N-ISOPROPYL-ACRYLAMIDE CONJUGATED POLYGLYCEROL AS A DELIVERY VEHICLE FOR IN VITRO SIRNA TRANSFECTION

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N-ISOPROPYL-ACRYLAMIDE CONJUGATED POLYGLYCEROL AS A DELIVERY VEHICLE FOR IN VITRO SIRNA TRANSFECTION

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LIST OF SYMBOLS

α	Alpha, significance level for statistical analysis
μg	Microgram, 10^-6 grams
mg	Milligram ,10^-3 grams
g	1 gram
kg	Kilogram 10^3 grams
μL	Microliter 10^-6 liters
mL	Milliliter 10^-3 liters
L	1 liter
[x]	Concentration of x
nM	Nanomolar, 10^-9 molar concentration
mM	Micromolar, 10^-6 molar concentration

LIST OF ABBREVIATIONS

AODN	Alexa488 tagged oligonucleotide
cDNA	Clonal deoxyribonucleic acids, created from reverse transcription of RNA
in vitro	Outside the body as in cell culture
in vivo	Inside the body as in an animal
Lipo	Lipofectamine 2000® or Lipofectamine RNAiMAX
N1	Negative control siRNA 1
N2	Negative control siRNA 2
NIPAM	N-isopropyl-acrylamide
P1	Positive control siRNA 1
P2	Positive control siRNA 2
PEI	Polyethylenimine
PG	Hyperbranched polyglycercol
PGNIPAM	NIPAM conjugated PG
pH	Potential hydrogen, a log measure of the concentration of hydrogen ions in a solution
pKa	Acid dissociation constant
RISC	RNA interference silencing complex
RNA	Ribonucelic acid
RNAi	RNA interference
rt-PCR	Real-time polymerase chain reaction
siRNA	Short-interfering RNA

SUMMARY

Gene expression knockdown using RNA interference has dramatically altered the ability to silence target genes without the need for a creation of a genetic knockout. The pitfalls surrounding successful siRNA gene expression knockdown fall in the broad category of delivery. This work focuses on the use of N-isopropyl-acrylamide conjugated polyglycerol (PGNIPAM) as a novel cationic vector of *in vitro* and possible *in vivo* delivery of siRNA. The hyper-branched structure of the PGNIPAM molecule bears a biocompatible core with cationic subunits on the surface, providing a less toxic alternative to other cationic polymers used in the past. Further PGNIPAM shows excellent binding and release characteristics over other comparable molecules and systems. Activity of the siRNA requires access to the cell cytoplasm, which in turn requires passage of the siRNA through the cell membrane and release into the internal environment with no degradation. PGNIPAM has shown the ability to traverse the endocytic pathway and release the siRNA directly into the cytoplasm where it can interact with cellular machinery. Knockdown of known oncogene survivin was observed *in vitro* both through mRNA expression reduction as well as through protein reduction in MDA-MB-231 human breast cancer cells. Additionally, early stage animal work with a human breast cancer model shows positive results for coupled treatment of tumors using siRNA against survivin and doxorubicin, an anticancer drug. PGNIPAM offers a safer alternative to other cationic delivery systems and has shown improvement over standard modes of knockdown from commercial products.

CHAPTER 1

INTRODUCTION TO SIRNA AND DELIVERY STRATEGIES

Gene expression knockdown to investigate physiology is fast becoming a significant portion of biological research. RNA interference (RNAi) technology represents the latest push to control cellular cues using very effective and focused treatments. Endogenously, animals as simple as nematodes and as complex as humans, utilize short RNA fragments to control various aspects of cell behavior and development. [1, 2] The ability to specifically knockdown gene expression allows researchers to create gene knock-out systems on a smaller scale, without the need for large scale genetic manipulation, as in the case of experimental knockout mice.

The hope to manipulate this pathway has encouraged the development of short interfering RNAs (siRNAs). These molecules are double stranded RNA of about 17 to 21 base pairs in length. The RNAi pathway is composed mainly of two components: siRNA and the RNA interference silencing complex (RISC), which is a protein complex that binds the siRNA, makes it single stranded by degrading one of strands, and has enzyme aspects that serve to bind the target messenger RNA (mRNA), and cut the molecule to prevent complete protein synthesis from taking place. By removing the mRNA from the population, the associated gene has effectively been knocked down in its expression level. As opposed to a system such as the antisense technology, siRNA can be reused for further knockdown; therefore less siRNA is required to see an effect.[3, 4] This knockdown is not necessarily total and is dependent on the target sequence chosen and the amount of siRNA delivered to the cell. [2, 4]

Assuming any given siRNA sequence has some level of efficacy, the meaningful quantity is how many siRNAs can be delivered successfully to a cell; the more siRNA delivered, the greater the chance of improved gene expression knockdown. The amount

of siRNA delivered is dependent on the efficiency of containing and release of the siRNA cargo, the toxicity of the treatment itself, and protection of the siRNA from endogenous and environmental RNase enzymes.

siRNA Delivery Strategies

Numerous directions have been taken in the area of delivery, but they each fall into one of three main categories: electroporation, viral, and non-viral.

Electroporation is based on the idea that applying a voltage across a cell's membrane triggers the formation of electropores. These "holes" in the cell membrane are due to a destabilization of the lipids that make up the membrane. This approach is extremely limited by distance from the electrodes and by the voltage that can safely be applied. The electrodes need to be put across the area of interest so this procedure is easiest to apply to skin problems but not ideal for internal organ or tissue problems, such as a tumor inside of the liver. As this process essentially destabilizes the cell membrane, it is possible that the cells will not recover to eventually expression the desired phenotypic change.[5]

Viral delivery has both a large upside and downside. Viruses have evolved to become very good gene delivery vectors. By replacing the viral genome with DNA or siRNA to elicit a cellular change, yields the possibility for very high transfection efficiencies.[4] However, as in the case of vaccines, it is not always possible to kill all the viruses. And even though the viruses may be killed and only carry the designed cargo, the viral protein shell still poses an antigenic threat to the immune system of the organism being injected. *In vitro* work may be spared any issues, however, any animal studies could prove to be problematic. [6]

The nonviral delivery methods fall into two distinct categories: lipids and polymers, mostly with some cationic characteristics. Essentially the same general principles govern both groups, however, they differ slightly in the method delivery. The

cationic aspect of the lipids serves to bind the siRNA and form a micelle around the molecules. The resultant vesicles fuse with the membrane of cells and the contents are spilled in to the cytosol. Products, such as Lipofectamine® from Invitrogen, follow this methodology and have very good *in vitro* results. *In vivo* problems persist due to serum and salt binding to the complex, leading to clearance from the circulatory system.[7]

Polymeric delivery relies on the same binding to the siRNA molecule, however, polymers cannot merge with a cell's membrane. Instead, the endocytic process is required to engulf the particles and bring them inside the cell within an endosome. Once inside the endosome, the polymeric carrier needs to escape and release its cargo, before the endosome develops into a lysosome and the contents are degraded.[7] This strategy has potential over lipid systems as the carrier polymer is not necessarily removed from the equation. Additional traits, such as cell labeling, could be conferred to the carrier to function independently of the delivered siRNA.

To break up the endosome, there are two distinct strategies. Chloroquine has been used to block lysosomal function by raising the pH and thereby preventing degradation by the lysosomal enzymes. The other strategy that has been employed is the concept of the proton sponge, which requires the polymers to possess sufficient cationic charge and buffering capacity.[6, 7]

Survivin

Cancer's resistance to therapy has prompted the idea of sensitizing the cancer prior to treatment. In the case of many cancers, the oncogene survivin has been shown to be up-regulated, and is considered a good target for cancer treatment.[8-10] Survivin is a 16.5 kDa protein which has roles in both cell division and inhibition of apoptosis. Cytoplasmic survivin interacts with and stabilizes the cell mitotic spindle, thereby supporting cell division necessary microtubule assembly. Survivin knockouts don't survive past embryo due to severe cell division problems. There is also a mitochondrial

supply of survivin which is released in tumor cells as a response cellular death signals. The survivin protein also inhibits caspase activation and blocks the apoptotic program. Survivin's anti-apoptotic program serves as a common mechanism for cancer cell survival. Due to this effect the use of survivin expression as a predictor of cancer prognosis is widely accepted for multiple cancers.[11-15] Survivin's dual roles in cell mitosis and apoptosis inhibition have been further shown to confer resistance to chemo- and radiotherapies, as well as to promote cell division.[9, 10] Knockdown using siRNA is contingent on the binding of the siRNA to the cellular RNAi silencing complex (RISC). The siRNA acts as the guide while the RISC serves as the enforcer, cutting any mRNA sequences that bind to the siRNA sequence with high affinity. Using this technology to treat a disease, such as cancer, where current problems exist with drug resistance, allows for sensitization of the cells to standard treatments. Survivin is a member of a family of apoptosis inhibiting proteins, whose mode of action is to prevent a cell from entering into an apoptotic, or planned cell death, stage. Knockdown of the survivin gene has been shown to sensitize many different cancers to chemotherapy.[9, 10, 15, 16] Can PGNIPAM complexed to an anti-survivin siRNA molecule effectively knockdown the gene expression levels better than commercially available modes of transfection, in an *in vitro* setting? By comparing PGNIPAM-siRNA to that of Lipofectamine RNAiMAX, a widely used standard for nucleic acid delivery now optimized specifically for siRNA delivery to cells, the improvement of PGNIPAM system over the standard is clearly demonstrated.

Project Introduction and Strategy

Cationic polymers such as NIPAM-conjugated polyglycerol have the ability to bind anionic molecules such as DNA and RNA, through simple electrostatic interaction. Association of the polymer with the nucleic acid molecule allows the polymer to serve as a vehicle for delivery of the nucleic acids to cells. The polymers readily associate with

cell membranes due to the positive charge and will be endocytosed by the cell over time. The first problem to overcome is release of the complex from the endosome before the vesicle becomes a lysosome and degrades the polymer and siRNA. In order for the siRNA to be actively used by the cell for knockdown, the molecule needs to be released in the cytoplasm. The proton sponge effect has been shown to effectively burst the endosomal membrane by swelling the vesicle with water until it can no longer sustain itself and bursts. [6, 7, 17] Cell imaging of fluorescent short oligonucleotides (ODN) should effectively demonstrate that this action is occurring within cells due to the PG NIPAM polymer.

The primary goal of this project is optimize siRNA association with the PGNIPAM and to demonstrate effective delivery and knockdown in the target cells. The best primer for understanding binding and release characteristics of the complexes is the N:P ratio, which represents the number of amine groups on the polymer and the number of phosphate groups on the siRNA. As the N:P ratio increases the electrostatic interaction of the polymer with the siRNA will also increase.[6, 18, 19] The latter result is that the release of the siRNA will become more difficult downstream, so there is a benefit to identifying the ideal ratio for delivery. Further, unlike DNA, RNA is more easily susceptible to degradation from a variety of forms, so protection of the siRNA on its journey to cells of interest is a critical aspect of the delivery process. Enzymes known as RNases are present everywhere and even a small contamination could lead to complete degradation of the siRNA. In order to maximize the protection of the siRNA, the binding ratio to the polymer needs to be high enough so that the siRNA is blocked from the RNase enzymes through steric effects or condensation.[6, 7] Comparisons to other cationic polymers, such as PEI, are essential to properly evaluate the PGNIPAM properties and put them in perspective with commercially available polymers delivery systems.[7, 20]

The natural next step is to study the toxicity of the polymer system to the cells that will be the basis for this aim as well as the following. PGNIPAM complexed to siRNA at varying N:P ratios will provide a clearer picture of which potential polymer and siRNA concentrations will be effective at delivery but also in minimizing nonspecific toxic effects to the cells. Cationic polymers and lipids, while serving as ideal binding agents for DNA and RNA due to their charge characteristics, are also typically responsible for higher levels of toxicity within cells. [6]

Once the binding and association traits of the polymer for the siRNA have been adequately established it is necessary to demonstrate that the polymer can effectively convey the siRNA to the target cells and release the cargo inside the cytoplasm. PGNIPAM complexed with fluorescently tagged oligonucleotides as models for siRNA, were delivered to cells and then fluorescent imaging was used to study distribution of the delivered nucleic acids. PGNIPAM's lower binding affinity due to its reduced charge density should confer improved delivery characteristics over other polymers with higher charge density and therefore stronger binding traits. *In vitro* and *in vivo* work using microscopy and gene expression knockdown will provide a more detailed understanding of the pathway followed by the PGNIPAM-siRNA complex.

CHAPTER 2

PGNIPAM AS A DELIVERY VECTOR FOR SIRNA

The polymer characteristics of PGNIPAM were chosen specifically for the role of siRNA or other small RNA/DNA delivery vehicles. The polymer possesses a non-toxic dendritic backbone with small cationic subunits on the perimeter (figure 4C). The overall size of the structure is on the order of 2.5 nanometers in diameter, putting it on a similar magnitude scale of siRNAs which fall at approximately 7 to 8 nanometers in length. The cationic NIPAM subunits provide the means of binding these small RNA molecules in an electrostatic fashion, thereby eliminating the need for covalent bonds, such as ester bonds (REF to other delivery vehicles) to maintain the link of the siRNA to the polymer. Cationic polymers offer the further potential for buffering and pH responsive charge alteration[21], thereby allowing a mechanism for transmembrane cellular delivery utilizing the proton sponge effect and release of the siRNA in to the cytoplasm. This chapter aims to explore this in more detail and attempt to elucidate the effectiveness of the polymer for siRNA delivery based on the inherent traits and qualities in the polymer structure.

Synthesis

The synthesis of PGNIPAM is described in Shen et al., with the addition conjugation of NIPAM subunits to a dendritic polyglycerol backbone. The reaction is carried out using a four step scheme, beginning with the necessary modifications to the hydroxyl side-chains of hyperbranched polyglycerol. Tosylation by p-toluenesulfonyl chloride protects the alcohol moieties on the polyglycerol and also makes them more susceptible to nucleophilic substitution. The exchange creates a better leaving anion for substitution, which is provided by the sodium azide addition. Sodium azide is used in a

substitution reaction to replace the tosylate protective group. The azide will then be hydrolyzed during the Staudinger reaction with triphenylphosphine, reducing the azide to a primary amine which leaves the polyglycerol with approximately 90% of the exposed hydroxyl groups converted to primary amines. [22] Finally, a Michael addition reaction is used to conjugate two NIPAM subunits to each of primary amine of prior product through nucelophilic substitution of the hydrogens. The PGNIPAM is now composed of an internal structure of polyglycerol, with approximately 90% of its side chains bearing two NIPAM subunit moieties, conferring cationic charge to the molecule. [21]

Cationic Nature

The cationic features of the PGNIPAM polymer, as is the case with other positively charge polymer structures is due to the amine residues in the sequence. Nitrogen elements in the polymer sequence gain a relative positive charge by further coordination past the neutral scheme. The coordination number of the amine, referred to by primary, secondary, etc, is equivalent to the number of R-groups that are bound to the nitrogen. For example, a primary amine is a nitrogen atom bound to only one R-group and to at least two hydrogen atoms. The coordination of the amine is also critical to the traits and pKa of that particular amine. The charge of the amine is dictated by the pKa and will therefore depend on the pH of the solution. This point is critical to the charge, buffering, and complexation of the PGNIPAM polymer as will be discussed in the remainder of the chapter. PGNIPAM contains a tertiary and secondary amine in its structure. The tertiary amine is equivalent to a triethyl amine and has an associated pka of approximately 10.65. The secondary amine is bound to an isopropyl group and a carboxyl group. The carboxyl group will lower the pKa of the amine by drawing away electronic charge. This will yield a pKa in the 7 to 8 range for the secondary amine. [23]

The pKa of a particular molecule or chemical group defines the pH where the charge of the molecule shifts through oxidation or reduction of the group. A single chemical group has one associated pKa. For example, a carboxyl chemical group has a pKa of approximately 6, which implies that below a pH of 6, the carboxyl groups are composed of a carbon bonded to two oxygen atoms where one of the oxygens is also bound to a hydrogen atom. If the pH is shifted basic, the carboxyl group loses the bound hydrogen through reduction of the group. This causes a neutrally charged chemical group to become negative. The same is true for amine groups, except that amines are positively charged below their pKa, and become neutral above this threshold. An amine group, at acidic pH, possesses a positive charge due to the extra coordinated hydrogen atom. At basic pH above the pKa, the extra proton is lost through reduction and the group becomes neutrally charged.

As all amines possess a relative positive charge in the biologically relevant range of pH 5-7.4 for cells, this group is functional for polymeric delivery of siRNA and other nucleic acids. This is by no means a new discovery as the majority of cationic polymers, such as polyethenimine (PEI) are based on amine groups to give them their characteristic positive charge. The pKa of PEI is 9.06 which implies that at biologically relevant pH the amines of the structure of protonated. [24] As was discussed earlier, the positive charge can be unacceptable due to toxic side effects. This has been a massive problem for cationic polymers and lipids in the delivery of siRNA or other nucleic acids. By covalently binding the siRNA to the polymer this can reduce the toxicity of the polymer by removing the charge, but this requires reduction of the bond once the complex reaches the cytoplasm of the cell. Electrostatic binding, however, allows for rapid self-assembly and disassembly of the complex due to pH. In addition, other features of amine group pose solutions to the delivery problems associated with endosomal uptake.[7, 20]

Particle Size

PGNIPAM is a cationic-charged hyper-branched polymer whereas siRNA is a double stranded nucleic acid with a distinct negative charge profile along the outside edge. In order to understand the relationship during binding and eventual release of the siRNA by the polymer, the N:P ratio associated with the complex needs to be understood. The N:P ratio is defined as the number of amines residues in the polymer for every phosphate group in the siRNA. The ratio will fluctuate based on the characteristics of the polymer in question, as opposed to the nucleic acids which are uniform regardless of whether you are working with RNA or DNA molecules. A polymer with high cationic charge density will require fewer molecules to bind a similar amount of siRNA to a polymer with lower charge density. This is case with PEI and PGNIPAM respectively. The PEI structure is composed entirely of tertiary and secondary amines, conferring high charge density. PGNIPAM's backbone is composed of ester bonds with the only charge conferring segments present on the NIPAM side-chains. Therefore, N:P ratios of higher order are required to adequately bind siRNA to the PGNIPAM structure. This can be observed in figure 3.[20]

Further characterization of the PGNIPAM-siRNA complex, such as particle size, will improve our understanding relationship that exists at the time of delivery and transfection. The polymer is composed of a dendritic backbone polymer composed of polyglycerol. The base polymer is a polyglycerol dendrimer where 90% of the hydroxyl groups have been replaced by N-isopropyl-acrylamide (NIPAM) subunits to confer the polymer with positive charge moieties. Due to the weak scattering of the polymer, and the low concentration available for testing, dynamic light scattering failed to provide a clear and consistent size profile for the polymer, alone or in siRNA-complexed form. FPLC was therefore employed to gauge the size of the complex and support the binding schematic demonstrated in Figure 2.

FPLC columns elute larger particles before smaller particles, due to the tortuous path that smaller particles must take through the superose gel within the column. It should be noted that the FPLC column used, was chosen for the range of particle sizes it could elute (~17 to ~3nm) linearly, and for the minimal amount of sample required for loading (100uL). The linear range of the column is more suited to determine approximate particle size than molecular weight due to the density differences that could make a comparatively light molecule (i.e. a dendrimer) seem larger diameter-wise than something heavier (i.e. a protein).

The pure polyglycerol (PG) polymer that was used as the backbone of the PGNIPAM polymer, was run as well as PGNIPAM to confirm that the there is no size fluctuations due to the preparation steps. It is anticipated that PGNIPAM should be approximately the same size as the PG polymer since the replacement of hydroxyl groups with NIPAM subunits will only cause a slight increase in size. This is confirmed by the FPLC with both polymers eluting at 45.3 minutes. This also confirms that there is no noticeable aggregation or cross-linking caused by the synthesis process.

The PGNIPAM-siRNA complex was prepared by titrating siRNA into an excess PGNIPAM solution so that the final concentrations would yield an N:P ratio of 40:1 which was previously determined to be an optimized N:P ratio for binding and release of siRNA. The siRNA was titrated into the solution to ensure that the siRNA molecules were encapsulated by the PGNIPAM polymer in a manner that would minimize the number of siRNA per polymer complex.

The anticipated structure of the PGNIPAM-siRNA complex is a single siRNA encapsulated within approximately 25 PGNIPAM polymers. This can be determined by converting the N:P ratio of 40:1 back to the number of the polymers per siRNA molecule. Table 1 displays the calculated values for the particle diameter based on the column elution time of the molecule in question.

The PGNIPAM-siRNA complex exits the column at approximately 35 minutes (figure 1), indicating a particle size of approximately 13.2nm in diameter and a molecular weight of 489.5kDa, respectively, based on the standard curve created from proteins and molecules with known sizes and molecular weights. The composite size of a single siRNA surrounded by a layer of the polymer would yield an estimated size of between 12.7 and 13.3nm in diameter based on the FPLC measurements of the individual components. This is in good agreement with the FPLC measurement of the PGNIPAM-siRNA complex at 13.2nm in diameter. Further, assuming a single siRNA is involved per complex, the calculated weight of 489.5kDa would imply that there are approximately 21 polymers molecules associated with it. At an N:P ratio of 40:1, the optimal ratio for binding and release of the siRNA, the theoretical ratio of polymer to siRNA is 25 to 1. These two values are very close and on the same level of magnitude so I believe this approximation is in good agreement with the FPLC measurement. Therefore, it can be concluded that the hypothetical binding schematic in figure 2, is a good approximation of the final complex structure.

However, it should be noted that this scenario may not be good representation of a general mixing of the PGNIPAM with siRNA. Under bulk mixing conditions the siRNA and PGNIPAM will rapidly associate and form larger aggregates for the majority of the solution. The idealized case I studied above does not include particles that possibly came out with void volume of the column. In order to better study these larger particles that would make up the remainder of the missing mass of polymer and siRNA, a column with a size range in the million molecular weight range should be used.

Toxicity

The downfalls of other cationic polymers such PEI and chitosan fall into several categories. The cationic nature of these polymers confers innate toxicity to the molecule and immediately lowers the effectiveness as a suitable transfection agent. Highly

cationic molecules, rapidly associate with cellular membranes due to the electrostatic attraction of the positive charged polymer structure for the relatively negatively charged outer surface of the cellular membrane. Cells actively pump sodium ions but of the

Table 1: Molecular weight and Stoke's radius calculation based on FPLC measurements. Column exit time refers to the time it takes for the sample to elute from the column be detected by the attached UV spectrometer and the refractive index detector. A standard curve created by proteins with known molecular weight and stoke's radius is used to convert the exit time (not shown). The PGNIPAM-siRNA complex elutes from the column 10 minutes prior to the individual components, conferring a molecular weight of 489.5kDa and a particle diameter of approximately 13.2nm.

Sample	*Column Exit Time (Minutes)*	*Molecular Weight (kDa)*	*Stoke's Diameter by Refractive Index (nm)*	*Stoke's Diameter by UV Spectrum (nm)*
Polyglycerol	45.38	20	2.66	2.62
PGNIPAM	45.3	22.5	2.52	2.72
siRNA (21 bp)	44.48	13.7	8.3	7.3
PGNIPAM-siRNA N:P=40:1	35.18	489.5	13.2	13.2

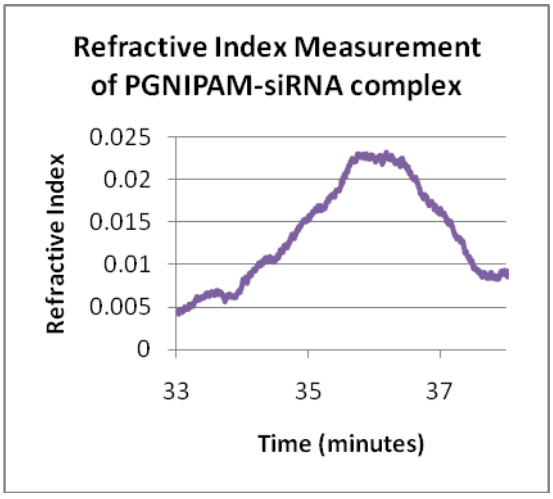

Figure 1: FPLC elution plot for PGNIPAM complexed with survivin siRNA. Survivin siRNA was titrated into an excess of PGNIPAM and run through an FPLC column. The polymer complex eluted at 35.18 minutes which is the average of the UV and the refractive index elution time. The excess polymer elutes outside the standard range.

cytoplasm and exchange them with potassium ions. While both ions are positively charged, the membrane is only permeable to sodium ions. Potassium ions that are actively pumped into the cell cannot leave, thereby increasing the positive charge of the cytoplasm relative to the outer surface of the membrane. This imparts a relative negative charge to the surface of the cellular membrane, allowing any positively charged species to become attracted and interact with the surface.

The proton sponge effect is observed in cells when a highly cationic molecule with the potential for pH buffering, draws negatively charged chloride ions towards it. The chloride ions in turn attract water molecules causing the water content of a vesicle, such as an endosome, to increase. As the water concentration increases, the osmotic pressure within the vesicle increases to the point where the membrane can no longer sustain itself. The membrane breaks apart and spills its contents into the cytosol. The ions and any other anionic molecules present in the cytosol, serve to neutralize the positive charge of the polymer and the siRNA can then be replaced and released from the electrostatic interaction.[17, 20, 25]

High cationic charge can cause increasing toxicity issues. Polymers such as polyethylenimine (PEI), is well known as an ideal transfection agent for DNA and siRNA but also an ideal example of how high density cationic charge can lead to nonspecific cell death. [6, 7, 26]

The RNase enzyme is specific for RNA and can degrade both single and double stranded molecules. Appropriate protection from these enzymes, which are actively present in the environment as well as inside cells, is necessary to maximize delivered treatment to the cells' cytosol. Containing the siRNA within a shell, whether it be made of polymer or lipid, is a good strategy because it separates the siRNA from any harmful enzymes. Another strategy, taken by the cationic polymers, such as PEI, is to condense the siRNA on the surface of the polymer structure so that it cannot interact with the RNase enzyme.

Most recent work has been focused on polymeric DNA and siRNA delivery systems, because of the vast possibilities that are presented by the ability to combine useful elements of different polymers into a co-polymer make up which suits multiple roles all at once. Polymers such as PEI, chitosan and cell penetrating peptides have been investigated by multiple groups in the hopes of creating an improvement over standard methods of transfection.[6, 7, 20, 27] While transfection efficiency is widely distributed between the different polymeric carriers, nonspecific toxicity is still the biggest cause for concern. [7]

There is still a need for a polymeric carrier which overcomes the toxicity problems but still maintains high transfection efficiency. PGNIPAM seems to possess these desired characteristics and was thoroughly explored in this work. This polymer is based on a polyglycerol backbone, which is completely biocompatible as well as NIPAM subunits at the ends of branches that provide the cationic character of the polymer. [21]

The objective of this work is predominantly to demonstrate the effectiveness of the PGNIPAM for siRNA delivery and its advantages over commercial standards of transfection, such as PEI and Lipofectamine. PGNIPAM combines aspects of both types of nonviral delivery systems with distinct advantages over both. Polyglycerol is biocompatible so the backbone itself presents no toxicity issues, as opposed to polymers like PEI, which are biocompatible but demonstrate marked toxicity as the both the internal and surface charge of the molecule is very dense.[7] The NIPAM subunits provide cationic charge to the surface of the polymer, through the teritiary amine groups and proton buffering with the secondary amine groups.[20, 21] This provides binding to the siRNA through electrostatic interaction.[7, 18] Additionally, the hydrophobic isopropyl elements of the NIPAM allow for additional interaction with a cell's membrane, than simply just the positive charge and nonspecific cell membrane interaction of other polymers.

Evaluation of the binding and release parameters of the PGNIPAM is necessary to compare the polymer to something more common, such as PEI. Like other cationic polymers, the amine to phosphate group ratio (N:P) of the polymer to the siRNA, is critical to determining the binding ability of the polymer. As PGNIPAM has a lower charge density than that of PEI, a higher N:P ratio is required to establish similar binding to that of PEI. This lower charge density will also assist in release of the siRNA cargo at the target site as less anionic charge is required to neutralize the polymer and release the cargo. Gel electrophoresis of PGNIPAM-siRNA complex will easily allow for optimization of N:P ratio. The release of the siRNA is also contingent on the charge of the polymer. PGNIPAM releases its cargo much earlier than that of PEI even though the N:P ratio will be much higher for PGNIPAM.

As the charge density is lower, the ability for the polymer to protect the siRNA from RNases needs to be evaluated. While the charge density is lower, and therefore more PGNIPAM polymer units may be required to bind all the siRNA, there will be less exposed cationic charge. The higher number of polymer units per siRNA is critical to understanding the binding interaction of the two; this demonstrates that a shell is forming around the siRNA rather than the siRNA forming a shell around the polymer, as in figure 2. The polymer provides adequate protection for the siRNA by essentially encapsulating it as opposed to the PEI where the siRNA is most likely condensed on the surface. The isopropyl groups allow for cellular membrane interaction so the reduced amount of cationic charge exposed to the cell is not an issue with binding. This further reduces the toxicity of the polymer system.

Intracellular Release

Delivery of the siRNA to the cytosol of the cell is critical to the effectiveness of RNAi. If the siRNA is not free of its polymer carrier it cannot interact with the RISC complex, so the quicker the release, the quicker the mode of action. [6, 7] Direct

comparison of PGNIPAM with PEI will provide a detailed understanding of the behavior of the polymer in a cellular environment. As PGNIPAM possesses less cationic charge density than PEI, the polymer should be neutralized by intracellular anionic charge quicker than PEI. Therefore, the time scale to release the siRNA cargo should be much shorter, which has implications for transfection efficiency. This prediction is confirmed by the cell imaging studies in figure 3. It was predicted can confirmed that free siRNA should present and highly distributed in the cytosol for PGNIPAM treated cells before free PEI-delivered siRNA will be present.

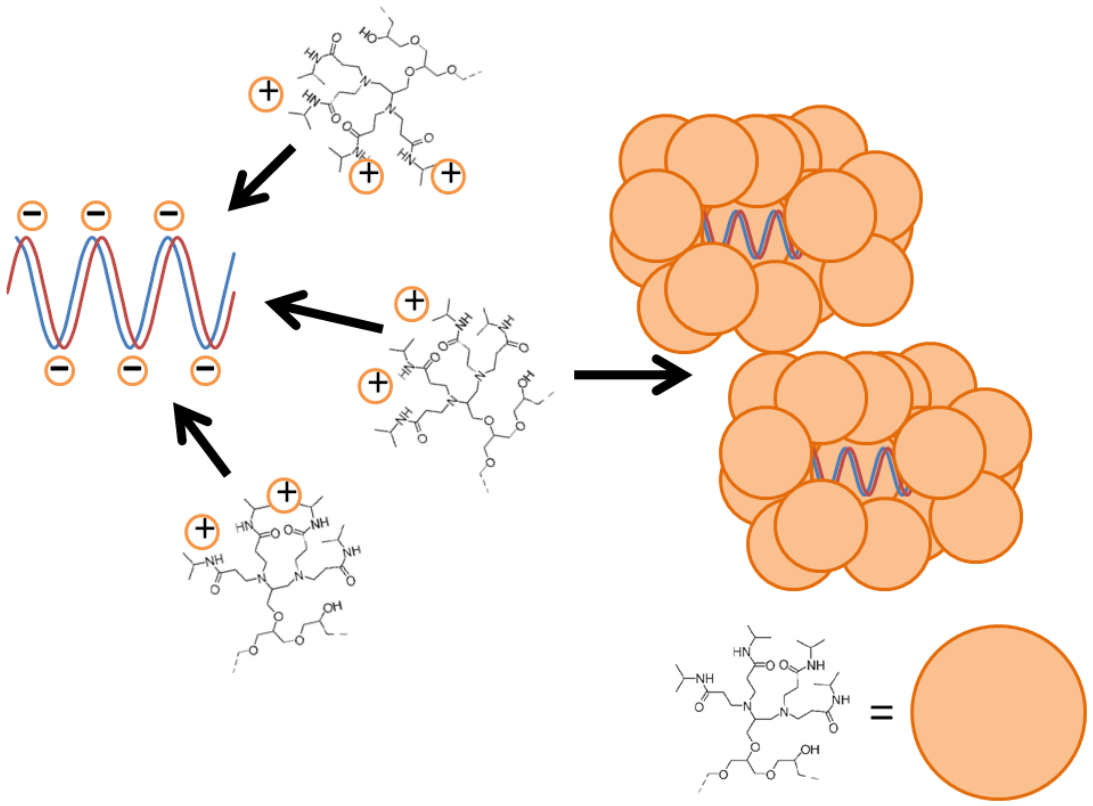

Figure 2: Schematic of PGNIPAM association with siRNA. The anionic siRNA will quickly interact with the positively- charged NIPAM moieties of the PGNIPAM polymer (left half of figure). Based on the binding results, the predicted final structure of the complex is shown on the right, with the PGNIPAM polymers forming a shell around the siRNA and shielding it from the outside environment.

Binding of Alexa488-tagged Oligonucleotide and siRNA

Agarose gel electrophoresis provides a simple method to study the interactions of nucleic acids with the PGNIPAM, as well as other polymers. In the case of PGNIPAM, the polymer is cationic, so it will be unable to travel through the agarose gel and will remain in the loading wells with any bound alexa488-tagged oligonucelotides (AODN) or siRNA. The AODN used is 21 bp long and double stranded just like a typical siRNA, the only difference is that it is made of DNA. By varying the N:P ratios between the PG NIPAM polymer and the nucleic acids, we are able to identify an appropriate binding ratio. Figure 3 A demonstrates how increasing N:P ratios lead to increasing amounts of AODN bound by the polymer. For the AODN, a 40:1 ratio seems to be appropriate (lane 5 in Figure 3A) for all of the nucleic acids to be bound up, with little improvement seen for the 80:1 ratio. The bands decrease in density from left to right indicating that higher N:P ratios lead to stronger binding by the polymer, and therefore less free nucleic acids to travel down the gel. Lane 2 contains the AODN and the PG backbone polymer confirming that the PG has zero interaction with the nucleic acids and that all binding is due to the NIPAM subunits. PEI with AODN at an N:P ratio of 6:1 was loaded in Lane 7 to demonstrate the strong binding attraction of the molecule even at a much lower N:P ratio. At a 40:1 N:P ratio of PGNIPAM to siRNA, there is approximately a 25:1 ratio (24.7:1 actual) of polymer molecules to siRNA molecules. This supports the conceptualization from figure 1 that multiple PGNIPAM molecules surround the siRNA and form a cocoon or shell, shielding the siRNA from the environment with the siRNA only serving to slightly lower the net charge.

Heparan Sulfate Release

Proper release of the siRNA is critical to delivery. If the siRNA is too tightly bound to the polymer, release will be slow and compromise the efficiency of the delivery. Samples of AODN were prepared in an identical manner to those of the binding

experiment, and were mixed with 5μg of heparin sulfate for 5 minutes. Following gel electrophoresis, it can be seen in figure 3B that all of the AODN has been released by the PGNIPAM at all N:P ratios (lanes 3-6). The spot intensity is approximately equal for all PGNIPAM conditions as compared to the control of AODN (lane 1) and the PG backbone control (lane 2). PEI, loaded in lane 7 of figure 3B, shows very little release of the bound AODN, demonstrating the superiority of the PGNIPAM over PEI for the ease of release. The faster the release of the siRNA at the target should lead to quicker interaction with the RISC complex and therefore knockdown action.

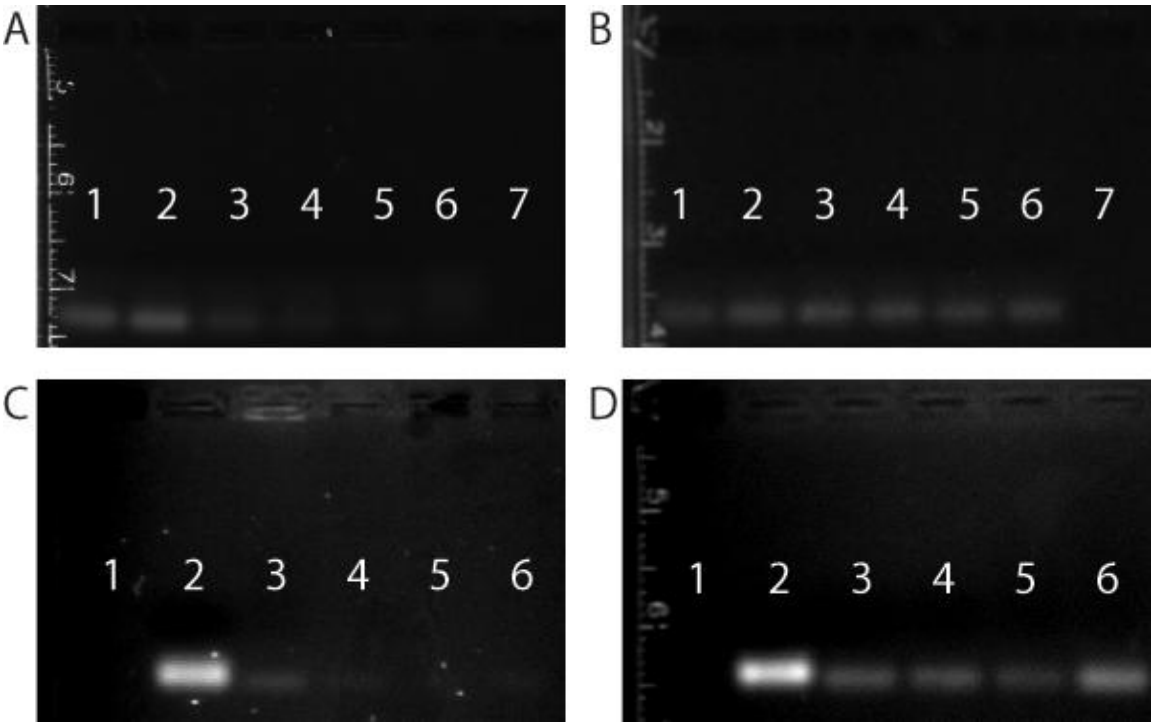

Figure 3: Agarose gel electrophoresis studies of PG NIPAM complexed with oligo DNA and siRNA. A) Binding gel of PGNIPAM complexed with alexa488 tagged 21bp-DNA (ADNA). Lane 1-ADNA, Lane 2-ADNA mixed with PG backbone polymer, Lane 3-ADNA complexed with PG NIPAM N:P ratio=10:1, Lane 4-N:P ratio=20:1, Lane 5-N:P ratio 40:1, Lane 6-N:P ratio=80:1, and Lane 7-PEI-N:P ratio 6:1. B) Release gel of PGNIPAM complexed with ADNA and released using 10 □g of heparan sulfate. C) Nuclease protection assay prior to release of Negative control #1 siRNA from complex. Lane 1-siRNA + RNase A, Lane 2-siRNA alone, Lane 3-PGNIPAM N:P ratio=10:1, Lane 4-N:P ratio=20:1, Lane 5-N:P ratio=40:1, and Lane 6-N:P ratio=80:1. D) Release of siRNA from PGNIPAM complex following nuclease protection, demonstrating protection of the siRNA by the polymer.

Cytotoxicity to MDA MB 231 cells

MDA MB 231 cells, a breast carcinoma cell line, were incubated with the PGNIPAM under varying N:P ratios and siRNA concentrations. Only negative control siRNA was used for these experiments. The cells were incubated for 4 hours to simulate common transfection procedures such as those for Lipofectamine 2000. While it has been shown that PG itself is not toxic as in Figure 4C, there is clear evidence that the NIPAM subunits confer some toxicity to the polymer at an N:P ratio of 80:1 and possibly higher. The toxicity is responsible for ~80% of observed cell death at the 80:1 N:P ratio, but little to no toxicity is observed for all other conditions and combinations of N:P ratio and siRNA concentration. The toxicity of the 80:1 ratio is similar to that of PEI at a ratio of 6:1. In light of this and in agreement with the binding results, the 40:1 N:P ratio of PGNIPAM to siRNA was chosen for all future experiments, to minimize toxicity, while maximizing binding efficiency.

Survivin Gene Expression Knockdown

The ultimate test of a novel transfection system is to perform a transfection and knock down the expression of a gene of importance. In the case of cancer, this entails knocking down an oncogene as a prelude to cancer cell death, whether it be direct or indirect. A direct target would cause the cell to either stop growing, or to go through programmed cell death, known as apoptosis. An indirect target, such as an apoptosis inhibitor, should lead to increased susceptibility of a cell to standard cancer treatments. The gene survivin is a member of the inhibitor of apoptosis gene family (IAP), and has been investigated by multiple groups for its role in cancer resistance. Survivin is up-regulated in many types of cancer.[8, 9, 14] Survivin is known to block caspase 3 activation, which is one the initial stages of apoptosis activation. By knocking down the gene's expression level, cancers have been shown to be sensitized to radio- and chemotherapies.[10, 15, 16]

Lipofectamine, an Invitrogen product, is widely used as the gold standard of transfection techniques for *in vitro* systems. Lipofectamine 2000® and Lipofectamine RNAiMAX® are cationic lipid-based products and function by encapsulating the siRNA and ferrying it across the cell membrane by membrane fusion, thereby releasing its cargo into the cell cytosol.[7] Comparing the PGNIPAM system to this widely used mode of transfection, allows for excellent evaluation of the transfection efficiency. The polymer's dual modes of interaction with the cell membrane give it a slight advantage over the liposome treatment. It will be demonstrated that this can improve the delivery efficiency over Lipofectamine.

Preliminary Western Blot

The gene survivin serves as an inhibitor of apoptosis and is over-expressed in many cancers. Knockdown of this gene has been shown to sensitize various cancers to radiation as well as chemotherapy. Transfection with anti-survivin siRNA was carried out for 48hours in MDA-MB-231 human breast cancer cells, at which point the protein was extracted, quantified and normalized using a Bradford assay. Western blotting was used to study the effect of the siRNA knockdown at increasing concentrations of anti-survivin siRNA complexed with PG NIPAM. Lipofectamine 2000 was used a control and the results can be seen in figure 4D. As the concentration of siRNA increases, the resulting concentration of survivin protein decreases by 90% with 100nM siRNA delivered by the PGNIPAM. Lipofectamine demonstrated no noticeable knockdown for the 100nM concentration of siRNA that was delivered. A negative control siRNA from Ambion was used to test for nonspecific knockdown, which was slightly observed for the Lipofectamine delivery but not for the PGNIPAM.

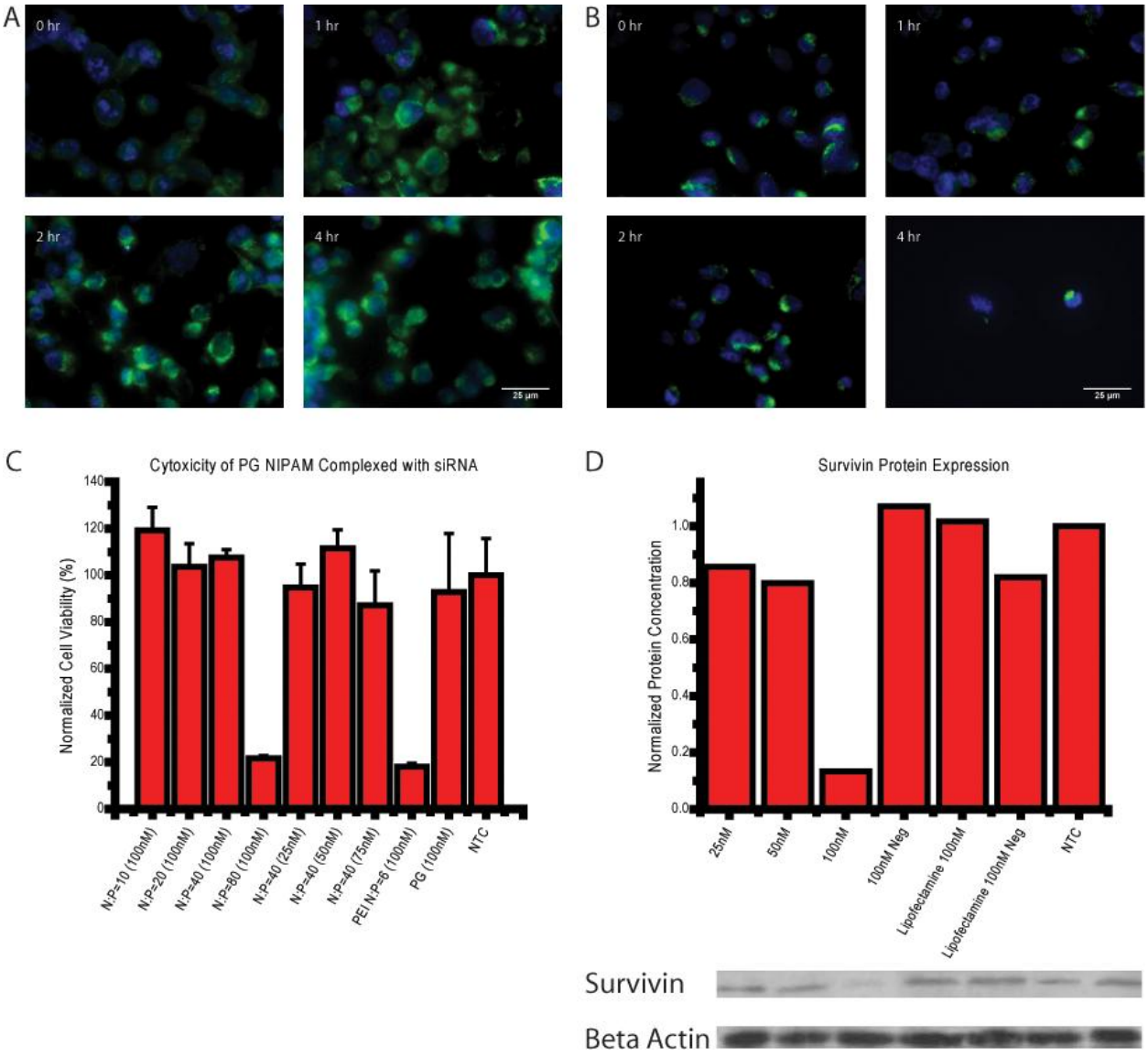

Figure 4: Cellular polymer delivery studies. A) PGNIPAM complexed with ADNA at a 200nM and an N:P ratio = 40:1, from 0 to 4 hours post transfection. B) PEI complexed with ADNA at 200nM and an N:P ratio=6:1, from 0 to 4 hours post transfection. Distribution of PGNIPAM delivered ADNA is throughout the cytoplasm while, PEI complexed ADNA is isolated to the MTOC, indicating that ADNA is still bound 4 hours after treatment. All nuclei are stained with DAPI. C) The cytoxicity of the PGNIPAM complexed with Negative control #1 siRNA at the concentration listed in parentheses, as compared to PEI and PG polymer. These results represent 4 hours post transfection. D) siRNA knockdown of protein expression of the IAP gene survivin. PGNIPAM was complexed with anti-survivin siRNA at the listed concentrations and at an N:P ratio = 40:1. Negative control #1 siRNA was used as a negative control and the PGNIPAM was compared to Lipofectamine 2000. Beta actin was used as a house keeping gene.

N:P ratio

Based on the binding and release data, as well as the insight yielded by the toxicity experiment, an N:P ratio of 40:1 was chosen and will be used for the remainder of this work. Based on the binding and release results, this ratio will provide similar binding and release features to that of 80:1, while minimizing the inherent and trademark toxicity of cationic polymers. At a ratio of 40:1, up to 100nM siRNA can be delivered with minimal toxic effects observed due to the polymer.

CHAPTER 3

CELLULAR UPTAKE AND IMAGING

Endosomal uptake and release

Once taken up by an endosomal vesicle, cellular membrane elements are recycled back to the surface or sorted into other vesicles for transport to various cellular machinery. Membrane proteins, such as receptors, fall into one of these categories and leave the endosomal environment by one path or another. Molecules within the endosomal sack are another matter. Any unbound material can be recycled back to the surface of the cell with receptors and other necessary membrane proteins that were endocytosed. At this time those molecules would be expelled from the cell and either float off or are endocytosed again at a later time point when the molecules re-associate with the membrane. The alternative and more likely scenario is that the molecules will remain within the confines of the endosome and, as the endosome matures into a late endosome and then into lysosome, the molecules will be digested by low pH.

In regards to siRNA delivery, the siRNA must reach the cytoplasm of the cell so that it can bind to the RISC complex and serve as a targeting template for mRNA digestion and subsequent gene expression knockdown. If the siRNA cannot exit the endosome it will be either digested or recycled out of the cell. Neither of these options is suitable and therefore a method of endosomal escape is required to release the siRNA into the cytoplasm before the endosome can mature into a lysosome.

As described earlier in the discussion of siRNA delivery systems, endosomal escape can be achieved through different outlets, each with inherent advantages and disadvantages. Avoiding the endosomes entirely using a method such as streptolysin or electroporation has advantages as this removes the limiting factor in the delivery process.

However, toxicity effects of these methodologies are very high when it comes to measuring risk versus reward. Chemical agents, such as chloroquinone, can be used to disrupt the endosomal membrane and cause release from the vesicle into the cytoplasm. This has been used by several groups to eliminate this problem, however it does require the co-delivery of the chloroquinione and therefore lowers the carrying capacity of the delivery vehicle. By using the vehicle itself to exit the endosome quickly before the structure can convert into a lysosome, the carrying capacity of the vehicle can be left intact. In the case of the PGNIPAM-siRNA complex, we are utilizing the proton sponge effect which is still not fully understood, but has potential for bursting or at the very least swelling the endosomes to the point of structural disruption.

Cell Transfection and Imaging

MDA-MB-231 cells were transfected with PGNIPAM and PEI both complexed with AODN at concentration of 100nM. Time points from 0 to 4 hours post transfection were prepared and fixed with 100% acetone to preserve cell structure and fluorescence of the AODN. All cells were stained with DAPI to show the location of the cell nucleus. Figures 4A and B shows cell images taken at 4 different time points encompassing the critical transfection time period of 0 to 4 hours, for PGNIPAM and PEI respectively. Within an hour all of the AODN has been taken up by the cells for both treatments. As the PG NIPAM and PEI are not conjugated to any targeting molecules, the uptake is completely driven by nonspecific endocytosis. Both substances possess cationic charge and therefore can elicit the proton sponge effect to burst the endosomes and spill in to the cytoplasm.[6] These images confirm that the PGNIPAM and PEI have left the endosomes due to their distribution in the cell. We would expect to see large green fluorescing vesicles inside the cells if the polymers were not able to escape. However, it should be noted that the PGNIPAM treatment shows quick release and distribution of the AODN through the cell cytoplasm by 1 hour following transfection. This is ideal for

siRNA because the molecule needs to be released from the polymer so it can interact with the cellular RISC complex for effective knockdown. PEI in Figure 4B appears to still be binding up most of the AODN, as it is confined to one part of the cytoplasm near the nucleus. This is believed to be the MTOC region where molecules are transported from the cell periphery for processing.

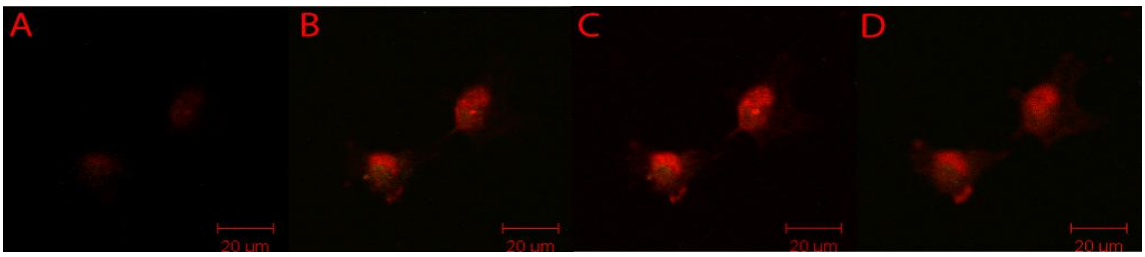

Figure 5: Confocal microscopy of FAMsiRNA transfected MDA-MB-231 cells with LAMP-1 protein stained by texas red. *The cells were transfected for 1 hour with PGNIPAM-FAMsiRNA complexes and then fixed with formaldehyde and stained using a texas red-linked antibody against LAMP-1, a late endosomal marker. Each image represents a slice of 0.5□m height resolution. The top and bottom slices (A and D, respectively) demonstrate an absence of the FAMsiRNA. Slices through the middle of cell (B and C) shows that the FAM-siRNA is distributed throughout the cytoplasm but not to regions that are high in expression of the LAMP-1 protein.*

To better elucidate the PGNIPAM-siRNA complex pathway for cellular entry, fluorescent tagging and co-localization can be employed. Cells will be treated with the complexes for select time periods in order to study both cellular location of the siRNA, but also the how this location changes with time relative to cellular endosomes. Antibodies against EEA-1 and LAMP-1, an early endosomal marker and a late endosomal marker, respectively, were used to stain the cells following fixation with formaldehyde. Secondary antibodies labeled with texas red dye was incubated to label the EEA-1 and LAMP-1 antibodies so that the location of the endosomes could be tracked, relative to the FAMsiRNA.

The time course of the experiment was from 0 minutes to 48 hours, during which cells were fixed to snap shot the process of cell binding, internalization through endocytosis, and distribution within the cell. For the siRNA to perform its function, it

needs to associate with the RNAi silencing complex (RISC) in the cytoplasm. Therefore, the first critical factor is whether the siRNA is clearly located within the cell cytoplasm, versus only located on the surface. Figure 5 shows two cells that have been fixed after one hour of transfection and then labeled with antibodies against LAMP-1. Panels A through D represents four different slices through the cells from top to bottom, respectively. There is no signal from the siRNA in the top slice (A), indicating that the siRNA is not located on the surface of the cell. Figure 5B through D all demonstrate that the siRNA is present throughout the cytoplasm of the cell.

We should expect to see a very early association of the siRNA complexes and the endocytic vesicles of the cells in question due to the cationic nature of the complex. In order to test this assumption, I used a co-localization scheme, encompassing fluorescently-labeled siRNA as well as protein labeling to elucidate this pathway.

To quantify the co-localization results, Pearson's and Mander's coefficients of co-localization will be used to analyze the fluorescent confocal images of the stained, fixed samples. Pearson's coefficient is a correlation of how well an image of only the green pixels overlaps with an image of only the red pixels (figure 6). Mander's coefficients represent the fraction of red pixels that overlap green and vice versa (figure 7). For the purposes of this study, the most appropriate Mander's coefficient is the green overlapping red measure, as there is only a limited amount of FAMsiRNA as compared to large and varyingly distributed range across the cell for the EEA-1 or LAMP-1 proteins.

Co-localization

In the Red-Green-Blue color scheme (RGB), when red and green light overlap, the resulting color is yellow. This is caused by the interference of the light waves created by the two sources of color. Because the wavelengths of the two light waves are different, destructive interference of the red wave by the green, lowers the wavelength

into the yellow portion of the visible spectrum. This effect can be beneficial in the field of fluorescence microscopy by allowing for the study of overlapping cellular features.

Pearson's coefficient of co-localization is calculated as in Figure 6. The correlation is a measure of the overlap of green pixels from one image and the red pixels from the other image.

$$R_r = \frac{\sum(R_i - \bar{R}) \times (G_i - \bar{G})}{\sqrt{\sum(R_i - \bar{R})^2 \times \sum(G_i - \bar{G})^2}}$$

Figure 6: Pearson's coefficient of co-localization. It is calculated as a correlation between the red and green pixels of two images. For pixels i in the images, R and G are intensities of the red and green channel respectively.

$$M_{red} = \frac{\sum_i R_{i,colocalized}}{\sum_i R_i} \quad M_{green} = \frac{\sum_i G_{i,colocalized}}{\sum_i G_i}$$

Figure 7: Mander's coefficients of co-localization. These are calculated as the fractional overlap of one color by the other. M_{green} represents the fraction of green pixels in one image that are overlapped by red in the other image, and vice versa. $R_{i,colocalized} = R_i$ if $G_i>0$ and $G_{i,colocalized}=G_i$ if $R_i>0$.

Mander's coefficients of co-localization are calculated slightly differently, where each coefficient represents a ratio of the co-localized pixels to the total number of pixels in that image. For example, M_{red} is the sum of the overlapping green and red pixels between images, divided by the total number of red pixels in the image. The reverse is true for M_{green}.

In the case of this work, it is necessary to understand the location of the siRNA during the process of transfection. As was stated earlier, siRNA needs to be located in the cytoplasm of the cell for it to act as a knockdown effecter of the RNAi pathway. Therefore, the cellular location of the siRNA during the transfection process is critical to our understanding of the cellular endocytic process, both through formation as well as escape.

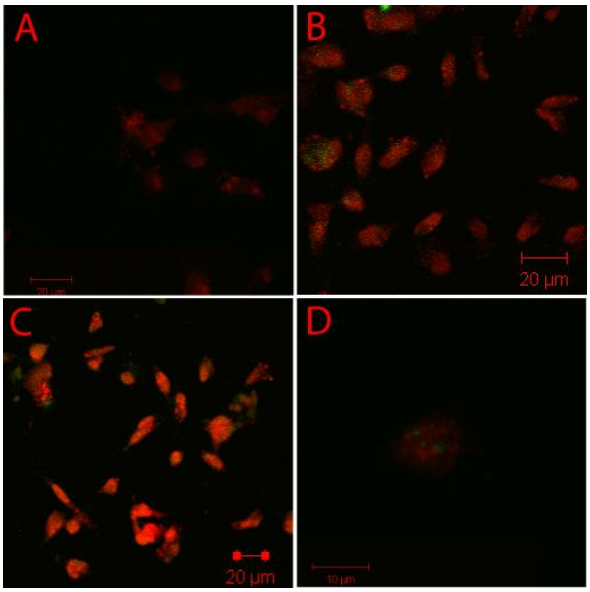

Figure 8: Confocal Microscopy of PGNIPAM-FAMsiRNA transfected into MDA-MB-231 cells with EEA-1 protein stained by texas red. *The transfections times for the images are 0 minutes (A), 1 hour (B), 4 hours (C), and 48 hours (D).*

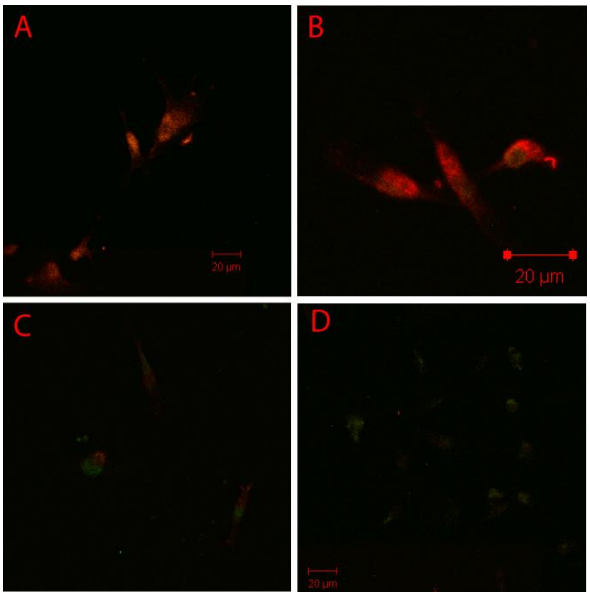

Figure 9: Confocal microscopy of PGNIPAM-FAMsiRNA transfected into MDA-MB-231 cells with LAMP-1 protein stained by texas red. *The transfection times for the images are 0 minutes (A), 1 hour (B), 4 hours (C), and 24 hours (D).*

Ideally the PGNIPAM-siRNA complex can escape from the endosomes through the proton sponge effect which will lead to endosomal swelling and either bursting of the endosomes or swelling to the point of leaking. There was no clear evidence detected for endosomal bursting in the described experiments.

As no clear imaging results are present to determine the exit strategy for the complex, I instead focused on confirmation that the PGNIPAM-FAMsiRNA is exiting the endosomes and entering the cell cytoplasm through reduced interaction with endosomal proteins. EEA-1 (figure 8) and LAMP-1 (figure 9) provide an early and late biomarker snapshot of the endosomal cycle and served as the markers for the co-localization studies. Time 0 for both EEA-1 and LAMP-1 protein stains, demonstrate no fluorescent signal due to the FAMsiRNA, which allows for the background auto fluorescence to be removed from the other images. After an hour of transfection and up to 48 hours for the EEA-1 cells, most of the cells demonstrate some FAMsiRNA signal

which is clearly defined. Also, the FAMsiRNA signal is very diffuse through the cells' cytoplasm following 4 hours of transfection, indicating that the FAMsiRNA is not still endosomally associated nor limited to the perinuclear region of the cell where endosomes

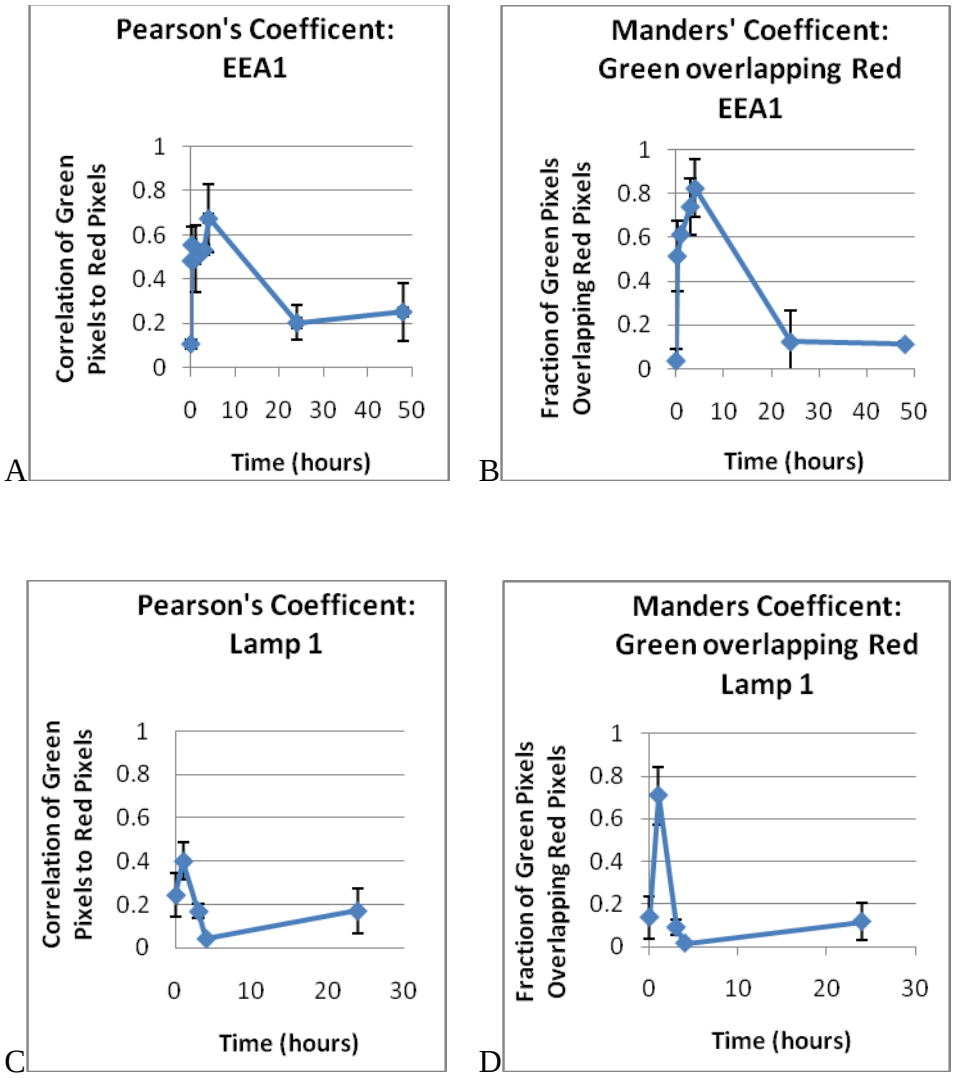

Figure 10: Coefficients of overlap for colocalization measurement. Pearson's and Mander's Coefficients are calculated for colocalization of EEA1 (A and B, respectively) or Lamp1 (C and D, respectively) and FAMsiRNA. EEA1 is highly correlated with the FAMsiRNA up to 4 hours post-transfection, but drops dramatically over the course of 24 hours and remains unchanged after 48 hours. LAMP-1 is correlated with the FAM siRNA at 1 hour post-transfection, but is unassociated completely after 3 hours. Data collected from 63x magnification confocal microscopy images of MDA-MB-231 cells.

are typically transported to. To confirm this, ImageJ was used to measure the Pearson's and Mander's coefficients for images taken at time points spanning 0 to 48 hours post-transfection.

The Pearson's and Mander's coefficients were measured and plotted against time for EEA-1 (figure 10A and C) and LAMP-1 (figure 10B and D) in order to observe how the localization of the two signals changes over time. Based on the EEA-1 results, the PGNIPAM-FAMsiRNA complex is mildly associated with the endosomes up to 4 hours post-transfection and then drops until by 24 hours and later, there is little to no association. Pearson's coefficients for EEA-1 and FAMsiRNA show between a 0.5 and 0.7 correlation from 1 to 4 hours, respectively, before dropping to a correlation of 0.2 by 24 hours. The Mander's coefficients fall between 0.6 and 0.8 for the same interval before dropping off to unassociated levels. Therefore FAMsiRNA is no longer co-localized with EEA-1, and therefore early endosomes by association.

However, to ensure that this is not caused by the endosomes maturing into pre-lysosomal stages before the PGNIPAM-siRNA complexes can escape, LAMP-1 co-localization was investigated. Along a similar, yet accelerated time line when compared to EEA-1, the LAMP-1 association with the complexes is highly correlated by 1 hour post-transfection. The correlation dramatically falls off afterwards to a correlation of less than 0.2 for the remainder of the study. This is clearly indicative of the weak association of the complex with early lysosomes and therefore very encouraging. Based on this data, the complexes are able to escape the endosomes before they mature to the lysosomal stages where degradation of the polymer and siRNA is very likely to occur.

CHAPTER 4

EXPRESSION KNOCKDOWN OF ONCOGENE IN HUMAN BREAST CANCER CELLS

mRNA Knockdown by Survivin siRNA

RNA interference is effected through the RNA Inference Complex (RISC). siRNA is bound by the RISC and used as a targeting sequence to identify the mRNA to be degraded. In order to test the effectiveness of PGNIPAM to deliver siRNA to cells and trigger gene knockdown, cells were transfected with both positive and negatively targeted siRNA for survivin, as well as Lipofectamine RNAiMax as control for transfection.

Forty-eight hours post-transfection, RNA was purified from the MDA-MB-231 cells and converted to cDNA. Real time PCR was performed to measure the relative gene expression of survivin mRNA as compared to 18s ribosomal RNA which was used as the housekeeping gene. The PGNIPAM polymer was complexed with either of two different siRNAs against the gene survivin (P1 and P2) or two different siRNAs that have negative sequences and serve as transfection controls (N1 and N2). Lipofectamine RNAiMAX served as a commercial transfection agent to compare the effectiveness of the polymer system. (SEE APPENDIX A for siRNA formulations)

Both positive siRNAs delivered with the PGNIPAM polymer significantly reduced survivin gene expression as compared to the non-treatment control, but also compared to the Lipofectamine delivery of the same siRNA molecules at the same concentration (figure 11). This indicates that the PGNIPAM delivery, over 48 hours, yields 76% and 89% knockdown of the survivin gene for siRNAs P1 and P2, respectively, whereas there is no apparent knockdown due to the Lipofectamine

RNAiMax delivery of the same siRNAs. The Lipofectamine-delivered siRNAs, positive and negative, are not significantly different from the non-treatment control.

The apparent up-regulation of the survivin gene for several of the other treatments as compared to the non-treatment control may be due to slight differences in cell density at the time of RNA extraction. The data represents the results of three separate and independent experiments so slight differences in cell density or number of cells in the late

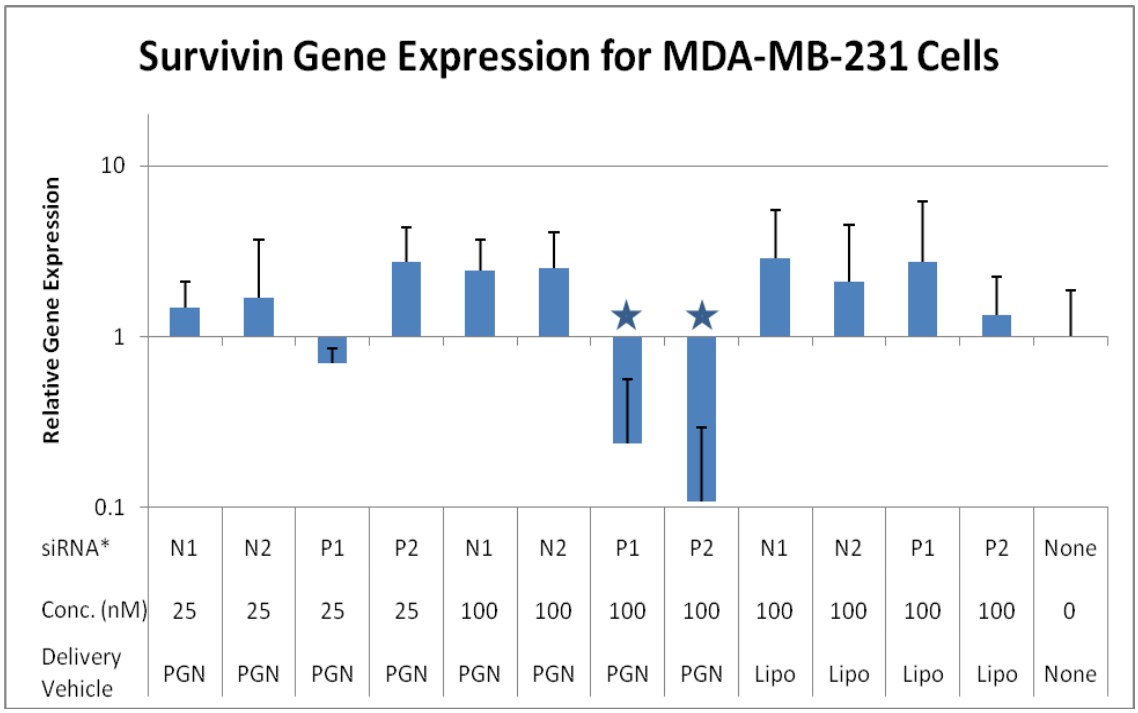

Figure 11: Survivin Gene Expression following 48 hour siRNA transfection. Survivin gene expression as measured by mRNA expression in MDA-MB-231 human breast cancer cells. Cells were transfected for 48 hours with either one of two positive (P1 and P2) or negative sequence (N1 and N2) siRNA molecules for survivin. PGNIPAM (PGN) and Lipofectamine RNAiMax (Lipo) were used for transfection delivery vehicles. The stars above the PGN 100 P1 and P2, represent significantly different knockdown from Lipofectamine P1 and P2, respectively, as well as from the non-treatment control at α=0.01. An ANOVA analysis shows the means are significantly different from each other at α=0.01.

stages of cell division could easily vary the survivin gene expression. The treatments in question represent between approximately 1.5 and 3.5 the mRNA concentration of the non-treatment control cells. The PGNIPAM delivered 25nM siRNA P2 is the only

treatment that is significantly different from the control cells for elevated survivin mRNA; the polymer delivered 100nM N1 and 100nM N2 are not significantly different from the control. Therefore, it can be inferred that the PGNIPAM delivery of positive siRNAs targeted against the gene survivin, is on average 80% more effective at eliciting gene expression knockdown that Lipofectamine RNAiMax, in MDA-MB-231 breast cancer cells.

Protein Knockdown by Survivin siRNA

Protein expression reduction is the final indicator of siRNA effectiveness. Western blotting allows protein to be separated based molecular weight and then blotted for using antibodies specific to the protein. Survivin protein expression was measured with beta actin as the house keeping gene control. Lipofectamine RNAi max was used as a gold standard for siRNA delivery in vitro. Two positive and two negative siRNAs (the same from the RNA expression knockdown study) were employed in order to study any differences in sequence and to confirm that negative siRNAs are not eliciting a false negative knockdown.

Spot intensity of the western blot was used to quantify the expression of the protein. A bradford protein assay was used to normalize the total protein concentration of each sample while the beta actin protein expression was used to normalize survivin expression. Figure 9 demonstrates that 100nM siRNA against survivin delivered with PGNIPAM is a 62% improvement over the Lipofectamine RNAiMAX for P1 siRNA in figure 9 and 26% more effective for P2 siRNA in figure 9. The P2 siRNA is significantly different from the control non treatment samples at a significance level of 0.01.

PGNIPAM delivered siRNA P1 at a concentration of 100nM and P2 at a concentration of 25nM and 100nM are significantly reduced from the non-treatment control group. Also, PGNIPAM delivered P1 siRNA is significantly different from the delivery of the same siRNA at the same concentration with Lipofectamine RNAiMAX.

These results are in agreement with the mRNA gene expression results for the same siRNA molecules delivered in the same methodology.

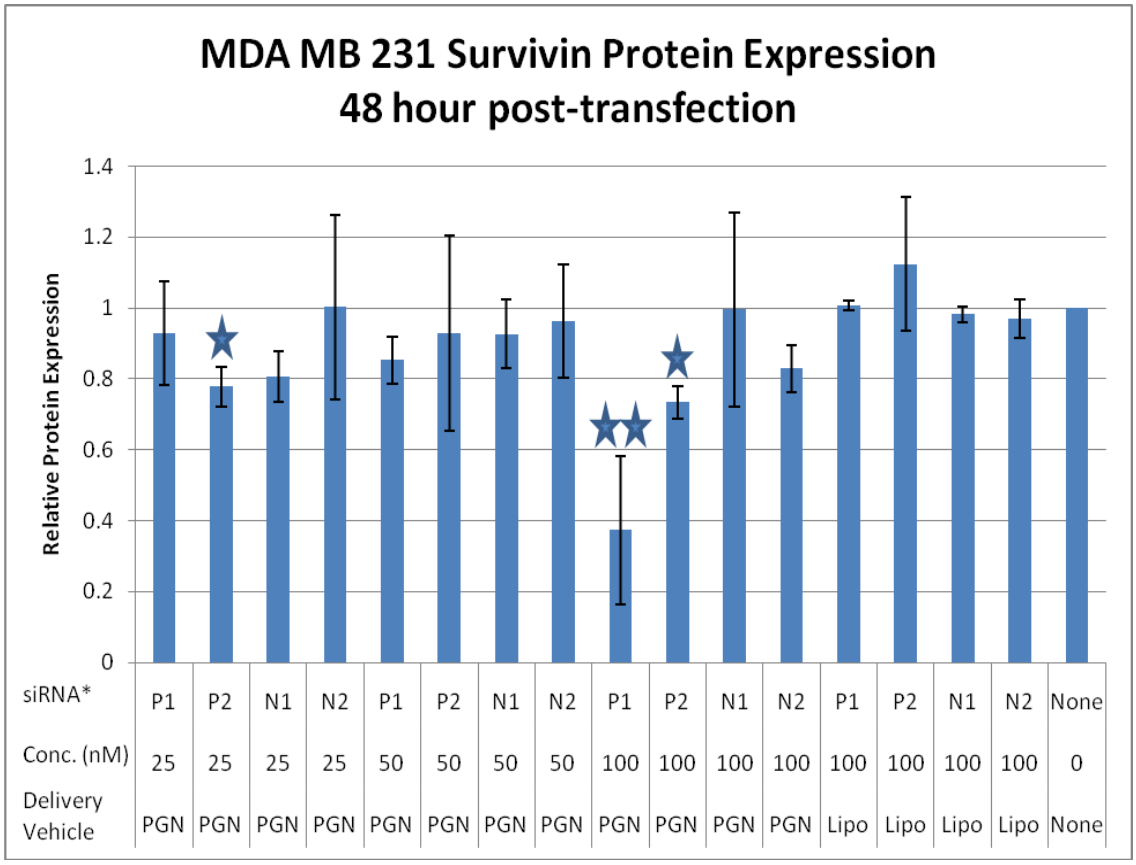

Figure 12: Western Blot Analysis of Survivin Protein Expression. Protein expression as measure by western blot and analyzed with spot intensity demonstrates significant knockdown of survivin protein expression as a result of survivin siRNA delivery. Expression of survivin protein is reduced by 60% for the P1 siRNA at 100nM concentration delivered with PGNIPAM (PGN) as opposed to no change for the Lipofectamine RNAiMAX (Lipo) delivered P1 siRNA. Significance (Stars) of protein expression knockdown from the control cells is measured with a t-test at an α=0.01 (t-test). One star indicates significant difference from non-treatment control cells, while a second star indicates significant difference from the Lipofectamine delivery of the same siRNA.

Coupled Delivery of siRNA and Doxorubicin with PGNIPAM

The survivin gene is up-regulated in MDA MB 231 cells, and these cells are capable of being implanted and grow a tumor in a mouse. *In vitro* studies to determine the effect of doxorubicin by itself as compared to a delivery with PGNIPAM were conducted to determine the dose dependency and any side effects. Figure 12

demonstrates that at a standard dose for human chemotherapy, 12mg/kg body weight, doxorubicin alone demonstrates no significant change from the non-treatment control. The PGNIPAM carrier is bound to negative siRNA at the nanomolar concentration denoted by the number adjacent. After four hours post treatment in figure 12A, there is an intense drop in cell viability for the higher concentrations of PGNIPAM for all conditions. This is indicative of apparent toxicity caused by doxorubicin being delivered with the PGNIPAM-siRNA complex. Doxorubicin is a negative charged molecule and may be interacting with the PGNIPAM in a similar fashion to the siRNA molecules already bound in the structure. This initial toxicity can be attributed entirely to efficient uptake of the doxorubicin drug through interaction with the PGNIPAM, as the doxorubicin itself shows no toxicity until double the recommended dose. At 48 hours post-transfection, figure 12B, the apparent toxicity to the cells for all doses of doxorubicin at a concentration of $1/100^{th}$ a standard dose and higher, is very dramatic. I believe this effect is due to toxicity of doxorubicin becomes evident over time as it interacts with the cells. However, the drop off from 4 hours for the PG 100 in particular, is still in effect, and the cell viability is significantly different from the PG 0 control with doxorubicin for both 0.01X and 0.1X doxorubicin concentrations. With the addition of an active siRNA against an oncogene, the potential for this system to quickly kill tumor cells and then target the remainder of the resistant cells through a separate pathway seems clear.

Immuno-compromised mice were injected with 2 million tumor cells each, and the tumor was allowed to grow to approximately $100mm^3$ in volume. Treatment injections of 100□L each were injected on days 0 and 10, as denoted in the methods. Figure 13 shows experimental treatment the tumors with regards to the change in tumor volume over time. Thirteen mice were injected with MDA MB 231 cells and after the average tumor size per group of 3 was approximately $100mm^3$, the tumors were treated. The percent volume change in tumor volume over time is evident from Figure 13A. The

PGNIPAM-antisurvivin siRNA with doxorubicin shows a negative change over time for tumor volume growth, indicating that the tumor size on average is shrinking. All other treatments are increasing in size on average, including the doxorubicin alone treatment. This can be expected because MDA-MB-231 cells represent a doxorubicin-resistant cell line.[28] Sensitization of the cells is required to allow doxorubicin to function as a treatment. The area of each bubble in the plot is proportional in size to the standard deviation associated with each particular data point. Figure 13B represents the average percentage change of tumor volume over time. PGNIPAM-antisurvivin siRNA with doxorubicin co-delivered is statistically significant as compared to all other treatments, using a one tailed t-test with a significance value of 0.05. This treatment showed a negative average daily change in tumor volume as opposed to all other treatments which demonstrated positive change in size. Doxorubicin appears to show some effect but it is not statistically significant compared to the control treatments. Negative siRNA was used to control for the siRNA itself, while the PGNIPAM-antisurvivin siRNA sample is used to show that there is no toxicity due to the polymer-siRNA complexes by themselves. This is to be expected, as the antisurvivin siRNA should only serve to sensitize the tumor cells, not lead directly to cells death. Direct evidence of siRNA against survivin, causing cell death is not currently known. The other effect of survivin knockdown, decreased mitosis, is not evident from the data based on the samples or the polymer-siRNA only treatment. Further studies need to be conducted using PBS and siRNA by itself as controls, to establish that there are no unforeseen toxic side effects.

An ANOVA analysis using Origin 6.1 revealed a p-value of 0.119, which shows the means are not statistically significant at an α=0.05. However, I feel that the trend is very strong and the significance issue is due to the sample size of the experiment. With an N=3 for each treatment group, and the higher variance due to tumor growth differences from mouse to mouse, a larger sample size would be extremely beneficial to

determining the effectiveness of coupling doxorubicin treatment with siRNA gene expression knockdown of the oncogene, survivin.

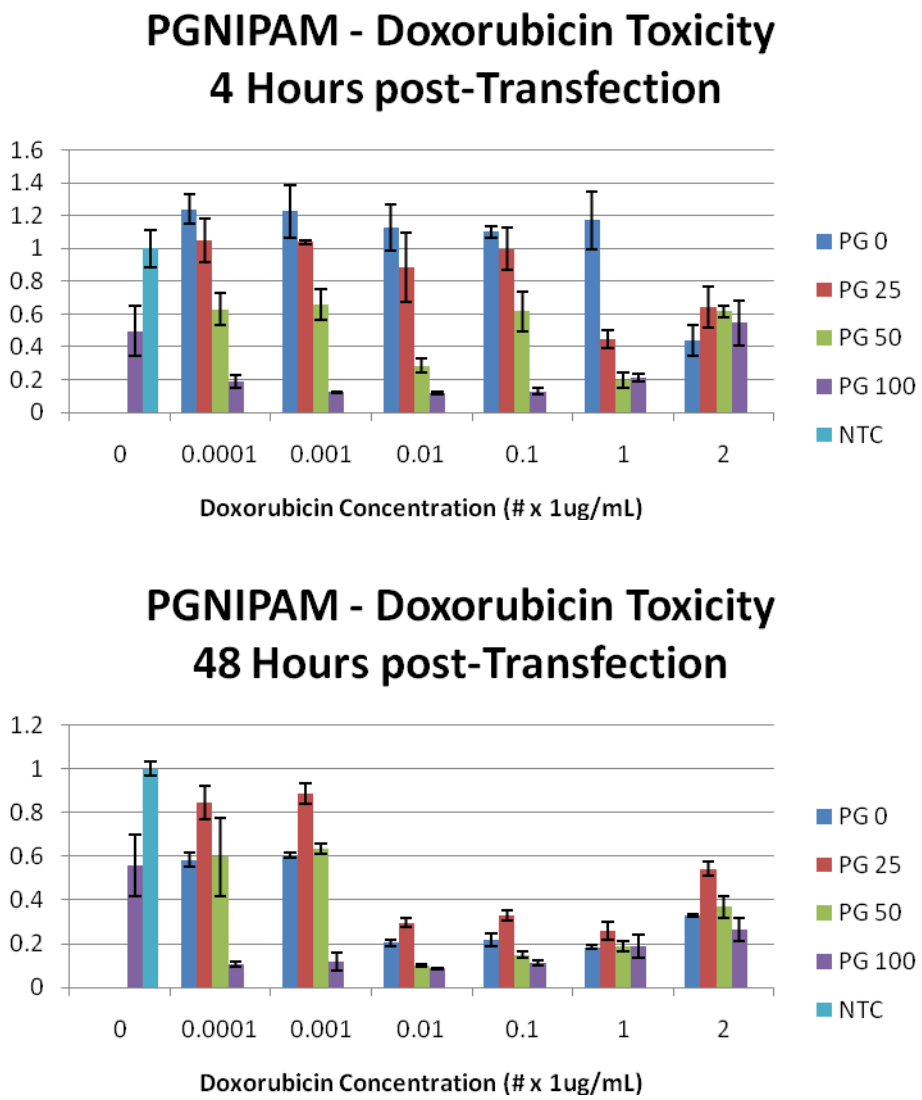

Figure 13: Cytotoxicity of Doxorubicin by delivery with PGNIPAM. Doxorubicin at a concentration of 1X is consistent by weight with a standard treatment dose administered to a human undergoing chemotherapy, at a concentration of 12mg/kg body weight. PG stands for PGNIPAM and the following number denotes a concentration of siRNA bound to the PGNIPAM in the experiment. For B, the PGNIPAM 50 and 100 are significantly different that PG 0 for 0.01X and 0.1X concentrations of doxorubicin at an α=0.05.

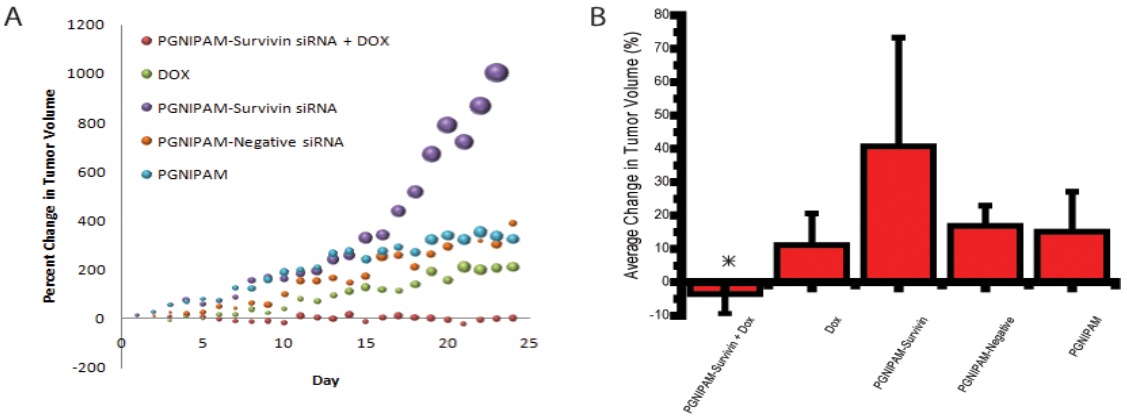

Figure 14: Subcutaneous MDA-MB-231 tumors in mice following treatment with PGNIPAM-siRNA complexes and doxorubicin. A) PGNIPAM-siRNA and doxorubicin (1ug/kg of mouse weight) is compared to doxorubicin treatment alone, and PGNIPAM-siRNA against survivin and a negative siRNA. The percent change in tumor volume is calculated based on initial tumor size measurements. B) The average daily percentage change in tumor volume for each treatment group. Significance is denoted by (*) compared to a P-value of 0.05. Tumor volume is measured in cubic millimeters and is tracked each day for 24 days. Injections took place on days 0 and 10.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

PGNIPAM offers several distinct features that can be exploited for successful siRNA cellular delivery. Structurally, the polymer is lower in toxicity than other polymeric delivery agents, such as PEI. The binding and release characteristics are indicative of polymer that offers the ability to easily vary the degree of binding through a larger range of N:P ratios. Further, despite the lower charge density, the polymer can navigate out of the endocytic pathway and deliver the siRNA to the cytoplasm where its effectiveness can be observed. Knockdown of gene expression and subsequent protein expression reveals significant improvement over Lipofectamine formulations. Finally, the preliminary animal studies indicate a potential for *in vivo* work, at least on a non-targeted scale. However, in evaluating this polymer, there are clear areas that require more in depth consideration for any future work to be carried out.

Characterization of PGNIPAM in regards to structure and function needs to be further evaluated. The currently measured polymer size represents an idealized scenario that does not exist for the bulk of the PGNIPAM-siRNA complexes which are more likely in an aggregated state. These particles were not observed on the FPLC column as they would have eluted with the void volume of the column. FPLC may still be the answer to this problem, however a column with a linear range at a larger size scale would be required to confirm. Charge of the complex prior to, during, and following formation will help to better understand the complexation relationship between PGNIPAM and siRNA as well. A relatively neutral charge on the final complex may help minimize issues such as serum binding during delivery, but this would also hinder cellular association.

Further characterization of PGNIPAM during delivery is required to understand the transfection process in greater detail than has currently been achieved. PGNIPAM is positively charged due to the secondary and tertiary amines on its surface and will associate with anionic serum proteins which may reduce delivery efficiency. All current studies have focused on OptiMEM reduced serum media delivery of the polymer in and *in vitro* setting. This was done to mimic the delivery strategy of Lipofectamine 2000 and Lipofectamine RNAiMAX to control for delivery. The procedure showed effectiveness, however, as the PGNIPAM rapidly associates with cell membranes, the 4 hour incubation window associated with Lipofectamine and other similar delivery systems, may not be required for the polymer. Tracking of the PGNIPAM within the cell would be useful to confirm co-localization studies.

As the pH of the solution decreases, the charge of the secondary amines of the NIPAM subunits will increase and cause the surface NIPAM units to spread apart. This occurs in the endosome and exposes the amines of the structure, allowing them to serve as buffering agents.[20] As this happens the isopropyl moieties on the ends of the NIPAM subunits will be exposed. These hydrophobic moieties will be able to interact with the hydrophobic portions of the cellular membrane.[25, 29] In the endocytic pathway this could lead to enhanced membrane disruption and trigger endosomal release as an additional element to the proton sponge effect. This feature was not investigated in this work but has the potential to support the endosomal escape and could be exploited in the future.

These scenarios point to modification of the PGNIPAM polymer as a necessity. Chemical group modification or addition of labeling agents could provide much needed insight into a mechanistic understanding the polymer. Fluorescent labeling of the PGNIPAM structure could allow for intracellular tracking and also co-localization with the siRNA during delivery. The isopropyl groups of the NIPAM subunits could be modified to another hydrophobic moiety and to a hydrophilic moiety which would help to

solve the endosomal disruption issue. These chemical group changes may alter the interaction between the polymer and siRNA, but would be useful to understanding the nature of the polymer's interaction with cells and also provide cellular localization of the polymer following delivery.

The mouse studies conducted during this work offer a new avenue for delivery of anticancer drugs coupled to siRNA gene knockdown. Some work has been shown by Saad et al. who used a cationic liposome to co-delivery siRNA against the multidrug resistance gene and also doxorubicin to trigger cell apoptosis for cancer cells. This was analyzed using an apoptosis assay to quantify the degree of cell apoptosis due to the doxorubicin. They found that the co-delivery of the siRNA and the doxorubicin created a cell death effect greater than that of each component individually.[30] I believe that the *in vitro* work and the preliminary animal studies I have conducted fall into a similar framework, with the different focus of survivin as the targeted gene for knockdown. The potential of this approach is that a single carrier is used for the treatment and no chemistry other than electrostatic interaction is required for the delivery itself. Other groups have delivered doxorubicin with cleavable linkers but this still requires downstream hydrolysis of the bond.[31] I feel that coupling the effects of anticancer drugs and siRNA can significantly improve treatment options for cancer.

In summation, the potential for PGNIPAM as an siRNA delivery agents is clear, however future work will need to focus on modifications to the polymer structure to better elucidate the mode of action. The animal studies offer the promise of a co-delivered doxorubicin and siRNA treatment, however the sample size was too small to prove a true coupled relationship for the two variables. To confirm the results of the animal studies, a larger sample size will be needed to achieve statistically significant results across treatment groups, as well as the use of more control groups for comparison.

APPENDIX A

EXPERIMENTAL DESIGN AND METHODS

Fast Protein Liquid Chromatography

Used an AKTAexplorer 10 system with a Superose 6 10/300 GL chromatography column. Ultraviolet measurement at 280□m and refractive index of the solution at elution time was measured. The equipment was calibrated using known protein size standards, to determine void volume elution and small molecule elution time.

siRNA and DNA

Negative control siRNA #1 (*cat. no. AM4611*) was purchased from Applied Biosystems to use as a negative control for cellular studies (Referred to as N1). Negative control siRNA #2 was purchased from Dharmacon (Referred to as N2). Anti-survivin siRNA was purchased from Dharamcon from the siGENOME library under the listing Human BIRC5 (*cat. no. D-003459-07-0010*) which is another name for surviving (Referred to as P1). Also, another positive siRNA for survivin was purchased from Sigma Aldrich (Referred to as P2). Anti-survivin siRNA sequence: 5'-CCACUGAGAACGAGCCAGA-3'. Alexa-488 tagged 21-bp double stranded DNA (ADNA) was purchased from Sigma Aldrich. The ADNA is a scrambled nonsense sequence designed to be the same length as a typical siRNA molecule for consistency in binding and release characteristics. The ADNA sequence: 5'-GATCGGCAGCTGGTACGGCGA-3' with the Alexa 488 dye attached to the 5' end via a 6 carbon linker.

PGNIPAM-siRNA and DNA Complex Preparation

The PGNIPAM polymer is prepared in a stock with 10mM Tris buffer. The tris buffer will serve to expand the PG backbone polymer and allow improved interaction of the positively charged NIPAM subunits with nucleic acids. This binding is through electrostatic interaction only. The PGNIPAM polymer was mixed thoroughly with siRNA or DNA in 10mM tris buffer and then incubated at 4°C for 2 hours. The lower temperature allows the complex to form quickly and stabilize.

Binding and Release Gels

Agarose Gel Electrophoresis serves as an ideal method to study the binding and release characteristics of PGNIPAM complex. The PGNIPAM polymer is positively charged while the nucleic acids are negative, therefore any unbound nucleic acid samples will travel towards the anode. The fully formed complex will remain in the wells due to the charge of the agarose. PGNIPAM was complexed with ADNA [1uM] at increasing N:P ratios to determine the most appropriate binding ratio. PG backbone polymer was used as a negative control at a concentration equivalent to PGNIPAM at an N:P ratio of 80:1. Polyethlyenimine 25K (PEI) (*Sigma Aldrich, cat. no. 408727*) at a N:P ratio of 6:1 was used as a positive control for binding and release due to its intense positive charge. Release of the ADNA is performed using anionic heparan sulfate (*Sigma Aldrich, cat. no. H7640*) at a concentration of 10ug/mL to displace the bound molecules. The agarose gels are prepared at 0.7% agarose concentration (*EMD Chemicals Inc., cat. no. 2010*) in 1X tris acetate EDTA buffer (TAE). All gels are run at 100V for 20 minutes in a Mini Sub-cell GT electrophoresis tank (*Biorad, cat. no. 170-4487*). All images were taken using a gel imager equipped with a green filter in the wavelength range of alexa 488 emission.

Nuclease Protection Assay

PGNIPAM is mixed with Negative control siRNA #1 at increasing N:P ratios and run alongside siRNA only controls. Following binding, all PGNIPAM complexes are incubated for 5 minutes with RNase A (*Sigma Aldrich, cat. no. R4875*) at a concentration of 1mg/mL as well as one of two siRNA-only samples for use as a positive control. After 5 minutes, all samples were heated to 100°C for 5 minutes to denature the RNase A. Samples were run on a 0.7% agarose gel stained with ethidium bromide at 100V for 20 minutes and imaged to confirm the degradation of all siRNA molecules. Heparan sulfate release under the same conditions as the ADNA release experiment are applied to the samples and they are run on a 0.7% agarose gel stained with ethidium bromide at 100V for 20 minutes. Imaging was conducted using a gel imager equipped with an ethidium bromide emission filter.

Cell Culture

MDA-MB-231 cells were cultured in RPMI (*Mediatech, Inc., cat. no. 10-040)* supplemented with fetal bovine serum *(ATCC, cat. no. 30-2020*) at 10%. Cells were split by aspirating off culture media, washing once with 1X phosphate buffered saline (PBS) (*Mediatech, Inc., cat. no. 21-040*), and incubating for 5 minutes with 0.05% Trypsin EDTA (*Gibco, cat. no. 25300*).

Cytotoxicity

MDA-MB-231 cells were plated at 10^4 cells per well in a 96-well plate (*Corning, cat. no. 07-200-91*). PGNIPAM and PEI complexes with Negative control siRNA #1 were prepared as well as PG backbone polymer at a concentration of 100ug/mL. PGNIPAM at an N:P ratio of 80:1 with 100nM siRNA is at a concentration of 100ug/mL so this control will measure the toxicity of the PG backbone as opposed to the full polymer. Samples were incubated with cells in OptiMEM (*Gibco, cat. no. 11058-021*) for 4 hours to simulate a typical transfection time, such as that for Lipofectamine 2000.

After 4 hours, all media was aspirated off the cells, the cells were washed twice with PBS and a Cell counting kit (Dojindo, cat. no. CK04) was used to measure cell viability. The substrate was prepared as directed and incubated with the cells for 2 hours. Absorbance was measured at 450nm and reference at 650nm was subtracted. All samples were normalized to a no-treatment control (NTC).

Cell Imaging

MDA-MB-231 cells were grown on two 8-well glass slides (*Nunc, cat. no. 154534*) at a cell density of 10^4 cells per well. PGNIPAM and PEI complexes were prepared with ADNA at a concentration of 200nM. Cells were incubated with the complexes for 0 to 4 hours and then incubated for 10 minutes with DAPI (*Sigma Aldrich, cat. no. D9564*) to stain the cell nucleus. Two PBS washes were used to remove excess media and DAPI. The cells were then fixed using 100% acetone (*Sigma Aldrich, cat. no. 154598*) at -20°C. Gel-Mount (*Electron Microscopy Sciences,cat. no. 17985-10*) was applied to the wells and the slides were coverslipped. Imaging was conducted using a CRI Nuance Multispectral Imaging System and an Olympus microscope equipped with a 100X oil immersion lens, and a FITC and DAPI filter set. The emission spectra for FITC and Alexa488 so a FITC emission filter will serve to capture the Alexa488 emission. Background was subtracted and Image J was used to merge the Alexa488 images with the DAPI images.

A FAM-tagged siRNA (FAMsiRNA) was delivered using PGNIPAM to MDA-MB-231 cells. FAM is a FITC derivative, with an excitation peak in the blue at 488nm and a fluorescence peak at 525nm. The cells were transfected using a standard procedure of up to a 4-hour incubation, followed by washing and replacement of media for samples studied after 4 hours. After incubation for the appropriate time point, the cells were washed with 1X PBS twice and then fixed using 4% formaldehyde. The samples were permeabilized with 0.1% triton X-100. The fixed cells were then labeled with primary

antibodies against either EEA1 and LAMP-1, an early endosomal marker and a late endosomal marker, respectively. Secondary antibodies labeled with texas red were incubated, the cells were washed and then cover-slipped. Confocal images were taken with a slice width of 0.5μm.

siRNA Transfection

PGNIPAM complexes were formed with anti-survivin siRNA at increasing concentrations from 25nM to 100nM and with Negative control siRNA #1 at 100nM. Lipofectamine 2000 (*Invitrogen, cat. no. 11668-019*) or Lipofectamine RNAiMAX (*Invitrogen, cat. no. 13778-075*) was used as a transfection control for both anti-survivin siRNA and Negative control #1 siRNA and the complexes were prepared as directed by the protocol from Invitrogen. MDA-MB-231 cells were plated in 6-well plates (*Corning, cat. no. 3516*) at 80% confluency one day. Media was aspirated and the cells were incubated with either the polymer complexes or the Lipofectamine complexes. The cells were then incubated for 4 hours at 37°C and then the media was removed and the cells were washed with PBS. Fresh full media was applied and the cells were placed in the incubator for 48 hours. At 48 hours, the media was aspirated off, and cells were washed twice with cold PBS. RIPA buffer (*Pierce, cat. no. 89900*) modified with 0.1% Triton X-100 (*Sigma Aldrich, cat. no. T9284*) and protease inhibitor (*Roche, cat. no. 11836153001*) was applied at the recommended volume and the cells were incubated for 10 minutes at 4°C. Protein was extracted by using a cell scraper to break up the cell debris so it could be collected. All samples were then sonicated for 30 minutes to release all protein from the cell mass. Protein concentration was determined using a bradford assay with Coomassie Plus Protein Assay Reagent (*Pierce, cat. no. 1856210*). All protein samples were normalized to 1 ug/uL concentration.

Western Blot

SDS PAGE was conducted to measure the protein concentration following transfection. A Mini-PROTEAN electrophoresis system from Bio-rad was used with a 10% Ready Gel Precast Tris-HCl gel (*Bio-Rad, cat. no. 161-1155*). All protein samples were mixed with 4X sample loading buffer (TrisCl/SDS, 40% glycerol, bromophenol blue) and then heated to 95°C for 5 minutes to denature the proteins and stabilize with SDS. Samples are loaded at 30ug protein per well and the gel is run in 1X tris glycine SDS electrophoresis buffer (*Bio-Rad, cat. no. 161-0772*) for 2 hours at 75V. The gels are then used with Immuno Blot PVDF membrane (*Bio-Rad, cat. no. 162-0177*) and inserted into a Bio-Rad TransBlot Cell with tris glycine transfer buffer (*MP Biomedicals, LLC, cat. no. 816200*). The blot was run at 30V for 2 hours at 4°C. The blot was removed and incubated in 10% blotting grade blocking buffer (*Bio-Rad, cat. no. 170-6404*) in tris buffered saline (TBS) (*Mediatech, Inc., cat. no. 46-011*) and 0.1% tween 20 (*Fisher, cat. no.*) for 1 hour. The primary antibody incubation was performed in 2% blocking buffer with the primary antibody for survivin (*Millipore, cat. no. AB16533*) at 1:500 dilution and b-Actin antibody (*Cell Signaling, cat. no. 4967*) at a 1:1000 dilution. Both antibodies are raised in rabbit. After 1 hour of incubation the blot was washed with TBS-tween 4 times for 5 minutes each. The blot was then incubated for 30 minutes in 2% blocking buffer with the secondary antibody, an anti-rabbit IgG horseradish peroxidase (HRP) (*Cell-signaling, cat. no. 7074*). Four more washes with TBS-tween and then the blot is incubated with ECLPlus western blotting reagent (*GE Healthcare, cat. no. RPN2132*) for 5 minutes and then imaged using a film developer. Exposure time for the Blue Devil Film (*Blue Devil, cat. no. 30-100*) is 10 minutes. Image J was used to measure the darkness of the protein spots and the intensity was background subtracted and normalized to the no-treatment control.

Gene Expression by Real-Time PCR

Purification of mRNA was conducted following the guidelines of the RNA Aqueous kit (*Applied Biosystems, cat. no. AM1912*). MDA-MB-231 cells were cultured to 80% confluence in 96-well plates, with the experiment being conducted in triplicate. Cell culture media was RMPI 1640 (*Mediatech, cat. no. 10-040*) with 10% FBS (*Atlanta Biologicals, cat. no. S11150*). PGNIPAM was complexed with one of four separate siRNAs, two different positive and two different negative siRNAs, at concentrations of either 25nM or 100nM. Lipofectamine RNAiMax (*Invitrogen, cat. no. 13778100*) treatments were prepared with each of the four siRNAs at a 100nM concentration to serve as knockdown controls for the experiment. Cells were incubated with PGNIPAM or Lipofectamine treatments for 4 hours in OptiMEM reduced serum media (*Invitrogen, cat. no. 51985034*). Following 4 hours, the media was aspirated and the cells were washed with 1xPBS prior to addition of fresh full media. Following 48 hours of incubations, cells were lysed using the included lysis buffer of the RNA isolation kit. Cell lysates were spun down and washed using spin cartridges and total RNA was eluted for each treatment sample of each experiment replicate. RT PCR was carried out using the iScript 1-step RTPCR kit with SYBR green (*BioRad, cat. no. 170-8890*) and an Applied Biosystems 7900 96-well plate PCR system. The gene 18S was used as the housekeeping gene and an RNA control was used as calibrator between plates. This was accomplished with the Quantum Classic 18S Internal Standard kit (*Applied Biosystems, cat. no. AM1716*). Data was collected an analyzed for significance using Origin 6.1, and a two-population t-test to test between groups for significance at an α=0.05.

Animal Studies

All mice used in these studies were purchased from Taconic. The strain nomenclature is CrTac:NCr-Foxn1nu, and specifically model number NCRNU-F homozygous, nude. These mice are a spontaneous mutant T-cell deficient strain causing the mice to be immunocompromised. All mice were housed in sterile cages with sterile

racked water bottles. Mice were injected with MDA-MB-231 human breast cancer tumors cells, cultured to passage 6at a concentration of 10*10^6 cells per 100uL injection. The injection volume consisted of 50% cells in cell culture media with 10% FBS, and 50% matrigel basement membrane (*BD, cat. no. 356231*). Subcutaneous injection was performed with a 21 gauge needle attached to a 1mL syringe. Injection was performed into the mammary tissue plate directly next to the right forelimb of each mouse to minimize site-specific effects. Tumor volume was measured each day until volume equaled or exceeded 100mm^3. Treatments were performed by direct tumor injection of 100uL of solution. Tumor size and volume were recorded each day for 30 days or until the mouse succumbed on its own. Animals were sacrificed using CO2 asphyxiation and tumors were extracted. Hematoxylin and Eosin staining was performed on tumor tissue to study cell morphology and tumor health.

REFERENCES

[1] Meister, G. and T. Tuschl, *Mechanisms of gene silencing by double-stranded RNA.* Nature, 2004. **431**(7006): p. 343-9.

[2] Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.

[3] Aoki, Y., et al., *RNA interference may be more potent than antisense RNA in human cancer cell lines.* Clin Exp Pharmacol Physiol, 2003. **30**(1-2): p. 96-102.

[4] Dorsett, Y. and T. Tuschl, *siRNAs: applications in functional genomics and potential as therapeutics.* Nat Rev Drug Discov, 2004. **3**(4): p. 318-29.

[5] Pang, W., et al., *Therapeutic protein production in vivo after electroporation-assisted intramuscular gene delivery.* Conf Proc IEEE Eng Med Biol Soc, 2005. **6**: p. 6229-32.

[6] Aigner, A., *Delivery systems for the direct application of siRNAs to induce RNA interference (RNAi) in vivo.* Journal of Biomedicine and Biotechnology, 2006: p. -.

[7] Mintzer, M.A. and E.E. Simanek, *Nonviral vectors for gene delivery.* Chem Rev, 2009. **109**(2): p. 259-302.

[8] Ambrosini, G., C. Adida, and D.C. Altieri, *A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma.* Nat Med, 1997. **3**(8): p. 917-21.

[9] Zaffaroni, N., M. Pennati, and M.G. Daidone, *Survivin as a target for new anticancer interventions.* J Cell Mol Med, 2005. **9**(2): p. 360-72.

[10] Croci, D.O., et al., *Silencing survivin gene expression promotes apoptosis of human breast cancer cells through a caspase-independent pathway.* J Cell Biochem, 2008. **105**(2): p. 381-90.

[11] Olie, R.A., et al., *A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy.* Cancer Res, 2000. **60**(11): p. 2805-9.

[12] Sarela, A.I., et al., *Expression of the antiapoptosis gene, survivin, predicts death from recurrent colorectal carcinoma.* Gut, 2000. **46**(5): p. 645-50.

[13] Sarela, A.I., et al., *Immunohistochemical detection of the anti-apoptosis protein, survivin, predicts survival after curative resection of stage II colorectal carcinomas.* Ann Surg Oncol, 2001. **8**(4): p. 305-10.

[14] Sarela, A.I., et al., *Expression of survivin, a novel inhibitor of apoptosis and cell cycle regulatory protein, in pancreatic adenocarcinoma.* Br J Cancer, 2002. **86**(6): p. 886-92.

[15] Yi, T., et al., *Enhancement radiosensitization of breast cancer cells by deguelin.* Cancer Biother Radiopharm, 2008. **23**(3): p. 355-62.

[16] Moriai, R., et al., *Survivin plays as a resistant factor against tamoxifen-induced apoptosis in human breast cancer cells.* Breast Cancer Res Treat, 2009. **117**(2): p. 261-71.

[17] Behr, J.P., *The proton sponge: A trick to enter cells the viruses did not exploit.* Chimia, 1997. **51**(1-2): p. 34-36.

[18] Thomas, M. and A.M. Klibanov, *Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells.* Proc Natl Acad Sci U S A, 2002. **99**(23): p. 14640-5.

[19] Oh, Y.K., et al., *Polyethylenimine-mediated cellular uptake, nucleus trafficking and expression of cytokine plasmid DNA.* Gene Ther, 2002. **9**(23): p. 1627-32.

[20] Paleos, C.M., et al., *Gene delivery using functional dendritic polymers.* Expert Opin Drug Deliv, 2009. **6**(1): p. 27-38.

[21] Shen, Y., et al., *Gold nanoparticles coated with a thermosensitive hyperbranched polyelectrolyte: towards smart temperature and pH nanosensors.* Angew Chem Int Ed Engl, 2008. **47**(12): p. 2227-30.

[22] Staudinger, H. and J. Meyer, *On new organic phosphorus bonding III Phosphine methylene derivatives and phosphinimine.* Helvetica Chimica Acta, 1919. **2**: p. 635-646.

[23] Hall, H.K., *Correlation of the Base Strengths of Amines.* Journal of the American Chemical Society, 1957. **79**(20): p. 5441-5444.

[24] Amara, M. and H. Kerdjoudj, *A modified anion-exchange membrane applied to purification of effluent containing different anions. Pre-treatment befoe desalination.* Desalination, 2007(206): p. 205-209.

[25] Duan, H. and S. Nie, *Cell-Penetrating Quantum Dots Based on Multivalent and Endosome-Disrupting Surface Coatings.* J. Am. Chem. Soc. , 2007.

[26] Godbey, W.T., K.K. Wu, and A.G. Mikos, *Poly(ethylenimine)-mediated gene delivery affects endothelial cell function and viability.* Biomaterials, 2001. **22**(5): p. 471-80.

[27] Zheng, F., et al., *Chitosan nanoparticle as gene therapy vector via gastrointestinal mucosa administration: Results of an in vitro and in vivo study.* Life Sciences, 2007. **80**(4): p. 388-396.

[28] Aroui, S., et al., *Conjugation of doxorubicin to cell penetrating peptides sensitizes human breast MDA-MB 231 cancer cells to endogenous TRAIL-induced apoptosis.* Apoptosis, 2009.

[29] Haba, Y., et al., *Comparison of thermosensitive properties of poly(amidoamine) dendrimers with peripheral N-isopropylamide groups and linear polymers with the same groups.* Angew Chem Int Ed Engl, 2007. **46**(1-2): p. 234-7.

[30] Saad, M., O.B. Garbuzenko, and T. Minko, *Co-delivery of siRNA and an anticancer drug for treatment of multidrug-resistant cancer.* Nanomedicine (Lond), 2008. **3**(6): p. 761-76.

[31] Teasdale, I., et al., *Multifunctional and biodegradable polyphosphazenes for use as macromolecular anti-cancer drug carriers.* Polymer Chemistry, 2010. **2**(4): p. 828-834.