Polymeric Micelles for SiRNA and AON Delivery

by

Dianna Chan

A thesis submitted in conformity with the requirements for the degree of Master’s of Applied Science

Department of Chemical Engineering and Applied Chemistry
University of Toronto

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2012

Abstract

Immuno-nanoparticles of poly(p, l-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-g-poly(ethylene glycol) (poly(LA-co-TMCC)-g-PEG) have been used to target breast cancer cells through the specific binding of trastuzumab antibodies to over-expressed human epidermal growth factor receptor 2 (HER2). Small interfering RNA (siRNA) and antisense oligonucleotides (AONs) disrupt the synthesis of select proteins. It is hypothesized that oligonucleotides coupled to polymeric immuno-nanoparticles can be used for gene silencing and specifically to target luciferase. The first objective is to demonstrate the capacity to create dual functional micelles with antibodies and oligonucleotides. The second objective is in vitro testing of the nanoparticle for gene silencing activity.

Oligonucleotides are conjugated to the nanoparticle by sequential click reactions of Diels Alder chemistry and copper catalyzed azide-alkyne cycloadditions, respectively. A luciferase assay is used to quantify knockdown of luciferase levels in SKOV-3luc cells (HER2+, luc+). When used in conjunction with a targeted drug delivery vehicle, the nanoparticles provide selective interactions with SKOV-3luc cells.
Acknowledgements

I would like to thank my supervisor, Dr. Molly Shoichet, for her motivation and advice during my Master’s degree. Her guidance has taught me to think critically within and outside of research problems and I am grateful for the opportunity to work among such bright minds. It has been a great pleasure to work with the lab members of the Shoichet group. In particular, I would like to thank ‘Team Nanoparticle’ and our collaborators, Dr. Masad Damha and Glen Deleavey, for their support and feedback on my thesis project. I would like to thank Dr. Shawn Owen for his continuous counsel and contributions to my thesis project. Lastly, I would like to thank my family and friends for their continuous support. I would also like to thank OGSST and NSERC for my funding.
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<th>Full Form</th>
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<tr>
<td>2′F-ANA, 2′F-RNA</td>
<td>2′-Deoxy-2′-fluoroarabino nucleic acid</td>
</tr>
<tr>
<td>AON</td>
<td>Antisense oligonucleotide</td>
</tr>
<tr>
<td>CuAAC</td>
<td>Copper catalyzed azide-alkyne cycloaddition</td>
</tr>
<tr>
<td>DA</td>
<td>Diels Alder</td>
</tr>
<tr>
<td>DBCO</td>
<td>Dibenzylcyclooctyne</td>
</tr>
<tr>
<td>Di</td>
<td>Dicer</td>
</tr>
<tr>
<td>Fu</td>
<td>Furan</td>
</tr>
<tr>
<td>LipA</td>
<td>Lipofectamine</td>
</tr>
<tr>
<td>MI</td>
<td>Maleimide</td>
</tr>
<tr>
<td>ON</td>
<td>Oligonucleotide</td>
</tr>
<tr>
<td>Poly(LA-co-TMCC)</td>
<td>Poly(d,l-lactide-co-2-methyl-2-carboxytrimethylene carbonate)</td>
</tr>
<tr>
<td>SiRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SPAAC</td>
<td>Strain promoted azide-alkyne cycloaddition</td>
</tr>
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1. Introduction

1.1 Rationale

Among women, breast cancer is the most commonly diagnosed cancer. It accounts for 14% of cancer-related mortality; the second deadliest cancer for women. In 2008, 1.38 million women worldwide were newly diagnosed with breast cancer [1]. Breast cancer is a slowly progressive disease and its costs to individuals are measured in years or decades. The cost of follow-up procedures and management of recurrent disease was estimated to cost over $454 million Canadian in one year [2].

Chemotherapeutic drugs, such as taxanes, are commonly used for the treatment of cancer. However, systemic toxicity can have negative side effects on healthy tissue. Based on literature review, gene therapy with siRNA and AONs are less toxic to the body than chemotherapeutics. These gene silencing strategies will only interrupt the translation of proteins in mRNA with matching base pairs [3]. Thus, the selectivity can minimize the negative effects of systemic drug distribution.

Typically gene therapy is limited by the instability of siRNA or AON in serum. However, the modified siRNA and AONs provided from the Damha Group at the University of McGill will address this problem [4, 5]. Combined with a biocompatible copolymer such as poly(LA-co-TMCC)-g-PEG-azide, the system can be used for targeted delivery. Our goal is to prevent cancer cell growth by using copolymer nanoparticles to deliver siRNA.

1.2 Hypothesis and objectives

It is hypothesized that siRNA or AONs coupled to polymeric immuno-nanoparticles will have targeted gene silencing activity. SiRNA or AONs can be used for selective gene knockdown of cells. Polymeric immuno-nanoparticles formed by poly(LA-co-TMCC)-g-PEG have been prepared to target HER2. HER2 is overexpressed in breast cancer cells and specifically binds to the antibody trastuzumab (Herceptin®). Poly(LA-co-TMCC)-g-PEG has also been used as a biodegradable delivery vehicle for the chemotherapeutic drug doxorubicin. Use of oligonucleotides for selective gene knockdown may provide an alternative to chemotherapeutics in the copolymer drug delivery vehicle. Gene knockdown can be modelled by selecting an oligonucleotide sequence specific to firefly luciferase. Measurements of bioluminescence of cells expressing firefly luciferase, SKOV-3luc cells, will demonstrate the effectiveness of gene knockdown.
The first objective is to demonstrate the capacity to create dual functional micelles. The second objective is to demonstrate that the immuno-nanoparticles have a gene knockdown effect.

### 1.3 Polymers for targeted drug delivery

Nanocarriers have shown promise for therapeutic applications that can exploit its controllable size and material properties to protect drugs or therapeutic moieties from degradation, increase drug solubility, enhance delivery through physiological barriers and modify its pharmacokinetics and distribution profile. The choice of material for the carrier can range from lipids, to metal nanoparticles and polymers [1]. Polymer therapeutics is a general term that includes the use of polymers in the form of micelles covalently bound to drugs, polymer-drug conjugates, polymer-protein conjugates, or multi-component polyplexes [2]. Drugs delivered by polymeric vehicles display features shown by macromolecular drugs, but include the versatility of synthetic chemistry. Adjusting the molecular weight or adding bioresponsive elements allow the therapeutic vehicle to be tailored [2]. Polymers are a logical option for targeted drug delivery because they can be modified to optimize size, hydrophobicity or hydrophilicity, and targeting properties. Most polymeric nanoparticles are amphiphilic; there is a hydrophobic core and hydrophilic shell. The core allows hydrophobic drugs to be encapsulated [3]. With cancer therapy, polymeric micelles face the challenge of increasing drug efficacy and reducing toxicity by controlling its biodistribution and improving intracellular penetration [1].

#### 1.3.1 Passive tissue targeting

Use of polymeric anticancer therapeutics is rationalized by the ‘enhanced permeability and retention’ (EPR) effect. Hyperpermeable angiogenic tumour vasculature allows long circulation times for polymer therapeutics [2]. There are two main features that result in the EPR effect: the disorganized pathology of tumour vasculature and the lack of effective tumour lymphatic drainage. As a result, solid tumours of breast cancer are hyperpermeable to circulating polymeric nanoparticles. Thus, the nanocarriers are able to accumulate [4, 5]. Polymeric micelles are promising drug carriers that can benefit from the passive targeting due to its size. However, the following design criteria are required for effective drug delivery: the micelle must be between 10-200 nm to penetrate into tissue, be biocompatible, be tuneable for stability and pharmacokinetic profiles, and be able to interact and locate targeted cells[6]. The micelles must circulate long enough to accumulate in the targeted tissue. Opsonin proteins in the blood serum can bind to nanoparticles to signal removal by the macrophages in the mononuclear phagocytic (MPS) system.
One common strategy to address this issue is the grafting of the biocompatible poly(ethylene glycol) (PEG) to decrease opsonization of the nanoparticles.

### 1.3.2 Active cellular targeting

Active targeting is another strategy used to deliver the nanoparticles and its cargo to tumour tissue. Specific ligand targeting proteins or antibodies are expressed on cancer cell membranes or cells lining the blood vessels of the tumour. Conjugation of proteins, antibodies, or ligands can improve the specificity of the drug delivery vehicle [1].

In order to improve drug targeting to breast cancer cells, a drug delivery system using poly(LA-co-TMCC)-g-PEG (Figure 1.1) to form nanoparticle micelles has been previously characterized. Poly(LA-co-TMCC)-g-PEG forms stable nanoparticles, has a low polydispersity index, and the carboxylate group can be used to graft amine-terminated PEG. The poly(LA-co-TMCC)-g-PEG molecules can self assemble into nanoparticles [8].

![Figure 1.1: Structure of poly(LA-co-TMCC)-g-PEG. Each polymer chain contains one pyrene molecule from the pyrene butanol initiator. Dispersed segments of d,l-Lactide (LA) and 2-methyl 2-carboxytrimethylene carbonate (TMCC) monomer units compose the polymer chain. Poly(ethylene glycol) is grafted onto approximately one TMCC unit per polymer backbone. X represents a functional group of furan or azide. The degree of polymerization is controlled by the quantity of monomers used for synthesis [8].](image-url)
The critical aggregation concentration (CAC) is the minimum polymer concentration required for self-assembled structures to form. The CAC reflects thermodynamic stability. The low values (50 nM for poly(LA-co-TMCC) and 200 nM for poly(LA-co-TMCC)-g-PEG) indicate that limited dissociation will occur. The polymers also successfully encapsulated docetaxel. This shows the ability of the nanoparticle to deliver a hydrophobic cancer drug [9]. Further modification through the attachment of antibodies for specific binding to cells overexpressing HER2 antigens to these micelles has demonstrated increased drug targeting [10].

1.4 Small Interference RNA (siRNA) and Antisense Oligonucleotides (AON)

Oligonucleotides have been shown to inhibit target RNA functions by interfering with translation of mRNA. Two strategies include the use of synthetic antisense oligonucleotides and RNA interference [11]. Antisense therapy uses antisense oligonucleotides (AONs) that are typically modified to improve serum stability. RNA interference (RNAi) uses small interfering RNA (siRNA). In both cases, the mechanism of action begins when AONs or siRNA bind to target RNA and activate nucleases that causes RNA degradation (Figure 1.2) [12].

![Figure 1.2: Mechanisms of action for siRNA and AONs.](image-url)
Antisense RNAs were discovered in 1981 when it was shown that RNA regulators could control the copy numbers of *Escherichia coli* plasmids [13, 14]. Antisense RNAs are sequences that target complementary sense RNA for translational blocking or activation of ribonuclease H (RNase H). RNase is found naturally in most cells and can act on immature and mature mRNA [12, 15].

RNAi was later discovered in 1998 by Fire et al. [16]. Post-transcriptional gene silencing was later shown in mammalian cells by Elbashir et al. for the specific knockdown of proteins in HeLa cells [17]. It has been demonstrated that synthetic short RNA duplexes can display high potency in gene knockdown. SiRNA is an oligonucleotide composed of approximately 20 to 25 base pairs [18]. It enters the cell and associates with an RNA induced silencing complex (RISC). There, it cleaves one of the RNA strands and leaves the single stranded RNA incorporated with the RISC. The complex downregulates specific genes [19]. The Dicer is the active nuclease that catalyzes the cleavage of dsRNA [20].

Oligonucleotide therapy is advantageous over small molecule drugs because the RNA and DNA sequences can be designed for specific inhibition of the target of interest [21]. AON and siRNA therapy display strong potency and specificity in cell culture once cellular uptake can be achieved. However, gene therapy faces the obstacles of poor nuclease stability leading to rapid degradation, poor unassisted cellular uptake, and unintended side-effects from “off-target” silencing and immunostimulation [21, 22]. Currently, these challenges are addressed through the use of delivery vehicles and chemical modifications to the oligonucleotide backbones to prevent nuclease degradation and undesired side effects [18, 19, 22].

### 1.4.1 Chemical modifications to siRNA and AONs

Phosphorothioate modifications to DNA can increase serum stability of AONs while maintaining their ability to activate RNase H [23]. One drawback of these modifications is their toxicity and non-specific binding to proteins [24]. The Damha group at McGill University has chemically modified oligonucleotides for improved stability. Modifications include changes to the sugar, phosphate linkage, base, overhangs, and termini [18]. Protection at the 2'-hydroxyl group has shown increased stability of siRNA. Chemical modifications include 2'-O'methyl, 2'-fluoro, and 2’-O-(2-methoxyethyl) groups [19]. Fluorination of the 2'-carbon of sugar rings (2’F-ANA) has become a useful chemical modification in both siRNA and AON gene silencing applications [22, 25-30]. With AON treatment, 2’F-ANA combined with the phosphorothioate backbone modification enhances nuclease stability [32-35]. For siRNA applications, 2’F-ANA improves siRNA nuclease stability and can be incorporated in the siRNA
passenger strand [26, 27]. When used in combination with 2′F-RNA, siRNAs exhibit improved potency and reduced immunostimulatory properties[28].

Another limitation of oligonucleotides is the inability of the negatively charged molecules to cross the cell membrane and begin the antisense or RNAi response [19]. Oligonucleotides alone have a low transfection efficiency, poor tissue penetration and non-specific immune stimulation. Nucleic acids are highly charged and will not cross the cell membrane by free diffusion [21].

Additionally, research by Santel et al. evaluated the biodistribution of naked siRNA and concluded that siRNA does not target any specific tissue. After injection, most of the siRNA was eliminated by renal clearance [21]. Thus, attachment to a delivery vehicle is necessary. Both viral and nonviral delivery methods have been demonstrated [19]. Viral vectors are very effective, but clinically limited. Viral vectors can have potential mutagenicity or host immune responses [19]. Non-viral delivery vehicles are a good alternative. Different systems include: chemical modification of siRNA or AONs, cationic polymers, lipids, and targeted delivery [19].

A hurdle with gene therapy is the ability to internalize siRNA or AONs specifically into the cell. Cell-specific delivery has been achieved using targeting ligands, such as antibodies and cell membrane receptors [21]. Methods used to deliver oligonucleotides in vitro are summarized in Table 1.1.
Table 1.1: Overview of oligonucleotide delivery through non-viral vehicles [31]

<table>
<thead>
<tr>
<th>Category</th>
<th>Carrier</th>
<th>Description</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid complex</td>
<td>Cationic liposomes (DOTAP, DOTMA, Lipofectamine) [32]</td>
<td>Cationic lipids form lipoplexes with negatively charged siRNA for high transfection.</td>
<td>Liposomes can also interact with serum proteins and aggregate. Lipofectamine also has dose-dependent toxicity.</td>
</tr>
<tr>
<td></td>
<td>Neutral liposome (DOPC, DOPE) [33, 34]</td>
<td>SiRNA is encapsulated using simple mixing and incubation. Ligands are conjugated for specific delivery.</td>
<td>Stability; fusion of liposomes to form large particles that clear quickly.</td>
</tr>
<tr>
<td></td>
<td>Lipoplexes (DOTAP and poly(arginine)) [32, 35, 36]</td>
<td>Lipoplexes are cationic liposomes complexed with nucleic acids. The lipoplex is delivered through the cellular membrane. Less toxicity is exhibited than cationic liposome alone.</td>
<td>Little control of interaction between siRNA and plasmid DNA or RNA used to deliver the lipoplex through the membrane. This leads to excessive sizes and low stability.</td>
</tr>
<tr>
<td>Conjugated polymers</td>
<td>Targeting molecule (peptides, antibodies, aptamers) [35]</td>
<td>Conjugation can be attached to either the sense or antisense strand without compromising the activity of siRNA silencing.</td>
<td>Antibodies and proteins are large molecules and alter the drug delivery vehicle size. For aptamers, the major challenge is internalization of cargo after the ligand interacts with the receptor.</td>
</tr>
<tr>
<td></td>
<td>Polymer-PEG [35, 37, 38]</td>
<td>PEG prevents non-specific binding of proteins. PEG can be linked to siRNA through a pH sensitive bond that breaks when internalized and exposed to the acidic endosome.</td>
<td>Pegylation can decrease the in vitro activity of proteins, but this is usually offset by its increased half-life.</td>
</tr>
<tr>
<td>Cationic polymers</td>
<td>Synthetic polymers (PEI) [21, 39]</td>
<td>The high cationic charge density allows PEI to exhibit a proton sponge effect to swell endosomes and cause release of siRNA in the cell.</td>
<td>PEI is cytotoxic in vivo. It may induce nonspecific interferon response.</td>
</tr>
<tr>
<td></td>
<td>Natural polymers (chitosan, atellocollagen) [36, 45]</td>
<td>The polymers are biodegradable. They form complexes with negatively charged siRNA.</td>
<td>Maximizing the activity of chitosan carriers is being investigated by Howard, et al.</td>
</tr>
<tr>
<td></td>
<td>Cyclodextrin [36, 46]</td>
<td>Cyclodextrin molecules have a hydrophilic outer surface and hydrophobic cavity. Molecules form vesicles that carry siRNA.</td>
<td>For improved uptake and release, modifications with polycations, targeting ligands and PEG are being investigated.</td>
</tr>
</tbody>
</table>

DOPC – 1,2-Oleoyl-sn-Glycero-3-phosphocholine  
DOPE – 1,2-Dioleoyl-sn-Glycero-3-phosphoethanolamine  
DOTAP – 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoazadiazol-4-yl)amino]hexanoyl]-3-trimethylammonium propane  
DOTMA – N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethyl-ammonium methyl sulphate  
PEI – polyethyleneimine

Although siRNA and AON both target mRNA for gene silencing, their differences in mechanism of action result in different levels of knockdown in similar conditions. SiRNA shows better inhibition of mRNA at lower doses; approximately 10-fold higher concentrations of AONs were required to achieve inhibition comparable to siRNA [47]. Nevertheless, both AON and siRNA provide potential strategies for
the therapeutic treatment of cancer. Antisense therapy has the benefit of being a more mature technology, while siRNA has the advantage of increased sensitivity at low concentrations.

1.5 Nanoparticle modification by click chemistry

Nanocarriers have often been surface modified to achieve targeted cellular and intracellular delivery or to monitor cell uptake and release [40]. The covalent coupling of antibodies, proteins, peptides, fluorescent molecules, or contrast agents provides additional functionality and versatility [41]. In contrast to the encapsulation of hydrophobic drugs in the core, targeting moieties require exposure to receptors on or within the cell. For diagnostic purposes, MRI contrast agents that require the chelation of metal atoms require exposure to the aqueous environment for enhanced contrast properties [42].

However, surface modification of micelles is not limited to targeting or diagnostic applications. Therapeutic molecules can also be conjugated to the micelle shell. Doxorubicin has been coupled to the PEG terminus of poly(LA-co-TMCC)-g-PEG-furan micelles using Diels Alder chemistry with maleimide-modified doxorubicin [43].

To maintain the bioactivity of these molecules, the chemistry required for conjugations have restrictions with pH, temperature and exposure to organic solvents [41]. Early chemistries include activated esters for condensation with amines[20], maleimide-thiol reactions [24], aldehydes and amines [11, 23], and biotin binding to avidin [44]. An increasingly popular approach uses click chemistry reactions [52-54].

1.5.1 Click chemistry

Click chemistry reactions are classified as reactions that are wide in scope, produce high yields, and generate mild byproducts. Two types of click chemistry reactions are Diels Alder chemistry and the Huisgen dipolar cycloaddition between azides and alkynes [45]. The reaction schemes are shown in Figure 1.3.
The Diels Alder reaction is a cycloaddition reaction between an electron-rich diene and electron-poor dienophile. Rideout and Breslow showed that the rate of Diels Alder reactions could be increased in aqueous environments by bringing together hydrophobic interactions of the reactive groups [46]. The mild reaction conditions and quantitative yields allow nanocarriers to be modified with antibodies, peptides, fluorescent probes, affinity tags and oligonucleotides [47, 48].

The most common click reaction is the copper catalyzed alkyne-azide (CuAAC) reaction. The addition of a copper catalyst increases the rate of reaction by forming a coordination complex with the acetylide. The rate of reaction has been shown to increase by seven orders of magnitude and the yield of CuAAC reactions in water range from 40-90% [49].

Alternatively, a copper-free reaction can be achieved using a strain-promoted alkyne-azide cycloaddition (SPAAC) reaction [53, 59]. SPAAC reactions overcome the potential cytotoxicity of metal ion contamination and degradation of oligonucleotides [50, 51]. Commonly, strained cyclooctynes are used to
10

enable the click reaction [50]. Fluorinated cyclooctyne rings or dibenzocyclooctynes are commonly used to react with azides [50, 52]. Azide modified peptides have been coupled to PEO-PCL micelles with dibenzocyclooctyne moieties with 58-76% yield [52].

1.5.2 Multi-functional nanoparticles

An ideal therapeutic carrier needs to incorporate multiple features: therapeutic cargo, ligands to facilitate cellular or subcellular targeting, the ability to maintain stable, and a safe profile [53]. To meet these criteria, multi-functional nanoparticles have been synthesized to combine various moieties onto a single vehicle. Weissleder first synthesized triple labelled nanoparticles with a magnetic core and fluorescent and isotopic tags [54]. More recently, “octa-functional” PLGA nanoparticles were used to deliver siRNA. The eight functions included: 1) the polymeric micelle vehicle, 2) the therapeutic moiety (siRNA), 3) PEG to improve nanoparticle circulation, 4) a small molecule enhancer for siRNA, 5) an endosomal escape agent, 6) cell penetrating peptides, 7) peptides for endosomal escape, and 8) tumor targeting peptides [53]. Most nanocarriers include variations of the first three functions listed.

With the addition of multiple ligands and cargo to micelles, there is added complexity to the synthesis process. One-step reactions and minimal purification steps are advantageous in terms of maximizing yields and minimizing reaction time. Double click reaction strategies combine orthogonal reactions for post-modification of polymers [55]. CuAAC and Diels Alder reactions have been used by Hizal, et al. in a one pot reaction to synthesize linear triblock copolymers. The orthogonal and independent reactions with azide-alkyne and anthracene-maleimide cycloadditions produced PMMA-b-PS-b-PEG [56]. Variations with CuAAC and Diels Alder double click reactions have been used to produce graft copolymers [57, 58], star polymers [59], dendrimers [60, 61] and cyclic polymers [62]. More recently, Gunay et al. synthesized a 3-arm star terpolymer with sequential Diels-Alder, then CuAAC and nitroxide radical coupling reactions. PEG with maleimide functionality, poly(ε-caprolactone) with tetramethylpiperidine-1-oxyl functionality and polystyrene with azide functionality were conjugated to a multifunctional core [63].

1.6 Cellular Uptake and Release

The charge of the delivery system will affect siRNA uptake. Highly charged particles can bind to proteins and lead to immune stimulation and rapid clearance. Another factor for cell uptake and clearance is size [21]. If a carrier system is used, it is typically taken up by endocytosis. After entering the cell, the siRNA must escape from the endosomes [64].
Escape from the endosome is not well understood, but it is hypothesized that the endosome undergoes a ‘proton sponge effect’: the acidic pH of the endosome interacts with the negatively charged nanoparticle to escape into the cytoplasm [21]. A common vehicle used for siRNA is polyethyleneimine (PEI). The PEI acts as a buffer in the endosome and supplies protons into the endosome. To maintain a charge balance, there is an influx of chloride ions. Osmotic swelling occurs and the endosome bursts. By escaping the endosome, the siRNA escapes the lysosomal trafficking pathway [21]. Negatively charged PLGA nanoparticles were also able to deliver siRNA after surface modification with positively charged PEI [65]. Thus, the charge of the polymer is a major factor which affects the endosomal escape [21].

Other types of co-polymers used to deliver siRNA have been shown by Kataoka et al. PEG-polycation di-block copolymers have been engineered to have high endosomal escape. A diamine side chain has a distinct pKa that results in siRNA encapsulation in polyplex micelles [21]. Sun et al. developed a cationic triblock copolymer mPEG$_{45}$-b-PCL$_{100}$-b-PPEEA$_{12}$ for the delivery of siRNA. The vehicle was biocompatible, biodegradable and demonstrated effective gene silencing in 40-70% of the targeted cells [76]. The choice of polymer will affect the biodistribution and release kinetics of the drug.

Liposomes have proven to be good carriers for oligonucleotides [28]. Cationic liposomes complex with negatively charged oligonucleotides to overcome the electrostatic repulsion of the cell surface and induce local destabilization of the plasma membrane [66, 67]. Anionic lipids in the endosome membrane complex with the cationic lipids. As a consequence, AONs are translocated into the cytoplasm [80, 81].

### 1.7 Summary of Research

Polymers can be effective vehicles for drug delivery due to their biocompatibility and potential for attaching targeting moieties. Attachment of antibodies, siRNA and AONs increase drug delivery to cancer cells and limit the exposure of toxic drugs to noncancerous cells. However, micellar drug delivery vehicles have limitations in stability. As well, endosomal escape must be considered when developing a drug carrier. If modifications are made to increase the stability of siRNA and the polymer delivery vehicle permits endosomal escape, then siRNA has the potential to increase the effectiveness of the drug delivery system.

An overview of the plan is shown in Figure 1.4. The first objective is to synthesize and characterize the nanoparticle. The second objective is in vitro testing of gene silencing activity.
Figure 1.4: Flowchart of plan. Two objectives have been defined. The first part requires the synthesis and characterization of a siRNA- or AON- conjugated nanoparticle. The second part tests the nanoparticle for its activity.

Using established protocols, the poly(LA-co-TMCC) backbone is synthesized. Next, PEG is coupled to the copolymer to produce poly(LA-co-TMCC)-g-PEG-X (X = azide or furan functional groups). Dual-functional nanoparticles are formed using the dialysis method. The nanoparticles can be characterized for particle size and polydispersity. Sequential click reactions to the furan and azide functionalities are completed with trastuzumab-SMCC and oligonucleotide-alkyne moieties. Figure 1.5 displays the schematic of the proposed nanoparticle.

Figure 1.5: Poly(LA-co-TMCC)-g-PEG nanoparticles coupled with siRNA or AONs. The hydrophilic PEG regions will interact with the aqueous environment. SiRNA or AONs and trastuzumab antibodies will attach to the PEG chains on the exterior of the nanoparticle.

The Damha group at McGill University has modified siRNA and AONs for increased stability. Additionally, the group has added an acetylene group and fluorescent dye to their oligonucleotides. There
is evidence that the use of CuAAC chemistry will allow the azide group on the nanoparticles to react with the acetylene group on the oligonucleotides. Thus, click chemistry will use the acetylene group to attach the siRNA to the nanoparticles. The coupling efficiency of siRNA or AONs to the nanoparticle can be evaluated using fluorescence. The oligonucleotides have a Cy5 dye that will allow quantification of siRNA or AONs.

The sequential double click reaction was achieved with model compounds of Alexa 488-dibenzylcyclooctyne and Alexa 647-SMCC. The coupling efficiency was measured by fluorescence. Later, dual functional nanoparticles were prepared with trastuzumab and FLAG peptides to monitor cell uptake.

The second part of the project involves quantifying the selective gene knockdown of the siRNA or AON immuno-nanoparticles to cells. An oligonucleotide sequence that targets firefly luciferase is coupled to the nanoparticle. Selectivity of the nanoparticles can be evaluated by introducing the siRNA-modified nanoparticles to cells expressing firefly luciferase. Gene knockdown decreases the bioluminescence from cells. Nanoparticles prepared with scrambled sequences are used as negative controls. Meanwhile, delivery using a common transfection reagent, Lipofectamine, is used as a positive control.

1.8 References


2. Double click: Dual functionalized polymeric micelles*

*This chapter is comprised of a paper submitted to Bioconjugate Chemistry.


Chan and Owen are co-first authors. Owen synthesized the FLAG peptide and stained the cells for confocal microscopy. Chan completed all other experiments outlined in this chapter. The discussion was written by Chan with input and editing by all other authors.

2.1 Abstract

Polymeric nanoparticle micelles provide a possible platform for theranostic delivery, combining role of therapeutics and diagnostics in one vehicle. To explore dual-functional micelles, the amphiphilic copolymer of poly(ᴅ,ʟ-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-graft-poly(ethylene glycol)-X (P(LA-co-TMCC)-g-PEG-X) was self-assembled to form micelles, with X representing either azide or furan. Micelles of P(LA-co-TMCC)-g-PEG-azide and P(LA-co-TMCC)-g-PEG-furan terminal functional groups were used to conjugate dibenzylcyclooctyne and maleimide-modified probes, respectively, taking advantage of orthogonal coupling chemistry. To verify the utility of the dual-functional micelles, trastuzumab-maleimide antibodies and FLAG- dibenzylcyclooctyne peptides were covalently bound by sequential click chemistry reactions. SKOV-3luc cells that were treated with the dual-functionalized micelles showed co-localization of the antibodies and peptides by confocal imaging, demonstrating the promise of this approach.

2.2 Introduction

Biodegradable copolymers that self assemble into nanoparticle micelles are promising drug delivery vehicles due to their tuneable physical properties [1]. Polymeric micelles can be tailored to improve the solubility of hydrophobic drugs, control the release rate of drugs, reduce the elimination rate associated with the host immune system, increase the circulation time and enhance the preferential delivery of drugs to solid tumors [2]. Polymeric micelles are unique drug delivery vehicles because they remain intact at concentrations above the critical micelle concentration (CMC) and dissociate slowly when below the CMC to release encapsulated drugs [2].
Polymeric micelles can capitalize on their unique amphiphilic properties by loading hydrophobic drugs into the core to improve drug solubility while incorporating targeting and/or imaging ligands on the surface as well. For example, micelles can be conjugated to antibodies, peptides, lectins, saccharides or hormones[3] for targeted drug delivery. Targeting cell surface receptors using antibodies is a promising method to improve the specific binding of micelles to desired cells [4]; however, intracellular trafficking may be required if the target is within the nucleus or organelles. Cell penetrating peptides and fusogenic proteins facilitate the targeting to intracellular locations and can be added to the drug carrier system [5, 6]. The co-delivery of additional therapeutics can also improve the overall efficacy [7]. Likewise, micelles that are labelled with a molecular probe or contrast agent can aid to diagnose disease state or track the kinetics of the drug delivery system [8]. A recent shift towards multi-functional “theranostic” treatments aim to use the polymeric micelle as a drug carrier with diagnostic capacity, thereby serving a dual purpose.

To covalently attach multiple, unique diagnostic agents, targeting ligands or therapeutic agents, the micelle must have multiple, accessible functional groups [2]. A mixed micelle system comprised of a blend of block copolymers could produce multi-functional micelles. Mixed micellar systems are formed by weak hydrophobic interactions, hydrogen bonding, stereocomplexation, ionic interaction or chemical crosslinking [1]. In one system of self-assembled micelles, a graft copolymer was combined with a diblock copolymer to conjugate two moieties, Cy5.5 and folate, with the size and polydispersity dependent on the proportion of each polymer[9].

Micelles have been surface-functionalized with a single type of functional group, using either Diels Alder or Huisgen 1,3-cycloaddition click chemistries [4, 10], but never both. While the most common click reaction is the copper-catalyzed alkyne-azide (CuAAC) reaction [11], the copper-free reaction using a strain-promoted alkyne-azide cycloaddition (SPAAC) reaction is gaining popularity as it overcomes the potential cytotoxicity associated with copper ion contamination[12]. The Diels Alder reaction similarly avoids potential metal ion contamination and is a clean and simple click reaction that is effective in aqueous conditions.
Poly(ᴅ,ʟ-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-graft-poly(ethylene glycol) (P(LA-co-TMCC)-g-PEG) is an amphiphilic polymer that self-assembles to form stable micelles with the capacity to encapsulate hydrophobic drugs and to be covalently modified with targeting ligands[13-15]. Here we show that a P(LA-co-TMCC)-g-PEG-X copolymer, where X represents one of two click-able functional groups (furan or azide), can self assemble into micelles and be functionalized with two distinct biomolecules (maleimide-trastuzumab and dibenzylcyclooctyne-FLAG peptide), thereby achieving dual functionalized micelles through click chemistry. In contrast to mixed micelles formed from two dissimilar block copolymers, the use of a single copolymer with two functional groups produces a stable micelle that retains the characteristics of a single population. We take advantage of the Diels Alder reaction between furan and maleimide and the copper-free reaction between azide and dibenzylcyclooctyne on pre-formed micelles in aqueous conditions. The strained alkyne bond of dibenzylcyclooctyne has increased electrophilicity over simple alkynes, allowing the reaction to proceed without a catalyst[16]. The versatility of binding different moieties with click chemistry reactions provides a platform for the incorporation of unique combinations of ligands with potential use in therapeutic and diagnostic applications or in primary/secondary targeting conditions. The ability to conjugate biomolecules on the surface of pre-formed micelles in aqueous conditions is particularly important for ligands that are sensitive to the organic solvents used during micellization, for targeting ligands that require direct binding to cells, or for MRI contrast agents that have enhanced contrast properties when exposed to aqueous environments[17].

2.3 Materials and Methods

Synthesis of 5-methyl-5-benzyloxy carbonyl-1,3-trimethylene carbonate (benzyl-protected TMCC, TMCC-Bn) was carried out as previously reported[4]. 3,6-dimethyl-1,4-dioxane-2,5-dione and 1-[3,5-bis(trifluoromethyl)phenyl]-3-[(1R,2R)-(−)-2(dimethylamino) cyclohexyl] thiourea (Strem Chemicals, Newburyport, MA) were used as received to synthesize P(LA-co-TMCC)[18]. Boc-NH-PEG(10K)-NHS (Rapp Polymere, Tübingen, Germany) was modified using previously reported protocols for furan and azide-functional groups[4, 10]. N,N'-diisopropyl carbodiimide, N,N'-diisopropylethylamine and hydroxybenzotriazole (TRC, Toronto, ON) were used as received. Amino acid derivatives were purchased from Novabiochem
Anti-FLAG (Rabbit) antibodies were purchased from Cell Sorting (Boston, MA), anti-human IgG (Rat) FITC from Sigma-Aldrich (St. Louis, MO), and anti-Rabbit (Goat) Alexa-647 from Invitrogen (Grand Island, NY). All solvents and reagents were purchased from Sigma-Aldrich and were used as received, unless otherwise noted.

The Alexa 488 C₅ maleimide was obtained from Invitrogen (Burlington, ON), the Alexa 488-dibenzylecyclooctyne (DBCO) was purchased from Click Chemistry Tools (Scottsdale, AZ) and sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) was purchased from Pierce (Rockford, IL). Trastuzumab was purchased through Hoffmann-La Roche Limited (Mississauga, ON). 5-((2-(and-3)-S-(acetylmercapto)succinoyl)amino) (SAMSA) fluorescein (Invitrogen, Eugene, OR) was used to quantify the amount of SMCC that coupled to the trastuzumab. The human ovary cancer cell line SKOV-3luc was purchased from Cell Biolabs, Inc (San Diego, CA).

The ¹H NMR spectra were recorded at 400 MHz at room temperature using a Varian Mercury 400 spectrometer. The chemical shifts are in ppm. The molecular weights and polydispersity of P(LA-co-TMCC) were measured by gel permeation chromatography (GPC) in THF relative to polystyrene standards at room temperatures on a system with two-column sets (Viscotek GMHHR-H) and a triple detector array (TDA302) at a flow rate of 0.6 mL/min. Polymeric micelle size and distribution were measured by dynamic light scattering (DLS) using the Zetasizer Nano ZS system (Malvern, UK). The NH₂-PEG-azide was characterized using the Perkin-Elmer FT-IR Spectrum 1000. The Sepharose CL-4B column was prepared by soaking the beads in distilled water overnight and packing the beads in a column (5 x 15 cm). The column was washed with distilled water for 1 h before use and the flow rate was determined by gravity. The Sephadex G-25 column was prepared using the same method, except PBS buffer (1x, pH 7.4) was used as the eluent and the beads were packed in a column (1.5 x 15 cm). FPLC of the micelles used a Superdex 200 column. The column was washed with distilled water for 20 min and PBS buffer (1x, pH 7.4) for 20 min at a flow rate of 1.5 mL/min before use. Fluorescence measurements were completed with the Tecan Infinite M200 Pro fluorescent plate reader and absorbance was quantified using the NanoDrop Spectrophotometer.
2.3.1 Polymer Synthesis

**Synthesis of NH$_2$-PEG-azide.** As previously shown, bifunctional tert-butyloxycarbonyl-protected amine-PEG activated acid (BocNH-PEG-(N-hydroxysuccinimide)), 0.523 g, 52 μmol) was modified to incorporate a terminal azide group[10] by reacting with 11-azido-3,6,9-trioxoanadecan-1-amine (Sigma Aldrich, 1.0 mL, 5 mmol) in DMF (Figure 2.1). The final product yielded NH$_2$-PEG-N$_3$ (0.3629 g, 80.6%). The presence of the terminal azide group (2101 cm$^{-1}$), terminal amine (3389 cm$^{-1}$), C–H alkane (2888 cm$^{-1}$), C=O amide (1692 cm$^{-1}$), and C–O ether (1101 cm$^{-1}$) was characterized by FTIR (Appendix Figure A 1).

![Figure 2.1. Synthesis of (a) NH$_2$-PEG-azide and (b) NH$_2$-PEG-furan by reacting NHS-activated PEG chains with furfurylamine or amine-terminated triethylene glycol-azide, respectively, followed by BOC deprotection.](image)

**Synthesis of NH$_2$-PEG-furan.** Similar to the procedure used to synthesize PEG-azide, BocNH-PEG-NHS (1.00 g, 0.1mmol) and furfurylamine (1.00 mL, 11.2 mmol) were reacted to yield bocNH-PEG-furan (0.8021 g, 80.2% yield). $^1$H NMR (CDCl$_3$, 400 MHz): δ 1.44 (s, Boc CH$_3$), 2.32 (t, $J$=14 Hz , –CH$_2$–), 3.61 (m, –OCH$_2$CH$_2$–), 4.42 (s, –CH$_2$-furan), 6.22 (d, $J$= 3Hz, furan CH-3), 6.30 (dd, $J$=5 Hz, furan CH-4) and 7.34 (d, $J$=3 Hz, furan CH-5). For NMR spectra, see
Appendix Figure A 2. The product was deprotected with treatment of 15% trifluoroacetic acid in CH₂Cl₂ for a final yield of NH₂-PEG-N₃ (0.6614 g, 81.5%).

**Grafting of PEG to P(LA-co-TMCC).** Synthesis of P(LA-co-TMCC) by ring opening polymerization has previously been shown [18]. ¹H NMR (CDCl₃, 400MHz): δ 1.22 (br s, CH₃ from TMCC), 1.59 (m, CH₃ from LA), 4.31 (br s, CH₂ from TMCC), 5.15 (m, CH from LA), 7.33 (m, benzyl Ar) and 8.02 (m, pyrene). Absence of the benzyl group peaks in the ¹H NMR after the hydrogenolysis of P(LA-co-TMCC-Bn) showed full deprotection of the polymer backbone. See Appendix Figure A 3 for ¹H NMR spectra.

The modified H₂N-PEG (PEG-X) was grafted onto the P(LA-co-TMCC) as previously shown (Figure 2.2) [18]. P(LA-co-TMCC) (100 mg, 8.3 μmol) was dissolved in 3 mL of DMF with N,N'-diisopropyl carbodiimide (DIC, 100 μL, 0.65 mmol), hydroxybenzotriazole (HOBt, 63.1 mg, 0.48 mmol) and N,N'-diisopropylethylamine (DIPEA, 0.52 mmol) and stirred for 30 min. The PEG-X (100 mg, 10 μmol) was dissolved in 3 mL of DMF and added to the P(LA-co-TMCC) solution dropwise. An additional 90 μL of DIPEA was added, the mixture was sealed under argon for 2 min and left to react overnight.

Borate buffer (400 μL, 500 mM, pH 9.0) was added to the P(LA-co-TMCC)-g-PEG-X solution before dialyzing against distilled water with a 2 kDa RC membrane. The P(LA-co-TMCC)-g-PEG-X was purified using the Sepharose CL-4B column, then freeze dried (37.5 mg, 38%). From the ¹H NMR spectra, it was calculated that there was an average of 0.9 PEG-azide chains per P(LA-co-TMCC) backbone. ¹H NMR (CDCl₃, 400 MHz): δ 1.25 (br s, CH₃ from TMCC), 1.57 (m, CH₃ from LA), 3.61-3.69 (m, –OCH₂CH₂–), 4.35 (m, CH₂ from TMCC) and 5.17 (m, CH from LA). See Appendix Figure A 4 for ¹H NMR spectra.

The same procedure was carried out to graft PEG-furan to P(LA-co-TMCC). An average of 0.7 PEG-furan chains per backbone was calculated using the ¹H NMR spectra. ¹H NMR (CDCl₃, 400 MHz): δ 1.22 (br s, CH₃ from TMCC), 1.56 (m, CH₃ from LA), 3.59-3.69 (m, –OCH₂CH₂–), 4.31 (m, CH₂ from TMCC) and 5.15 (m, CH from LA). See Appendix Figure A 4 for ¹H NMR spectra.
2.3.2 Micellization

The micelles were prepared by co-self-assembly of P(LA-co-TMCC)-g-PEG-furan and P(LA-co-TMCC)-g-PEG-azide by membrane dialysis, as previously shown for single functionalized chains [19]. To form the dual-functional micelles, P(LA-co-TMCC)-g-PEG-furan (2.2 mg, 18.2 kDa) and P(LA-co-TMCC)-g-PEG-azide (1.8 mg, 20.2 kDa) were combined for an average of 80 nmol each of furan and azide functional groups. The average number of furan and azide functional groups per micelle accounted for the difference in grafting efficiency (0.7 PEG and 0.9 PEG per backbone, respectively) by adjusting the ratio of each polymer dissolved in 1mL of DMF. Borate buffer (50 μL, 0.5 M, pH 9.0) was added dropwise to the solution and an additional 500 μL of distilled water was added to form the micelles. The solution was dialysed against...
distilled water using a dialysis membrane with MWCO of 2 kDa at room temperature overnight to form micelles. The water was changed every 2 h for the first 6 h. The micelles were filtered using a 0.45 μm nylon filter and brought to a concentration of 50 μM of polymer before characterization by DLS.

Azide-only and furan-only micelles were prepared using the same dialysis method as controls for the coupling chemistry. P(LA-co-TMCC)-g-PEG-azide (4.0 mg, 20.2 kDa) was used for the azide micelles and P(LA-co-TMCC)-g-PEG-furan (4.0 mg, 18.2 kDa) was used for the furan micelles. The average diameter was measured by DLS of the furan micelles at 66.43 nm with PDI=0.218; the average diameter of the azide micelles was measured at 102.9 nm with PDI=0.165.

2.3.3 Conjugation of Trastuzumab and FLAG Peptides to the Nanoparticles

Synthesis of Trastuzumab-SMCC. Trastuzumab (6.6 mg, 45.5 nmol) was dialyzed overnight (2 kDa MWCO) against Tris buffer (100 mM, pH 8.5) to remove the storage solution of sodium azide. Sulfo-SMCC (0.5 mg, 1.15 μmol) was added to the trastuzumab solution and left to react for 4 h. The SAMSA fluorescein (10 mg/mL in DMSO) was dissolved in 500 μL of NaOH (0.1 M) and incubated for 15 min at room temperature. The solution was neutralized using 7 μL of HCl (6 M) and buffered with 100 μL Na₂HPO₄ (0.5 M). From this solution, the SAMSA fluorescein (0.55 μmol, 63 μL) was mixed with trastuzumab-SMCC (55 nmol, 100 μL) and incubated at room temperature for 30 min. The mixture was dialyzed against Tris buffer overnight. A standard curve was prepared using the activated SAMSA fluorescein solution and was used to quantify the trastuzumab-SMCC at an excitation wavelength of 480 nm and emission at 530 nm. An average of 1 fluorescein molecule per antibody translated to 1 SMCC group available per antibody.

Synthesis of dibenzylcyclooctyne-modified FLAG peptide. The FLAG peptide (DYKDDDDDK) was synthesized by standard Fmoc-based solid phase peptide chemistry on a CEM Liberty 1 microwave peptide synthesizer. The resin-bound FLAG peptide (100 mg, 1.1 meq/g, 0.11 mmol) was swollen in CH₂Cl₂ and then rinsed several times with N,N-
diisopropylethylamine (DIPEA). Dibenzylcyclooctyne-NHS (80 mg, 0.165 mmol) was dissolved in DMF (~2 mL), added to the resin, and allowed to react overnight. The resin was washed several times with DMF and CH$_2$Cl$_2$. The product was deprotected and cleaved from the resin with treatment of 95% trifluoroacetic acid in CH$_2$Cl$_2$. Solvent was removed from the resulting product and peptide precipitated in cold Et$_2$O. Product was purified by HPLC and analyzed by electrospray ionization mass spectrometry (ESI+ MS). Expected 1327.5, found 1329.6 (M+2H).

**Coupling of Alexa 488-maleimide (MI) on Furan Micelles.** P(LA-co-TMCC)-g-PEG-furan micelles (2 mg/mL, 50 nmol) were reacted for 24 h with Alexa 488-C$_5$-MI (2 mg/mL, 100 nmol) in MES buffer (100 mM, pH 5.5, 100 μL). Using the G-25 Sephadex column, the unreacted Alexa 488-MI was separated. Fractions containing the micelles were detected using Bradford reagent and quantified by absorbance at 260 nm. Fluorescence was used to quantify the Alexa 488 that conjugated to the micelle.

Controls were prepared by quenching the Alexa 488-MI (100 nmol) with furfurylamine (10 μmol) and cysteine (10 μmol) in MES buffer (100 mM, pH 5.5, 100 μL) before the coupling reaction. To fully quench the maleimide with the cysteine, tris(2-carboxyethyl)phosphine) (80 μmol) was added for the 48 h reaction. The same purification procedure was followed to remove the unreacted dye.

The Diels Alder (DA) reaction was characterized by reacting NH$_2$-PEG-furan (100 nmol) with Alexa 647-MI (150 nmol) overnight. The solution was dialyzed to remove the unreacted Alexa 647-MI with 2 kDa MWCO dialysis tubing in distilled water. The sample was lyophilized before forming a KBr pellet. The reacted NH$_2$-PEG-furan was compared with an unreacted sample to confirm the DA reaction. A decrease of the characteristic peaks of furan C=C (1466 cm$^{-1}$) and the appearance of the C=C Diels Alder adduct peak (1459 cm$^{-1}$) indicate that the Diels Alder reaction occurred. See Appendix Figure A 5 for FTIR spectra.

**Coupling of Alexa 488-dibenzylcyclooctyne (DBCO) on Azide Micelles.** P(LA-co-TMCC)-g-PEG-azide micelles (2 mg/mL, 100 nmol) were reacted for 24 h with Alexa 488-DBCO (0.5
mg/mL, 200 nmol). Using the G-25 Sephadex column, the unreacted Alexa 488-DBCO was removed.

As a control, the reaction was repeated with Alexa 488-DBCO where the Alexa 488-DBCO (200 nmol) was quenched by incubation with 11-azido-3,6,9-trioxaundecan-1-amine (400 µL, 2 mmol) prior to the click chemistry reaction. The micelles were purified using the same procedure with a size exclusion column.

To characterize the SPAAC reaction, NH₂-PEG-N₃ (100 nmol) was reacted with Alexa 488-DBCO (500 nmol) overnight. Similar to the procedure outlined previously, the sample was prepared for FTIR analysis to compare the unreacted and reacted NH₂-PEG-N₃. The presence of the terminal azide group was characterized by the FTIR analysis. See Appendix Figure A 6 for FTIR spectra.

**Coupling of Alexa 488-DBCO and Alexa 647-MI on Dual-Functional Micelles by Click Chemistry.** P(LA-co-TMCC)-g-PEG micelles with azide and furan functional groups (50 µM) were sequentially reacted with maleimide and dibenzylcyclooctyne-functional moieties. First, Alexa 647-MI (100 nmol) was added at room temperature with MES buffer (100 mM, pH 5.5) for 24 h. The solution was dialyzed overnight against PBS buffer (1x, pH 7.4) using 2 kDa MWCO dialysis tubing. The buffer was changed every 2 h for the first 6 h. Then, the solution was transferred to a glass vial to react with the Alexa 488-DBCO (100 nmol) at room temperature for 24 h. The reacted micelles were purified using size exclusion chromatography on a Sephadex G-25 column with PBS buffer (1x, pH 7.4) as the eluent. Fractions were collected in 1 mL volumes for a total of 20 fractions. Fluorescence of the Alexa 647 (ex. 640 nm, em. 675 nm) and Alexa 488 (ex. 490 nm, em. 525 nm) were used to quantify the conjugation efficiency. The concentrations of the micelles were measured by absorbance at 260 nm. Controls were repeated by quenching either the Alexa 647-MI or Alexa 488-DBCO with three orders of magnitude excess of furfurylamine or 11-azido-3,6,9-trioxaundecan-1-amine, respectively, before reacting with the micelles. See Appendix Figure A 7 for fluorescence and absorbance measurements from the size exclusion column.
Coupling of FLAG-DBCO and Trastuzumab-SMCC on Dual-Functional Micelles by Click Chemistry. The dual-functional micelles were prepared using the same procedure as other micelles described. As with the Alexa dyes, the trastuzumab-SMCC and FLAG-DBCO were conjugated sequentially. Micelles were purified by FPLC (GE Healthcare AKTA Purifier) monitoring absorbance at 215 and 280 nm.

2.3.4 Cell Culture and In Vitro Assessment of Dual Functionalized Micelle Uptake
SKOV-3luc cells were cultured in McCoy’s 5A media containing 10% FBS, 10 UI/mL penicillin, and 10 µg/mL streptomycin under standard culture conditions (37°C, 5% CO₂, 100% humidity). Cells were seeded in 16-well chamber slides at 10,000 cells/well and allowed to adhere for 22 h. Dual functionalized micelles having both trastuzumab and FLAG peptides (100 µL, 100 nM trastuzumab) were added. Cells were supplemented with an additional 200 µL of media and incubated for 3 h. Cells were fixed in 4% paraformaldehyde before staining and analysis. Cells were incubated with 1% BSA in PBS with 0.05% Tween 20 (PBST) for 30 min to block unspecific binding of the antibodies. Cells were then incubated with the primary antibody (anti-FLAG or anti-human IgG) diluted in 1% BSA in PBST for 1 hr at room temperature. Secondary antibodies were incubated in the same manner. Cells were then washed several time with PBS and mounted with medium containing DAPI. Images were collected by confocal microscopy (Olympus FV1000) - for DAPI, excitation at 405 nm, emission at 460; for FITC, excitation at 485 nm, emission at 520 nm; for Alexa-647, excitation at 650nm, emission at 675nm.

2.4 Results

2.4.1 Polymer Synthesis
Synthesis of P(LA-co-TMCC)-g-PEG-X. The number average molecular weight and polydispersity of P(LA-co-TMCC) was determined by GPC to be 11.2 kDa and 1.12, respectively. Based on ¹H NMR integrated peak areas for TMCC methylene (4.35 ppm) and LA methyl (5.15 ppm), the molar percentage of TMCC in the copolymer was determined to be 7.1%. The copolymer composition was calculated using the ¹H NMR spectra based on the integrated
peak areas of LA methyl groups and TMCC methylene groups relative to the single pyrene initiator group (8.02 ppm) present on each polymer chain: P(LA)_{130}-co-(TMCC)_{10}. These data are consistent with the GPC measurement.

Using DIC chemistry, we grafted NH\textsubscript{2}-PEG-furan or NH\textsubscript{2}-PEG-azide onto the carboxylic acid groups present on the TMCC segments of the copolymer. We estimated the ratio of grafted PEG to P(LA-co-TMCC) by comparing the integrated peak areas of the PEG methylene (3.61-3.64 ppm) to the TMCC methylene (4.35 ppm). On average, there was approximately 0.7 PEG-furan or 0.9 PEG-azide chains grafted to every P(LA-co-TMCC) backbone. The number average molar mass of P(LA-co-TMCC)-g-PEG was calculated according to equation (1):

\[ M_n(P(LA-co-TMCC)-g-PEG) = M_n(\text{backbone}) + (M_n(PEG) \times \text{# of PEG per copolymer backbone}) \]  

Thus, the P(LA\textsubscript{130}-co-TMCC\textsubscript{10})-g-PEG\textsubscript{10kDa}-furan had a number average molar mass of 18.2 kDa and the P(LA\textsubscript{130}-co-TMCC\textsubscript{10})-g-PEG\textsubscript{10kDa}-azide had a slightly larger number average molar mass of 20.2 kDa.

### 2.4.2 Micellization

**Estimation of the Aggregation Number of Micelles.** The micelles formed from P(LA-co-TMCC)-g-PEG-X have an average diameter of 96.2 nm and distribution is 0.166 as determined by dynamic light scattering. The aggregation number (N\textsubscript{agg}) was estimated for these micelles, as previously described\cite{10}, according to equation (2), where k is a scaling factor between the radius of the polymeric micelle, R\textsubscript{micelle}, and the aggregation number.

\[ N_{agg} = kR_{micelle}^2 \]  

For these micelles, with average diameter of 96.2 nm, the aggregation number is approximately 3500.

### 2.4.3 Conjugation of Trastuzumab and FLAG Peptides to the Nanoparticles

**Coupling of Alexa 488-DBCO and Alexa 647-MI on Dual-Functional Micelles by Click Chemistry.** We first verified the coupling reactions in our system using two functionalized dye
molecules, Alexa 647-MI and Alexa 488-DBCO, as surrogate ligands for the trastuzumab and FLAG peptide, respectively. The NH$_2$-PEG-furan was reacted with Alexa 647-MI to demonstrate the DA reaction. In order to verify the specificity of the two click reactions with our polymeric micelles, we tested multiple combinations of reactants and controls (Table 2.1). The first reaction, (a), showed efficient binding from sequential click reactions between nanoparticle micelles and Alexa 647-MI followed by Alexa 488-DBCO. Three controls demonstrated the selectivity of the DA and SPAAC reactions: the functional groups of either the Alexa 647-MI, Alexa 488-DBCO, or both, were quenched prior to the click chemical reaction with dual functionalized micelles that had an equal number of azide and furan groups.

For one control (b), both dye molecules were quenched prior to the double click reaction. The results confirmed that there was little non-specific binding between the dyes and micelles. When only the DBCO was quenched (c), we observed the selective reactivity between the maleimide moiety and available furan groups on the micelle: the average number of Alexa 647-MI groups that bound to each micelle was similar to reaction (a). By contrast, when only the maleimide was quenched (d), the number of Alexa 488-DBCO which bound to the micelle was greater than reaction (a), where the available furan sites were occupied by the Alexa 647-MI, indicating that the DBCO reacted with both azide and furan groups.

<table>
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<th>Average R$_1$-MI/micelle</th>
<th>Average R$_2$-DBCO/micelle</th>
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<td>(a) Micelles + R$_1$-MI + R$_2$-DBCO</td>
<td>88 ± 11</td>
<td>270 ± 28</td>
</tr>
<tr>
<td>(b) Micelles + R$_1$-MI (quenched) + R$_2$-DBCO (quenched)</td>
<td>11 ± 7</td>
<td>18 ± 18</td>
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<tr>
<td>(c) Micelles + R$_1$-MI + R$_2$-DBCO (quenched)</td>
<td>110 ± 42</td>
<td>14 ± 11</td>
</tr>
<tr>
<td>(d) Micelles + R$_1$-MI (quenched) + R$_2$-DBCO</td>
<td>18 ± 11</td>
<td>510 ± 7</td>
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</tbody>
</table>
We confirmed that the conjugations were due to DA and SPAAC reactions with two additional control reactions: (1) between a furan-only micelle and Alexa 647-MI; and (2) between an azide-only micelle and Alexa 488-DBCO. The DA reaction with the furan micelle and Alexa 488-MI showed an average of 1 dye for every 83 polymer chains (i.e. 1:83 dye/polymer). When the Alexa 488-MI was first quenched with either furfurylamine or cysteine prior to reaction with furan-micelles, the average number of dyes per polymer decreased significantly to 1:1200 dye/polymer chains for furfurylamine pre-quenched controls and 1:8200 dye/polymer chains for cysteine pre-quenched controls. These demonstrate that there is little non-specific binding between Alexa 488 and our polymeric micelles, which is consistent with previous reports of the DA reaction between furan and maleimide groups, as confirmed by FTIR [20].

For the SPAAC reaction with the azide micelle and Alexa 488-DBCO, we detected an average of 1 dye for every 13 polymer chains (i.e 1:13 dye/polymer). When the DBCO was first quenched with excess 11-azido-3,6,9-trioxaundecan-1-amine, the number of dyes per polymer dropped to an average of 1:200 dye/polymer chains, demonstrating that there was little non-specific binding between Alexa 488 and the polymeric micelles.

**Coupling of FLAG-DBCO and Trastuzumab-SMCC on Dual-Functional Micelles by Click Chemistry.** To demonstrate the functional utility of a dual-labelled polymeric micelle, we performed sequential click reactions with the SMCC-functionalized antibody (trastuzumab-SMCC) and DBCO-functionalized peptide (FLAG-DBCO). Trastuzumab is a humanized antibody that binds human epidermal growth factor receptor 2 (HER2). A FLAG peptide was selected as a simple, model peptide sequence that can be recognized by an antibody. We chose SKOV-3luc cells, a human ovarian cancer cell line that overexpresses HER2, as a target to verify cell binding of the dual-functionalized micelles, through anti-HER2 and HER2 interactions. After the double-click reaction described above, micelle constructs were separated from free, unreacted ligands by size-exclusion FPLC. Micelle elution peaks were completely resolved from unreacted material (Figure 2.3)
Figure 2.3 FPLC purification of nanoparticles with trastuzumab and FLAG peptide. Nanoparticles conjugated with trastuzumab and FLAG were separated from the unreacted moieties. Fractions between 8 and 12 mL were incubated with SKOV-3luc cells to observe cell uptake.

Cells were treated with fluorescently-labeled antibodies against trastuzumab/FLAG to reveal the cellular localization of the antibody and peptide. Fluorescence images show trastuzumab and FLAG co-localized on the cell surface and inside the cells (Figure 2.4). Cells treated with non-functionalized micelles showed no uptake (Appendix Figure A 8), thereby demonstrating receptor mediated cell targeting. If trastuzumab was denatured or was not accessible on the micelle surface, no binding would occur. Likewise, positive staining for the FLAG peptide indicated that the peptide remained intact and accessible on the micelle surface.
Figure 2.4 Confocal images of SKOV-3luc cells. (a) Cell nuclei are stained with DAPI (blue); (b) trastuzumab is fluorescently-tagged with Alexa Fluor 488 (green); (c) FLAG peptide is fluorescently-tagged with Alexa Fluor 647 (purple); and (d) Overlay of all channels showed cell nuclei surrounded by trastuzumab/FLAG-tagged micelles.

2.5 Discussion

Polymeric nanoparticle micelle chemical modifications are limited to aqueous reaction conditions, yet many of these involve cytotoxic catalysts or result in undesirable side reactions[12]. The dual functional micelles synthesized herein provide a platform for facile orthogonal conjugation chemistry, resulting in clean and versatile ligand modification. By forming the micelles prior to the conjugation reaction and using aqueous-based chemistry, we are able to preserve the native structure and activity of the ligands and micelles. Furthermore, a single purification step after ligand bonding is beneficial for maximizing yields and maintaining micelle stability.

Interestingly, we observed a difference in the reactivity of Diels Alder and azide-alkyne click chemistries. When the dual functionalized micelles were reacted sequentially with MI and then
DBCO, more DBCO conjugated to the micelle than MI. This was also observed for micelles with a single functional group: the SPAAC reaction between the DBCO and azide functionalized micelles was more efficient than the DA reaction between the MI and furan micelle. While SPAAC reactions can be completed at pH 7.4, DA reactions between the furan and SMCC moiety are most efficient at acidic pH. Hydrolysis of the maleimide group will form maleamic acid that will not react with the furan [21].

Using either click reaction, not all functional groups on the micelle were reacted - only approximately 7.7% of azide groups and 1.2% of furan groups reacted on average. Although the functional groups are on the PEG termini, they are not all accessible at the micelle surface for reaction, possibly because the terminal groups are buried within the flexible PEG chain. Importantly, the click conjugated ligands remain active to facilitate interactions with cell surface receptors and antibody probes.

For the double labelling to be most effective, the DA reaction had to be completed prior to the SPAAC reaction because the DBCO was able to bind to both azide and furan whereas maleimide only reacted with furan (see Appendix Table A1 for a summary of possible reactions). Notwithstanding this requirement for the sequential DA and then SPAAC reactions, importantly, both reactions were carried out in the same vessel and only one purification step was required after modification.

The use of two reactive groups improves control of the chemical modifications and composition. In a micelle system with multiple ligands binding to a single reactive group, the reactions are competitive. With dual functional micelles, each ligand reacts independently with a specific receptor, eliminating competition. In addition, multiple functionalities allow for the most effective reaction partners to be selected, based on the properties of each ligand. Importantly, the aqueous-based coupling chemistries used in our system result in bioactive functionalized micelles: trastuzumab interacts with HER2 receptors expressed on SKOV-3 cells and FLAG provides a marker of the micelle for cell trafficking. Since there is, on average, less than one functional group available on each polymer chain, if the micelles were to dissociate, the FLAG peptides would not co-localize with trastuzumab labeled micelles. Thus, the co-localization of
trastuzumab and FLAG in the cells suggest that the micelles remain intact after receptor-mediated endocytosis, which is consistent with previous reports.

Nanoparticle micelles have shown potential as drug delivery vehicles due to their tuneable physical properties. One of the challenges with modifying micelles is controlling surface chemistry while maintaining micelle stability [4, 22]. In our system, the aqueous-based click reactions are completed after the micelles have formed, preserving the size of the micelles and the bioactivity of the conjugated ligands[4]. The approach of coupling multiple ligands onto a single micelle in a simple manner provides the versatility needed to achieve: selective cellular and subcellular targeting, cell targeting with therapeutic delivery, or facilitated co-delivery of therapeutic agents with diagnostic probes.

2.6 Conclusion

Using two independent, sequential click reactions, we were able to prepare dual-functional micelles. Sequential reactions with maleimide- and dibenzylcyclooctyne- Alexa dyes allowed us to quantify the conjugation reactions and demonstrate the specificity between the DA and SPAAC reactions. To further demonstrate applicability of the dual-functional micelle, we conjugated trastuzumab antibodies and FLAG peptides onto the self-assembled, polymeric micelles by DA and SPAAC reactions, respectively. The ability to selectively bind two different moieties onto the same micelle results in a platform for future combination strategies, such as multi-functional micelles of therapeutic moieties, diagnostic agents, targeting ligands and cell penetrating/trafficking peptides.

2.7 References


3. Delivery of SiRNA and Antisense Oligonucleotides using Dual Functional Polymeric Immuno-nanoparticles*

*This chapter comprises a paper submitted to Angewandte Chemie International.


Chan and Deleavey are co-first authors. Deleavey synthesized and characterized the oligonucleotides. Chan completed all other experiments outlined in this chapter and contributed to the discussion relating to siRNA and AON delivery using nanoparticles and Lipofectamine. The manuscript was written by Chan and Deleavey with input from all authors.

3.1 Abstract

Efficient and targeted cellular delivery of gene silencing oligonucleotides (small interfering RNAs (siRNAs), antisense oligonucleotides (AONs)) is a major challenge facing oligonucleotide-based therapeutics. The majority of current delivery strategies employ either conjugated ligands or oligonucleotide encapsulation within delivery vehicles to facilitate cellular uptake. Chemical modification of the oligonucleotides (ONs) themselves can improve potency and duration of activity, usually as a result of improved nuclease resistance. Here, dual functionalized polymeric immuno-nanoparticles have allowed formation of nucleic acid-functionalized polymeric micelles enabling antibody-targeted delivery of siRNAs or AONs for gene silencing. ONs are carried on the exterior PEG corona of the functionalized nanoparticles, and results show that phosphorothioate (PS), 2′F-ANA, and 2′F-RNA backbone chemical modifications improve siRNA and AON potency and duration of activity. Gene silencing potencies of nanoparticle-delivered nucleic acids were comparable to those observed with Lipofectamine transfections.

3.2 Introduction

RNA interference (RNAi) and antisense gene silencing strategies are ON-based therapeutic approaches that utilize small interfering RNAs (siRNAs) or antisense oligonucleotides (AONs), respectively, to silence the expression of a target gene through sequence-specific mRNA knockdown[1-3]. Both strategies feature excellent potency and specificity once cellular uptake can be achieved. Unfortunately, potential nucleic acid therapeutics are disadvantaged by poor nuclease stability leading to rapid degradation, poor unassisted cellular uptake, and unintended side-effects from “off-target” silencing[4] and immunostimulation [5]. Many of these obstacles are addressed through the use of effective new delivery technologies and chemical modifications to the ON backbones to prevent nuclease degradation and unwanted side effects [1, 3, 6, 7].
2′-Deoxy-2′-fluoroarabino nucleic acid (2′F-ANA) has proven a particularly useful chemical modification in both siRNA and AON gene silencing applications[7-13]. In AON applications, when combined with the phosphorothioate (PS) backbone modification [14-16], 2′F-ANA enhances nuclease stability[7, 8], is fully compatible with RNase H-mediated cleavage[7, 17], and can improve binding stability, duration of activity, and potency [7, 12, 13]. 2′F-ANA modified PS-AONs can also function in gyomotic cellular delivery, in which unassisted AON cellular uptake can be achieved in certain cell lines at high concentrations and extended incubation time[18]. In siRNA, 2′F-ANA enhances siRNA nuclease stability [9, 10], can be readily incorporated in the siRNA passenger strand[9, 10], and can be used in combination with the rigid North-type nucleoside analogues 2′F-RNA and locked nucleic acid (LNA) to create fully modified siRNAs with improved potency, reduced immunostimulatory properties, and a thermodynamic bias for antisense strand RISC loading [10].

ON delivery methods[19-21] typically employ nucleic acid conjugates or delivery vehicles to complex with ONs to facilitate uptake. Examples include liposomes [22, 23], cationic polymers[24-27], and polyvalent siRNA structures[28]. ON encapsulation typically requires polycation block copolymers to complex with the negatively charged siRNA[11, 26, 29]. Lipofectamine is a widely used transfection reagent in cell culture; however, dose dependent toxicity[22] has been observed, and delivery by cationic polymers is non-specific and requires a mechanism for endosomal escape.

Polymeric micelles with controllable size and material properties can be modified to achieve active tumor targeting for therapeutic applications[30]. As well, PEGylated surfaces have the ability to decrease opsinization of plasma proteins [31]. Although small molecule drugs are typically encapsulated within micelles for drug delivery, there are examples in which conjugation to the surface of NPs can be beneficial for achieving cellular uptake[32]. Therapeutics with low affinity for the hydrophobic NP core are good candidates for surface conjugation, since encapsulation strategies result in rapid diffusion out of the core[32]. The caveat for this strategy is the release of the therapeutic entity at the site of action. This can be achieved by designing linkers to be susceptible to the microenvironment[33-35] or endogenous proteins in the cell[29].

Here, the efficient cellular delivery of ONs conjugated to the polymeric NP shell of poly(δ,δ-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-graft-poly(ethylene glycol) (P(LA-co-TMCC)-g-PEG) NPs is demonstrated. Because the ONs are on the NP shell, delivery of natural and chemically modified siRNAs and AONs was investigated to observe possible improvements in nuclease resistance. Using orthogonal
click reactions (Diels-Alder and copper catalyzed azide-alkyne cycloadditions (CuAAC)), NPs were conjugated first with maleimide-modified antibodies, followed by 3’-alkyne-modified gene silencing ONs. ON sequences were designed to facilitate enzyme-mediated release upon cellular uptake. Results demonstrate that the combination of chemically modified ONs, P(LA-co-TMCC) micelles, and trastuzumab (Herceptin®) antibodies provides effective targeted gene silencing. All three components are required for cell-specific targeted ON delivery and gene silencing comparable to Lipofectamine. These strategies are amenable to the delivery of both siRNAs and AONs, as evidenced by knockdown of firefly luciferase expression from a pGL3 vector in SKOV-3luc cells (HER2+, luc+).

3.3 Materials and Methods

5-methyl-5-benzyloxy carbonyl-1,3-trimethylene carbonate (benzyl-protected TMCC, TMCC-Bn) was synthesized as previously reported [36]. 3,6-dimethyl-1,4-dioxane-2,5-dione and 1-(3,5-bis(trifluoromethyl)phenyl)-3-[(1R,2R)-(−)-2(dimethylamino) cyclohexyl] thiourea (Strem Chemicals, Newburyport, MA) were used following previous protocols to synthesize P(LA-co-TMCC) [37]. Boc-NH-PEG(10K)-NHS (Rapp Polymere, Tübingen, Germany) was modified as previously reported protocols for shown furan and azide-functional groups [36, 38]. N,N′-diisopropyl carbodiimide, N,N′-diisopropylethylamine and hydroxybenzotriazole (TRC, Toronto, ON) were used as received. The human ovary cancer cell line SKOV-3luc was purchased from Cell Biolabs, Inc (San Diego, CA). The Lipofectamine LTX® and Plus Reagent were purchased from Invitrogen (Burlington, ON) and the Luciferase Assay System was from Promega (Madison, WI). All solvents and reagents were purchased from Sigma-Aldrich and were used as received, unless otherwise noted.

The 1H NMR spectra were recorded at 400 MHz at room temperature using a Varian Mercury 400 spectrometer. The chemical shifts are in ppm. The molecular weights and polydispersity of P(LA-co-TMCC) were measured by gel permeation chromatography (GPC) in THF relative to polystyrene standards at room temperatures on a system with two-column sets (Viscotek GMIHR-H) and a triple detector array (TDA302) at a flow rate of 0.6 mL/min. Polymeric micelle size and distribution were measured by dynamic light scattering (DLS) using the Zetasizer Nano ZS system (Malvern, UK). The NH2-PEG-azide was characterized using the Perkin-Elmer FT-IR Spectrum 1000. The Sepharose CL-4B column was prepared by soaking the beads in distilled water overnight and packing the beads in a column (5 x 15 cm). The column was washed with distilled water for 1 h before use and the flow rate was determined by gravity. The Sephadex G-25 column was prepared using the same method, except PBS buffer (1x, pH 7.4) was used as the eluent and the beads were packed in a column (1.5 x 15 cm). FPLC of
the micelles used a Superdex 200 column. The column was washed with distilled water for 20 min and PBS buffer (1x, pH 7.4) for 20 min at a flow rate of 1.5 mL/min before use. Fluorescence measurements were completed with the Tecan Infinite M200 Pro fluorescent plate reader and absorbance was quantified using the NanoDrop Spectrophotometer. Luminescence was measured with the MicroLumat Plus LB96V (EG&G Berthold, Bad Wildbad, Germany).

3.3.1 Polymer Synthesis and Characterization

Synthesis of P(LA-co-TMCC), modification of NH$_2$-PEG-NHS for furan and azide functionalities and grafting of the PEG onto the polymer backbone, have all been previously shown [37]. Detailed characterization for the batches of polymer used in these experiments are shown in Chapter 2.

**Synthesis of (P(LA-co-TMCC)).** $^1$H NMR (CDCl$_3$, 400MHz): δ 1.22 (br s, CH$_3$ from TMCC), 1.59 (m, CH$_3$ from LA), 4.31 (br s, CH$_2$ from TMCC), 5.15 (m, CH from LA), 7.33 (m, benzyl) and 8.02 (m, pyrene).

**Synthesis of NH$_2$-PEG-azide.** Perkin-Elmer FT-IR Spectrum 1000: terminal azide group (2100 cm$^{-1}$), terminal amine (3389 cm$^{-1}$), C–H alkane (2888 cm$^{-1}$), C=O amide (1692 cm$^{-1}$), and C–O ether (1101 cm$^{-1}$).

**Synthesis of NH$_2$-PEG-furan.** $^1$H NMR (CDCl$_3$, 400 MHz): δ 1.44 (s, Boc CH$_3$), 2.32 (t, $J$=14 Hz , –CH$_2$–), 3.61 (m, –OCH$_2$CH$_2$–), 4.42 (s, –CH$_2$-furan), 6.22 (d, $J$= 3Hz, furan C-3), 6.30 (dd, $J$=5 Hz, furan C-4) and 7.34 (d, $J$=3 Hz, furan C-5).

**Synthesis of P(LA-co-TMCC)-g-PEG-X.** For X=azide, $^1$H NMR (CDCl$_3$, 400 MHz): δ 1.25 (br s, CH$_3$ from TMCC), 1.58 (m, CH$_3$ from LA), 3.59-3.69 (m, –OCH$_2$CH$_2$–), 4.35 (br s, CH$_2$ from TMCC) and 5.17 (m, CH from LA). For X= furan, $^1$H NMR (CDCl$_3$, 400 MHz): δ 1.22 (br s, CH$_3$ from TMCC), 1.56 (m, CH$_3$ from LA), 3.59-3.69 (m, –OCH$_2$CH$_2$–), 4.31 (br s, CH$_2$ from TMCC) and 5.15 (m, CH from LA).

**Table 3.1.** Characterization of poly (LA-co-TMCC) copolymer backbone

<table>
<thead>
<tr>
<th>Copolymer backbone</th>
<th>Mn (GPC)$^a$ (kDa)</th>
<th>Mw/Mn (GPC)$^a$</th>
<th>TMCC content (theoretical)$^b$ (mol%)</th>
<th>TMCC content (cal)$^c$ (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(LA-co-TMCC)</td>
<td>11.2</td>
<td>1.12</td>
<td>10</td>
<td>7.1</td>
</tr>
</tbody>
</table>

$^a$ Determined by GPC measurements in THF with poly(styrene) standards;
b At quantitative monomer conversion
c Determined using $^1$H NMR measurements in CDCl$_3$. Calculated by comparing the integral of the methylene groups in TMCC (4.31 ppm) with the methyl groups of LA (5.15 ppm)

<table>
<thead>
<tr>
<th>Graft copolymer</th>
<th>Average PEG$^a$ grafts/copolymer backbone</th>
<th>Mn ($^1$H NMR) (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(LA-co-TMCC)-g-PEG-azide</td>
<td>0.9</td>
<td>18.2</td>
</tr>
<tr>
<td>P(LA-co-TMCC)-g-PEG-furan</td>
<td>0.7</td>
<td>20.2</td>
</tr>
</tbody>
</table>

$^a$ PEG: Mn= 10kDa from manufacturer

$^b$ Estimated from $^1$H NMR and calculated by comparing the integral of the methylene groups of the PEG (3.61-3.64 ppm) and the methylene groups of TMCC (4.31 ppm)

### 3.3.2 Oligonucleotide Synthesis and Characterization

All oligonucleotides were synthesized by Glen Deleavey; a PhD student working in Dr. Masad Damha’s Lab at McGill University. Details relating to the procedure and characterization of the oligonucleotides are in Appendix B.

### 3.3.3 Nanoparticle Formation and Characterization

**Micelle formation.** The micelles were prepared by co-self-assembly of P(LA-co-TMCC)-g-PEG-furan and P(LA-co-TMCC)-g-PEG-azide by membrane dialysis, as previously shown for single functionalized chains [39]. P(LA-co-TMCC)-g-PEG-furan (2.5 mg, 18.2 kDa) and P(LA-co-TMCC)-g-PEG-azide (1.3 mg, 20.2 kDa) were combined to form the dual-functional micelles with an average ratio of 1:2 furan to azide functional groups available. Dynamic light scattering (DLS) measurements of the micellar nanoparticles determined a hydrodynamic diameter of 63.35 nm and polydispersity of 0.186.

**Double-click reaction.** P(LA-co-TMCC)-g-PEG micelles with azide and furan functional groups (680 nmol, 200 μM) were sequentially reacted with maleimide and alkyne-functional moieties. First, trastuzumab-SMCC (250 nmol, 80 mg/mL) was added at room temperature with MES buffer (1mL, 100 mM, pH 5.5) for 24 h. The solution was dialyzed overnight against PBS buffer (1x, pH 7.4) using 2 kDa MWCO dialysis tubing. The buffer was changed every 2 h for the first 6 h. The trastuzumab-nanoparticles were concentrated to 100 μM using centrifuge concentrators at 1600 rpm.
The trastuzumab-nanoparticles (50 nmol, 40 µM) were transferred to a glass vial to react with the alkyne-modified oligonucleotide (35 nmol, 30 µM), copper sulphate (CuSO₄, 40 µM), sodium ascorbate (NaAsc, 113 µM) and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 60 µM) in 3% methanol at room temperature for 24 h. Micelles were purified by FPLC (GE Healthcare AKTA Purifier) monitoring absorbance at 215 and 280 nm. Free oligonucleotides were eluted at 7-10 mL and purified nanoparticles were eluted at 15-20 mL. Fluorescence of the Cy5 (ex. 640 nm, em. 675 nm) was used to quantify the conjugation of the oligonucleotides. Absorbance at 260 nm was used to determine the micelle concentration after subtracting absorbance contributions from the oligonucleotides. The aggregation number of the micelles have previously been calculated to be 3500. There was approximately 30 ± 10 trastuzumab and 120 ± 40 siRNA molecules per micelle (Table 3.3).

<table>
<thead>
<tr>
<th></th>
<th>Number of oligonucleotides per micelle</th>
<th>% of azide groups reacted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-siRNA</td>
<td>165</td>
<td>9.2</td>
</tr>
<tr>
<td>Mod-Di-siRNA</td>
<td>95</td>
<td>5.3</td>
</tr>
<tr>
<td>D-AON</td>
<td>145</td>
<td>8.1</td>
</tr>
<tr>
<td>Mod-D-AON</td>
<td>90</td>
<td>5.0</td>
</tr>
</tbody>
</table>

### 3.3.4 Luciferase Characterization

**Cell Culture with oligonucleotide-nanoparticles.** SKOV-3luc cells were cultured in McCoy’s 5A media containing 10% FBS and 1% penicillin/streptomycin under standard culture conditions (37°C, 5% CO₂, 100% humidity). Cells were seeded in 96 well plates at 7000 cells/well and allowed to adhere overnight. The cell media was replaced with serum free media and dual functionalized micelles having both trastuzumab and oligonucleotides were added. The cells were incubated for 24h. For each type of nanoparticle, 8 replicates were prepared. Untreated cells were prepared simultaneously as a baseline for comparison. Controls of unmodified nanoparticles and trastuzumab-nanoparticles were used to observe the effect of the nanoparticles on luciferase activity. As well, oligonucleotides without nanoparticles were delivered using a cationic lipid delivery system, Lipofectamine, to confirm the effectiveness of the AON and siRNA sequences. Scrambled sequences of oligonucleotides were also included as a negative control.

**Gene silencing response to nanoparticles.** The cells were treated with three concentrations of AON or siRNA. After the coupling reaction with the oligonucleotides, the number of AON or siRNA per
nps were calculated. The nanoparticles were diluted with unmodified immuno-nanoparticles for a final nanoparticle concentration of 1.7 µM and AON or siRNA concentrations of 56 nM, 14 nM or 2.8 nM (100 µL final volume in each well). Concentrations of siRNA were based on protocols targeting firefly luciferase in HeLa X1/5 cells [10]. Each well was treated with 50 µL of nanoparticles and an additional 50 µL of serum free McCoy’s 5A media was added. For the positive controls, the AON or siRNA (5 µL) were complexed with the Lipofectamine (10 µL) and Plus reagent (10 µL) for 30 min, then added to the cells. β-galactosidase was co-transfected with the free siRNA so the luminescence readings could be normalized to account for transfection efficiency. The final volume of cell media was adjusted to 100 µL using serum free media.

**Persistence of gene silencing duration.** Following the procedure outlined above, the cells were treated with AON- or siRNA-nanoparticles for 1, 3, 4 and 7 days. 7 x 10^5 cells were seeded into T-25 flasks and allowed to adhere overnight. The cells were treated with 5 mL of serum free media and 5 mL of nanoparticles (final concentrations of 1.7 µM nanoparticles, 56 nM AON or siRNA) for 5 h of transfection. Then the cell media was replaced with complete media. The cells were split when confluent and cell media was replaced every 2 days. 5 repeats of each sample were prepared. At each of the time points, the luciferase activity was measured by the luciferase cell assay.

**Luciferase cell assay.** The cell media was removed and each well was washed three times using PBS buffer (1x, pH 7.4) before adding 20 µL of lysis buffer. After 20 min, the 10 µL of lysate was transferred to a white bottom 96 well plate. Following the Promega protocol, the luciferase assay reagent was prepared. The auto-injector was used to add 25 µL of luciferase reagent to each well and measure luminescence.

For each sample where β-galactosidase was co-transfected, another 10 µL of lysate was transferred to a clear 96 well plate. A fresh mixture of Na₂HPO₄•7H₂O (81 mM), NaH₂PO₄•H₂O (18 mM), MgCl₂ (2.3 mM), β-mercaptoethanol (49 mM) and ortho-nitrophenyl-β-galactoside (ONPG, 3.3 mM) was prepared. 90 µL of the ONPG mixture was added to the lysate and incubated for 30 min. The absorbance of the o-nitrophenol formed was measured by the Tecan plate reader at 420 nm.

**3.4 Results**
P(LA-co-TMCC)-g-PEG-X micelles (D = 63.35 ± 6.95 nm, PDI= 0.186 ± 0.03) with X as both furan and azide reactive groups on the PEG-X corona were sequentially reacted with trastuzumab-SMCC and
alkyne-functionalized ONs, respectively, as shown in Figure 3.1. For siRNA delivery, nanoparticles were prepared using Dicer substrate [40] 27nt versions of both native unmodified siRNAs (Di-siRNA) and 2′F-ANA/2′F-RNA-modified dicer substrate siRNAs (Mod-Di-siRNA) corresponding to potent designs observed in Deleavey et al. (2010) (sequences and modifications are outlined in Figure 3.2). Negative control sequences consisted of non-targeting dicer substrate (i.e. scrambled) siRNAs: unmodified (Sc-Di-siRNA) and modified (Mod-Sc-Di-siRNA) (Figure 3.2). For comparisons to standard ON transfection methods, the activity of 21-mer siRNAs (siRNA) and modified siRNAs (Mod-siRNA) delivered by Lipofectamine were measured. siRNA sense strands were modified with an alkyne functionality at the 3′ terminus for reaction with the azide groups on the micelle via CuAAC reactions [41]. A Cy5 label was added to the 5′ end of the sense strands for quantification, revealing an average of 120 ± 40 siRNA molecules and 30 ± 10 antibodies per micelle.

Figure 3.1 siRNA and AON Delivery Strategies. (a) siRNA delivery constructs. Immuno-NPs carry dicer-substrate type siRNA constructs. Unmodified dicer-substrate siRNAs (Di-siRNA) are shown on top; 2′F-ANA/2′F-RNA modified Di-siRNAs (Mod-Di-siRNA) corresponding to active designs from Deleavey et al. (2010) are shown below. (b) AON delivery constructs. Immuno-NPs carry AONs annealed to RNA complement strands. Both a PS-DNA AON (D-AON, top) and a PS-2′F-ANA gapmer AON (Mod-D-AON, bottom) were utilized. (c) NPs were dual functional, allowing antibody attachment through Diels
Alder cycloaddition, and ON attachment via a CuAAC reaction. (d) Chemically modified ON backbones used in these siRNA and AON constructs.

SKOV3-luc cells expressing HER2 and firefly luciferase were treated with the siRNA- and trastuzumab-functionalized immuno-nanoparticles (Her-NPs for Herceptin®-Nanoparticles). Knockdown was quantified by measuring luminescence, where a lower luminescent signal corresponds to greater gene knockdown (Figure 2). The 2'F-ANA/2'F-RNA-modified Mod-Di-siRNA was equally effective when delivered via NPs or Lipofectamine; however, the unmodified Di-siRNA showed somewhat greater potency using Lipofectamine delivery (p<0.05), perhaps due to nuclease degradation and underscoring the importance of Di-siRNA modification. When comparing NP delivery of modified and unmodified siRNA, Mod-Di-siRNA demonstrated greater knockdown (p<0.05) whereas with Lipofectamine transfection, there was no statistical difference (p>0.05). All statistics were completed with a one-way ANOVA, unless otherwise noted. When 56 nM and 14 nM of a targeting siRNA sequence were delivered, a knockdown effect was observed.

Among non-targeting negative controls, there was no statistical difference in luciferase levels regardless of treatment type (unmodified/modified ON, Her-NP or LipA) or concentration (p>0.05, two-way ANOVA). Targeting sequences resulted in lower luciferase levels when compared to relevant non-targeting sequences at the same concentration (p≤0.012) with the exception of 2.8 nM of siRNA delivered by Lipofectamine (p=0.213) and 2.8 nM of Di-siRNA Her-NP. While at 2.8 nM, the siRNA is near the minimum concentration required to see a significant knockdown effect, the knockdown observed with modified Mod-Di-siRNA (at 2.8 nM), by both immuno-nanoparticle and Lipofectamine, demonstrates its enhanced stability and potency.
**Figure 3.2** Modified and unmodified siRNA (sequences in Table) were delivered to SKOV3-luc cells by conjugation to immuno-nanoparticles (Her-NP). Positive controls were prepared by delivering 21mer siRNA with Lipofectamine (LipA), and negative controls used non-targeting, scrambled (Sc) sequences. Luciferase levels were normalized to untreated cells. Each treatment was repeated 8 times (mean ± standard deviation plotted). Bars with the same lower case letter denote no statistical significance between pairs letters (one-way ANOVA, p>0.05). Bars with different letters are statistically different (one-way ANOVA, p<0.01). Statistical significance (one-way ANOVA) is denoted by * (p≤0.05), ** (p≤0.01), *** (p≤0.001). Scrambled sequences were compared to active sequences by a two-way ANOVA and
showed a statistical difference (p<0.05). Legend for sequences: RNA, 2′F-ANA, 2′F-RNA, Cy5 = Cyanine 5, alkyne = 3′ alkyne modification, p = 5′ phosphate.

SKOV3-luc cells were also treated with AON-functionalized immuno-nanoparticles and tested to luciferase knockdown relative to controls. In this construct, PS-DNA (D-AON) and PS-2′F-ANA gapmer (Mod-D-AON) were annealed to complementary RNA strands, which were modified with 3′ alkyne and 5′ Cy5 functionalities, to allow for click-coupling to azide-functionalized NPs and subsequent quantification. Upon cellular uptake, ON duplexes may be substrates for RNase H, resulting in RNA strand cleavage and release of active AONs. Firefly luciferase gene silencing experiments using PS-DNA and PS-2′F-ANA AONs with Lipofectamine and Her-NP mediated delivery are shown in Figure 3.3. Lipofectamine transfections were completed with the RNA-AON duplex, which showed similar activity to the single strand AONs. The D-AON and Mod-D-AON sequences showed greater knockdown potency using Lipofectamine delivery (p<0.001) when compared to the Her-NPs; however, at the lowest AON concentration (2.8 nM), there was no statistical difference between the two methods of delivery for Mod-D-AON and D-AON (p>0.05).

Immuno-nanoparticle delivery of Mod-D-AON was significantly more potent than that with D-AON (p<0.001), underscoring the importance of stability achieved with modification; however, this difference was not observed with Lipofectamine delivery (p>0.05). Both D-AON and Mod-D-AON were more effective at luciferase knockdown with Lipofectamine vs. NP delivery. The diminution of D-AON (PS-DNA) potency, and retention of potency for the Mod-D-AON (PS-2′F-ANA), suggests that nuclease degradation prior to mRNA cleavage may play a role in determining gene silencing success when AONs are delivered on the surface of these Her-NPs, although uptake and AON release efficiency may also contribute to the observed differences.

Luciferase levels reflected a gene silencing effect to AON treatments. In all cases, active AONs showed greater gene knockdown/lower luciferase levels when compared to equivalent treatments using non-targeting PS-DNA (Sc-D-AON) or PS-2′F-ANA gapmer (Sc-Mod-D-AON) AONs, with the exception of the 2.8 nM concentrations of AON delivered by either Lipofectamine or Her-NPs (p>0.05). At 2 nM, the AON is likely outside the effective concentration range. There is no statistical difference between delivery methods and scrambled sequences at this concentration.
**Figure 3.3** Mod-D-AON and unmodified D-AON were delivered to SKOV3-luc cells by immuno-nanoparticles (Her-NP). Positive controls were prepared by delivery with Lipofectamine (LipA), and negative controls used scrambled sequences. Luciferase levels were measured by luminescence and normalized to untreated cells. Each treatment was repeated 8 times (mean ± standard deviation reported). The same lower case letter denotes no statistical significance between pairs (one-way ANOVA, p>0.05). Bars with different letters are statistically different (one-way ANOVA, p ≤ 0.05). Statistical significance (one-way ANOVA) is denoted by * (p≤0.05), ** (p≤0.01), *** (p≤0.001). Scrambled sequences were compared to active sequences by a two-way ANOVA and showed a statistical difference (p<0.05).

To understand the role of the nanoparticle, siRNA and trastuzumab to the gene knockdown observed with immuno-nanoparticle delivery of siRNA, SKOV-3luc cells were treated with siRNA delivered by the Her-NP system vs. NPs alone, NPs derivatized with only trastuzumab or siRNA (not both), and a mixture of Her-NPs and siRNA (without covalent attachment of the siRNAs) (Figure 3.4), and measured average luciferase levels of were observed. In all cases, luciferase levels of these controls were 0.891 or greater, which is significantly greater than the 0.416 ± 0.087 measured for Mod-Di-siRNA Her-NPs (p<0.001).
demonstrating the importance of these three components in achieving a transfection-competent gene silencing NP complex. There was no statistical difference among the controls (p>0.05).

\[
\begin{align*}
\text{Relative Luciferase Level} & \quad \text{Di-siRNA} & \quad \text{Her-NP} & \quad \text{NP} & \quad \text{Her-NP} & \quad \text{Di-siRNA NP} & \quad \text{Di-siRNA mixed with Her-NP} \\
0 & 0.2 & 0.4 & 0.6 & 0.8 & 1 & 1.2
\end{align*}
\]

**Figure 3.4** Luciferase levels for NP controls demonstrate targeting effects of the immuno-nanoparticle. From left to right, treatments consisted of unmodified Di-siRNA functionalized Her-NPs, NP alone, Her-NP alone, Di-siRNA functionalized NPs, or Di-siRNA mixed (but not chemically conjugated) with Her-NPs. In all treatments, the polymer concentration was maintained at 1.7 µM and the siRNA concentration was 14 nM. 5 repeats were collected for each control group, and 8 repeats for the siRNA Her-NP (mean ± standard deviation shown).

Chemical modification with phosphorothioates, 2’F-ANAs, and 2’F-RNAs is expected to not only protect AONs and siRNAs from degradation during cellular delivery, but also extend the duration of gene silencing activity. To observe differences in duration of effect, NP-delivered siRNA activity was monitored over 7 days (Figure 3.5). Both modified and unmodified siRNAs showed a persistence of effect for up to 4 days, after which the cells were split due to cell confluency, thereby possibly diluting out the knockdown. The modified siRNA and siRNA delivered with immuno-nanoparticles demonstrated greater knockdown effect relative to scrambled controls and the modified siRNA-Her-NP showed the greatest knockdown at all time points until full rebound at day 7 (p≤0.02).
Figure 3.5. Persistence study for siRNA NPs at 56nM concentration. SKOV-3luc cells were treated with siRNA-carrying Her-NPs for 24h. Luciferase levels were measured at 1, 3, 4 and 7 day intervals following treatment. The knockdown effect for siRNA-NPs was persistent for up to 4 days. Confluent cells were split at day 4. By day 7, all treatment groups ceased to report a gene silencing effect. Each sample was repeated 5 times.

Differences in the duration of activity for D-AON and Mod-D-AON AONs delivered via NPs were also observed. Compared to the D-AON NPs and NPs with non-targeting sequences, the Mod-D-AON NPs displayed a persistent effect (Figure 3.6). Gene knockdown was observed for up to 4 days after which the cells were split. At day 7, luciferase levels returned to untreated levels.

The cells were split at day 4 due to cell confluency. The modified siRNA demonstrated a greater knockdown effect at all time points until full rebound at day 7 (p≤0.02). Non-targeting controls did not affect expression levels.
Figure 3.6 Persistence study for AON NPs at 56nM concentration. SKOV-3luc cells were treated with AON-modified Her-NPs for 24h. Luciferase levels were measured after 1, 3, 4 and 7 days. The knockdown effect for Mod-D-AON NPs was persistent for up to 4 days. Confluent cells were split at day 4. By day 7, all treatment groups all treatment groups ceased to report a gene silencing effect. Each sample was repeated 5 times (mean ± standard deviation shown).

3.5 Discussion

Both unmodified and 2′F-ANA/2′F-RNA-modified Di-siRNAs were effectively delivered by Her-NPs in an antibody-targeted fashion. Modified Di-siRNA showed similar levels of activity when delivered by either Lipofectamine or Her-NPs, demonstrating that Her-NPs covalently modified with Mod-Di-siRNAs are equipotent to Lipofectamine delivery and cell-specific. By achieving equipotency without the toxicity often associated with Lipofectamine, the Her-NP-Mod-Di-siRNA provides a more desirable siRNA delivery vehicle. This delivery strategy requires a combination of 3 components to achieve effective ON delivery: a biodegradable[36] polymer-based nanoparticle core, antibody functionalization for active targeting, and stabilizing ON chemical modifications (PS, 2′F-ANA, 2′F-RNA). Furthermore, chemical modification of siRNAs with 2′F-ANA and 2′F-RNA has been shown to reduce siRNA-triggered immunostimulation[10], which can be particularly important when siRNAs are introduced to cells via a delivery vehicle[5].

AONs can also be delivered using the Her-NP strategy, although Her-NP-mediated delivery was only successful with the PS-2′F-ANA gapmer AON, and not with a PS-DNA AON. Even with the PS-2′F-ANA AON, gene silencing levels were slightly reduced compared with Lipofectamine-mediated delivery (as expected, PS-DNA and PS-2′F-ANA AONs both effected potent silencing when delivered by
Lipofectamine). Results indicate that the reduction in potency of the PS-2′F-ANA AONs when delivered by Her-NPs was only approximately 2-fold compared with Lipofectamine, indicating that Her-NP-mediated delivery can maintain AON potency within a very reasonable concentration range when chemically modified AONs are used. On the Her-NPs, AONs are partially protected by the PEG corona, but exposure to nucleases could cause degradation and/or premature AON release prior to uptake. In contrast, Lipofectamine complexes with the negatively charged ONs, protecting them from nucleases in addition to facilitating uptake. Kim, et al. (2010) have shown that liposomes and PEGylated liposomes can efficiently protect siRNA from RNase degradation[42]. These differences could help explain the observed differences in gene silencing potency between PS-DNA and PS-2′F-ANA AONs.

The antibody-targeted delivery strategy implemented here is particularly interesting for the delivery of ON therapeutics, where tissue-specific uptake is highly desirable. Studies on the pharmacokinetics and tumor localization of siRNA delivered by polycationic carriers showed little difference versus unformulated siRNA [43]. When siRNA was delivered with targeted NPs to mice, Bartlett et. al saw evidence that more functional siRNA could be delivered to the tumor, even though non-targeted NPs also accumulated in the tumor microenvironment to a similar extent [44]. This underscores the importance of targeted delivery to the cell and not just the tumour. Based on previous studies where Her-NPs enter the cell via receptor-mediated endocytosis [32, 36, 45], ON immuno-nanoparticles also likely accumulate in the cell cytoplasm after endosomal escape, thereby achieving target specificity through a specific cell surface receptor[32, 46, 47]. This cell-specific delivery differs from that of Lipofectamine which complexes with the ONs to overcome the electrostatic repulsion of the cell surface, and induces local destabilization of the plasma membrane allowing translocation into the cytoplasm in a non-specific fashion [23, 48]. The Her-NP delivery strategy we have identified features active antibody-targeting, and gene silencing potencies comparable with Lipofectamine delivery but with greater cell specificity and without the associated cytotoxicity.

Transfected ONs are internalized by endocytosis into early and late endosomes, and subsequently lysosomes. While the mechanism of endosomal escape by Her-NP delivered siRNAs and AONs is still under investigation, potential explanations include lysosomal degradation or endo-lysosomal escape[18, 49]. Poly(δ,1-lactide-co-glycolide) (PLGA) NPs have a negative charge in physiological pH, but acquire a positive charge in the acidic endo-lysosomal compartments and escape with a similar mechanism to cationic lipids [49]. Our nanoparticles have a similar backbone comprised primarily of lactide monomers and net negative charge [37] and may follow a similar mechanism. Endosomal escape of the lipoplex is better understood: the complex destabilizes the endosomal membrane by switching the anionic lipids in
the membrane with the cationic lipid. As a consequence, AONs are translocated into the cytoplasm [50, 51].

It is hypothesized that active siRNA is released from the Her-NPs when Dicer processes the dicer substrate siRNA sequences [51, 52]. In our experiments, we attributed gene silencing to the effective release of siRNA by Dicer cleavage. However, Dicer efficiency could also be measured by labelling the 5′ and 3′ ends of siRNA with a fluorescent donor and quencher. Upon cleavage by the Dicer, the fluorescence intensity of the donor would increase [53]. Monitoring Dicer cleavage could provide insight into whether the knockdown efficiency is hindered by incomplete release of siRNA. AONs might be released following RNase H-mediated cleavage of the RNA complement strands covalently attached to the NPs. Sequence designs for the siRNA and AON delivery strategies are illustrated in Figure 1. The dicer substrate was designed to allow siRNA release in the cellular environment: Di-siRNAs on the surface of the NPs are a potential substrate for cellular nucleases, which would furnish active siRNAs following enzymatic processing. Dicer substrate siRNA designs were selected featuring a 2nt 3′-CA overhang on the antisense strand of the construct. It has been shown that this overhang facilitates better Dicer processing versus blunt ended duplexes, although this overhang is also associated with increased degradation in serum [50, 51]. The 3′ sense strand terminus was selected as the point of attachment to the NPs, again to facilitate Dicer processing to produce the desired active siRNA[52].

Unmodified and modified siRNAs (and modified AONs) delivered by Her-NPs have a duration of activity up to at least 4 days. While the effect was attenuated by day 7, a dilution effect likely contributes to observed diminution of activity because the cells reached confluency at day 4 and were split. Indeed, SKOV3-luc cells double every 20 h, so the number of cells increases exponentially and the concentration of ONs are likely below an effective concentration at day 7. The full recovery of luciferase levels confirms that the NPs re non-cytotoxic.

3.6 Conclusion

P(LA-co-TMCC)-g-PEG NPs, targeted with trastuzumab and carrying unmodified or modified siRNAs or AONs, are potent vehicles for tissue specific targeted gene silencing through either an RNAi or an antisense mechanism. siRNA NPs achieved potencies comparable in effectiveness to the standard in siRNA cell transfection protocol: Lipofectamine 2000. Although AON NPs showed reduced potency compared with Lipofectamine delivery, gene silencing can still be readily achieved when PS-2′F-ANA AONs are used. We have shown two new ON delivery systems based on antibody-targeted polymeric
micelles carrying chemically modified ONs conjugated on their exterior shell. The described chemical modifications improve the stability of ON sequences, and the attachment of antibodies to the micelle allow for targeted delivery, both of which are essential for the success of this delivery strategy. The use of NPs as an alternative to cytotoxic cationic transfection reagents for the delivery of siRNA or AONs demonstrates the benefits achieved through incorporation of targeting ligands and chemically modified ONs with a competent drug delivery vehicle.

3.7 References


4. Conclusions

The overall purpose of this work was to determine whether oligonucleotides (siRNA or AONs) could be coupled to the exterior PEG shell of polymer nanoparticles and maintain gene silencing activity. First, we developed a strategy to sequentially label dual functional poly(LA-co-TMCC)-g-PEG micelles by double click reactions. Secondly, we employed these methods to covalently attach antibodies and siRNA or AONs to the nanoparticles. Cell assays used to measure luciferase levels of SKOV-3Luc cells overexpressing HER2 and luciferase validated the activity of the nanoparticles. The following conclusions can be derived from this work:

1. Dual functional micelles can be formed by the co-dialysis of poly(LA-co-TMCC)-g-PEG-furan and poly(LA-co-TMCC)-g-PEG-azide. The functional groups can be modified using sequential Diels Alder and strain promoted azide-alkyne cycloadditions with maleimide- and dibenzylcyclooctyne-modified moieties, respectively.

2. Immuno-nanoparticles formed using dual functional micelles can be further modified with peptide sequences. A FLAG peptide was covalently coupled to trastuzumab-nanoparticles. Confocal microscope imaging showed that the dual functional micelles remain intact to deliver the peptide to the cells that overexpress HER2.

3. The siRNA and AON sequences target firefly luciferase in SKOV-3Luc cells. A gene silencing effect was observed when the oligonucleotides were delivered by immuno-nanoparticles or Lipofectamine. In contrast, scrambled sequences did not demonstrate gene silencing activity.

4. Knockdown activity was only observed when active oligonucleotide sequences and trastuzumab were covalently bound to the nanoparticle.

5. Of all the sequences used for gene silencing, modified 2’F-ANA siRNA was the most effective. Modified siRNA showed equally effective levels of knockdown when delivered by immuno-nanoparticles or by Lipofectamine.

6. Unmodified sequences delivered by the nanoparticle showed loss of activity and require 2’F-ANA modification for effective gene silencing.
5. Recommendations and Future Directions

In this work, we reported the gene silencing activity of siRNA and AON sequences delivered by immuno-nanoparticles. Some recommended studies for future research:

1. **Monitor cell uptake and release of nanoparticles using confocal microscopy.**
   
   Both the siRNA and AON mechanism require entry into the cell cytoplasm for Dicer and RNase enzymes to be activated. Based on our knowledge of receptor-mediated endocytosis of immuno-nanoparticles, it is hypothesized that the nanoparticles are taken up by the cells into endosomes or lysosomes [1]. However, it is unclear how our negatively charged nanoparticle escapes the endosome. Literature has shown that cationic liposomes or polymers can escape through the “proton sponge effect” [2]. One potential explanation for endosomal escape stems from research by Panyam, *et al.* with PLGA nanoparticles [3]. Our poly(LA-co-TMCC)-g-PEG nanoparticle backbone is primarily composed of lactide units, thus its net charge is similar to PLGA. PLGA nanoparticles were able to escape the endosome by selective reversal of the surface charge from anionic to cationic in acidic endo-lysosomal compartments [3].

   One proposed study uses confocal microscopy to gain a mechanistic understanding oligonucleotide delivery using micelles. The siRNA and AONs have been modified with a Cy5 fluorophore that can be used to track the nanoparticle. Unreacted TMCC units on the backbone can also be modified with fluorophores to label the micelle. Confocal microscopy can monitor both the nanoparticle and the siRNA to determine how both components are taken into the cell. No co-localization of oligonucleotides and micelles would indicate separation of the siRNA/AON from the micelle.

   A potential challenge is the low concentration of Cy5 since siRNA and AONs are delivered in picomolar concentrations. If the concentration of oligonucleotides is too high, there could be cell cytotoxicity. In the case where the Cy5 signal is too weak, the nanoparticles could be further modified with fluorescent dyes. Unreacted azide and furan groups could be coupled with maleimide- and dibenzylcyclooctyne- modified labels. The cells could be co-stained with endosomal markers to observe endocytosis of the nanoparticles. This could address the question of whether the nanoparticles are taken into early/late endosomes or lysosomes.

   Since trastuzumab nanoparticles are shown to bind to the cell membrane after one hour and have receptor-mediated uptake within 3 hours [4], it would be interesting to monitor the cells at these time points.
2. Deliver clinically relevant modified Di-siRNA and D-AONs with immuno-nanoparticles.

Having demonstrated the gene silencing capability of modified siRNA and AON-nanoparticles, one logical direction involves the use of clinically relevant oligonucleotide sequences for breast cancer. From our results, we concluded that the fluorinated modification (2‘F-ANA) improves siRNA and AON stability and potency and is necessary for nanoparticle delivery. RNAi technology provides a potential therapeutic strategy because of its high efficacy and specificity for down-regulating gene expression [5]. Current research with siRNA for cancer therapy involves knockdown of genes involved in oncogenesis pathways, apoptosis, tumor-host interaction and resistance to conventional therapies [6]. Some potential protein targets are shown in Table 5.1. These sequences could be modified by the Damha group and protein levels can be quantified by qPCR [7].

<table>
<thead>
<tr>
<th>Approach</th>
<th>Target Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncogenesis</td>
<td>C-raf [5, 8]</td>
<td>- C-raf plays a key role in a mitogen-activated protein kinase cascade to promote tumor progression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- SiRNA against c-raf was tested by transfection to A549, PC-3, MDA-MB-231 and SKOV-3 cancer cells with liposomes</td>
</tr>
<tr>
<td>Heat shock protein 90 (HSP90)</td>
<td>[9]</td>
<td>- HSP90 plays a role in the conformational stabilization and maturation of mutant oncogenic signalling proteins.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Knockdown of HSP90 leads to MIF (migration inhibitory factor) degradation and triggers apoptosis of cancer cells</td>
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<tr>
<td></td>
<td></td>
<td>- In ErbB3 transgenic model of human HER2+ breast cancer, genetic knockdown delays tumor progression and prolongs overall survival of mice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- MDA-MB231 and MDA-MB468 cells were cultured to observe gene knockdown</td>
</tr>
<tr>
<td>Her2/neu (HER2) [7]</td>
<td></td>
<td>- Overexpression of HER2 gene provides mitogenic signals to tumour cells to increase growth potential and resistance to apoptotic factors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- siRNA sequences used against SKBR3, SKOV3, MCF-7 cell lines</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Bcl-2 and xIAP [10]</td>
<td>- Bcl-2 or xIAP may be responsible for apoptotic resistance to cytotoxic drugs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Use of siRNA to downregulate bcl-2 or xIAP enhances the effects of etoposide and doxorubicin treatments</td>
</tr>
<tr>
<td>Tumor-host interactions</td>
<td>Ras homologous A (RhoA) and Ras homologous C (RhoC) [11]</td>
<td>- RhoA and RhoC promote cell proliferation and cell invasion</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Aggressiveness of cancer is associated with increased angiogenesis, which displays RhoA signalling</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- siRNA used to inhibit cell proliferation and invasion in MDA-MB-231 breast cancer cells in vitro</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Inhibited growth and angiogenesis of xenografted MDA-MB-231 tumors in a nude mouse model</td>
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</table>
3. Conjugate siRNA or AONs using SPAAC chemistry.

In these studies, the oligonucleotides were conjugated using copper catalyzed azide-alkyne cycloadditions. The copper was removed with the unreacted antibodies and oligonucleotides during the purification step. However, the concentration of copper in the purified nanoparticle solution was not measured. Trace amounts of copper could be quantified by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) or Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).

The strain promoted azide-alkyne reaction provides an alternative strategy to avoid problems of cell cytotoxicity associated with copper. The Damha group has been working on the synthesis of dibenzylcyclooctyne terminated oligonucleotides. These sequences could be used for a copper-free reaction. SPAAC reactions also have the added benefit of reducing the complexity of the click reaction; the reducing agent and ligand used to protect the oligonucleotide from reactive copper species is no longer required.

4. Use nanoparticles to encapsulate agents for the improvement of siRNA knockdown.

Previous studies have shown that our polymeric nanoparticle can encapsulate small molecule drugs. The siRNA immuno-nanoparticle system could make use of the hydrophobic core to load and deliver small molecules to improve cellular translocation or endosomal escape of siRNA. SiRNA can passively transmit through nuclear pore complexes from the cytoplasm to the nucleus. However, permeability of the nuclear membrane can be improved with the delivery of trans-cyclohexane-1,2-diol (TCHD). TCHD is an amphipathic alcohol that can improve delivery efficiency by 6 times [12, 13]. Alternatively, endosomal escape can be improved by the encapsulation and delivery of agents like chloroquine. Chloroquine buffers the endosomal pH and has improved delivery efficiency by 7 times [14]. The encapsulation of small molecules could strengthen the siRNA effects for complete gene knockdown.

References


Appendix A: Supplemental Information

A1. Characterization of NH$_2$-PEG-azide

![Characterization of NH$_2$-PEG-azide by FTIR. Terminal azide group (2100 cm$^{-1}$), terminal amine (3389 cm$^{-1}$), C–H alkane (2888 cm$^{-1}$), C=O amide (1692 cm$^{-1}$), and C–O ether (1101 cm$^{-1}$).]
A2. Characterization of NH$_2$-PEG-furan

(a)
Figure A 2. Characterization of NH₂-PEG-furan by $^1$H NMR (a) before boc deprotection. $^1$H NMR (CDCl₃, 400 MHz): $\delta$ 1.44 (s, boc protecting group), 2.32 (t, $J=$14 Hz, $-$CH$_2$), 3.64 (m, $-$OCH$_2$CH$_2$), 4.42 (s, $-$CH$_2$-furan), 6.21 (d, $J=$ 3Hz, furan CH-3), 6.30 (dd, $J=$5 Hz, furan CH-4) and 7.33 (d, $J=$3 Hz, furan CH-5). (b) NH₂-PEG-furan after deprotection. $^1$H NMR (CDCl₃, 400 MHz): $\delta$ 2.33 (t, $J=$14 Hz, $-$CH$_2$), 3.64 (m, $-$OCH$_2$CH$_2$), 4.43 (s, $-$CH$_2$-furan), 6.22 (d, $J=$ 3Hz, furan CH-3), 6.30 (t, $J=$5 Hz, furan CH-4) and 7.34 (d, $J=$3 Hz, furan CH-5)
A3. Characterization of P(LA-co-TMCC)
Figure A.3. Characterization of P(LA-co-TMCC) by $^1$H NMR: (a) P(LA-co-TMCC-Bn) shows presence of the benzyl group. $^1$H NMR (CDCl$_3$, 400MHz): $\delta$ 1.23 (br s, CH$_3$ from TMCC), 1.58 (m, CH$_3$ from LA), 4.31 (br s, CH$_2$ from TMCC), 4.98 (s, CH-Bn), 7.33 (m, benzyl Ar) and 8.02 (m, pyrene). (b) Absence of the benzyl group indicates complete deprotection. Due to the large polymer size, the pyrene group is indistinguishable at the polymer concentration sampled. $^1$H NMR (CDCl$_3$, 400MHz): $\delta$ 1.19 (br s, CH$_3$ from TMCC), 1.51 (m, CH$_3$ from LA), and 4.26 (br s, CH$_2$ from TMCC).
A4. Characterization of P(LA-co-TMCC)-g-PEG

(a)
Figure A 4. Characterization of P(LA-co-TMCC)-g-PEG-X by $^1$H NMR: (a) Azide-functionalized PEG grafted to the polymer backbone. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 1.25 (br s, CH$_3$ from TMCC), 1.58 (m, CH$_3$ from LA), 3.59-3.69 (m, –OCH$_2$CH$_2$–), 4.35 (br s, CH$_2$ from TMCC) and 5.17 (m, CH from LA). (b) Furan-functionalized PEG grafted to the polymer backbone. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 1.22 (br s, CH$_3$ from TMCC), 1.57 (m, CH$_3$ from LA), 3.59-3.69 (m, –OCH$_2$CH$_2$–), 4.37 (br s, CH$_2$ from TMCC) and 5.15 (m, CH from LA). Integrated peak areas of the PEG methylene to TMCC methylene was used to determine the grafting efficiency: 0.7 PEG-furan or 0.9 PEG-azide per backbone.
A5. DA Reaction with NH$_2$-PEG-Fu

Figure A 5. DA Reaction with NH$_2$-PEG-Fu. The decrease of the characteristic peaks of maleimide C=C (1466 cm$^{-1}$) and the appearance of the C=C Diels-Alder adduct peak (1459 cm$^{-1}$) indicate that the Diels Alder reaction has occurred.

A6. SPAAC Reaction with NH$_2$-PEG-N$_3$

Figure A 6. SPAAC Reaction with NH$_2$-PEG-N$_3$ and Alexa 488-DBCO. Decrease in the azide peak (2109 cm$^{-1}$) indicates that the SPAAC reaction has occurred between the dibenzylcyclooctyne and azide.
A7. Size Exclusion Column Purification of Micelles

Figure A 7. Size exclusion column purification of mixed micelles with Alexa 647-MI and Alexa 488-DBCO. Separation was completed using a Sephadex G-25 column. Fluorescence of the Alexa 647 (ex. 640 nm, em. 675 nm) and Alexa 488 (ex. 490 nm, em. 525 nm) was used to quantify the conjugation efficiency. The concentration of the nanoparticles was measured by absorbance at 260 nm. Fractions 6 and 7 contain the dual labelled micelle.
Figure A8. Confocal images of SKOV-3luc controls. (a) Cells without nanoparticles show neither background signal of the Alexa Fluor 647 nor Alexa Fluor 488; (b) SKOV-3luc cells and Trastuzumab-nanoparticles show no background signal of the Alexa Fluor 647 used to stain the FLAG (i) Cell nuclei are stained with DAPI (blue); (ii) FLAG peptide (Alexa Fluor 647, purple); (ii) trastuzumab (Alexa Fluor 488, green); (iv) Overlay of all channels.
### A9. Click Chemistry Reactions

**Table A1.** Click Chemistry Reactions. In this system, the micelle was functionalized with azide and furan groups. Potential binding partners include: maleimides, acetylenes and strained alkynes. Our studies used the maleimide and strained alkyne.

<table>
<thead>
<tr>
<th></th>
<th>Azide</th>
<th>Furan</th>
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<tr>
<td><strong>Maleimide</strong></td>
<td><img src="image" alt="Maleimide" /></td>
<td>No reaction</td>
</tr>
<tr>
<td><strong>Acetylene</strong></td>
<td><img src="image" alt="Acetylene" /></td>
<td>CuAAC [2] + Cu</td>
</tr>
<tr>
<td><strong>Strained alkyne</strong></td>
<td><img src="image" alt="Strained alkyne" /></td>
<td>SPAAC [3]</td>
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</tbody>
</table>

*Although there is no reaction between an acetylene group and furan, Martin-Matute, et al. have shown that the addition of a PtCl₂ catalyst will result in a Diels Alder reaction. The electrophilic catalyst forms a Pt carbine intermediate which initiates a nucleophilic attack of the furan.


Appendix B: siRNA and AON Synthesis and Characterization

B1. Synthesis of siRNA and AON sequences

All oligonucleotides (ONs) were synthesized on an Applied Biosystems (ABI) 3400 DNA synthesizer at 1µmol scale. Unylink CPG (ChemGenes) was used for the syntheses of all ONs, except those modified with 3′ alkyne functionality, which was introduced using a 3′ alkyne-modifier serinol CPG, which is commercially available from Glen Research (Figure S2). 2′F-ANA, 2′F-RNA, Cyanine 5 (Glen Research) and RNA phosphoramidites were prepared as 0.15M solutions in dry acetonitrile (ACN), DNA as 0.1M. RNA amidites were 5′-DMT, 2′-TBDMS protected, and base protection was benzoyl (A), i-Bu (G) or acetyl (C). 5-ethylthiotetrazole (0.25M in ACN, ChemGenes) was used to activate the phosphoramidites from coupling. Detritylations were accomplished with 3% trichloroacetic acid in dichloromethane for 110s. Capping of failure sequences was achieved with acetic anhydride in tetrahydrofuran, and 16% N-methylimidazole in tetrahydrofuran. Oxidations were done using 0.1M I₂ in 1:2:10 pyridine:water:tetrahydrofuran, except for those following Cyanine 5 addition to ON 5′ termini, which was accomplished with 0.02M I₂ instead. AONs containing phosphorothioate linkages were sulfurized using a 0.1M solution of Xanthane Hydride (TCI) in 1:1 vol/vol pyridine/ACN (anhydrous). The sulfurization step was allowed to proceed for 2.5min, with new sulfurization reagent added after 1.25min. Phosphoramidite coupling times were 600s for 2′F-ANA, 2′F-RNA, and RNA, with the exception of the guanosine phosphoramidates, which were allowed to couple for 900s. DNA coupling times were 110s, and 270s for guanosine. Cy5 coupling times were extended to 20min.

B2. Cleavage and Purification

Base deprotection and cleavage from the solid support was accomplished with 1mL of 3:1 aqueous NH₄OH:EtOH for 48 hours at room temperature (for modified sequences), after which samples were chilled on dry ice for 15min and subsequently lyophilised to dryness in a speedvac concentrator (Savant). Standard RNA sequences were base deprotected with 1mL of 40% (w/v) aqueous methylamine at 65°C for 10min, chilled on dry ice, and lyophilized to dryness. 2′-TBDMS protecting groups were removed with 250µL neat triethylamine trihydrofluoride (TreatHF) for 48hr at room temperature (modified sequences), or with 300µL of TreatHF/N-methyl pyrrolidinone (NMP)/triethyl amine solution (prepared by adding 0.75mL NMP, 1mL TEA, and 1.5mL TreatHF together at 65°C) at 65°C for 3 hours. Following desilylation, ONs were precipitated by the addition of 25µL 3M NaOAc and 1mL of n-butanol followed by cooling on dry ice. The ON pellets were lyophilized to dryness.
Oligonucleotides were desalted on NAP-25 sephadex size exclusion columns (GE Healthcare) according to manufacturer protocol to prepare for HPLC purification. ONs were purified by either anion exchange or reverse phase HPLC, on either a Waters 1525 or Agilent 1200 HPLC, using a Varian Pursuit 5 semipreparative reverse phase C18 column, or a Waters Protein Pak DEAE 5PW semipreparative anion exchange column. For reverse phase purifications, a stationary phase of 100mM triethylammonium acetate in water with 5% ACN (pH7) and a mobile phase of HPLC-grade ACN (Sigma) were used (with a gradient of 0%-35% over 30min). Purified ONs were lyophilized to dryness, which also served to remove excess triethylammonium acetate salts. For anion exchange purifications, a stationary phase of milliQ H2O and a mobile phase of 1M LiClO4 in milliQ water was used (with a gradient of 0%-38% over 38min). Following anion exchange purification, excess LiClO4 salt was removed using a second desalting with NAP-25 sephadex size exclusion columns (GE Healthcare) according to manufacturer protocol.

B3. Quantitation and characterization
All oligonucleotides were quantitated by UV (extinction coefficients were calculated using the online IDT OligoAnalyzer tool (www.idtdna.com/analyzer/Applications/OligoAnalyzer); 2′F-ANA extinction coefficients were calculated using DNA values). Oligonucleotides were characterized by LC-MS on a Waters Q-TOF2 using an ESI NanoSpray source. A CapLC (Waters) with a C18 trap column was used for LC prior to injections. Thermal denaturation measurements were performed for select siRNA sequences on a Cary 300 UV/Vis spectrophotometer, by ramping from 15°C- 95°C at a rate of 1°C/min using common ON buffer (140mM KCl, 1mM MgCl2, 5mM NaHPO4, pH 7.2). The Tm for the unmodified 21mer siRNA targeting sequence is 63.8°C, well above the temperatures encountered during the click reaction to attach siRNAs to the nanoparticles. To determine if ON backbone damage could be expected following the copper-mediated click reaction to attach ONs to the nanoparticles, a Cy5-labelled 21mer poly-dT ON was treated with Sodium L-ascorbate, CuSO45H2O, and tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA), and followed at regular time points over a 30hr period. Strand integrity was observed using 24% PAGE, and bands were visualized by both UV shadowing and using Stains-all reagent (Sigma) in isopropanol (50mL formamide, 125mL isopropanol, 325mL water, soak gel overnight). No strand cleavage was detected.

Equimolar amounts of the sense and antisense strands (or AON and RNA complement strands for antisense gene silencing) of each oligonucleotide duplex were combined in annealing buffer (140 mM
KCl, 1mM MgCl₂, 5 mM NaHPO₄, pH 7.2) for a final concentration of 28 µM for each strand. The vial was heated at 90°C for 1 min, then cooled to room temperature over an hour. The UV visible light spectrophotometer was used to confirm annealing of the two strands by measuring absorbance at 260 nm.

References


Appendix C: Statistical Analysis

C1. SiRNA Delivery

In Figure 3.2, lower case letters were grouped bars with no statistical difference (one-way ANOVA, $p>0.05$). Statistics were also completed between groups (labelled ‘a’, ‘b’, ‘c’ and ‘d’) and are summarized in Table C1.

Table C1. P-values calculated by one-way ANOVA analysis of luciferase levels of SKOV-3Luc cells treated with chemically modified and unmodified siRNA with immuno-nanoparticles or Lipofectamine.

<table>
<thead>
<tr>
<th>Label</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
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<td>b</td>
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<td>&lt; 0.001</td>
<td></td>
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<tr>
<td></td>
<td>c</td>
<td></td>
<td>&lt; 0.001</td>
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<tr>
<td></td>
<td>d</td>
<td></td>
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<td>&lt; 0.001</td>
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C2. AON Delivery

In Figure 3.3, lower case letters were used to group bars with no statistical difference (one-way ANOVA, $p>0.05$). Statistics were also completed between groups (labelled ‘a’, ‘b’, ‘c’ and ‘d’) and are summarized in Table C2.

Table C2. P-values calculated by one-way ANOVA analysis of luciferase levels of SKOV-3Luc cells treated with chemically modified and unmodified siRNA with immuno-nanoparticles or Lipofectamine.

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