Methylated and Unmethylated pDNA Delivery Comparison in Mammalian Cells

by

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ABSTRACT

In this study, the differences in delivery of methylated and unmethylated prokaryotic DNA in mammalian cells was investigated. 3 plasmids, DH5-α, ER2925 and GM272 were extracted and transformed from E. coli bacteria. DH5-α is the regular methylated plasmid, however,ER2925 and GM272 lack Dam and Dcm enzymes which methylate adenine and internal cytosine in prokaryotes respectively, hence they are unmethylated. The 3 plasmids were delivered via different delivery vectors in two cell lines, UMUC3 and MDA-MB-231 which are human bladder cancer cell line and human triple negative breast cancer cell line, respectively.

Luciferase and BCA assay were carried out to quantify transgene expression to compare the efficacy of gene delivery in three aforementioned plasmids. In addition, hydrodynamic diameter and zeta potential was measured for all delivery vectors, to correlate with other transgene expression data. The results show that methylated plasmid has significantly higher transgene expression in mammalian cell lines. This can be either a result of smaller size and more positive zeta potential that the methylated plasmid had, or a result of having Dam and Dcm enzymes which enhance binding of DNA and transcription factors and enhance gene expression. Having smaller size and more positive zeta potential was proven to be the case for the methylated plasmid in this study. However the latter hypothesis should be investigated furthermore.

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TABLE OF CONTENTS

Page

LIST OF TABLES iv

LIST OF FIGURES v

CHAPTER

1 INTRODUCTION 1

1.1 Gene Delivery 1

1.2 Intercellular Barriers for Non-viral Gene Delivery 3

1.2.1 Cellular Uptake and its Barriers 4

1.2.2 Endosomal Escape 6

1.2.3 Nuclear Entry 7

1.2.4 Delivery Vector Dissociation 7

1.3 DNA and Histone Modification 8

1.3.1 Histone Modification 8

1.3.2 DNA Methylation 8

2 PREPARATION, TESTING METHODS AND RESULTS 14

2.1 Methodology 14

2.1.1 Materials 14

2.1.2 Preparation and Testing Methods 14

2.2 Results 22

3 CONCLUSION 35

REFERENCES 36

LIST OF TABLES

Table Page

1.1 Examples of Loci Under Dam Methylation Control in Gamma-proteobacteria Sánchez-Romero *et al.* (2015) 12

LIST OF FIGURES

Figure Page

1.1 Intercellular Barriers to Gene Delivery Christensen (2016) Used with Permission 4

2.1 Plasmid pGL4.5 Schematics (sketched Using SnapGene Software)..... 15

2.2 Polymer Monomers Potta *et al.* (2014) 17

2.3 $\frac{LUV}{\mu gofProtein}$ for Each Plasmid-PG Polymer Polyplex Was Measured in UMUC3 and MDA-MB-231 Cell Lines 22

2.4 $\frac{LUV}{\mu gofProtein}$ for Each Plasmid-PGC18 (1:5) Lipopolymer Polyplex Was Measured in UMUC3 and MDA-MB-231 Cell Lines 23

2.5 $Log\frac{LUV}{\mu gofProtein}$ for Each Plasmid-NR Polymer Polyplex Was Measured in UMUC3 and MDA-MB-231 Cell Lines 24

2.6 Viability Was Measured for Each Condition in Both Cell Lines 25

2.7 Hydrodynamic Diameter and Zeta Potential of Each Plasmid-PG Polymer Polyplex 26

2.8 Hydrodynamic Diameter and Zeta Potential of Each Plasmid-PGC18 (1:5) Lipopolymer Polyplex 27

2.9 Hydrodynamic Diameter and Zeta Potential of Each Plasmid-NR Polymer Polyplex 28

2.10 Hydrodynamic Diameters and Zeta Potentials of 3 Polymers 29

2.11 Hydrodynamic Diameters and Zeta Potentials of 3 Plasmids 30

Chapter 1

INTRODUCTION

1.1 Gene Delivery

Gene delivery is the process of transferring exogenous nucleic acid into the host cell nucleus.It has several applications including expression of therapeutic proteins, gene therapy, and is a technique to understand biological pathways Christensen (2016). These applications are discussed below.

Gene delivery can be used to employ the cells to produce recombinant proteins in large scale for downstream use. These proteins can be used directly as therapeutics or in protein vaccines Jin *et al.* (2014); Baldi *et al.* (2007). Potential treatment of several genetic disorders, either inherited or acquired, is now considerable by gene therapy. Cancer, AIDS and cardiovascular disease are some examples of such deceases. The principle that this approach is based on, is to deliver the foreign DNA to alter defective gene expression at the origin of the defect. This alteration is done with two approaches. The first approach is to correct the genetic malfunctions and ultimately rescue the cell and the organ. The second approach though, leads the host cells to self destruction. This approach is mainly used in cancer gene therapy. The advantage of gene therapy against others is that the limitations of some protein drugs can be bypassed with this technique Jin *et al.* (2014); Liu and Huang (2002); Christensen (2016); Siu *et al.* (2012). In molecular biology, in vitro gene delivery is used as a study tool to study biological pathways. For example, it is used to study gene functions via manipulative protein expression. Silencing a specific gene ,which can be carried out by delivering siRNA or shRNA, can help us determine specific protein functions Jin

et al. (2014); Christensen (2016).

There are many intercellular barriers to nucleic acid transfer that are thoroughly discussed in the next section. However, in this section, we talk about two methods that are employed to circumvent the intercellular barriers. Gene delivery methods should ideally have high transfection efficiency and low toxicity to the cells Jin *et al.* (2014). Currently, three main categories of gene delivery methods are employed. viral vector based, non-viral vector based and physical Mali (2013).

Virus vectors are either replication-deficient viruses, which contain our nucleic acid of interest but the decease causing sequence is deleted from their genome,or genetically modified viruses Kamimura *et al.* (2011); Mali (2013).

Non-viral gene delivery methods are done using natural or synthetic compounds to bound with DNA and form a complex with it. Complexes of DNA with protein, lipids and polymers are examples of non-viral vectors Kamimura *et al.* (2011); Mali (2013). Usually, the transgene expression of the plasmids delivered via cationic vectors have two phases. In the first few days the transgene expression is dramatically high and after that transgene expression continues at a lower intensity Al-Dosari and Gao (2009). This can be due to several reasons. Most of the times, the transfered plasmid stays in the cell nucleus episomal DNA molecule and do not integrate with the target cell's genomic DNA. During cell division, these plasmids are not replicated and as the population percentage of newly divided cells grow and old cells die, transgene expression lowers. Another reason for this decline in transgene expression may be the programmed death of transfected cells or that the transfected cells simply die because of the injuries caused by tansfection Nguyen *et al.* (2007). In addition, transfected cells may be eliminated due to the immune response to producing foreign proteins Wolff *et al.* (1992).

Physical methods focuses on overcoming membrane and intercellular barriers us-

ing physical principles. For examples, one of the physical methods is to inject the DNA directly to the target tissue with a needle. Injection into muscle cells, skin, cardiac muscle, liver and solid tumor had been successfully done. Some other are Electroporation, Sonoporation, Photoporation, Photoporation, etcKamimura *et al.* (2011); Mali (2013).

All these methods have their advantages and disadvantages in comparison to one another. Virus vectors are the most efficient, however there are some safety issues with them and they are very costly as well. Non-viral vectors are much less toxic than the viral vectors. Another advantage of them is that they are more likely to be used for delivering long nucleic acid sequences and they are less immunogenic. Advantage of physical methods in comparison to the two methods previously mentioned is its simplicity since DNA is delivered straightly to the target without the usage of other substances which can be toxic to the cell or immunogenic and is the method utilized in DNA vaccination. In addition, non-endocytic strategies such as microinjection and electroporation allow the nucleic acid to circumvent many intercellular barriers such as escaping the endocytic vesicles. However, the disadvantages of this method is its low efficiency and the fact that gene delivery is only restricted to the injected cells. Thus their application is limited in gene therapy and industrial scale protein production Kamimura *et al.* (2011); Mali (2013); Christensen (2016).

Some advantages and disadvantages and characteristics of different non-viral methods are listed in the table below:

1.2 Intercellular Barriers for Non-viral Gene Delivery

There are many intercellular barriers that gene delivery vectors should circumvent for a successful transfection. They are demonstrated in 1.1 and in the following subsections, we will discuss them in detail.

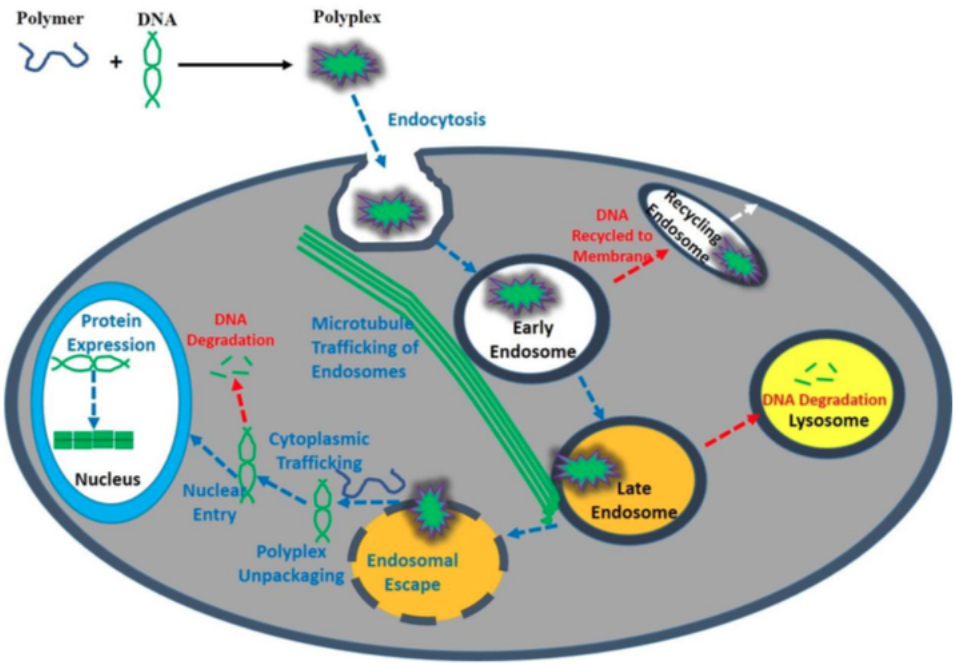


Figure 1.1: Intercellular Barriers to Gene Delivery Christensen (2016) Used with Permission

1.2.1 Cellular Uptake and its Barriers

In encodytic cellular uptake, the cargo can enter the cell via four different pathways, clathrin-mediated endocytosis, caveolae, phagocytosis and macropinocytosis. Size and surface charge of the cargo have a huge impact on cellular uptake as well as the cell itself. Moreover, multiple mechanisms may occur simultaneously due to the heterogeneity of the cargoes.

Clathrin-mediated Endocytosis

Clathrin-mediated endocytosis is mediated by a large protein named Clathrin. On the inner surface of the cell plasma membrane, a coated pit is formed with the help of this protein. The ligands of this protein complex bind to the plasma membrane

receptors and lipids and clatherin forms a coat around the cargoIrajizad *et al.* (2017). Then from this pit the cargo comes into the cytoplasm of the cell as a coated vesicle. Later, the vesicles lose their clatherin coating. the fusion a similar vesicles ultimately form early endosomes DeB (1998); Christensen (2016).

Caveolaes

Caveolaes are cave like structures in the membrane. Many cell types have caveolaes on their surface and they can take up to a third of plasma membrane surface. The pits are relatively narrow with a diameter of approximately 50 nm. They consist of a cholesterol binding protein called Vip21 and a bilayer of cholesterol and glycolipids Christensen (2016). cellular uptake is mediated via receptors in caveolaes however it's clatherin independent. Unlike clatherin mediated uptake which the vesicles then goes to form an early endosome, the cargo taken up via cavolaes can go directly to Golgi or endoplasmic reticulum for sorting. As a result, they bypass the pathway in which are lead to degradation by lysosome which can affect the delivery of nucleic acid's efficacy Parton and Simons (2007).

Phagocytosis

Phagocytosis, is the cellular uptake pathway most commonly used by the immune system cells to take up pathogens and other large particles (larger than 500 nm in diameter)Christensen (2016). Phagocytosis can also occur in cells other than immune system cells and has the potential to be used for delivery of particles that are too large to be taken up by caveolaes or clatherin mediated pathways. Despite its applications in gene delivery, for in vivo gene delivery, phagocytic cells in the immune system might take up the delivered nucleic acid in the blood and affect gene delivery efficacy Dash *et al.* (1999). The takeup vesicle with this pathway is called phagosome. It's

ultimately lead to fusion with lysosome for degredation Falcone *et al.* (2006).

Macropinocytosis

Macropinocytosis is another pathway of cellular uptake. This pathway involves uptake of large particles which results in 500-5000 nm vesicle diameter. Another difference of this pathway in comparison to caveolae and clatherin mediated is that no receptors are involved. The vesicle later forms early endosomesFalcone *et al.* (2006).

Delivery vectors can be taken up and trafficked in the cells in via different pathways. Even subtle differences in delivery vectors can change the pathway in which the cargo is taken up. The hydrodynamic diameter of the cargo its surface charges have tremendous effects on cellular take-up. For example, a study shows that in murine melanoma cell line, different cellular take-up pathways were utilized depending on the size of the latex bead cargoes. Cargoes smaller than 200 nm in diameter were taken up via clatherin mediated pathway. However, cargoes larger than 500 nm were taken up by caveolae. Since in caveolae takeup, the cargo circumvents degradation by lysosomes, it can be relatively more efficient in this case Christensen (2016).

1.2.2 Endosomal Escape

Sorting endocytosed materials before they are degraded by lysosomes are done by endosomes. Internalized cargoes from the plasma membrane can either go to lysosome for degradation or be recycled back to the plasma membrane. Depending on the stage after endocytosis, endosomes are classified as early, sorting, or late endosomes. Different stages of endosomes have different mythologies. Early endosomes have tubules however late endosomes mostly don't and are spherical. This is due to early endosome's loss of tubules to recycling pathways back to the plasma membrane Barua and Rege (2009). Endocytic vesicles fuse into early endosomes after being un-

coated. Later, early endosomes mature into late endosomes and ultimately fuse with lysosomes. In the stage of maturation of early endosomes into late endosomes, the pH level lowers. The drop in pH level can assist endosomal escape for gene delivery before endosomal maturation and ultimately fusion with lysosome Mellman (1996); Suh *et al.* (2003).

1.2.3 Nuclear Entry

After overcoming the barriers discussed before, cargo has to enter the cell nucleus. As we know, The nucleus of the cell is constantly dividing due to cell division. The Nucleus membrane of a dividing cell is much more permeable than a non-dividing nucleus due to the nuclear membrane breakdown prior to cell division. As a result, cargo can relatively easily penetrate dividing nuclei where as it is almost impossible for it to enter the nucleus at any other time Jones and Takai (2001). Diffusion through cell nucleus pore complexes only allows particles smaller than 9 nm to penetrate a non-dividing cell nuclei. To summarize, this step of gene delivery is relatively much more inefficient for non-viral gene delivery vehicles Christensen (2016); van der Aa *et al.* (2006).

1.2.4 Delivery Vector Dissociation

When designing gene delivery vectors, we must take into account that this step works against the previous ones. For gene transfer into the nucleus, the binding of the nucleic acid and delivery vector should be strong. However, in this step DNA should unbind with the delivery vector for it to be accessible to the host cell's transcription machinery Huth *et al.* (2006); Schaffer *et al.* (2000). Cationic carrier has can be removed from the exogenous nucleic acid by the chromosomal DNA in the nucleus in this step. However, removal of the cationic carrier of delivery vector in the cytoplasm

by host cell RNA can make gene delivery more inefficient Christensen (2016); Kircheis *et al.* (2001).

1.3 DNA and Histone Modification

1.3.1 Histone Modification

In eukaryotic cells, DNA is wrapped around some alkaline proteins called histone. The structural units that DNA and histone make are called nucleosomes. Histone not only orders the DNA into structural nucloesomes, but has other roles specially in gene expression regulations which affects whether a gene is activated or repressed due to the interactions between DNA and histone tails. These interactions result from the bounds between histone and DNA getting tighter or looser. Histone proteins have tails that can be covalently modified. Some of the most important covalent modifications are acetylation and methylation. Acetylation has an activation role for genes. However, methylation can have either activation or repression role depending which histone tail is activated and how many methyl groups are attached to the histone tails. These modifications do not make a change in genomic DNA sequence. Delpu *et al.* (2013)

1.3.2 DNA Methylation

DNA Methylation in Eukaryotes

DNA methylation is very abundant in eukaryotic systems, specially in plants and vertebrates. Methyl groups are added to DNA at two times. First, they can be added to newly replicated DNA. Modification in methylation pattern, can later be carried out by adding and removing methyl groups from various sites. Methylation pattern in cancer cells is very different from normal ones as it is largely modified in them.

This difference can be used to distinguish cancer cells. Delpu *et al.* (2013); Schübeler (2015)

Methylation is carried out by the enzyme methyl transferase. For DNA methylation, the enzyme is called DNA methyl transferase (DNMT). DNA methylation sites are very specific. CpG islands in plants and vertebrates are mostly located near the promoter of the genes. They provide many DNA methylation sites since they are usually about 1000-2000 base pairs long Klose and Bird (2006); Jones and Takai (2001).

Usually, the activity of DNA methylation is to silence genes and inhibit their transcription long term. This regulatory action is specially done in tissue specific genes. These genes help our tissues have different functions despite the genomic DNA being the same in all of them. Tissue specific gene silencing helps prevent genes to be expressed in the wrong tissue Bird (2002).

DNA methylation, inhibits gene transcription in two ways. First, for the initiation of the transcription, activator proteins are transcription factors that bind to the enhancer sequence present behind the promoter of the gene to be transcribed. In eukaryotic system, transcription factors play an important role in initiation of transcription. In normal situation, the activator protein attaches to the enhancer sequence and will send some signal which will recruit the polymerase and the transcription will go on Li *et al.* (1993); Jones and Takai (2001). However, due to methylation in the CpG island, the activator protein can no longer bind to the enhancer element. As a result, the activator protein can no longer provide any signal to call upon the polymerase. Hence the transcription will not take place Klose and Bird (2006); Razin (1984).

The second way that DNA methylation can inhibit transcription in by a protein known as "methyl CpG binding protein" (MBD). This protein binds to methylated

sequencesBird (2002). These proteins are recruited to the methylated sites in DNA and attach to them by covalent binds. As a result, activator protein cannot bind to the DNA. So the transcription will not be initiated. This kind of MBD attachment will recruit Histone Deacetylase (HDAC) protein to detach the acetyl group from the histone and further inhibit gene transcription Li *et al.* (1993); Razin (1984).

DNA Methylation in Prokaryotes

Similar to eukaryotes, DNA methylation plays important roles in prokaryotes and is specially very prevalent among prokaryotes. Using single molecule, real-time sequencing (SMRs) to map DNA modifications for 230 diverse bacterial and archaeal species, DNA methylation was found in 93% of them. In that study, 840 distinct methylated motifs and 620 620 DNA Methyltransferases binding sites were found leading to acknowledge diverse presence and functions of DNA methylation in prokaryotes Blow *et al.* (2016). Along with m5C (C^5-Methyl-cytosine) which is most prevelant in eukaryotes, m6A (N^6-methyl-adenine) and m4C (N^4-methyl-cytosine) are also found in bacteria. m6A is also found in lower eukarytes (not in vertebrates) but m4C is only found in bacteria Sánchez-Romero *et al.* (2015). Despite the abundance, patterns and functions of methylation in prokaryotic DNA is still relatively unknown.

DNA methylation along with restriction enzymes are components of restriction-modification (RM) systems. The main function of this system is to protect the integrity of prokaryotic genome. For example, they are linked to DNA base pair mismatch repair. In addition, restriction endonuclease degrades DNA from exogenous sources (eg. viruses) while methyl transferase binds to restriction endonuclease sites in the host genome to prevent cleavage Blow *et al.* (2016); Modrich and Lahue (1996); Sánchez-Romero *et al.* (2015). To further study RM systems in prokaryotes, a comprehensive database of restriction enzymes, DNA methyl transferases and other

proteins involved in the process has been gathered. This database is refereed as "REBASE" Roberts *et al.* (2010).

As mentioned before, DNA methylation has an important role in eukaryotic genome regulation. However, its role in prokaryotic genome regulation and the extent of it is still relatively unknown. What we know is that in prokaryotes, if a regulatory region (eg pomoter region) is methylated, the methyl groups can help binding of transcription factors (eg RNA polymerase) with cell DNA. Some examples of such regulation involves two methyltransferase enzymes Dam (Deoxyadenosine methylase) and CcrM (cell-cycle regulated methyltransferase). These enzymes both methylate adenine bases in DNA and if they methylate DNA at a regulatory region (eg promoter region), they can regulate transcription. Methylation-dependent transcription controls are divided into two groups: Clock-like and Switch-like controls. In clock-like controls, gene methylation (which activates expression) is linked to the stage of the cell cycle. However, switch-like controls work in a different manner. Before DNA methylation patterns are formed, they turn on and off gene methylation, hence gene expression Sánchez-Romero *et al.* (2015); Han *et al.* (2004). Some examples of Dam methylation control in Gamma-proteobacteria -which E. coli is a part of- are mentioned in the table 1.1.

Table 1.1: Examples of Loci Under Dam Methylation Control in Gammaproteobacteria Sánchez-Romero *et al.* (2015)

Type of control	Locus	Methylation-sensitive protein or protein complex	Number of regulatory GATC sites	Location of GATC site(s)	Position of GATC sites[a]
Clock-like	*tnp(IS10)*[b]	RNA polymerase	1	Promoter	−14
	traJ[c]	Lrp	1	UAS[d]	−52
Switch-like	*papBA*[b]	Lrp	2	UAS	−155, −53
	agn43[b]	OxyR	3	Promoter	+1, + 20, +33
	std[c]	HdfR	3	UAS	−242, −229, −220
	sciH[b]	Fur	3	UAS	Not determined[e]
	gtr[f]	OxyR	4	UAS	−110, −97, −46, −33
	opvAB[c]	OxyR	4	UAS	−174, −124, −101, −51

[a] Each number indicates the position of the G moiety of a GATC on the coding DNA strand.
[b] *E. coli*.
[c] *Salmonella enterica*.
[d] Upstream activating sequence.
[e] GATC sites are in the UAS but their precise location cannot be established because the transcription start point has not been determined.
[f] *S. enterica* bacteriophage P22.

Like adenine methylation, cytosine methylation in prokaryotes also have regulatory effects on transcription, like enhancement. Inoue *et al.* (1997) However, unlike adenine methylation which typically enhances transcription, cytosine methylation can have both enhancement and repression effect Militello *et al.* (2014). two studies on E. coli suggest that lack of Dcm (DNA cytosine methyltransferase) increased expression of stress response sigma factor RpoS and drug resistance transporter SugE. However, relationship between m5C and transcription is not yet fully known. In these studies, Dcm did not have an effect on E. coli growth rate or the ability to enter or persist stationary phase Kahramanoglou *et al.* (2012); Sánchez-Romero *et al.* (2015).

Plasmid Methylation and Immune System Response

Since plasmid DNA is from a prokaryotic source, it is considered a foreign entity in mammalian cells Yew (2005); McLachlan *et al.* (2000). As mentioned in the previous sections, mammalian DNA have very abundant methylated CpG sequences. Since CpG sequences are mostly unmethylated in prokaryotic DNA compared to mammalian DNA, the mammalian innate immune system recognizes the plasmid DNA.

Krieg (1999); Reyes-Sandoval and Ertl (2004); Li and Huang (2000) As a result, following the administration of polyplexes, an inflammatory response occurs. Unmethylated CpG sequences activate a receptor called "Toll-like receptor 9" which then activated immune response from the cell **??**Reyes-Sandoval and Ertl (2004). Another reason for this immune system response may be due to the non-linear nature of plasmids which again distinguish them from mammalian DNA Yew (2005); Zhou *et al.* (2004). There are ways that we can reduce this immune response from the cell in order to increase the efficiency of pDNA delivery. In a study it was shown that the use of pDNA vectors which contain a reduced number of CpG motifs, results in a reduction of inflammatory response. In that study, pDNAs were developed in a way that all the CpG motifs from the enhancer, promoter, intron, poly- adenylation signal, and antibiotic resistance gene were eliminated. The only remaining CpG sequences were then found in plasmid replication origin which was about 20% of CpG sequences in the original unmodified DNA. Delivery of this modified pDNA vector resulted in a decrease in proinflammatory cytokines Yew *et al.* (2002).

Chapter 2

PREPARATION, TESTING METHODS AND RESULTS

2.1 Methodology

2.1.1 Materials

dimethyl sulfoxide (DMSO), resorcinol diglycidyl ether (RDE) were purchased from Sigma-Aldrich, St. Louis, USA. Aminoglycoside monomers, neomycin sulphate, and paromomycin sulphate were purchased from Gold Biotechnology, Inc., USA. The BCA protein assay kit was purchased from Thermo Scientific, Inc. (Rockford, IL). The pGL4.5 control vector, which encodes for modified firefly luciferase protein under the control of an SV promoter, and the Bright-GloTM luciferase assay system were purchased from Promega Corporation (Madison, WI). acetone was purchased from VWR international LLC, USA suppliers and was used without further purification. MDA-MB-231 human triple-negative breast cancer cells, and UMUC3 human bladder cancer cells were procured from the American Type Cell Culture (Manassas, VA).

2.1.2 Preparation and Testing Methods

Bacterial Transformation and Preparation of Plasmids

Plasmid pGL4.5 was used for this study. A schematic of this plasmid is shown in **??**. This plasmid codes for Luc2 gene which is the firefly luciferase. Hygromycin resistance gene is used as a biological marker in this plasmid. The plasmid has a replication origin after the hygromycin resistance gene and also codes for ampicillin resistance gene in order to survive after adding ampicilin to its media. Ampicillin is

added to the media in order to eliminate all the other microorganisms in the media and only our bacteria of interest which contains ampicillin resistance gene can survive.

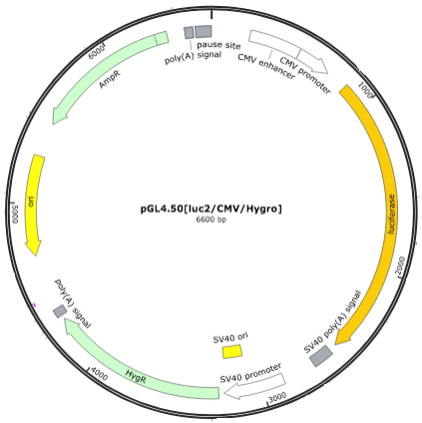


Figure 2.1: Plasmid pGL4.5 Schematics (sketched Using SnapGene Software)

For this study, 3 variations of plasmid DNA pGL4.5 were prepared: DH5-α, K12 ER2925 and GM272. K12 ER2925 lacks Dam5 and GM272 lacks Dam3 and Dcm6 enzymes which as previously discussed, methylate adenine and cytosine bases in prokaryotic DNA MacNeil (1988); Sun *et al.* (2010). Hence, These strains are relatively unmethylated. On the contrary, DH5-α is the regular methylated pGL4.5 strain. Dam enzyme methylates Adenine in GATC sequences and Dcm enzyme methylates the second cytosine in CCAGG and CCTGG sequences. In pGL4.5 plasmid, there are 17 GATC and 12 CCWGG sequences in luc2 gene and its promoter. In the whole plasmid, there are 34 GATC and 20 CCWGG sequences respectively.

Bacterial transformation of pDNA pGL4.5 was carried out in order to prepare ER2925 and GM272 E. coli strains. For this process, 2 μl of pGL4.5 with concentration of $\frac{150ng}{\mu l}$ was mixed with 20 μl of component cells. The mixture was then incubated on ice for 20 minutes. For the heat shock, mixture was put in 42°C water bath for 45 seconds and then incubated on ice for an additional 3 minutes. 800 μl LB media was added to the mixture and they were placed on a shaker at 37°C ,200

rpm for 3 hours. Then, cells were centrifuged for 1 minute at 10000 rcf. Most of the supernatant was discarded, however, cells with the remaining supernatant were plated on LB + Ampicillin (100 mg/L) agar plates. 24 hours later, the colonies had grown. Individual colonies were injected in LB + Ampicillin (100 mg/L) liquid media and were placed on shaker at 37°C ,200 rpm for 24 hours. Plasmid DNA were then extracted.

Competent E. coli strains were prepared using the Mix and Go E. coli Transformation Kit and Buffer Set and plasmid DNA extraction was done using the Qiagen Plasmid Maxi Kit.

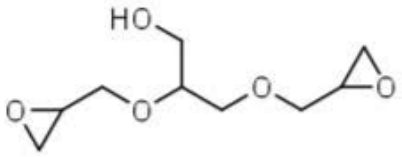
After extraction, plasmid DNA concentration and purity were determined using a NanoDrop Spectrophotometer (ND-1000; NanoDrop Technologies) by measuring absorbance at 260 and 280 nm. Plasmid stocks were made with concentration of $\frac{25ng}{\mu l}$ in EB buffer (Qiagen, Germany) to make polyplexes in the future.

Polymers

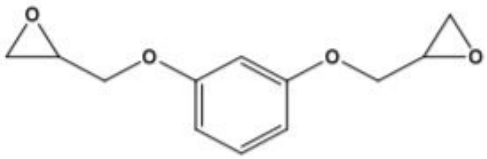
PG and NR

The two polymers used in this project, paromomycinGDE(PG) and neomycinRDE(NR)(GDE: Glycerol diglycidyl ether and RDE: Resorcinol diglycidyl ether) were synthesized by Dr. Sudhakar Godeshala according to Godeshala et al. (2016). In short, a solution of sulphate-containing aminoglycosides neomycin and paromomycin in nanopure water was ion exchanged in a Cl-ion exchange resin for 3 hours. After the sulphates were removed, the resultant aminoglycosides were reacted with GDE (in case of PG) or RDE(in case of NR) in a solution containing 1:2.2 molar ratio of water and N,N-dimethylformamide (DMF)), (1.5:1 v/v) for 5 hours at 60C. Acetone was used to precipitate the reaction mixture and further purification was carried out by dialysis

using a 3.5 kDa molecular weight cutoff(MWCO) dialysis membrane for 48 hr to remove unreacted aminoglycosides and diglycidylethers. To obtain the purified polymer after dialysis, the material was lyophilized. The M_W values for these polymers are 4,889 and 3,965 respectivelyPotta *et al.* (2014). The monomers of these polymers are shown in 2.2.



(a) GDE



(b) RDE

Figure 2.2: Polymer Monomers Potta *et al.* (2014)

PG-C18(1:5)

A solution of 0.01 mmol PG in 2ml DMSO was stirred at room temperature for 30 minutes, after which 0.1 mmol triethyl amine(TEA) was added to the solution, and was stirred for an additional 30minutes. The solution was then cooled to 4°C and a 1:5 molar ratio of alkanoyl chloride (with respect to PG) was added in a dropwise manner. The mixture was stirred at RT for 12 hours, then precipitated in excess ether, dialyzed against water for 48 hours using a 3500 molecular weight cutoff (MWCO)

membrane. To obtain the lipopolymer product, the dialyzed material was further luophilized. he M_W value for this lipopolymer is 4,519Miryala *et al.* (2015).

Gel Permeation Chromatography

Gel Permeation Chromatography (GPC) was used to measure the molecular weights of PG, PG-C18(1:5) and NR. The system used was Waters 1515 GPC system, equipped with an ultrahydrogel 250 column (Waters Corporation, MA) and a refractive index detector (Waters 2410) with Waters Millennium 32 GPC software. A solution of 0.1% trifloroacetic acid and 40mobile phase which was operated at 0.5 mL/min flow rate and the temperature was set at 358C. Poly(2-vinyl- pyridine) standards (MWs: 3,300, 7,600, 12,800, 35,000, and 70,000Da) were employed for molecular weight (MW) calibration. To further investigate the MW range, purified samples after dialysis were subjected to ninhydrin analysis which reports for reactive amine content. To determine the theamine content of the polymers, a calibration using glycine was carried out, and the relative amounts of residual amines were compared to determine the molecular weight range of the polymers Potta *et al.* (2014).

In order to make polyplexes for the experiments, polymer solution stocks were prepared with concentration of $\frac{1.5mg}{\mu l}$ in 1XPBS and were filtered through 2μm filters.

Cell Culture

UMUC3 and MDA-MB231 cells were cultured in DMEM media containing 10% v/v fetal bovine serum (FBS) and 1% penicillin streptomycin (10,000 units/mL) solution in an atmosphere of air (95%) and carbon dioxide (5%) in an incubator at 37 °C.

Transgene Expression in Cells

When approximately 80% confluent, cells were trypsinized with 0.25% trypsin-EDTA and plated in 96 well plates (Corning,Corning, NY, USA) with density of 10000 cells/well in DMEM media. Plates were incubated in previously mentioned situation (Atmosphere: 95% air, 5% carbon dioxide at 37°C) for 18-24 hours. Polyplexes were prepared by adding the polymer solution to the DNA (with the following w/w DNA:Polymer ratios: 1:4, 1:5, 1:10, 1:15, 1:20, 1:25, 1:50, 1:75, 1:100) and letting them sit for 30 minutes at room temperature. Ploypleхes were then added to each well (75 ng of pDNA per well). Plates were then incubated in the previous situation for 48 hours before screening to allow for transgene delivery and expression.

Luciferase and BCA Assay

48 hours after the transfection, media was removed from the cells and they were washed with 1x PBS. After removing PBS, cell culture lysis reagent (Promega) was added to the cells. Plates were incubated for 15 minutes in 37°C for the cells to be lysed. 30 μl of the luciferin solution (Bright GloTM Luciferase assay kit (Promega)) was added to 15 μl of cell lysate and luminescence (LUM) was immediately measured using Synergy 2 plate reader (Biotek, Winooski, VT). Using the BCA Protein assay kit (Pierce, Rockford, IL, USA), total protein content was determined from the cell lysates .

Quantification of Luciferase Expression

For quantification, LUV values were determined by dividing luminescence values (LUM) by relative cell proliferation (PRO). LUV values were normalized by the total protein content values from BCA assay. These normalized values (LUV/ μg of protein) are used to compare different conditions.

Cytotoxicity of Polyplexes

48 hours after the transfection is done (same time as the Luciferace assay), MTT assay is carried out on the transfected cells to make sure that the polyplexes were not cytotoxic. 10 μl of MTT reagent ((3-(4,5-Dimethylthiazol-2-yl)22,5-diphenyltetrazolium bromide)) was added to each well including the controls which are the untreated and dead cells. Then the plate was incubated in the previous situation for 4 hours. Then, the media was removed from the wells and 50 μl of 1:1 v/v solution of DMSO and methanol were added to each well to dissolve remaining crystals. After being incubated in room temperature for 30 minutes, the absorbance of each well at 570 nm and 670 nm was determined using the plate reader (Bio-Tek Synergy 2). Cell viability is determined by the following formula:

$$\%viability = \frac{A_{\text{well}} - A_{\text{dead}}}{A_{\text{live}} - A_{\text{dead}}} \times 100$$

$A_{\text{well}} = A_{\text{absorbance at 570}} - A_{\text{absorbance at 670}}$

Determination of Hydrodynamic Diameter and Zeta Potential

Polyplexes were prepared in a similar manner as previously mentioned in transfection section. Size and zeta potential of the polyplexes were measured in triplicates using a Zetasizer Nanosystems Nano-ZS instrument (Malvern Instruments, Mission Viejo, CA).

For the size, 50 μl of polyplex diluted in 950 μl of 1xHEPES buffer (10 mM, pH 7.4) were used for the measurements and For measuring zeta potentials, 50 μl of polyplex diluted in 950 μl of 1xPBS (pH 7.4) were used for the measurements.

In addition to polyplexes, size and zeta potential of 3 plasmids and 3 polymers were measured. Polymer and plasmid solutions were prepared with conc. of 75 $\frac{\mu g}{ml}$ in 1xHEPES buffer (10 mM, pH 7.4) and in 1xPBS (pH 7.4) and were used to measure

size and zeta potential of them respectively.

All the measurements were done in room temperature.

Zeta potentials and size distribution measurements were carried out at least three times after Polyplex formation. Positive charges on the surface of polymer and negative charges on the surface of DNA interact to form nanoscale polymer-DNA complexes. Moreover, positively charged polyplexes can be delivered more efficiently.

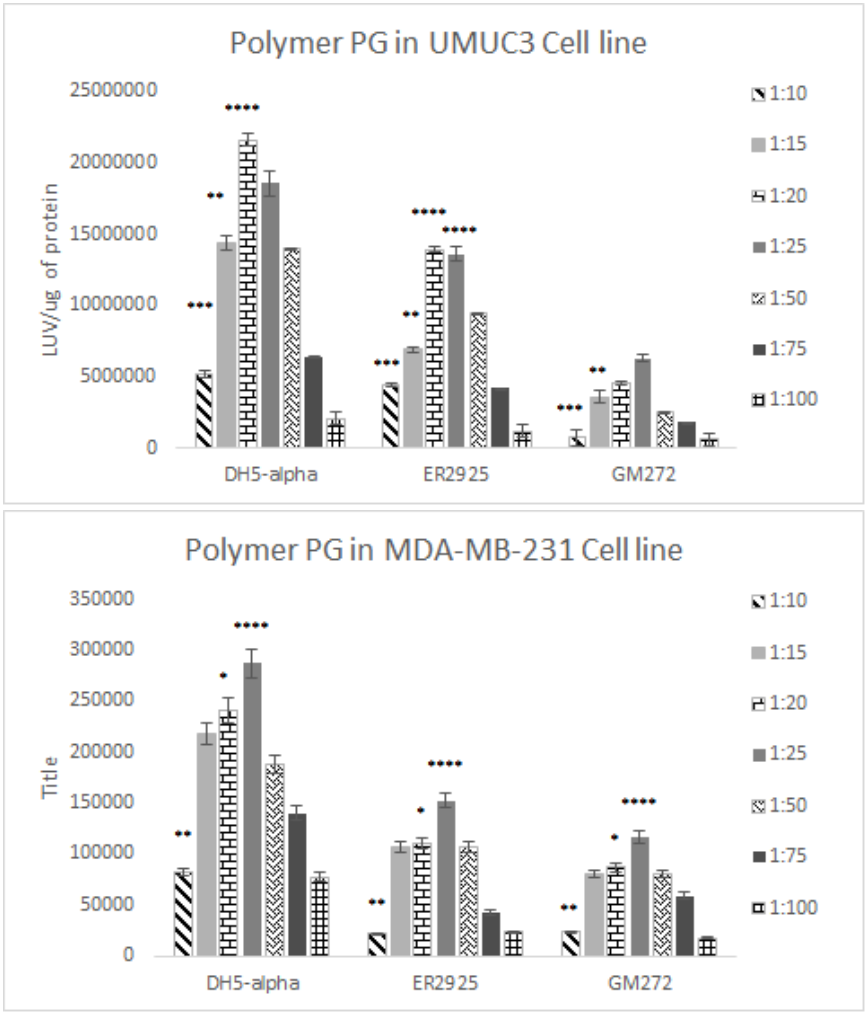


Figure 2.3: $\frac{LUV}{\mu g of Protein}$ for Each Plasmid-PG Polymer Polyplex Was Measured in UMUC3 and MDA-MB-231 Cell Lines

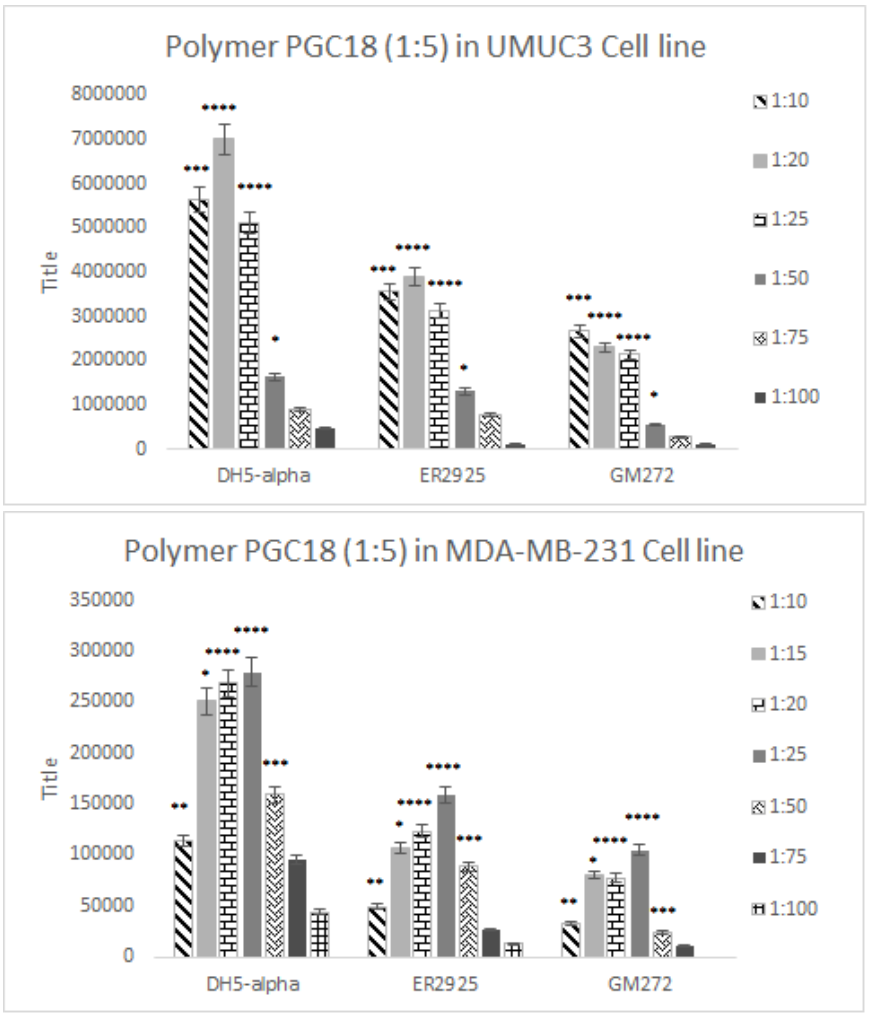


Figure 2.4: $\frac{LUV}{\mu g of Protein}$ for Each Plasmid-PGC18 (1:5) Lipopolymer Polyplex Was Measured in UMUC3 and MDA-MB-231 Cell Lines

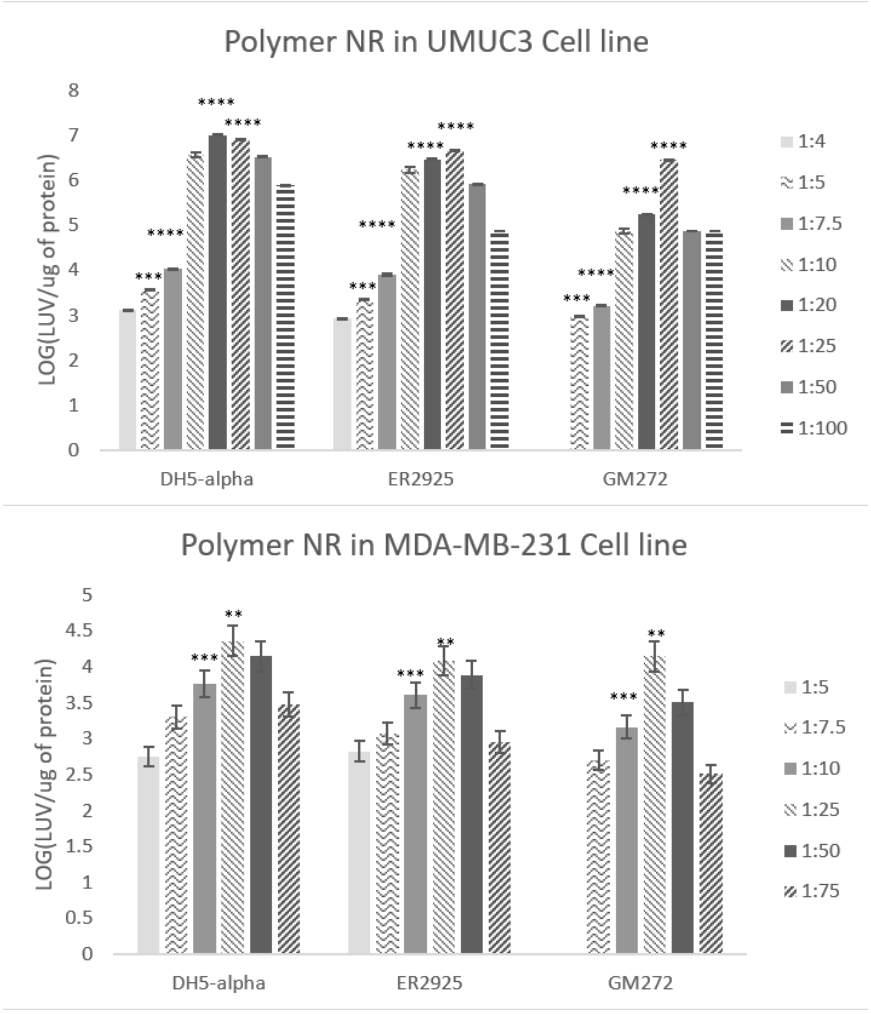


Figure 2.5: $Log\frac{LUV}{\mu g of Protein}$ for Each Plasmid-NR Polymer Polyplex Was Measured in UMUC3 and MDA-MB-231 Cell Lines

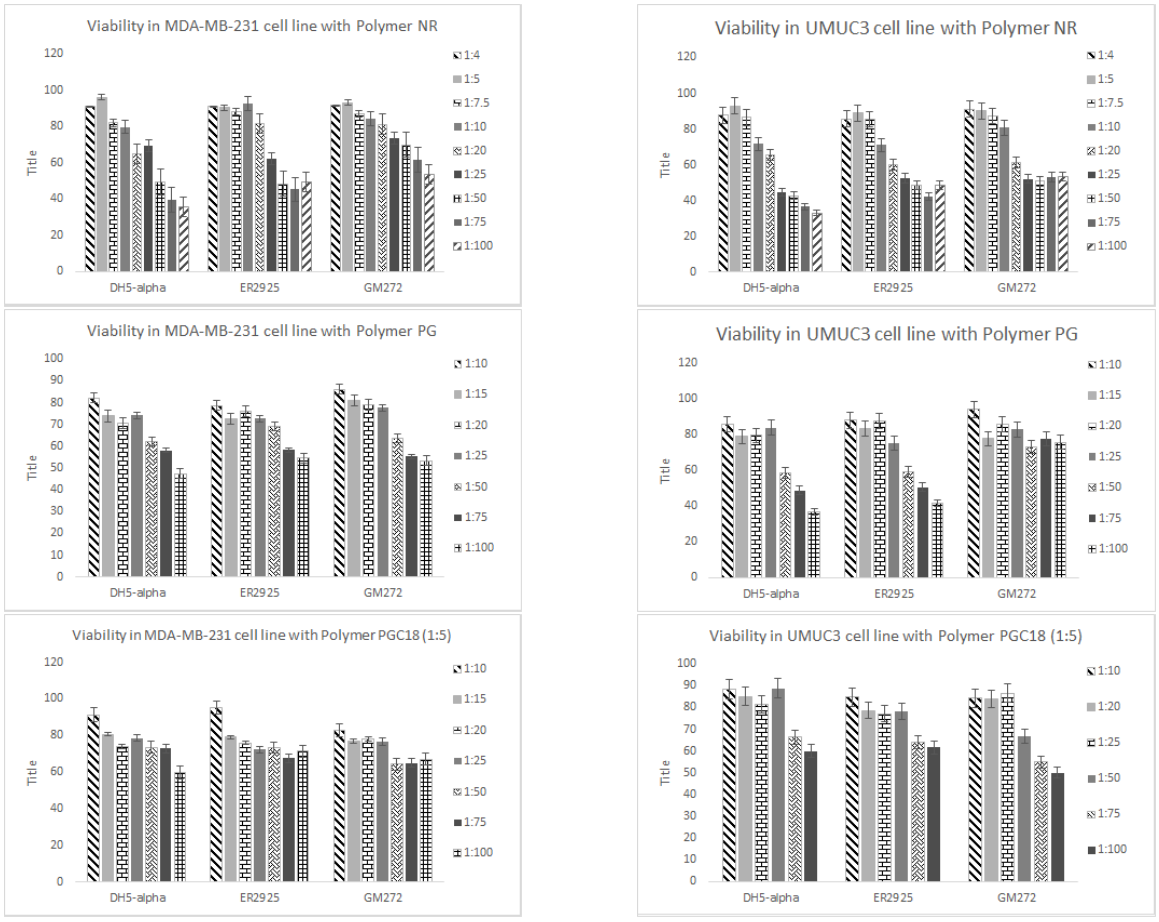
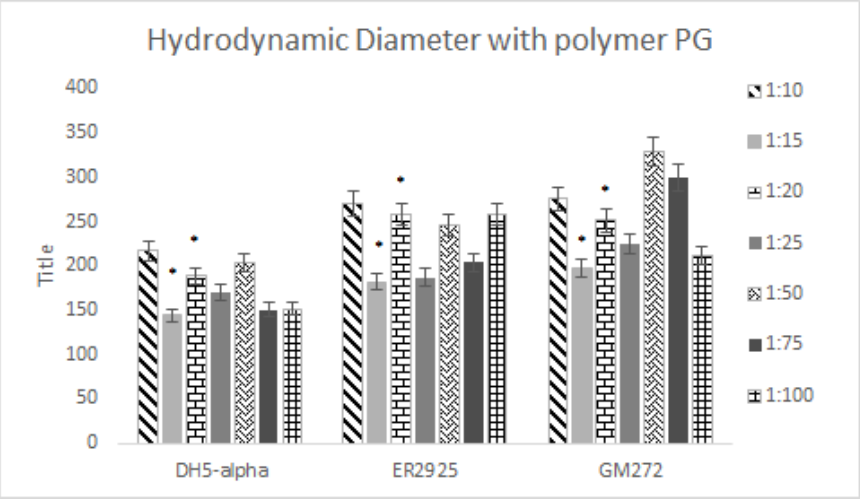
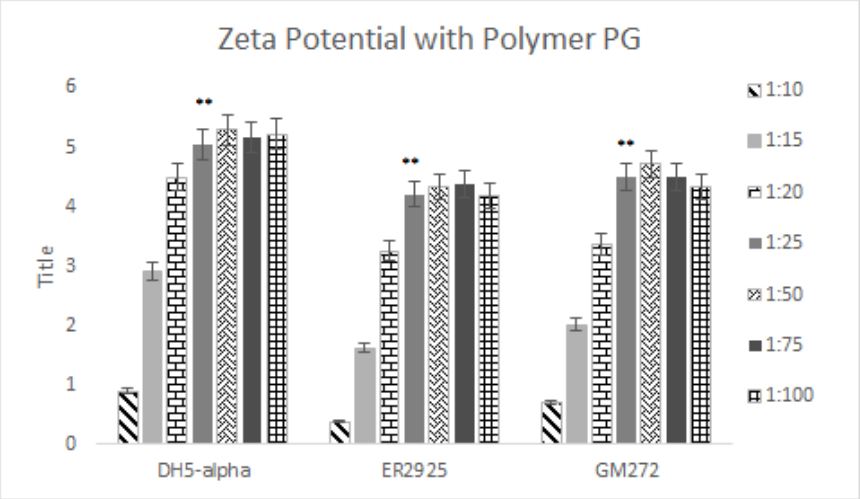


Figure 2.6: Viability Was Measured for Each Condition in Both Cell Lines

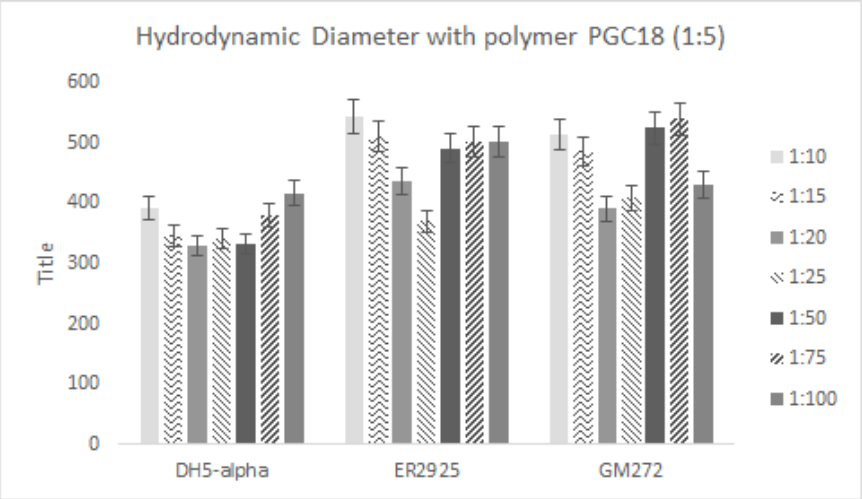


(a) Hydrodynamic Diameter in 1xHEPES Buffer

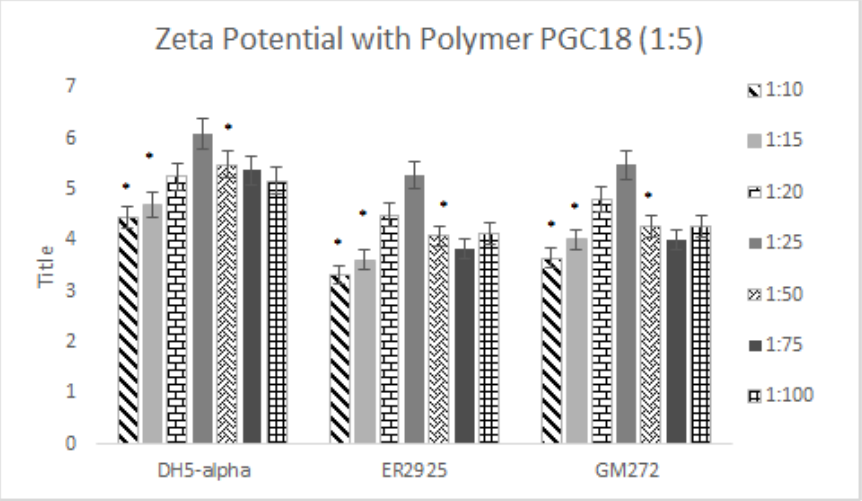


(b) Zeta Potential in 1xPBS Buffer

Figure 2.7: Hydrodynamic Diameter and Zeta Potential of Each Plasmid-PG Polymer Polyplex

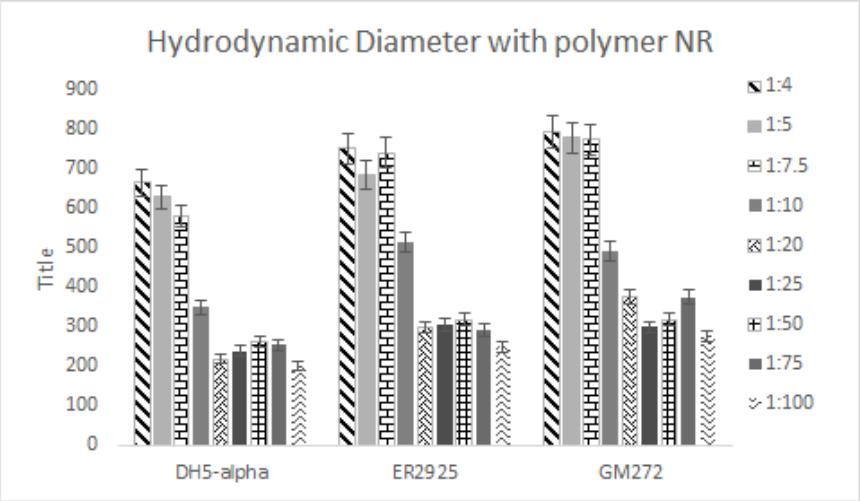


(a) Hydrodynamic Diameter in 1xHEPES Buffer

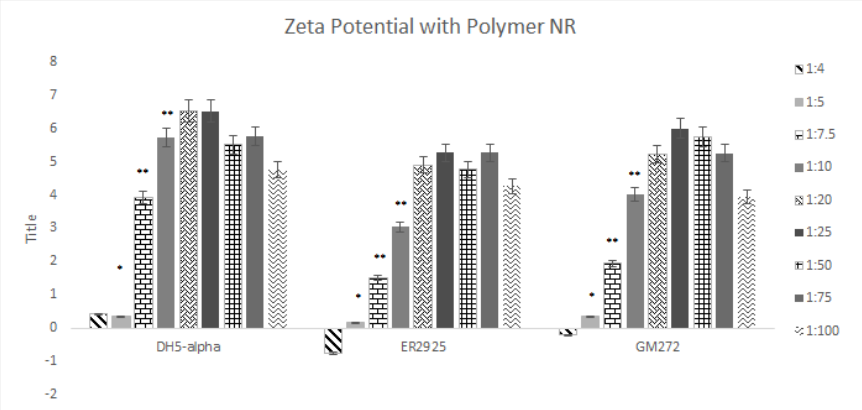


(b) Zeta Potential in 1xPBS Buffer

Figure 2.8: Hydrodynamic Diameter and Zeta Potential of Each Plasmid-PGC18 (1:5) Lipopolymer Polyplex

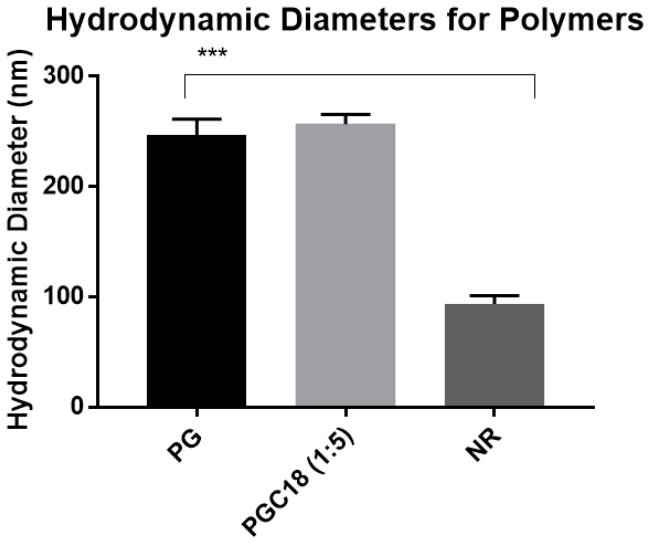


(a) Hydrodynamic Diameter in 1xHEPES Buffer

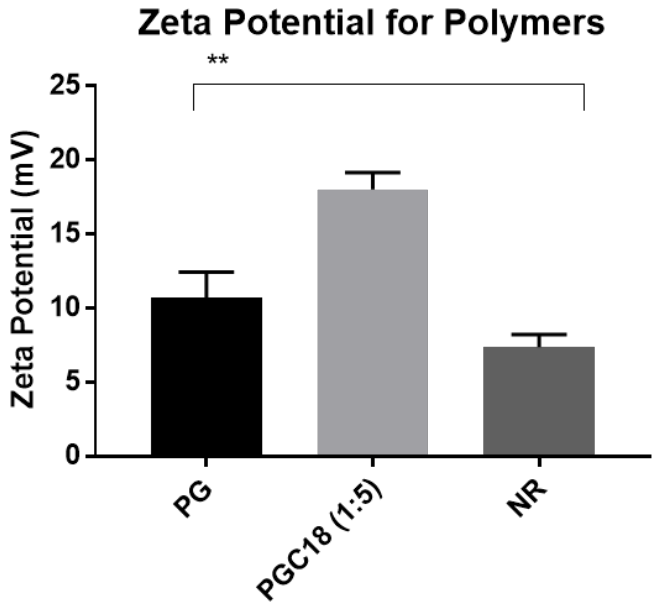


(b) Zeta Potential in 1xPBS Buffer

Figure 2.9: Hydrodynamic Diameter and Zeta Potential of Each Plasmid-NR Polymer Polyplex

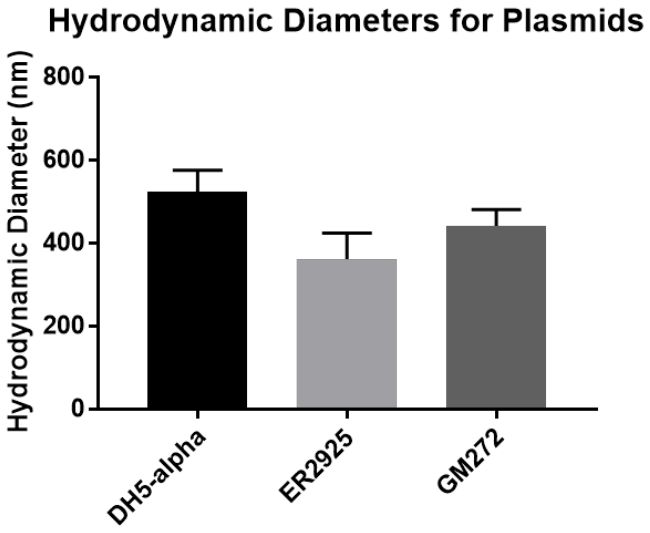


(a) Hydrodynamic Diameter in 1xHEPES Buffer

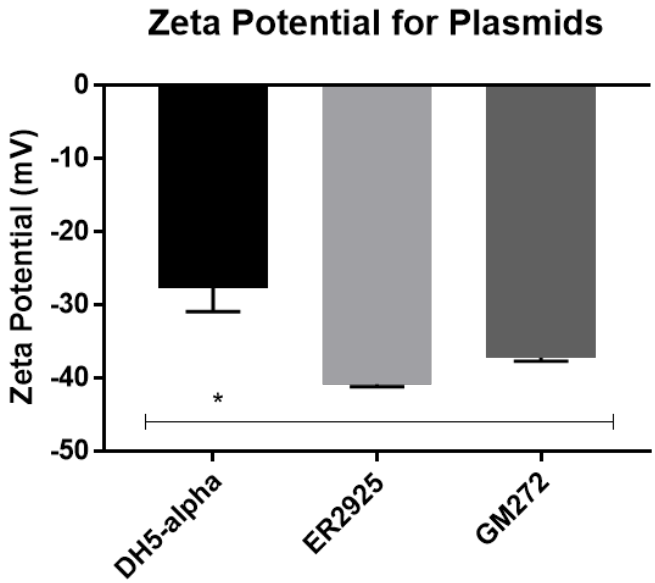


(b) Zeta Potential in 1xPBS Buffer

Figure 2.10: Hydrodynamic Diameters and Zeta Potentials of 3 Polymers



(a) Hydrodynamic Diameter in 1xHEPES Buffer



(b) Zeta Potential in 1xPBS Buffer

Figure 2.11: Hydrodynamic Diameters and Zeta Potentials of 3 Plasmids

To quantify the difference of gene delivery between methylated and unmethylated plasmids, transfections were carried out in 2 different cell lines, UMUC3 and MDA-

MB-231. The extent of expression of luciferase protein in each condition (different cell line, carrier polymer, DNA:polymer weight ratio and different methylated and unmethylated plasmids) determined how well the gene was delivered and transcribed.

To examine the first set of conditions, polyplexes were formed by slowly adding different concentrations of PG polymer solution to three different plasmid solutions (DH5-α, ER2925 and GM272) to make different weight ratio polyplexes. After adding the polymer, the mixture is incubated for 30 minutes in room temperature for the polyplexes to form. Different DNA:Polymer weight ratios tested were 1:10, 1:15, 1:20, 1:25, 1:50, 1:75, 1:100. Polyplexes were then added to UMUC3 and MDA-MB-231 cells for gene delivery.

figure 2.3 and show $\frac{LUV}{\mu gofProtein}$ for each plasmid-PG polyplex in UMUC3 and MDA-MB-231 cell lines. As seen in figure 2.3 , in all conditions, the methylated plasmid (DH5α) had considerably higher expression in comparison to the other two unmethylated plasmids. Gene expression enhances as the DNA:Polymer increases at first and then declines. The peak of transgene expression for DH5α and ER2925 plasmids are in the 1:20 and 1:25 ratios in UMUC3 and MDA-MB-231 cell lines respectively. For GM272 plasmid, the peak of expression is in 1:25 ratio in both cell lines.

Similarly, to examine the second set of conditions, polyplexes were formed using the 3 plasmids and PGC18 (1:5) lipopolymer at DNA:Polymer weight ratios of 1:10, 1:15, 1:20, 1:25, 1:50, 1:75, 1:100. Figure 2.4show $\frac{LUV}{\mu gofProtein}$ for each plasmid-PGC18 (1:5) polyplex in UMUC3 and MDA-MB-231 cell lines. Similar to figure 2.3 the methylated plasmid DH5α has significantly more expression than the other two unmethylated plasmids. The peaks and trend are also the same where we see transgene expression enhancement first and then it declines and the peaks are in 1:20-1:25 area.

Figure 2.5 shows LOG$\frac{LUV}{\mu gofProtein}$ for conditions of making plasmid-NR polyplex in different DNA:Polymer weight ratios (1:4, 1:5, 1:7.5, 1:10, 1:20, 1:25, 1:50, 1:75, 1:100)

in the 2 different cell lines, UMUC3 and MDA-MB-231. The scales of $\frac{LUV}{\mu g of Protein}$ for the measurements in low DNA:Polymer ratios (1:4, 1:5, 1:7.5) were very different from the rest of the values. In order to be able to see all the y-values and the trend, y-axis in these graphs are plotted logarithmic. Staying consistent with the previous two sets of conditions in figures 2.3 and 2.4, it shows the same peaks and trend were we first see an increase in $\frac{LUV}{\mu g of Protein}$ and then it declines and the peak is around 1:20-1:25 for all conditions. In addition, the methylated plasmid has significantly more transgene expression in all the conditions than the other two unmethylated plasmids. The reason why the DNA:Polymer weight ratios are different here in comparison to PG and PGC18 (1:5) polymers is that according to figure 2.6 after 1:25 weight ratio, the toxicity of the polyplex is too high and most likely affects the $\frac{LUV}{\mu g of Protein}$ values. In order to be able to see an accurate trend, lower DNA:Polymer weight ratios were used for experiments in addition to previous ones.

In conclusion, figures 2.3-2.5 all show the same trend where the methylated plasmid shows the highest transgene expression, hence the highest delivery with all three polymers in both cell lines in comparison to the two unmethylated plasmids. Also the highest transgene expression happens in 1:20-1:25 DNA:Ploymer ratios.

To make sure that the transgene expression data is accurate, cytotoxicity of each polyplex, was investigated through MTT assay. MTT assay was carried out for each of the conditions in figures 2.3-2.5 to calculate cell viability. Low viability shows that the polyplex was too toxic for the cells, resulting in less accurate data. For this study, cell viabilities more than 70% were considered to give accurate results. In figure 2.6, cell viability for each condition has been graphed as a reference for figures 2.3-2.5.

As previously discussed, in order to compare gene delivery, the transgene expression was compared. However, in order to investigate gene delivery more thoroughly, we can compare the cellular uptake of different polyplexes by measuring the size

and zeta potential of all the polyplexes. As discussed before in sections 1.1 and 1.2, smaller hydrodynamic diameter and higher (more positive) zeta potential can facilitate cellular uptake, hence the delivery of the transgene. However, we know that the relationship between hydrodynamic diameter, zeta potential and gene delivery efficacy is not always that simple. For example, as discussed in section 1.2, cellular uptake may be different in a cell line depending on the particle, or as much as positive zeta potential is beneficial for cellular uptake, before entering the nucleus, polymer and plasmid must be able to unbind and overly positive zeta potential may make this stage a bit harder.

Figures 2.7a, 2.8a and 2.9a show the hydrodynamic diameter for each polyplex. As visible in these figures, DH5α has the lowest diameter in comparison to the other two in all conditions which correlates with trends in figures 2.3-2.4 showing that smaller nanoparticles have better chance of cellular uptake and delivery. Also, the smallest size for all the polyplexes is happening around 1:20-1:25 ratio which correlates with the peak of transgene expression inprevious transgene expression data in 2.3-2.4.

Similarly for zeta potential, figures 2.7b, 2.8b and 2.9b demonstrate the zeta potential for each polyplex. These figures demonstrate that DH5α has the most positive zeta potential in comparison to the other two plasmids in all conditions which again correlates to the trends we had in figures 2.3-2.4 for transgene delivery. Also, the most positive zeta potential is in ratios 1:20-1:25 which correlates to the peaks in 2.3-2.4.

Furthermore, size and zeta potential of the polymers and plasmids themselves were measured using DLS for comparison with polyplex data. **??** show zeta potential values for each plasmid at 75 $\frac{ng}{\mu l}$. These data, show the boundaries for previous size and zeta potential measurements in 2.7-**??**. We can see in 2.11 the methylated plasmid has the biggest size and the most positive zeta potential due to the attachment of the

methyl groups.

In addition to size and zeta potential, other factors may contribute to the trends and values in figures 2.3-2.4 which correlate to transgene expression. In section 1.3.2, the effects of Dam and Dcm in prokaryotic gene expression were discussed. Since adenine and internal cytosine methylation (which is caused by Dam and Dcm) may enhance the binding of transcription factors and RNA polymerase, hence enhancing transcription, lacking Dam and Dcm may be a contributing factor that transgene expression was less in unmethylated plasmids in comparison to the methylated one. However, this hypothesis must be further examined.

Chapter 3

CONCLUSION

In conclusion, methylated plasmid had significantly higher efficacy in transgene delivery and transgene expression in comparison to the two unmethylated plasmids. This was partially due to the smaller size and more positive zeta potential that polyplexes with methylated plasmid. As mentioned in previous section, smaller size and more positive zeta potential can help the efficacy of cellular uptake. Another hypothesis is that methylation in adenine and internal cytosine also contribute to higher transgene expression in methylated plasmid. As previously mentioned, methylation in adenine and internal cytosine can help the binding of DNA and transcription factors such as RNA polymerase, hence enhance transcription. However, this hypothesis should be tested in future studies.

In addition, The highest transgene expression was found to be within 1:20-1:25 DNA:Polymer weight ratio in all the conditions.

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