapment of siRNA in endocytic compartments not only prevents siRNA from reaching the cytosol, but also accelerates siRNA degradation due to the harsh acidic environment encountered during endosome-lysosome trafficking (Figure 2) (Wang *et al.* 2010).

This barrier represents a major impediment in the delivery of biological therapeutics to the cytoplasm, especially when packaged with peptide transduction domains (El-Sayed *et al.* 2009). Strategies to achieve endosomolysis have traditionally been based on osmotic agents, fusogenic lipids, and fusogenic peptides. The application of these methodologies to peptide-mediated siRNA delivery will be reviewed here. In addition, novel strategies enabled by the membrane-disruptive properties of melittin appear to negate the need for secondary endosomolytic methodologies and will be presented as an alternative to traditional peptide-based siRNA delivery strategies.

2 Cationic Peptides for siRNA Transfection

Given the challenges associated with traditional siRNA delivery methodology, there is clearly a need for siRNA delivery technology to enable endosomal escape with minimal

cytotoxicity. With the observation that the Trans-Activator of Transcription (TAT) peptide from HIV could directly translocate across cell membranes to trans-activate the viral promoter in tissue culture, cell penetrating peptides (CPPs) have become a widely utilized tool for delivery of therapeutics (Frankel *et al.* 1988). CPPs have been recruited for delivery of cargoes ranging from small molecules to large proteins (Heitz *et al.* 2009). With their hypothesized ability to bypass the cellular membrane, CPPs were expected to enable cytoplasmic delivery of siRNA, while avoiding the endosomal compartment. Although this hope has ultimately proven unfounded, peptides remain a viable option for siRNA delivery thanks to a relative lack of cytotoxic effects.

2.1 Covalent Formulations

Initial attempts to harness peptides for siRNA transfection focused on direct chemical conjugation to known cell penetrating peptides, penetratin and transportan. These studies reported an IC50 of 25nM, and with minimal cytotoxicity (Chiu *et al.* 2004, Davidson *et al.* 2004, Muratovska *et al.* 2004). Unfortunately, these initial studies were later shown to be confounded by poor purification as excess unconjugated peptide augmented the transfection efficiency. Turner *et al.* demonstrated that after careful purification, siRNA conjugated to cell penetrating peptides had minimal transfection capacity, requiring 5μM to achieve significant knockdown (Turner *et al.* 2006, Turner *et al.* 2007, Endoh *et al.* 2008). They attributed the limited transfection capacity to endosomal entrapment based on the findings that endosomolytic agents such as chloroquine were able to release siRNA into the cytoplasm (Shiraishi *et al.* 2006). When tested for intratracheal delivery of siRNA to the lung, these purified conjugates did not exhibit any improvement over naked siRNA alone (Moschos *et al.* 2007). In addition, work by other groups revealed that peptide-siRNA conjugate uptake was limited in the presence of serum proteins (Turner *et al.* 2005). However, all studies investigating the use of peptide-oligonucleotide conjugates have shown minimal cytotoxicity with doses up to 5-10μM *in vitro*, indicating at least a high degree of safety on a cellular level (Lundin *et al.* 2008).

2.2 Noncovalent Formulations

Due to the limited efficacy of peptide-siRNA conjugates and the finding that excess peptide imbues improved transfection efficiency, peptide-based siRNA vectors have more commonly been utilized in non-covalent formulations (Table 1). Initial studies examining siRNA delivery via electrostatic packaging by TAT, penetratin, and transportan all gave minimal siRNA mediated knockdown despite high levels of siRNA internalization (Lundberg *et al.* 2007, Endoh *et al.* 2008, Meade *et al.* 2008). Further treatment with chloroquine increased siRNA-mediated knockdown, revealing that some of these peptides were unable to achieve sufficient endosomal escape (Mäe *et al.* 2009). Interestingly, penetratin and poly-arginine peptides continued to exhibit minimal knockdown in the presence of chloroquine indicating that endocytosed siRNA was not released from the peptide vector. These initial studies point out the requirement for peptide vectors to both release siRNA and promote endosomal escape in order to achieve maximal siRNA transfection efficiency.

Despite these initial difficulties, noncovalent siRNA delivery via cell penetrating peptides has achieved some success, with much higher efficiencies (IC50<1nM) than covalent means (IC50>5μM) (Meade *et al.* 2008, Okuda *et al.* 2009, Hassane *et al.* 2010). Moreover, peptide vectors have demonstrated efficacy in a variety of cell types in tissue culture as well as successful abatement of cancer progression in mouse models (Endoh *et al.* 2009). Importantly, peptide vectors have demonstrated remarkable safety, with minimal toxicity even at high μM concentrations. Unfortunately, the limiting factor for peptide-based vectors appears to be endosomal entrapment as well as decreased transfection in the presence of serum proteins (Mäe *et al.* 2009, Crombez *et al.* 2011). Selected peptides for non-covalent siRNA delivery are reviewed below.

2.2.1 Noncovalent Formulations: MPG—Initial success with peptide mediated siRNA delivery was achieved with MPG, a hybrid peptide consisting of the fusion sequence from HIV glycoprotein 41 and the nuclear localization sequence of the SV40 virus. This peptide, originally developed for plasmid DNA transfection, achieved knockdown of target mRNA with an IC50 of 20-50nM (Simeoni *et al.* 2003, Deshayes *et al.* 2004, Deshayes *et al.* 2008). Initial work indicated that MPG mediated siRNA transfection via direct translocation through the cell membrane. However, later studies concluded that MPG/siRNA complexes enter cells via macropinocytosis (Veldhoen *et al.* 2008). Further sequence refinement led to a truncated form, MPG-8, as well as MPGα which has increased membrane inserting properties (Veldhoen *et al.* 2006, Crombez *et al.* 2009). These peptides are characterized by sub-nanomolar IC50 when targeting luciferase. However, detailed work by Veldhoen *et al.* demonstrated that MPGα is not maximally efficient, required almost two orders of magnitude more siRNA to achieve the same knockdown as Lipofectamine 2000 (Veldhoen *et al.* 2006). This finding may be attributable to endosomal entrapment, as treatment with chloroquine improved transfection by nearly 20%. Despite these difficulties, MPG has been licensed to Sigma-Aldrich as the N-TER siRNA transfection system designed for *in vitro* research applications (Sigma Aldrich, 2015).

2.2.2 Noncovalent Formulations: CADY—CADY is the first peptide designed to specifically promote both siRNA binding and membrane permeability. With an alpha-helical secondary structure, CADY has a siRNA binding face with cationic residues, and a membrane binding face with tryptophan residues (Deshayes *et al.* 2012). To date, CADY is the most efficient peptide based siRNA vector with an IC50 <0.5nM (Crombez *et al.* 2008). Interestingly, CADY appears to function by direct membrane translocation as neither ATP depletion nor incubation at 4°C inhibits siRNA transfection (Rydström *et al.* 2011). Nevertheless, CADY may experience limited *in vivo* utility due to decreased transfection in the presence of serum proteins (Crombez *et al.* 2011).

2.2.3 Noncovalent Formulations: dsRBD—Dowdy *et al.* further improved upon peptide-mediated transfection by utilizing conjugates of TAT and double stranded RNA binding domains (dsRBD) (Eguchi *et al.* 2009, Kim *et al.* 2009). dsRBD sequences were modified from protein kinase R, a cytoplasmic protein which plays a crucial role in the detection of viral infection. These TAT-dsRBD conjugates were shown to transfect a variety of difficult to transfect cell lines such as Jurkat T-cells and endothelial cells in tissue culture

with an IC50 near 400nM, and were able to yield significant siRNA knockdown *in vivo*. Unfortunately, later studies demonstrated that due to low binding affinities, a single dsRBD is unable to bind siRNA efficiently, suggesting that TAT-dsRBD mediated transfection is attributable to electrostatic TAT/siRNA interactions (Geoghegan *et al.* 2012). These peptides were initially commercialized by Traversa Therapeutics and were licensed to IDT as research tools (2009). Unfortunately, there has been limited progress after Traversa shut down in 2012 after failing to secure funding. However, progress has been made recently with further *in vivo* studies focused on inner ear transfection in chinchillas (Qi *et al.* 2014).

2.3 Modified Peptides for siRNA Transfection

Modifications to cell penetrating peptides that promote increased endosomal escape have centered on three design features: combination with buffering agents to promote osmotic rupture of endosomes, fusion to pH sensitive fusogenic viral peptides, conjugation to lipid moieties, and (Table 2) (Varkouhi *et al.* 2011, Erazo-Oliveras *et al.* 2012).

2.3.1 Osmotic Endosomolysis—Lysosomotropic agents are taken up selectively into lysosomes. One example is chloroquine, which is a weak base that accumulates in endosomes and lysosomes after being protonated (Wibo *et al.* 1974). Chloroquine's functions include inhibiting endosomal acidification and causing endosomolysis. Cells incubated with chloroquine exhibit extensive endosomal swelling and vacuolation suggestive of endosomal disruption (Ohkuma *et al.* 1981, Erbacher *et al.* 1996). Moreover, biologic therapeutics including plasmids exhibit greater degrees of cytoplasmic delivery when delivered in the presence of chloroquine (El-Sayed *et al.* 2009). The proposed mechanism involves chloroquine's ability to buffer endosomal protons leading to accumulation of counterions and osmotic rupture of endosomes.

The importance of endosomal pH buffering as a release mechanism has led researchers to take advantage of histidine residues as a potential trigger for endosomal escape (Yu *et al.* 2004). Histidine residues are unique for their ability to be protonated at acidic pH (pKa ∼6) while remaining uncharged at neutral pH. By incorporating high percentages of protonatable histidine residues, poly arginine and TAT have been modified to increase their ability to delivery nucleotides to the cytoplasm. For instance, the addition of 10 histidine residues to TAT improves plasmid DNA transfection by 7000 fold over TAT itself (Lo *et al.* 2008). Unfortunately, the use of histidine-augmented peptides has yet to provide the efficiency of lipid or polymer vectors despite their lack of cytotoxicity (Tanaka *et al.* 2010).

2.3.2 Fusogenic Peptides—Standard CPPs such as penetratin and TAT have been fused with portions of influenza proteins hemagluttin-2 (HA2), LK15, or N-E5L which mediate viral escape from the endosome when exposed to acidic pH (Lundberg *et al.* 2007, Neundorf *et al.* 2009, Arthanari *et al.* 2010, Hoyer *et al.* 2012). This functional pH triggered structural change increases the alpha helical content of the peptide and promotes insertion into the endosomal membrane. *In vitro*, HA2 fusions have shown the ability to improve siRNA transfection when packaged by CPP (Lundberg *et al.* 2007). Unfortunately, this modification increases the cytotoxicity of the peptide inducing significant cell death at 1μM (Lundberg *et al.* 2007). Furthermore, these fusion peptides are not maximally efficient as treatment with

chloroquine improves siRNA transfection indicating that endosomal entrapment is a problem that is not completely addressed by these methods (Hoyer *et al.* 2012).

2.3.3 Lipid Conjugation—Additional attempts to increase the membrane disruptive potential of cell penetrating peptides come in the form of conjugation to lipids, most commonly stearyl moieties (Mäe *et al.* 2009, Nakase *et al.* 2012). Originally developed for plasmid DNA, stearylated peptides also improve the transfection of small oligonucleotides such as siRNA and splice correcting oligos (Futaki *et al.* 2001, Lehto *et al.* 2010). For these purposes, stearyl-TP10 has proven to be more effective than stearyl-penetratin, stearyl-TAT, or stearyl-R_8 (Mäe *et al.* 2009, Ren *et al.* 2012). Studies utilizing unstearylated peptides suggest that differences in these stearylated variants may be due to the ability of the peptide to release siRNA. Moreover, in the case of stearyl-TP10, authors noted that improved transfection may be due to improved particle stability and increased siRNA uptake instead of improved endosomal release (Mäe *et al.* 2009). In fact, treatment with chloroquine doubled siRNA-mediated knockdown, again revealing poor endosomal release. This finding is not surprising given that stearyl moieties are not considered fusogenic lipids, with the majority of membrane disruption typically attributed to the lipid headgroup. The extent to which stearylation improves peptide disruption of membranes remains unclear. However the ability of peptide stearylation to increase siRNA transfection is clearly demonstrated across multiple studies.

2.3.4 PepFect – Combining Endosomolytic Strategies—The group of Langel et al. has further modified cell-penetrating peptides to overcome known endosomal entrapment of CPP-based oligonucleotide delivery agents. Their efforts have centered on the CPP TP10, the shortened version of transportan. Modifications made to TP10 include stearylation, replacement of lysines by ornithines, addition of trifluoromethylquinolone side chains, chemical modification with UV light responsive side chains and oligomerization (Regberg *et al.* 2014). These modifications have resulted in a range of “PepFect” and “NickFect” peptides, which deliver siRNA, splice correcting oligos, and even plasmid DNA through a mechanism that involves Scavenger Receptor A mediated endocytosis (Hassane *et al.* 2011, Ezzat *et al.* 2012, Arukuusk *et al.* 2013).

By taking advantage of stearylation and conjugation to the proton buffering agent chloroquine, Andaloussi *et al.* have created Pepfect6, which has an IC50 of <10nM in a variety of cell types (Andaloussi *et al.* 2011). They have further demonstrated that stearylation improves particle stability and also increases the peptides amphipathicity to promote insertion into model membranes. This presumably contributes to membrane destabilization. Additionally, the incorporation of three chloroquine moieties per peptide likely contributes to improved endosomal escape via the proton sponge effect.

In analogous work, PepFect14 and PepFect 15 were developed for the transfection of splice-correcting oligos (Ezzat *et al.* 2011, Lindberg *et al.* 2013). Starting with stearylated TP10, lysines were replaced with ornithines to improve siRNA binding and also provide a higher transfection efficiency. This peptide, Pepfect14, was able to transfect SCO into HeLa cells with an EC50 of 86-104nM depending no the presence of serum, however, even with these modifications, significant transfection efficacy was lost due to endosomal entrapment (Ezzat

et al. 2011). Transfection in the presence of chloroquine augments SCO transfection by over 2 fold at 50nM siRNA concentration. Given this evidence supporting inadequate endosomal escape, PepFect14 was further modified to include the chloroquine analog trifluoromethylquinolone. This new peptide PepFect15 exhibits increased transfection efficacy over PepFect14 and its transfection efficiency is not augmented by the presence of additional chloroquine.

Taken together with studies of PepFect 6, which also includes stearylation of TP10 combined with chemical conjugation to 3 chloroquine moieties, obvious limitations to peptide- mediated transfection begin to emerge. Stearylation is required for improved particle stability and increased transfection efficiency, while incorporation of protonatable structures improves endosomal escape presumably via the proton sponge effect. These changes highlight the importance of endosomal escape strategies in developing peptides for siRNA delivery. Recent strategies to further increase nucleotide delivery capacity include UV light to disrupt endosomes via free radical production by incorporating hydrophobic dyes Alexa Fluor 633 or Texas Red into peptide/siRNA complexes followed by excitation with 545-580nm light *in vitro* (Räägel *et al.* 2013).

In vivo data are currently limited. However, early results reveal *in vivo* efficacy and limited toxicity. Langel *et al.* have published *in* vivo studies involving hydroinjection of PepFect6/ siRNA particles to yield effective transfection of hepatocytes (Andaloussi *et al.* 2011). However, further analysis reveals that Pepfect6-based particles demonstrate an increase in size and a mild decrease in transfection in the presence of serum proteins highlighting the need for further peptide development and additional *in vivo* analyses (Andaloussi *et al.* 2011). Subsequent toxicity analyses have revealed minimal *in vivo* toxicity at peptide concentrations of up to 5mg/kg as witnessed by a lack of increase in IL-1B and TNFa serum levels 24 and 48 hours after a single intravenous injection (Suhorutsenko *et al.* 2011). In addition, work by Ren *et al.* has also revealed the ability to impart cell-specific uptake in cancer cells via incorporation of LyP1 ligands suggesting the promise for effective *in vivo* siRNA transfection (Ren *et al.* 2012). However, further studies to better elucidate serum stability, pharmacokinetics, biodistribution and immune response with repeated dosing are required before the ultimate utility of these agents can be established.

3 Melittin as a Basis for Endosomal Escape

It is apparent that peptide-mediated transfection is hampered by poor efficiency as a consequence of endosomal entrapment. Methods to overcome this limitation have focused on peptide modification to include membrane active peptides, lipids as well as chemical conjugation to proton buffering moieties. Other strategies focus on leveraging the membrane lytic properties of melittin to enable endosomal release of siRNA. Melittin is a 26 amino acid alpha helical peptide first purified from the European honeybee in 1958, which has a high affinity for lipid membranes and ultimately causes membrane lysis (Sessa *et al.* 1969). Although the mechanism of membrane disruption has not fully been clarified, melittin has shown the ability to lyse red blood cells, as well as model membranes (Pratt *et al.* 2005, Bogaart *et al.* 2008).

Currently, melittin is hypothesized to disrupt membrane bilayers via a two-step "detergent-like" mechanism (Ladokhin *et al.* 2001, Shai 2002, Bechinger *et al.* 2006, Lee *et al.* 2008). The first step involves electrostatic interaction of melittin with negatively charged lipid headgroups. After the concentration of the peptide on the lipid surface reaches a critical concentration, melittin peptides rearrange to form pore like structures that disrupt the membrane bilayer (Figure 3). Data for the initial electrostatic interaction are derived from studies in which melittin is shown to bind with lower affinity to membrane bilayers with a lower component of anionic headgroups (Sessa *et al.* 1969).

Although the efficiency with which melittin binds to and disrupts membranes depends on many variables including pH, salt concentration, and membrane composition, melittin's ability to disrupt membrane structures under myriad conditions is without question. However when considered in the context of endosomal disruption, it is critical that melittin also has the ability to disrupt membrane bilayers while exposed to an acidic environment. This could pose difficulties given melittin's initial electrostatic attraction to negatively charged lipid components. In fact, detailed biophysical studies have revealed that melittin's ability to bind to and disrupt model membranes actually *decreases* as pH decreases (Tan *et al.* 2012). Nonetheless, melittin has been shown to be capable of altering endosomal membrane integrity after cellular uptake under sub-toxic concentrations, suggesting that melittin does bind to and alter endosomal membranes despite the acidic environment (Rozema *et al.* 2003).

Given the known hemolytic properties of melittin, its use as a potential therapeutic may include significant *in vivo* toxicity. However, previous work has shown that appropriate sequestration of melittin in nanoparticles, or reversible blocking of melittin's hydrophobic residues can ameliorate any significant *in vivo* toxicity of naked melittin (Rozema *et al.* 2003, Meyer *et al.* 2007, Meyer *et al.* 2009, Soman *et al.* 2009). These studies suggest that if melittin can be appropriately modified or delivered to the target cell in an inactive form, its membrane disruptive properties can be harnessed for endosomal escape without either cellular or systemic toxicity given an appropriate mechanism for activation or deprotection (Figure 4).

3.1 Melittin for the Augmentation of Oligonucleotide Delivery

Given its lytic nature, melittin itself has been recruited for the treatment of cancer, bacterial infections, and recently for augmentation of oligonucleotide delivery either indirectly as an excipient for endosomal escape of directly as an oligonucleotide transfection agent (Raghuraman *et al.* 2007, Oršolić 2012). Work by Wooddell et al. at Arrowhead Pharmaceuticals has provided critical evidence for the role of melittin in release of therapeutics from the endosomal compartment (Wooddell *et al.* 2013). In their work, melittin has been recruited for its membrane lytic properties to serve as an excipient for hepatocyte targeted siRNA therapy. In these studies, melittin or melittin like peptides are protected by acid labile groups which prevent membrane insertion until exposed to the acidic endosomal environment (Figure 4a) (Rozema *et al.* 2003). Activation of melittin in the hepatocyte endosome has proven to be robust and yielded a 500-fold increase in siRNA-mediated knockdown *in vivo*. These studies are particularly illuminating because they reveal

that melittin can be delivered safely in the blood stream if protected from inserting into red blood cell membranes, and are able to avoid degradation if inaccessible to serum proteases. More importantly, these protected melittin peptides can improve endosomal escape providing definitive evidence that melittin is active within acidified endosomal compartments in an *in vivo* setting. This strategy is now in Phase IIa clinical trials for treatment of chronic Hepatitis B. Preliminary dose escalation data reveal HBsAg decrease of 39% and 51% at single doses of 1mg/kg and 2 mg/kg respectively without any side effects (Arrowhead 2014).

3.2 Dynamic Polyconjugates

The development of functionalized polymer vectors incorporating masked melittin derivatives for siRNA delivery was first demonstrated by Meyer *et al.* (2007). Their efforts yielded a functionalized polylysine backbone which could be covalently modified for acid labile PEG functionality along with masked melittin groups which could be activated in acidic compartments to impart improved endosomal escape capacity. This polylysine backbone could then be used to bind siRNA electrostatically. These constructs demonstrated a lack of cytotoxicity *in vitro* providing evidence for the safety of protected melittin structures. Unfortunately, these constructs cause substantial liver necrosis with abdominal bleeding in mice (Meyer *et al.* 2007, Meyer *et al.* 2008, Meyer *et al.* 2009). It is hypothesized that, the presence of a polycationic polymer backbone with a polyanionic oligonucleotide leads to aggregation and micron sized particles with a propensity for hepatic uptake and resultant liver toxicity.

Almost simultaneously Rozema *et al.* published initial studies regarding the utility of dynamic polyconjugates which follow a similar strategy (Rozema *et al*. 2007) (Figure 4b). One large distinction is the use of poly butyl and amino vinyl ethers to generate a masked endosomolytic polymer modeled after melittin along with PEG functionality with liver targeting via the apolipoprotein B receptor. These particles had a size of 10±2nm and single dose tail vein injections produced prolonged knockdown in hepatocytes with liver specific biodistribution. This technology was initially published with support from Mirus Bio Corporation which was later absorbed by Roche who published prolonged (>3 week) knockdown of genes in rhesus monkeys in 2009 (Babiss 2009). This technology was ultimately transferred to Arrowhead. Further development identified a masked melittin like peptide, which provided an appropriate backbone for dynamic polyconjugate assembly. This iteration of the technology has demonstrated >90% knockdown in hepatocytes at doses as low as 3mg/kg in non-human primates at a dose (Arrowhead 2012). Given the small size of DPCs, they are also being investigated for subcutaneous injections as well as antibody conjugation for additional targeting strategies. The utility of such technology is further bolstered by work initiated at Merck by Parmar *et al.* which focused on development of new polymer backbones via high throughput screening of a library of amphiphilic polymers (Parmar *et al.* 2013).

3.3 Direct siRNA Binding

Melittin's utility is not limited to acting solely as an endosomolytic agent, but can also directly serve as an oligonucleotide transfection agent. With its hydrophobic N-terminus and

cationic C-terminus, melittin features a similar amphipathic sequence to that of siRNA transfecting peptides. Although melittin only contains five cationic charges in its native sequence, it has been utilized as a transfection agent to delivery plasmid DNA (Chen *et al.* 2006). In these studies, melittin was able to bind DNA, but only gave poor condensation due to an inadequate number of basic residues. Polymerization of melittin was found to improve DNA condensation and yielded moderate transfection. Of note, these studies relied on oxidation of free thiols with incorporation of both N and C terminal Cysteine residues allowing for depolymerization and release of free melittin peptides in the reducing environment of the endosome. This work provides another mechanism for endosomal activation of melittin to avoid the hemolytic toxicity associated with naked melittin peptides.

In separate work, melittin was modified to function as an siRNA transfection agent without requiring additional chemical modification or polymerization (Figure 4c). This work centered around the use of sequence modifications designed to decrease cytotoxicity and improve siRNA binding (Hou *et al.* 2013a, 2013b). The first modification involves N-terminal truncation of melittin, which is known to decrease melittin's cytotoxicity by over two orders of magnitude (Pan *et al.* 2010). The peptide is then augmented with additional basic residues for improved siRNA condensation. The lead candidate p5RHH was shown to bind siRNA to form peptide/siRNA particles with a diameter of <50nm with considerable temporal stability when incubated with albumin post formulation. These particles are capable of transfection *in vitro* in the presence of serum without any loss of efficiency and exhibit no noticeable endosomal entrapment as demonstrated by a lack of improved GFP knockdown with addition of chloroquine unlike previously published peptides. These data suggest that despite the reduced cytotoxicity of modified-melittin peptides compared to melittin itself, they retain a high capacity for endosomal disruption, likely due to the high effective endosomal concentration of peptides after macropinocytosis.

In depth analysis of the function of modified melittin demonstrate that p5RHH/siRNA particles disassemble under acidic conditions found in the endosome, likely due to protonation incorporated histidine residues, allowing liberated p5RHH to disrupt membrane bound compartments releasing siRNA to the cytoplasmic compartment (Figure 5 and 6). With the incorporation of only a two histidine residues, histidine protonation is unlikely able to generate the required osmotic forces required to disrupt endosomal integrity as histidine dependent strategies have traditionally required augmentation with 10 or more histidines (Lo *et al.* 2008). Instead, the authors suggest that histidine protonation drives dissociation of the peptide and siRNA allowing free peptide to disrupt the endosomal membrane (Hou *et al.* 2013b).

In additional work, p5RHH/siRNA nanoparticles were utilized to completely halt the progression of collagen antibody induced arthritis in mouse models of rheumatoid arthritis by targeting the p65 component of NFkB (Zhou *et al.* 2014) (Figure 5). These studies further demonstrated accumulation in arthritic paws and kidney clearance. Furthermore, the authors carefully demonstrated a lack of immunostimulatory affects of p5RHH/siRNA particles packing non-targeted siRNA. Of equal importance, this work also revealed a lack of observable decrease in the native immune function of p65 siRNA treated mice suggesting localized action in inflamed paws. A considerable amount of work remains before clinical

trials can be considered including detailed pharmacokinetic and pharmacodynamics analysis in animal models. One area of interest that has yet to be clarified includes the increased renal clearance reported by these authors with a relative lack of reticuloendothelial clearance. However, the apparent *in vivo* efficacy make peptides derived from melittin a potential candidate for future therapeutic success in clinical settings.

5 Conclusions

Successful application of siRNA as a therapeutic requires the development of vectors that can promote cytoplasmic delivery of siRNA without inducing cytotoxicity, but providing adequate endosomal escape. Peptides are a relatively new strategy for siRNA transfection, which appear to be limited by endosomal entrapment. Traditional methods to overcome the barrier of endosomal entrapment rely on augmenting existing vectors with osmotic agents, lipid moieties, or fusogenic peptides. These strategies have provided modest improvements to peptide mediated transfection, and have provided simple guidelines to further improve peptide-mediated siRNA transfection.

With a focus on endosomal escape, melittin, a naturally occurring, membrane lytic peptide can provide an alternative to peptide transduction domains in the development of peptide-mediated transfection. Despite the hemolytic nature of naked melittin, existing data using protected or packaged melittin have demonstrated no systemic toxicity. Moreover, new strategies based on melittin have recently been shown safe and effective delivery of siRNA in *in vivo* settings. As a barrier to successful siRNA delivery, endosomal escape is undeniably a considerable hurdle that must be overcome to enable maximally efficient siRNA delivery. Modified peptide transduction domains and melittin-based technologies are uniquely designed to target this barrier and have enabled new nanoscale strategies to accomplish the formidable task of *in vivo* siRNA delivery. Ongoing development and clinical trials will ultimately determine the therapeutic relevance of these strategies.

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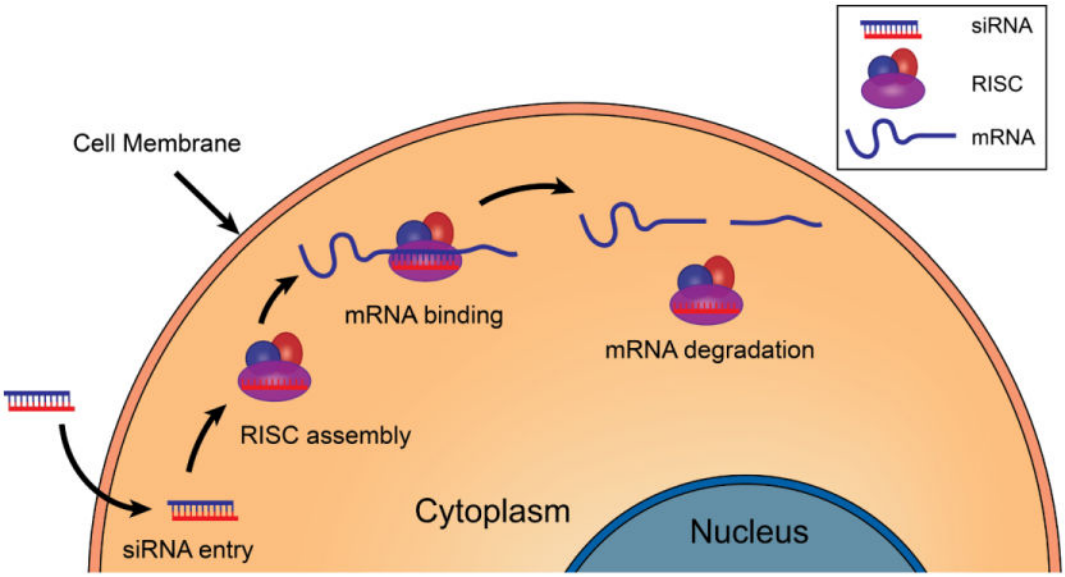

Figure 1.
siRNA can induce mRNA degradation and gene silencing if delivered into the cytoplasm.

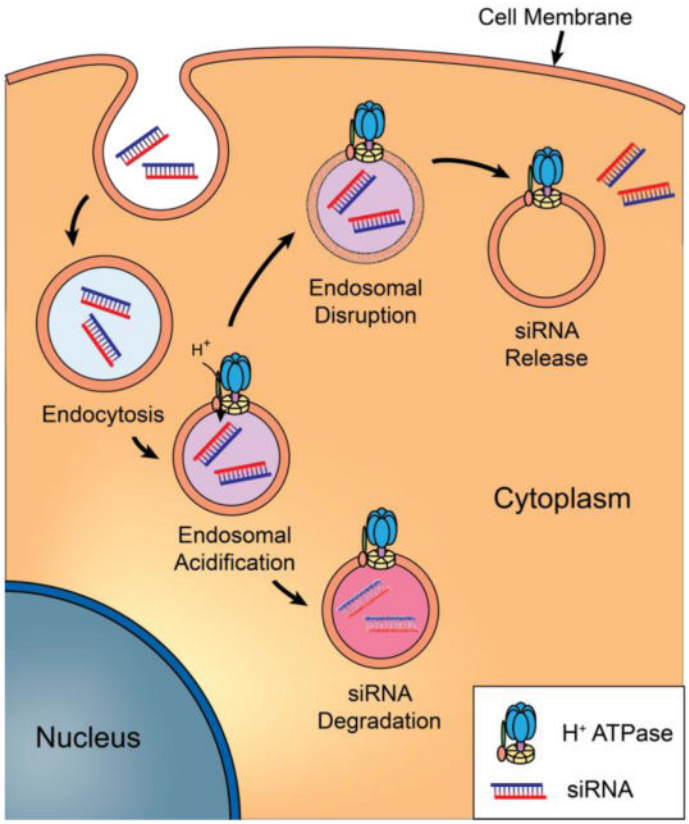

Figure 2.

siRNA entering the cell via endocytosis is degraded in lysosomes by the acidic environment before reaching the cytoplasm due to endosomal entrapment. However, appropriate methods for achieving endosomal disruption can release siRNA into the cytoplasm.

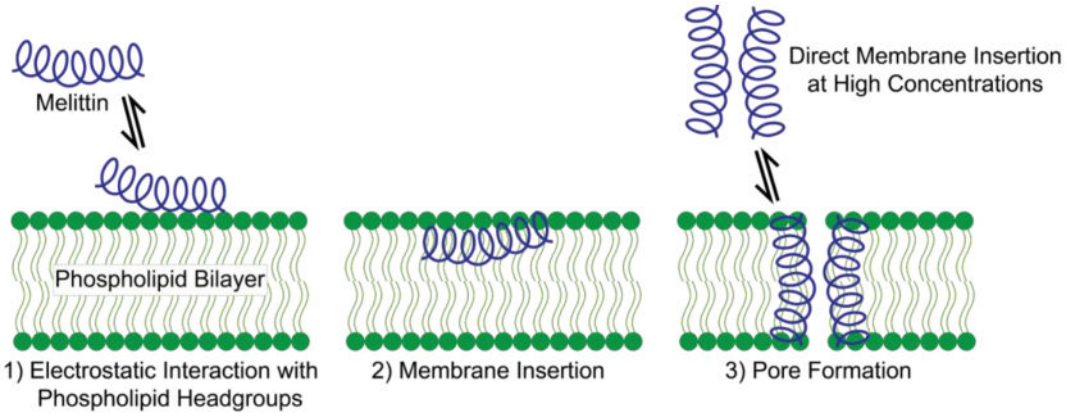

Figure 3.

Melittin initially binds to membrane bilayers by electrostatics before inserting into the membrane and forming pores. At high melittin concentrations, there is evidence for direct membrane insertion and pore formation without the initial electrostatic interactions, which can explain continued membrane disruption even in acidic environments.

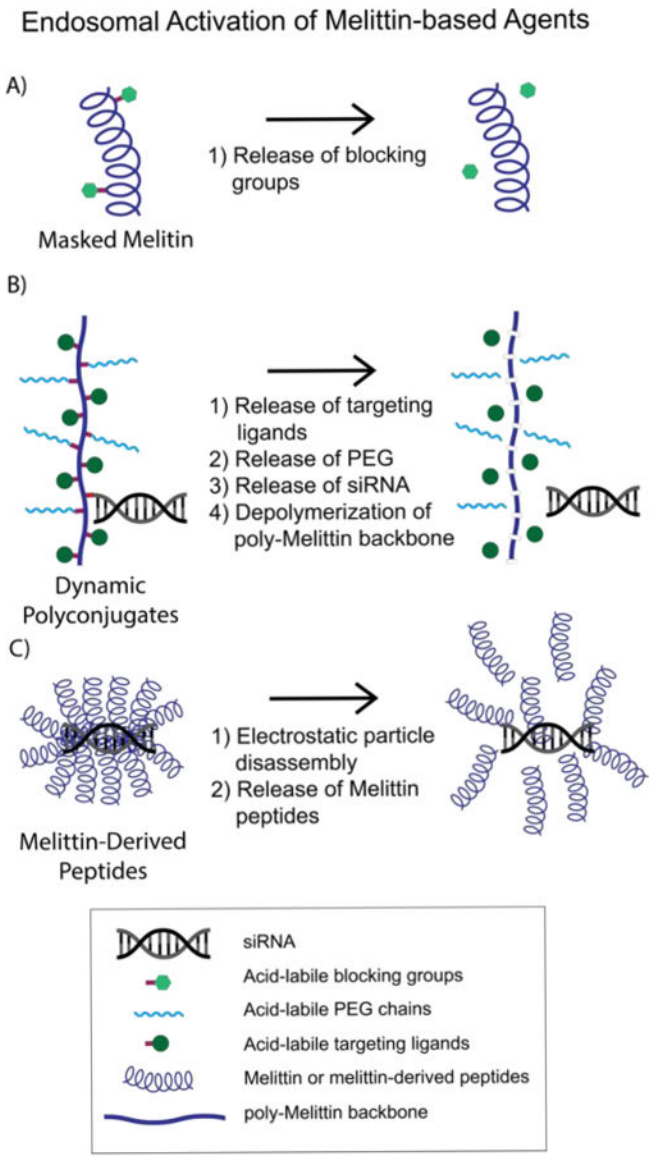

Figure 4.

Melittin based siRNA delivery strategies must be activated in the endosome to avoid cytotoxicity. (a) Acid-activated melittin contain acid-labile blocking elements that are released in an acidic environment. (b) Dynamic polyconjugates are composed of acid-labile targeting ligands, acid-labile PEG chains, reducibly bound siRNA, and poly-melittin backbones which disassemble in the acidic, reducing environment of the endosome. (c) Melittin-derived peptides for direct siRNA binding disassemble based on electrostatic repulsion secondary to protonation in the endosome.

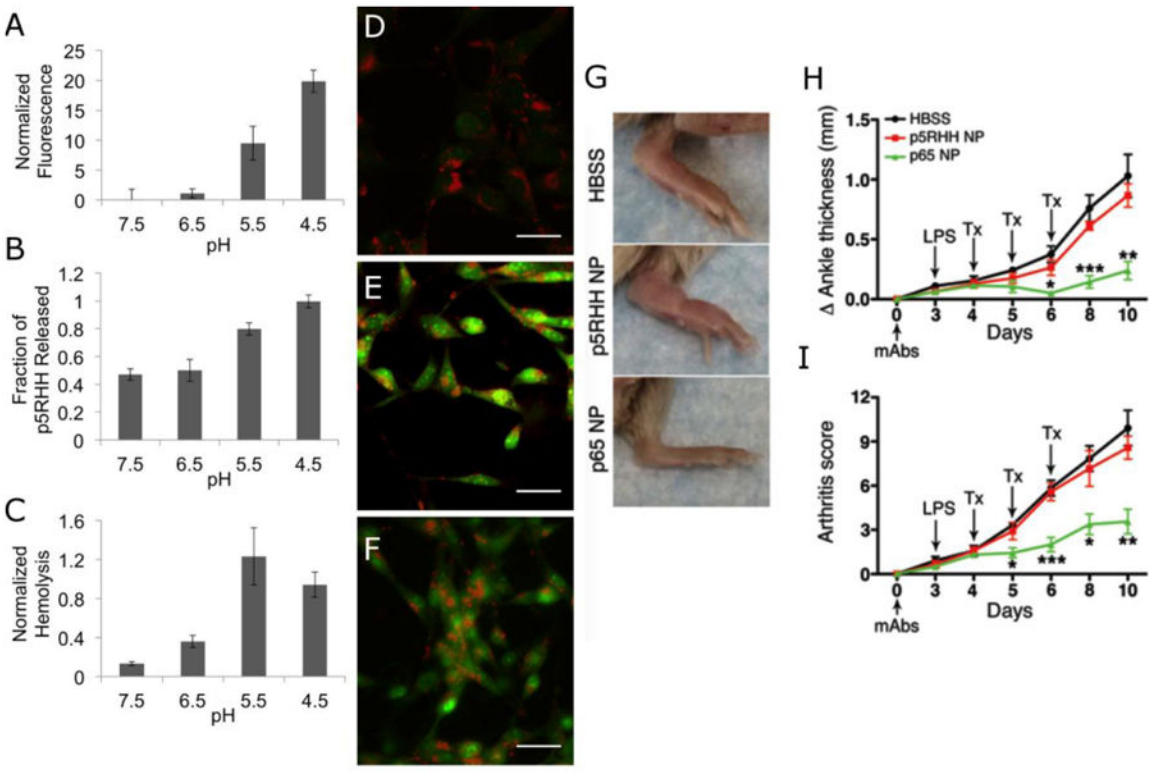

Figure 5.

Melittin-derived peptides for siRNA delivery are pH responsive, with siRNA release (a), peptide release (b), and membrane disruption measured by hemolysis (c) all triggered by pH < 5. This pH responsiveness allows endosomal disruption *in vivo* as witnessed by acridine orange dye release assays. Control (d) cells only show dye accumulation in lysosomes where as chloroquine (e) and particle (f) treated cells demonstrate dye leakage as a result of endosomal disruption. p5RHH/siRNA nanoparticles are able to decrease ankle thickness (g, h) and arthritic score (i) in a murine model of rheumatoid arthritis when delivering NFkB siRNA.

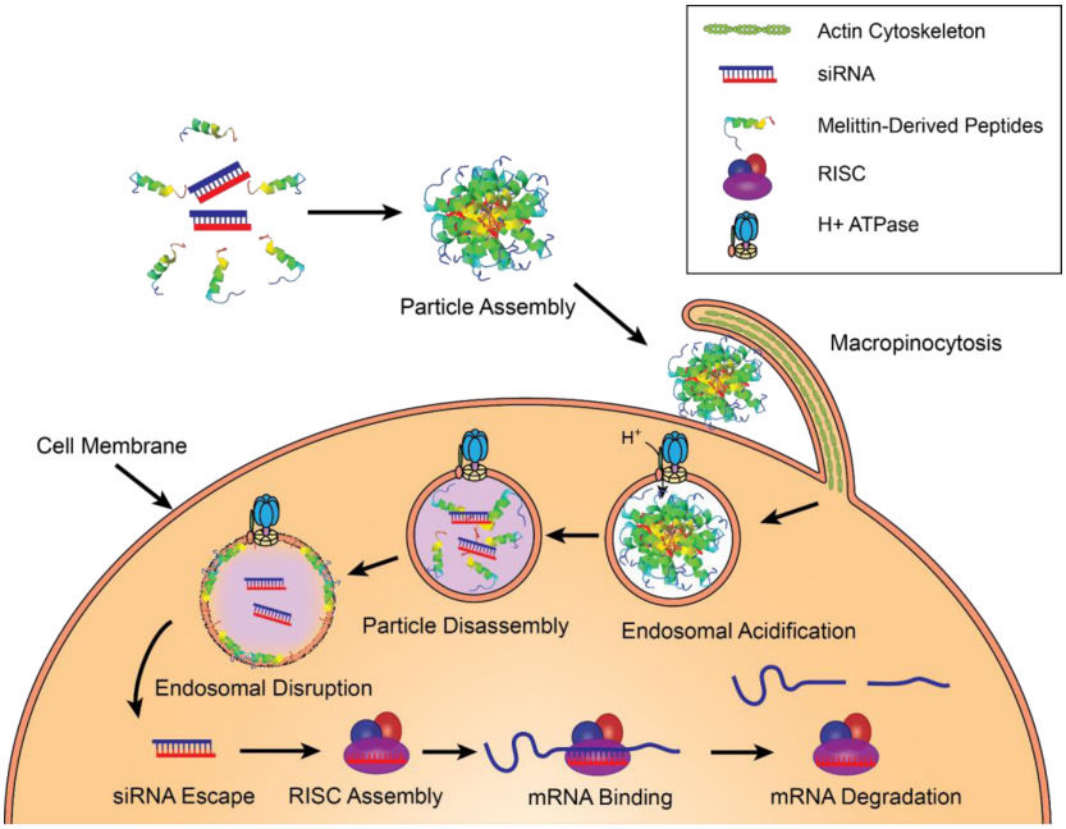

Figure 6.

Melittin-derived peptides promote endosomal escape after particle disassembly triggered by endosomal acidification.

Table 1
Cell penetrating peptides for siRNA transfection

Peptide	Target Gene	IC50	Ref
TAT_{47-57} (YGRKKRRQRRR)	eGFP	none	(Meade *et al.* 2007, Endoh *et al.* 2008)
Penetratin (RQIKIWFQNRRMKWKK-amide)	Luciferase	none	(Lundberg *et al.* 2007, Åmand *et al.* 2008, Mäe *et al.* 2009)
Transportan (GWTLNSAGYLLGKINLKALAALAKKIL-amide)	Luciferase	none	(Lundberg *et al.* 2007)
TP10 (AGYLLGKINLKALAALAKKIL-amide)	Luciferase	none	(Lundberg *et al.* 2007, Mäe *et al.* 2009)
R_n (8<n<15)			
R_9-RVG	GFP	100nM	(Kumar *et al.* 2007)
R_9	VEGF	none	(Kim *et al.* 2006, Mäe *et al.* 2009)
R_{15}	VEGF	100nM	(Kim *et al.* 2010)
Dermaseptin S4	--	none	(Trabulo *et al.* 2010)
MPG (Ac-GALFLGFLGAAGSTMGAWSQPKKKRKV-amide)	Luciferase	>100nM	(Simeoni *et al.* 2003)
$MPG_{\Delta NLS}$ (Ac-GALFLGFLGAAGSTMGAWSQPKSKRKV-amide)	Luciferase, GAPDH, Cyclin B1	30-50nM	(Simeoni *et al.* 2003, Crombez *et al.* 2009)
MPG8 (Ac-βAFLGWLGAWGTMGWSPKKKRK-amide)	Cyclin B1	1nM	(Crombez *et al.* 2009)
MPGα (Ac-GALFLAFLAAALSLMGLWSQPKKKRKV-amide)	Luciferase	1nM	(Veldhoen *et al.* 2006)
CADY (GLWRALWRLLRSLWRLLWRA-amide)	GAPDH	<1nM	(Crombez *et al.* 2008, Crombez *et al.* 2011, Rydström *et al.* 2011, Deshayes *et al.* 2012)
dsRBD		none	(Kim *et al.* 2009)
TAT-dsRBD		400nM	(Eguchi *et al.* 2009)

Table 2

Modified Peptide Vectors

Modified Peptides	Benefit	Ref
TAT		
Stearylation	Improved siRNA uptake/Endosomal escape	(Futaki *et al.* 2001)
HA2 conjugation	pH triggered endosomal escape	(Wadia *et al.* 2004)
LK15 conjugation	Endosomal escape	(Arthanari *et al.* 2010)
$Histidine_{10}$ conjugation	pH triggered endosomal escape	(Lo *et al.* 2008)
Penetratin		
HA2 conjugation	pH triggered endosomal escape	(Lundberg *et al.* 2007)
Stearylation	pH triggered endosomal escape	(Mäe *et al.* 2009)
Poly-Arginine		
Stearylation	Endosomal escape	(Mäe *et al.* 2009, Lehto *et al.* 2011)
Myristoylation	Brain targeted delivery	(Ifediba *et al.* 2010)
Cholesterol conjugation	Increased particle stability/Endosomal escape	(Kim *et al.* 2006)
Stearylation and Insertion of histidine	Endosomal escape	(Tanaka *et al.* 2010)
Poly-Lysine		
Insertion of histidine	Endosomal escape	(Yu *et al.* 2004, Langlet-Bertin *et al.* 2010, Chou *et al.* 2011)
TP10		
EB1	Endosomal Escape	(Lundberg *et al.* 2007)
Stearylation	Improved siRNA uptake/Endosomal escape	(Mäe *et al.* 2009)
Stearylation and chloroquine conjugation	pH triggered endosomal escape	(Andaloussi *et al.* 2011, Ezzat *et al.* 2011, Ezzat *et al.* 2012, Lindberg *et al.* 2013)
MPG8		
Cholesterol conjugation	Improved particle stability/Endosomal Escape	(Crombez *et al.* 2009)
Calcitonin-derived peptides		
Myristoylation	Improved siRNA uptake/Endosomal Escape	(Hoyer *et al.* 2012)
HA2 conjugation	Improved siRNA uptake/pH triggered endosomal escape	(Hoyer *et al.* 2012)