

# **Cationic Gelatin/Pluronic-based Nanoparticles as Novel Non-Viral Delivery Systems for Gene Therapy**

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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## **Abstract**

A delivery system is essential to protect the DNA from degradation because of the extracellular and intracellular barriers. Two main types of delivery systems have been used to deliver genes into the body: viral and non-viral vectors. Although viral vectors are still superior over non-viral vectors, non-viral types are a better and safer alternative due to the safety concern of viral vectors. The low transfection efficiency of non-viral vectors remains a challenge due to the barriers that must be tackled. An effective non-viral vector must fulfill many conditions in order to be able to handle these barriers. The vector should be biocompatible, biodegradable, able to interact with DNA and cell membrane, able to successfully escape the endosome, and finally, capable of entering the nucleus to express the required protein. Cationic polymers are one of the most effective non-viral delivery systems. Cationic polymer/DNA (also called polyplexes) are able to interact with the DNA through electrostatic interaction, which results in DNA complexation and condensation. Gelatin is a natural polymer that can be easily modified by increasing the positive charge to effectively interact with the DNA and the cell membrane. In this project, gelatin nanoparticles were prepared using the two-step desolvation method, and were modified with cholamine as a cationic agent. Pluronic block copolymers were subsequently added to protect the cationic gelatin/DNA from degradation, and to increase the circulation time. The interactions of gelatin/Pluronic/DNA with the model membranes DPPC-CHOL and POPC-CHOL were studied using Langmuir's monolayer study, as well as Brewster's angle microscopy at the air/water interface. Transfection efficiency and cell viability were then evaluated at COS-7 cells. The results revealed that gelatin nanoparticles were successfully modified, at which

point the positive charge increased from +11 to +32. Additionally, cationic gelatin (CG) was able to interact with and neutralize the negative charge of the DNA. CG/Pluronic/DNA complexes were characterized by size and zeta potential, showing a small particle size and a positive charge. The interaction of CG/Pluronic/DNA complex with the model membranes demonstrated a fluidization effect, especially with CG and Pluronic, whereas DNA showed an ineffective and negligible condensation effect. With respect to transfection efficiency, CG results were poor compared to positive control jetPEI<sup>®</sup>, with no improvement after adding Pluronic. Gemini surfactant (GS) was also used in the transfection experiments in hope that it might improve the transfection efficiency of cationic gelatin. However, CG/GS showed some aggregation, and the positive charge decreased by increasing the CG, which resulted in lower transfection efficiency. Cell viability of the cells containing cationic gelatin was very high (similar to non-treated cells), which was confirmed by the safety of the gelatin, even after modification. More investigational studies and optimizations are required to understand the low transfection efficiency of cationic gelatin. These studies will help design more effective delivery vectors, either with gelatin or with any other non-viral system.

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## Dedication

*To My Parents ... Ali Madkhali and Aysa Madkhali*

*To My Wife ... Dikra Haddadi*

*To My Children ... Ayham, Aws and Maria*



## Table of Contents

<b>Examining Committee Membership .....</b>	<b>ii</b>
<b>Author's declaration .....</b>	<b>iii</b>
<b>Abstract.....</b>	<b>iv</b>
<b>Acknowledgements .....</b>	<b>vi</b>
<b>Dedication .....</b>	<b>viii</b>
<b>Table of Contents .....</b>	<b>ix</b>
<b>List of Figures.....</b>	<b>xiii</b>
<b>List of Tables .....</b>	<b>xvii</b>
<b>List of Abbreviations .....</b>	<b>xix</b>
<b>Chapter 1 Introduction and Literature Review .....</b>	<b>1</b>
1.1 History and General Introduction to Gene Therapy .....	1
1.2 Classifications of Gene Therapy Delivery Systems .....	4
1.2.1 Viral vectors .....	4
1.2.2 Non-viral vectors .....	7
1.3 Barriers Facing Non-viral Gene Delivery .....	8
1.4 Naked DNA .....	12
1.5 Physical Methods of Non-viral Systems .....	13
1.5.1 Electroporation .....	13
1.5.2 Gene gun.....	14
1.5.3 Sonoporation.....	15
1.5.4 Photoporation .....	16
1.5.5 Magnetofection.....	16
1.6 Chemical based Non-viral Systems.....	16
1.6.1 Inorganic particles .....	17
1.6.2 Organic synthetic/natural materials .....	18
1.7 Cationic Lipids as Non-viral Systems for Gene Delivery .....	18
1.7.1 Helper lipids .....	20
1.7.2 Stealth liposomes.....	21
1.7.3 Gemini surfactants.....	24
1.8 Polymers as Non-viral Systems for Gene Delivery.....	26

1.8.1 Polyethyleneimine (PEI) .....	28
1.8.2 Poly-L-lysine (PLL) .....	28
1.8.3 Chitosan.....	29
1.8.4 Dextran .....	29
1.9 Gelatin Nanoparticles for Gene Therapy Applications .....	31
1.9.1 Introduction to gelatin .....	31
1.9.2 Chemical structure of gelatin.....	34
1.9.3 Gelatin nanoparticles for gene therapy.....	36
1.9.4 PEGylated gelatin nanoparticles.....	38
1.9.5 Thiolated gelatin nanoparticles.....	40
1.9.6 EGFR-targeted gelatin nanoparticles.....	42
1.9.7 Cationic gelatin and its importance for gene delivery .....	44
1.10 Pluronic Block Copolymers for Gene Therapy Applications.....	45
1.10.1 Pluronic block copolymers .....	45
1.10.2 Pluronic block copolymer for gene therapy applications .....	47
1.11 Gelatin-Pluronic-based Nanoparticles as Non-viral Vectors for Gene Therapy .....	49
1.12 Scope of Thesis .....	50
<b>Chapter 2 Hypothesis and Objectives .....</b>	<b>53</b>
2.1 Aim of the Thesis .....	53
2.2 Hypothesis .....	53
2.3 Objectives.....	53
<b>Chapter 3 Characterization of Gelatin Nanoparticles and Cationic Gelatin</b>	
<b>Nanoparticles.....</b>	<b>55</b>
3.1 Introduction .....	55
3.2 Materials.....	57
3.3 Methods.....	57
3.3.1 Preparation of gelatin nanoparticles .....	57
3.3.2 Preparation of cationic gelatin.....	59
3.3.3 Particle size measurement .....	60
3.3.4 Zeta potential measurement.....	61
3.3.5 Scanning electron microscopy (SEM).....	62
3.3.6 Transmission electron microscopy (TEM).....	62

3.3.7 Fourier transform infrared spectroscopy (FT-IR).....	63
3.3.8 Extraction and purification of plasmid DNA (pTGIFN-GFP) .....	63
3.4 Results and Discussion.....	64
3.4.1 Preparation of nanoparticles.....	64
3.4.2 Stability of nanoparticles after freeze-drying .....	71
<b>(w/w%).....</b>	<b>72</b>
3.4.3 Cationic gelatin.....	72
3.4.4 Size and zeta potential of cationic gelatin .....	73
3.4.5 FT-IR .....	78
3.4.6 Optimization of cationic gelatin and DNA.....	81
3.5 Summary .....	82
<b>Chapter 4 : Understanding the Interaction of Gelatin/Pluronic/DNA System with Model</b>	
<b>Membranes: A Langmuir Monolayer Study .....</b>	<b>84</b>
4.1 Introduction to Langmuir Monolayer Studies .....	84
4.2 Cell Membranes .....	87
4.3 Materials.....	90
4.4 Methods.....	91
4.4.1 Monolayer formation and surface pressure measurements .....	91
4.4.2 Imaging the monolayer.....	93
4.4.3 Analysis of the $\pi$ - A isotherms.....	93
4.4.4 Compressibility modulus.....	94
4.5 Results and Discussion.....	95
4.5.1 DPPC-CHOL and POPC-CHOL.....	95
4.5.2 DPPC-CHOL and POPC-CHOL with DNA .....	98
4.5.3 Interaction of cationic gelatin with model membrane .....	102
4.5.4 DPPC-CHOL and POPC-CHOL with Pluronics, CG, and DNA.....	106
4.6 Summary .....	120
<b>Chapter 5 Transfection Efficiencies and Cell Viability of Gelatin/Pluronic/DNA System</b>	
<b>in COS-7 Cells .....</b>	<b>122</b>
5.1 Introduction .....	122
5.2 Materials.....	123

5.3 Method.....	123
5.5 Results and Discussion.....	128
5.5.1 Particle size and zeta potential.....	128
5.5.2 Transfection efficiency and cell viability of cationic gelatin/Pluronic/DNA.....	129
5.5.3 Gemini surfactant and cationic gelatin.....	132
<b>Chapter 6 Summary and Future Studies.....</b>	<b>140</b>
6.1 Summary.....	140
6.2 Future Studies.....	143
<b>Letter of Copyright Permission.....</b>	<b>146</b>
<b>References.....</b>	<b>154</b>

## List of Figures

Figure 1.1-1: Timeline highlighting some significant milestones of gene therapy .....	4
Figure 1.2-1: Vectors used in gene therapy clinical trials (wiley.co.uk) .....	5
Figure 1.3-1: Schematic presentation of barriers required to be overcome by nucleic acid using non-viral vectors.....	10
Figure 1.5-1: Basic concept of electroporation. Reproduced from ( <a href="https://igtrcn.org/injectoporation-for-gene-delivery/">https://igtrcn.org/injectoporation-for-gene-delivery/</a> ).....	14
Figure 1.5-2: Ballistic Gene Delivery .....	15
Figure 1.7-1: Schematic illustration representing the structure of liposome .....	20
Figure 1.7-2: General structure of gemini surfactant (A), structure of the m-s-m gemini surfactants (B). Adapted from (Wettig, Verrall et al. 2008).....	24
Figure 1.8-1: Packaging of DNA using polymer. The three main strategies employed to package DNA using polymers are <i>via</i> (1) electrostatic interaction, (2) encapsulation within or (3) adsorption onto biodegradable nano- or microspheres. Reproduced with permission from (Wong, Pelet et al. 2007) .....	27
Figure 1.9-1: Extraction of gelatin from collagen. The production of type A (cationic) gelatin is shown on the right of the figure; the production of type B (anionic) gelatin is shown on the left of the figure. Reproduced with permission from (Hosseinkhani, Abedini et al. 2015)....	32
Figure 1.9-2: General chemical structure of gelatin .....	35
Figure 1.10-1: General chemical structure of Pluronic block copolymers .....	45
Figure 3.3-1: Schematic illustration of the preparation of cationic gelatin nanoparticles using one-step desolvation method.....	59
Figure 3.4-1: Size (bars) and PDI (dots) of gelatin nanoparticles and reported as mean. A) before adding GA; and B) after adding GA. Samples were measured in triplicate and the average reported - errors are equal to standard deviation. PDI represents polydispersity index. GA represents glutaraldehyde.....	65
Figure 3.4-2: Scanning-electron-microscopy (SEM) images of the gelatin nanoparticles A) Two-step desolvation, B) Nanoprecipitation. The particle size was determined about 60-100 nm. ....	68

Figure 3.4-3: Schematic illustration of surface cationization of gelatin nanoparticles using cholamine. Reproduced from (Zwiorek 2006).....	75
Figure 3.4-4: SEM (top) and TEM (bottom) images of cationic gelatin nanoparticles .....	77
Figure 3.4-5: Zeta potential values at different pH conditions for both native and cationic gelatin. Samples were measured in triplicate.....	78
Figure 3.4-6: ATR-FTIR spectra of native and cationic gelatin.....	80
Figure 4.1-1: A schematic of the Langmuir trough used to determine Langmuir isotherms; a) frame, b) barriers, c) the trough, d) surface pressure sensor, e) Dipping mechanism (LB option), and f) Interface unit. Reproduced from ( <a href="http://www.ksvnima.com">http://www.ksvnima.com</a> ).....	85
Figure 4.1-2: Theoretical $\pi$ -A isotherm obtained by compressing an insoluble lipid monolayer formed at an air-water interface. Reproduced from (Eeman and Deleu 2010)....	87
Figure 4.2-1: Structure of the human cell membrane. Reproduced from ( <a href="http://ruleofsix.fieldofscience.com/2013/01/hiv-finds-cellular-door-knob-siglec1.html">http://ruleofsix.fieldofscience.com/2013/01/hiv-finds-cellular-door-knob-siglec1.html</a> ) ....	88
Figure 4.2-2: Chemical Structures of A) 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), B) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), C) Cholesterol ...	90
Figure 4.5-1: Isotherms (A and B) and compressibility modulus plots (C and D) for DPPC-CHOL (A, C) and POPC-CHOL (B, D) in the presence and absence of DNA.....	96
Figure 4.5-2: BAM Images of DPPC-CHOL (left) and POPC-CHOL (right) in the presence and absence of DNA at different surface pressure.....	101
Figure 4.5-3: Isotherms (A, B) and compressibility modulus plots (C, D) of the monolayer alone, monolayer and CG, and monolayer, CG, and DNA. DPPC-CHOL (A, C) and POPC-CHOL (B, D). .....	104
Figure 4.5-4: BAM Images of CG with DPPC-CHOL (left), and POPC-CHOL (right) in the presence and absence of DNA at different surface pressure. A) <1 mN/m, B) 5 mN/m, C) 30 mN/m, D) 50 mN/m for DPPC-CHOL and 40 mN/m POPC-CHOL. CG: cationic gelatin.	106
Figure 4.5-5: Isotherms (A, B) and compressibility modulus plots (C, D) of the monolayer alone, and monolayer and Pluronics. DPPC-CHOL (A, C) and POPC-CHOL (B, D). .....	111
Figure 4.5-6: BAM images of all Pluronics with DPPC-CHOL and POPC-CHOL at different surface pressures. A) <1 mN/m; B) 5 mN/m; C) 30 mN/m; D) 50 mN/m for DPPC-CHOL and 40 mN/m for POPC-CHOL.....	113

Figure 4.5-7: Isotherms (A, B) and compressibility modulus plots (C, D) of the monolayer alone, and monolayer with Pluronics, CG, and DNA. DPPC-CHOL (A, C) and POPC-CHOL (B, D). CG: Cationic gelatin. .... 116

Figure 4.5-8: BAM images of all Pluronics with CG and DNA with DPPC-CHOL and POPC-CHOL at different surface pressure. A)..... <1 mN/m, B) 5 mN/m, C) 30 mN/m, D) 50 mN/m for DPPC-CHOL and 40 mN/m for POPC-CHOL. CG: cationic gelatin ..... 119

Figure 5.5-1: Particle size and zeta potential of nanoparticles containing cationic gelatin, DNA, and the indicated Pluronic at concentrations corresponding to 0.1×CMC, 1×CMC, and 2×CMC. Bars represent particle size, dots represent zeta potential. Samples were measured in triplicate, errors are equal to standard deviation. .... 129

Figure 5.5-2: Transfection efficiency in % (expressed in terms of EGFP expression of live cells) (A), and cell viability in % (expressed in terms of dead cells stained by PI) (B) of cationic gelatin and DNA using different w/w ratios in COS-7 cells..... 130

Figure 5.5-3: Particle size (A) and zeta potential for nanoparticles containing different ratios of DNA to cationic gelatin in water (black) and buffer (grey). Samples were measured in triplicate, errors are equal to standard deviation. .... 131

Figure 5.5-4: Transfection efficiency in % (expressed in terms of EGFP expression of live cells) (A), and cell viability in % (expressed in terms of dead cells stained by PI) (B) of different w/w ratios of gemini surfactant and cationic gelatin (1:1, 1:2, 1:3, and 1:4) in COS-7 cells. GS is significantly different to CG at all ratios (\*\*P<0.0001). .... 134

Figure 5.5-5: Size (A) and Zeta potential (B) of gemini surfactant and cationic gelatin at different w/w ratios (1:1, 1:2, 1:3, and 1:4). Samples were measured in triplicate, errors are equal to standard deviation. .... 135

Figure 5.5-6: Schematic illustration of the interaction between gemini surfactant and cationic gelatin. Gelatin forms a shield around the gemini surfactant, which results in aggregation and decreases the positive charge of gemini surfactant. .... 135

Figure 5.5-7: Transfection efficiency in % (expressed in terms of EGFP expression of live cells) (A), and cell viability in % (expressed in terms of dead cells stained by PI) (B) of different combinations of delivery system in COS-7 cells. NG represents native gelatin, CG represents cationic gelatin, and GS represents gemini surfactant. GS alone with DNA is significantly different to all other nanocomplexes containing Pluronics, CG, or both (\*\*P<0.0001). .... 136

Figure 5.5-8: Transfection efficiency in % (expressed in terms of EGFP expression of live cells) (A), and cell viability in % (expressed in terms of dead cells stained by PI) (B) of the positive control (JetPEI) and GS/DNA complex in COS-7 cells . Positive control is

significantly different than the highest transfection obtained with GS and DNA alone  
(\*\*\*P<0.0001). ..... 138



## List of Tables

Table 1.2-1: The main viruses used as gene delivery systems, with their advantages and disadvantages .....	7
Table 1.2-2: Comparison between viral and non-viral vectors (Li and Huang 2007) .....	8
Table 1.7-1: Chemical structures of most common cationic and helper lipids used for gene delivery .....	23
Table 1.8-1: Chemical structure of the common polymers used in gene therapy.....	30
Table 1.9-1: Typical specification for commercial gelatin.....	33
Table 1.9-2: Amino acid composition of pigskin gelatin .....	35
Table 3.4-1: Optimization of the amount of sucrose added to gelatin nanoparticles before freeze-drying. Samples were measured in triplicate, errors are equal to standard deviation. PDI represents polydispersity index. ....	72
Table 3.4-2: Particle size and zeta potential of native and cationized gelatin. Samples were measured in triplicate, errors are equal to standard deviation. PDI represents polydispersity index.....	74
Table 3.4-3: IR absorption frequencies of amides groups .....	80
Table 3.4-4: Particle size and zeta potential of different ratios of plasmid DNA and cationic gelatin (W/W ratios). Samples were measured in triplicate, errors are equal to standard deviation. PDI represents polydispersity index. ....	82
Table 4.3-1: Physiochemical Characteristics of Pluronic Block Copolymers (Kabanov, Zhu et al. 2005) .....	91
Table 4.5-1: Monolayer properties derived from isotherm and compressibility modulus plots, for untreated DOPC-CHOL or POPC-CHOL monolayers, and for monolayers treated with DNA.....	97
Table 4.5-2: Monolayer properties derived from isotherm and compressibility modulus plots, for untreated DOPC-CHOL or POPC-CHOL monolayers, and for monolayers treated with CG in the presence and absence of DNA.....	102
Table 4.5-3: Monolayer properties derived from isotherm and compressibility modulus plots, for untreated DOPC-CHOL or POPC-CHOL monolayers, and for monolayers treated with CG and Pluronic in the presence and absence of DNA.....	109

Table 5.3-1: Conditions used for transfection experiments ..... 125

Table 5.3-2: Transfection formulations template for each well..... 127

## List of Abbreviations

### A

AAV .....Adeno- associated virus

Ad .....Adenovirus

AIDS ..... Acquired immunodeficiency syndrome

ATR..... Attenuated total reflection

### B

BAM. ....Brewster's Angle Microscopy

### C

CF.....Cystic Fibrosis

CG.....Cationized gelatin

Chol.....Cholestrol

CLs.....Cationic liposomes

CMC.....Critical micelle concentration

COS-7.....African green monkey kidney fibroblast-like

CS.....Chondroitin sulfate

### D

DLC.....Dynamic Light Scattering

DNA.....Deoxy ribonucleic acid

DOPE.....1, 2-diolelyl-snglycerophosphatidlyethanolamine

DPPC.....1,2-dipalmitoylsn-glycero-3-phosphocholine

DS.....Dextran sulfate

### E

EDA.....Ethylenediamine

EDS .....Energy Dispersive X-ray Spectroscopy

EPR.....Enhanced permeability and retention

EGFR.....Epidermal Growth Factor Receptor

### F

FACS.....Fluorescence-activated cell sorting

FBS.....	Fetal bovine serum
FDA.....	Food and Drug Administration
<b>G</b>	
GS.....	Gemini surfactant
GFP.....	Green fluorescent protein
GNPs.....	Gelatin nanoparticles
GRAS.....	Generally regarded as safe
GSH.....	Glutathione
GT.....	Gene Therapy
<b>H</b>	
HIV.....	Human Immunodeficiency Virus
HLB.....	Hydrophilic-Lipophilic Balance
<b>I</b>	
IBD.....	Inflammatory bowel disease
<b>K</b>	
Kb.....	Kilobase
<b>L</b>	
LC.....	Liquid condensed phase
LDE.....	Laser Doppler Electrophoresis
LE.....	Liquid expanded phase
LE-LC.....	Liquid expanded-liquid condensed phase
LLC.....	Lewis lung carcinoma
<b>M</b>	
MDR.....	Multi-drug resistance
MPS.....	mononuclear phagocytic system
<b>N</b>	
NiMOS.....	Nanoparticles-in-microsphere oral system
<b>O</b>	
OC.....	Ovarian cancer

OTC.....Ornithine transcarbamylase

**P**

PAMAM.....Polyamidoamine

PBC.....Pluronic block copolymers

PC.....phosphatidylcholine

PCL.....Poly (epsilon-caprolactone)

PCTP..... phosphatidylcholine transfer protein

PE..... phosphatidylethanolamine

PEG.....Poly-ethylene glycol

PEI.....polyethyleneimine

PEO.....Polyethylene oxide

PLD.....PEGylated liposomal doxorubicin

PLL.....poly-L-lysine

PPO.....polypropylene oxide

PS.....phosphatidylserine

**R**

RES.....Reticuloendothelial system

**S**

SEM.....Scanning electron microscopy

**T**

TEM.....Transmission electron microscopy

TH.....Thiol

**X**

X-linked SCID.....X-linked combined immune deficiency

## **Chapter 1 Introduction and Literature Review**

### **1.1 History and General Introduction to Gene Therapy**

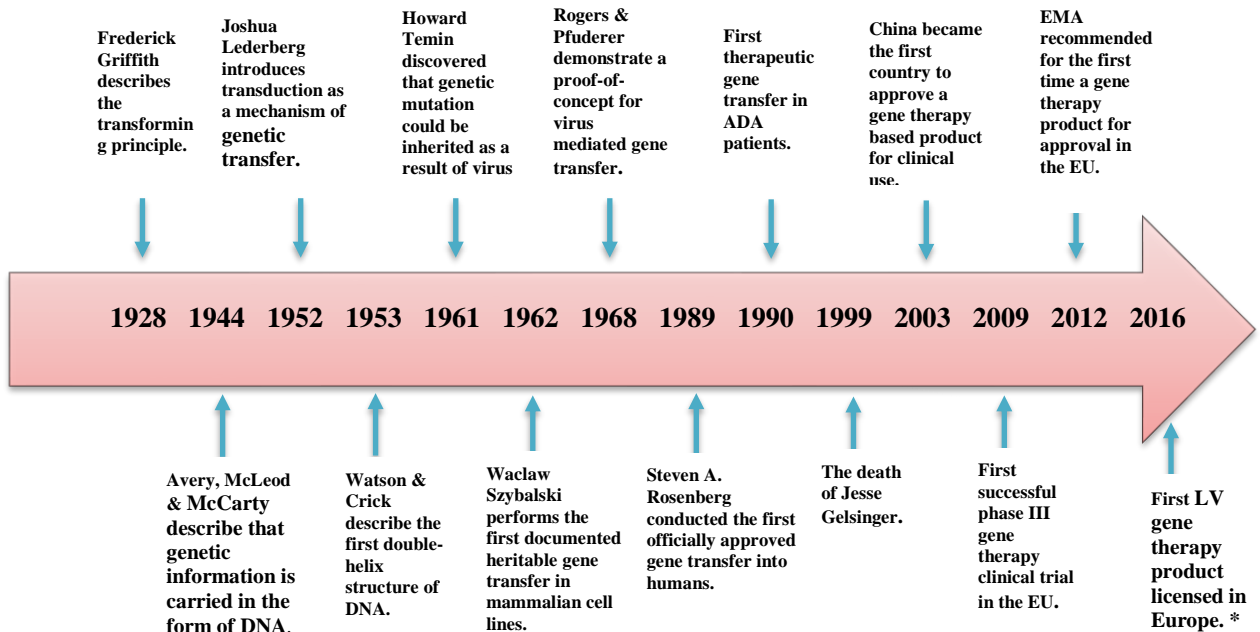
Gene therapy (GT) has received much attention due to its great potential for the treatment of both acquired and inherited diseases such as cancer, cystic fibrosis (CF), acquired immunodeficiency syndrome (AIDS), X-linked combined immune deficiency (X-linked SCID), emphysema, retinitis pigmentosa, sickle cell anemia, hemophilia, Duchenne Muscular Dystrophy (DMD), certain some autosomal dominant disorders, vascular disease, neurodegenerative disorders, polygenic disorders, inflammatory conditions, and other infectious diseases (Stone 2010, Nayerossadat, Maedeh et al. 2012, Keeler, Elmallah et al. 2017). According to the US Food and Drug Administration (FDA), gene therapy is defined as products “that mediate their effects by transcription and/or translation of transferred genetic material and/or by integrating into the host genome and that are administered as nucleic acids, viruses, or genetically engineered microorganisms. The products may be used to modify cells *in vivo* or transferred to cells *ex vivo* prior to administration to the recipient” (Wirth, Parker et al. 2013). A gene therapeutic should fulfil two characteristics: (a) it should contain an active substance containing or consisting of a recombinant nucleic acid that is administered into the nucleus in order to regulate, repair, replace, add, or delete a defective gene; (b) its therapeutic, diagnostic, or prophylactic effect should relate to the recombinant nucleic acid it contains (Wirth, Parker et al. 2013). When gene therapy is used in the treatment of genetic diseases, it restricts these diseases by introducing genes coding for functional proteins to cells; thus, it normalizes the cells and even organs in question (Jin, Zeng et al. 2014). Gene therapy is also used to produce large quantities of secreted proteins

that can be utilized for direct therapeutic applications or vaccine production (Jin, Zeng et al. 2014).

Since the first attempt of gene therapy made by Fredrick Griffith in 1928 (Griffith 1928), there have been enormous and significant changes have occurred in the development and improvement of gene therapy (Figure 1.1-1). The most important change has been the development of delivery systems. Deoxyribonucleic acid (DNA) requires a delivery vehicle in order to efficiently travel through extra- and intra-cellular barriers, finally entering the nucleus to express the required protein to correct or moderate specific diseases. Two main types of delivery systems have been developed for the purpose of transferring plasmid DNA into the nucleus: viral and non-viral vectors. Gendicine™, developed by SiBiono Gen Tech Co. in Shenzhen, is a non-replicative adenovirus used for the treatment of head and neck cell squamous carcinoma (HNSCC) that was approved by China's State Food and Drug Administration (SFDA) in 2003 as the first gene therapy based product for clinical use (Peng 2005, Wilson 2005). Two years later, SFDA approved Oncorine™, another gene therapy-based product. In contrast to Gendicine™, Oncorine™ is a replicative adenovirus developed by Sunway Biotech Co. Ltd for the treatment of late-stage refractory nasopharyngeal cancer in combination with chemotherapy (Liang 2012). To date, the US FDA has not approved any gene therapy product. However, Cerepro®, developed by Ark Therapeutics Group plc in 2009, was the first adenoviral vector gene therapy-based product to complete a phase III clinical trial in the European Union (EU) (Wirth, Samaranayake et al. 2009). Cerepro® is intended for use in the treatment of malignant brain tumor. Then, in 2012, the European Medicines Agency (EMA) recommended a gene therapy product (Glybera) for approval in

the EU for the first time (Wirth, Parker et al. 2013). Glybera, developed by Amsterdam Molecular Therapeutics, is an adeno-associated viral vector engineered to express lipoprotein lipase to treat severe lipoprotein lipase deficiency. Lastly, Strimvelis, another gene therapy product, was approved in Europe for the treatment of ADA-SCID (severe combined immunodeficiency due to adenosine deaminase deficiency) in May 2016 (Keeler, Elmallah et al. 2017, Touchot and Flume 2017). Strimvelis, which is the first ex vivo stem cell gene therapy developed by GlaxoSmithKline in collaboration with a charitable organization (Italian's Fondazione Telethon) and an academic center in Milan (Ospedale San Raffaele). These new approvals and recommendations along with a high number of ongoing clinical trials (Figure 1.1-2) have effectively demonstrated the enormous potential effectiveness of gene therapy; thus, it is very likely that gene therapy will find its place among clinical therapeutics.





**Figure 1.1-1:** Timeline highlighting some significant milestones of gene therapy

Reproduced with permission from (Wirth, Parker et al. 2013)

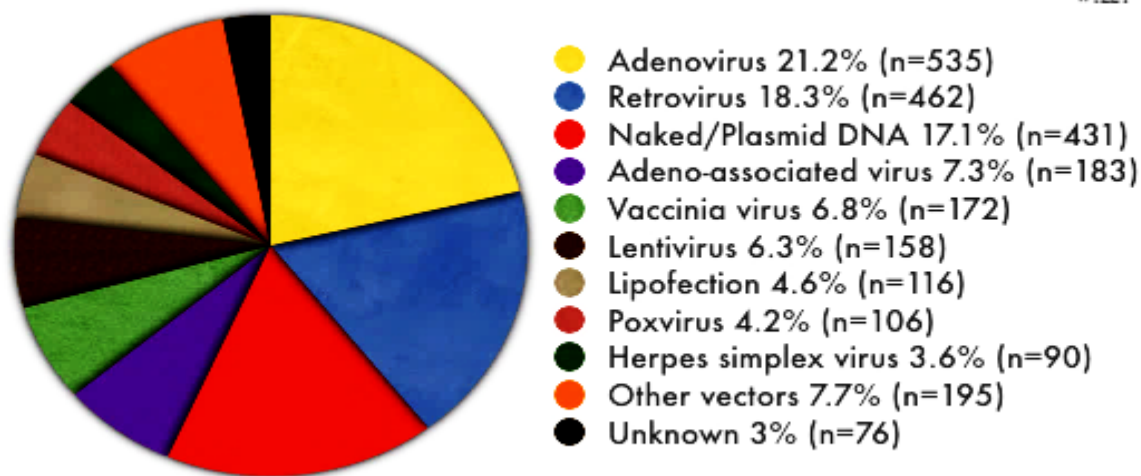
\* Reproduced from (Keeler, Elmallah et al. 2017, Touchot and Flume 2017)

## 1.2 Classifications of Gene Therapy Delivery Systems

### 1.2.1 Viral vectors

Viral vectors involve the packaging of DNA into a virus particle. Gene transfer occurs by normal viral infection (mechanisms) and is both efficient and cell selective. There are currently more than 1700 approved gene therapy clinical trials that utilize viruses as vectors for either gene therapy or vaccines applications (Figure 1.2-1)

## Vectors Used in Gene Therapy Clinical Trials



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[www.wiley.co.uk/genmed/clinical](http://www.wiley.co.uk/genmed/clinical)

**Figure 1.2-1:** Vectors used in gene therapy clinical trials ([wiley.co.uk](http://wiley.co.uk))

With their distinct biological keys (e.g., glycoprotein in a viral envelope (Grandi, Spear et al. 2004)), viruses are able to infect cells and cross both the cell membrane and nuclear barrier via a rapid cytosolic passage along dynein-based active linear translocation microtubules (De Laporte, Rea et al. 2006). This feature enables viruses to achieve high transfection efficiency in most of the transfected cells *in vitro*. However, several concerns with respect to viral vectors have been raised. These include: a strong immune response in host cells due to viral protein(s), which causes induction of inflammatory system resulting in degenerating of transduced tissues and toxic production; insertional mutagenesis; limitation to the size of loaded DNA; and the high cost of production. With respect to insertional mutagenesis, an ectopic chromosomal integration of viral DNA may cause malignant transformation due to the disruption of the expression of tumor suppression or due to the oncogene activation

(Ramamoorth and Narvekar 2015). In 1999, the tragic death of Jessie Gelsinger, an 18-year-old patient who was being treated for ornithine transcarbamylase (OTC) (Stolberg 1999) with an adenovirus vector, as well as the two out of eleven children who were developing a blood disorder similar to leukemia following adenoviral treatment against SCID, placed viral vectors under severe inspection and led researchers to explore alternative, safer vectors that can replace viruses (Teichler Zallen 2000). Adenovirus (Ad), retrovirus, adeno-associated virus (AAV), lentivirus, herpes simplex virus, and poxvirus are the most common viruses used in clinical trials as delivery systems for gene therapy. Table 1.2-1 summarizes the advantages and disadvantages of some of these viruses.

**Table 1.2-1:** The main viruses used as gene delivery systems, with their advantages and disadvantages

Reproduced with permission from (Ratko, Cummings et al. 2003)

Vector	Advantages	Disadvantages
<b>Adenovirus (Ad)</b>	Very high titers ( $10^{12}$ pfu/ml) * High transduction efficiency <i>in vitro</i> and <i>in vivo</i> Transduction many cell types Transduces proliferation and nonproliferating cells Production easy at high titers	Remain episomal Transient expression Requires packaging cell line Immune-related toxicity with repeated administration Potential replication complex No targeting Limited insert size: 4-5 kb
<b>Adeno-associated Virus (AAV)</b>	Integration on human chromosome 19 to establish latent infection Transduction does not require cell division Small genome, no viral genes	No well characterized No targeting Requires packaging cell line Potential insertional mutagenesis High titers ( $10^{10}$ pfu/ml) but Production difficult Limited insert size: 5 kb
<b>Herpes Simplex Virus</b>	Large insert size Neuronal tropism Latency expression Efficient transduction <i>in vivo</i> Replicative vectors available	Cytotoxic No targeting Requires packaging cell line Transient expression does not integrate into genome Moderate titers ( $10^4$ - $10^8$ pfu/ml)
<b>Lentivirus</b>	Transduction proliferating and nonproliferating cells Transduces hematopoietic stem cells Prolonged expression Relatively high titers ( $10^6$ - $10^7$ pfu/ml)	Safety concern: from human immunodeficiency virus origin Difficult to manufacture and store Limited insert size: 8 kb Clinical experience limited
<b>Retrovirus</b>	Integration into cellular genome Broad cell tropism Prolonged stable expression Require cell division for transduction Relatively high titers ( $10^6$ - $10^7$ pfu/ml) Large insert size: 9-12 kb	Inefficient transduction Insertional mutagenesis Requires packaging cell line Requires cell division for transduction No targeting Potential replication competence

\*Plaque-forming unit (PFU): a measure of the number of particles capable of forming plaques per unit volume, such as virus particles.

### 1.2.2 Non-viral vectors

Due to the large number of clinical trials using viral vectors that have failed for safety reasons (or for failure to meet clinical endpoints), non-viral vectors are viewed as an

appropriate alternative method. Non-viral vectors are divided into three methods: naked DNA, physical based and chemical based. Examples of physical methods includes electroporation, gene gun, sonoporation, photoporation, and magnetofection while chemical methods include cationic lipids and cationic polymers. Non-viral vectors are safe (generally non-toxic, and non-immunogenic), easy to produce in large scale, cheap, and able to deliver large pieces of DNA in contrast to viral vectors (Li and Huang 2007) (Table 1.2-2). However, non-viral vectors demonstrate low transfection efficiency compared to viral vectors because of the numerous extra- and intra-cellular obstacles required to be crossed before reaching the nucleus to express the necessary protein (Pathak, Patnaik et al. 2009).

**Table 1.2-2:** Comparison between viral and non-viral vectors (Li and Huang 2007)

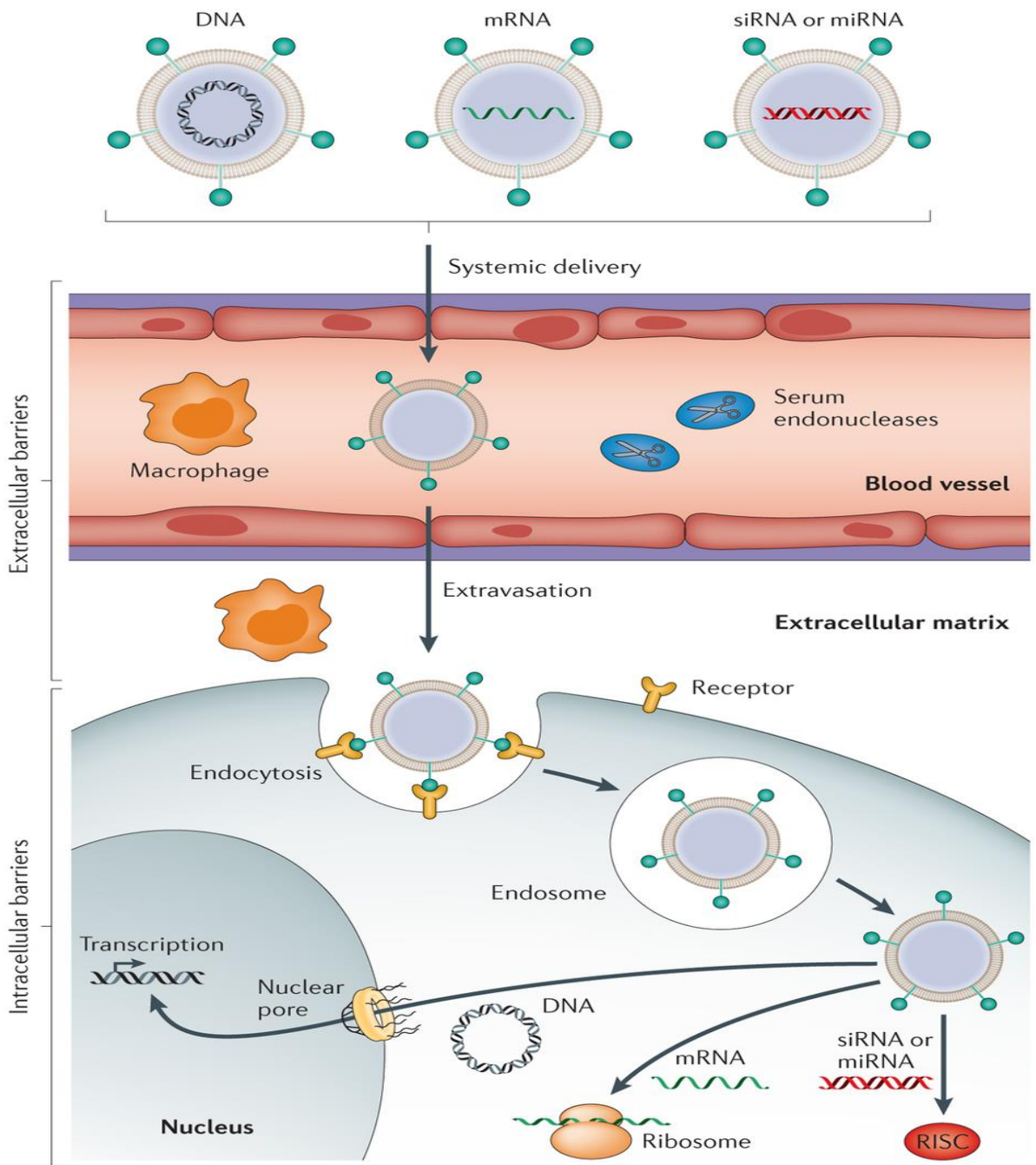
<b>Viral vectors</b>	<b>Non-viral vectors</b>
High production cost	Low production cost
Immunogenic	Low immunogenicity
Limitation in size loaded of DNA	Easily produced in large scale
Potential for oncogenesis	Very low toxicity
High transfection efficiency	Low transfection efficiency

### **1.3 Barriers Facing Non-viral Gene Delivery**

Nucleic acids (DNA or RNA) must cross several barriers before they are reaching the nucleus and expressing the required protein. These barriers can be divided into extracellular and intracellular barriers. Extracellular barriers include skin, blood cells, plasma components,

the reticuloendothelial system (RES), and the immune system response. Intracellular barriers include the plasma membrane, endosomes, trafficking with the cytosol, nuclear localization, and nuclear membrane (Figure 1.3-1). As a result, several researchers who have studied these barriers and have attempted to overcome them by using various techniques. Regardless of the method by which non-viral vectors are administered into the body (e.g., inhalation, IV, IM, gavage, etc.), they should come to the extracellular environment (Gottfried and Dean 2013). There, the vector (either cationic lipid or cationic polymer) may either be degraded or cleared rapidly from circulation due to multiple factors existing within the extracellular milieu, such as the presence of serum proteins, blood cells, and enzymatic degradation (Gottfried and Dean 2013). An effective strategy to overcome these barriers and to protect the DNA from degradation is the use of PEGylated polymer. Polyethylene glycol (PEG) has been demonstrated to enhance the stability of the complexes in serum and to substantially increase the vector's half-life in the systemic circulation (Crawford 2002, Kaul and Amiji 2002, Otsuka, Nagasaki et al. 2003, Kaul and Amiji 2005, Kou, Wang et al. 2014, Ran, Liu et al. 2014, Jones, Chen et al. 2015).

The presence of a positive charge is significant, both to interact with the negatively charged phosphate group on in the DNA, and also with the negatively charged proteoglycan contained in the cell membrane (Halama, Kuliński et al. 2009). The electrostatic interactions between plasmid DNA and cationic polymer help by condensing the DNA, and protecting the complex from degradation by nucleases (Ogris and Wagner 2002, Halama, Kuliński et al. 2009).



**Figure 1.3-1:** Schematic presentation of barriers required to be overcome by nucleic acid using non-viral vectors  
 Reproduced with permission from (Yin, Kanasty et al. 2014)

Once the polyplex is taken up by endocytosis, it has to escape from the endosome and travel toward the nucleus before it is degraded at the lysosomal level. Cationic polymers achieve endosomal escape mainly through a “proton sponge” mechanism. The proton sponge hypothesis is that release of the DNA occurs when low pH in the endosomal environment results in protonation of the entrapped agents with a high buffering capacity. Protonation induces an extensive inflow of ions and water into the endosome. This leads to osmotic swelling and rupturing of the endosomal membrane; therefore, releasing of the entrapped components (Varkouhi, Scholte et al. 2011). Cationic lipids can escape the endosome by destabilizing the endosomal membrane, resulting in a flip-flop reorganization of phospholipids, which then dissociate the DNA into the cytoplasm (Xu and Szoka 1996). Afterwards, the free nucleic acid must cross the nuclear membrane for transcription and expression of the encoded protein.

The nuclear membrane is considered a major barrier for DNA delivery. Depending on the cell type, only 1 to 10% of the transfected DNA can enter the nucleus (Cohen, van der Aa et al. 2009, Glover, Leyton et al. 2010). Pollard et al. (Pollard, Remy et al. 1998) stated that PEI-DNA polyplexes showed higher transfection efficiency when microinjected in cytoplasm compared to naked DNA or DOTAP/DNA lipoplexes. This means that PEI has the ability to facilitate DNA translocation into the nucleus (Tros de Ilarduya, Sun et al. 2010). Designing non-viral delivery vehicles that overcome these barriers is the primary goal in obtaining effective transfection efficiency. Numerous non-viral vectors can be used to deliver DNA, mRNA and short double-stranded RNA, including small interfering RNA (siRNA) and microRNA (miRNA) mimics. These vectors require to prevent degradation by serum



endonucleases and evade immune detection. These objectives can be achieved through chemical modifications of the nucleic acids and the encapsulation of vectors. These vectors also require avoiding renal clearance from the blood and prevent nonspecific interactions by using polyethylene glycol (PEG) or through specific characteristics of particles. In addition, these vectors need to extravasate from the bloodstream to reach target tissues, which requires certain characteristics of particles and specific ligands. Finally, these vectors require to mediate cell entry and endosomal escape by specific ligands and key components of carriers. siRNA and miRNA mimics must be loaded into the RNA-induced silencing complex (RISC), whereas mRNA must bind to the translational machinery. DNA must be further traveled to the nucleus to express the required protein (Yin, Kanasty et al. 2014) (Figure 1.3-1).

#### **1.4 Naked DNA**

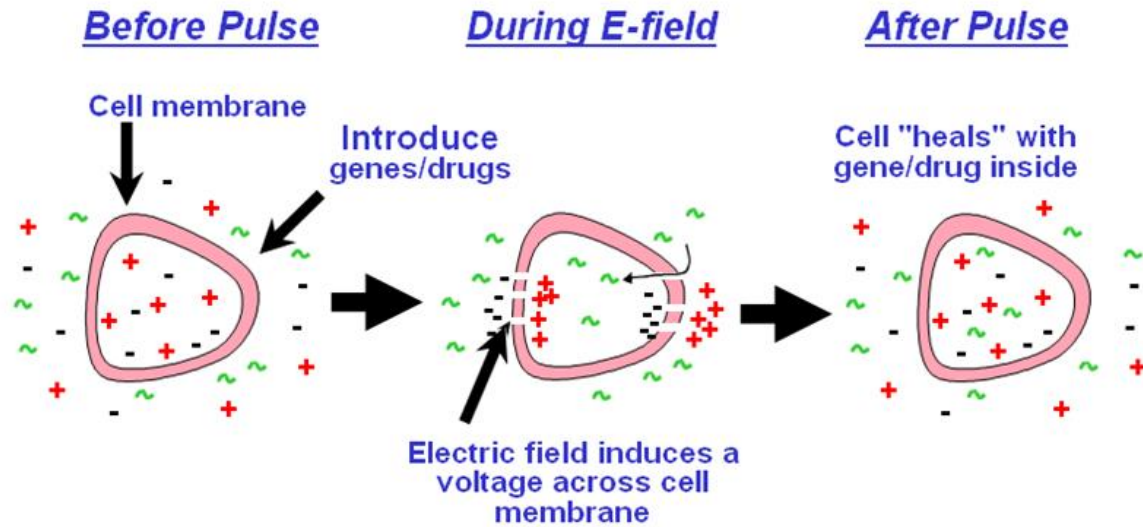
Administration of naked DNA is considered the least difficult strategy for non-viral transfection. Although clinical trials completed of intramuscular injection of a naked DNA plasmid have had some success, expression has been very low in contrast with different strategies for transfection. In addition to trials with plasmids, there have been trials with naked polymerase chain reaction (PCR) product, which have had relative or more prominent success. This achievement, nonetheless, does not measure up to that of alternate techniques, prompting research into more proficient strategies for the conveyance of the naked DNA, for example, electroporation and the utilization of a "gene gun", which shoots DNA coated gold particles into the cell utilizing high pressure gas.

## **1.5 Physical Methods of Non-viral Systems**

These methods depend on using physical force in order to destabilize the cellular membrane, therefore facilitating the entry of gene therapeutic materials into the cells. These methods are simple and straightforward.

### **1.5.1 Electroporation**

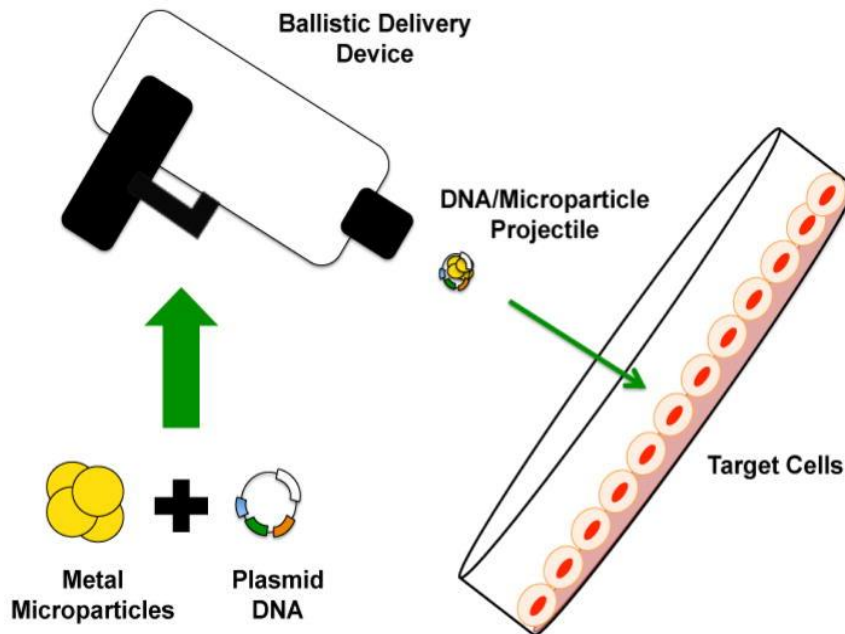
Electroporation is known as gene electro injection, gene electro transfer, electrically mediated gene therapy, or electro gene transfer (Ramamoorth and Narvekar 2015). It works by applying an electric field greater than the membrane capacitance into the targeted tissue cell membrane, resulting in a pore that allows the molecules to pass through it (Figure 1.5-1). As a result, the previously injected DNA can enter into the cytoplasm and nucleoplasm of the cell (Nayerossadat, Maedeh et al. 2012). This method is very effective and safe when it applied *in vivo* in comparison to other non-viral methods. However, the complexity of surgical procedures, and high voltage [ $>700\text{V/cm}$ ] applied to the tissues, as well as the difficulty of reaching some internal tissues makes this method inappropriate for delivering DNA (Young and Dean 2015).



**Figure 1.5-1:** Basic concept of electroporation. Reproduced from (<https://igtrcn.org/injectoporation-for-gene-delivery/>)

### 1.5.2 Gene gun

The gene gun (also known as particle bombardment, micro projectile gene transfer or ballistic DNA - Figure 1.5-2) delivers DNA coated heavy metal particles into the target tissue at a particular speed using high voltage electronic discharge, spark discharge, or high pressure inert gas, usually helium (Mali 2013). The most common metal particles used in this method are gold, tungsten, and silver, which all typically measure 1  $\mu\text{m}$  in diameter. Gene transfer is affected by several parameters such as gas pressure, particle size, and dose frequency. Precise delivery of DNA is the most important advantage using the gene gun method, and it most commonly used in gene therapy that targets ovarian cancer (OC) cells *in vitro* (Ramamoorth and Narvekar 2015).



**Figure 1.5-2:** Ballistic Gene Delivery

Reproduced with permission from (Mellott, Forrest et al. 2013)

### 1.5.3 Sonoporation

Sonoporation is a noninvasive technique using ultrasound wave to permeabilize the cell membrane; thus, allowing the uptake of DNA. Genetic materials of interest are first administered into the circulation using microbubbles, followed by the application of the ultrasound waves. The ultrasound waves cavitate and break up the microbubbles within the microcirculation of target tissue, leading to the disruption of the nearby cell membrane that results in targeted transfection of the therapeutic gene (Omata, Negishi et al. 2015). The major advantages of sonoporation include safety, noninvasiveness, and the ability to reach internal organs without the necessity of surgery; consequently, it is used in the brain, cornea, kidney, and peritoneal cavity, as well as in muscle and heart tissues (Ter Haar 2007, Ramamoorth and Narvekar 2015).

#### **1.5.4 Photoporation**

This technique works by using a single laser pulse in order to generate a pore in the cell membrane allowing the DNA to enter into the cells. The effectiveness of this method depends on the focal point and pulse frequency of the laser. The major advantage of this approach is its safety, in which the pore that is formed by the laser can be healed in less than a second. However, the lack of documented evidence limits the use of this technique (Li and Huang 2007).

#### **1.5.5 Magnetofection**

The magnetofection technique is based on coupling therapeutic gene to magnetic nanoparticles, which are then introduced into the cell culture (Jones, Chen et al. 2013). The field gradient is produced by adding rare, earth electromagnets under the cell culture, which then result in increasing transfection speed that arises from increasing the complex sedimentation. The therapeutic gene-magnetic particle complex is administered intravenously when it used *in vivo*. With the help of enzymatic cleavage of cross linking molecules, charge interaction, or charge degradation, the genetic material is released (Plank, Schillinger et al. 2003). This method is considered to be an alternative for certain primary cells, as those transfections are difficult when using other techniques.

### **1.6 Chemical based Non-viral Systems**

Chemical methods of transfection are divided into two categories: inorganic particles (such as calcium phosphate, silica, and gold particles); and organic synthetic/natural materials (such as cationic lipid and cationic polymers).

### **1.6.1 Inorganic particles**

The most common examples of these particles include calcium phosphate, gold, and silica.

They have small nanoparticles that are capable of protecting the DNA from degradation and avoiding most of the physiological barriers (RES in particular).

#### **1.6.1.1 Calcium phosphate**

Calcium phosphate particles are biodegradable, biocompatible, non-toxic, and non-immunogenic (Choi, Cui et al. 2015). Calcium phosphate, as a delivery system, works by co-precipitating the calcium phosphate and DNA in the aqueous core of the reverse micellar droplets (Roy, Mitra et al. 2003). They play a vital role in endocytosis, and they are readily absorbed due to their high nucleic acid binding affinity (Ramamoorth and Narvekar 2015, Bakan, Kara et al. 2017). About 85% of the added DNA was found in the matrices of calcium phosphate nanoparticles (Roy, Mitra et al. 2003). Due to the problem of calcium phosphate crystal growth, the presence of magnesium is occasionally required (Ramamoorth and Narvekar 2015).

#### **1.6.1.2 Silica**

These nanoparticles are commonly made of amino silicane especially for gene delivery due to its low toxicity (Kneuer, Sameti et al. 2000). They can also be produced from sand and glass (Ramamoorth and Narvekar 2015). Silica nanoparticles can be easily modified with aminosilanes at lower pH to produce a net positive charge, and are able to electrostatically interact with the DNA (Kneuer, Sameti et al. 2000). The interaction with serum protein is the major disadvantage of silica particles because it leads to a decrease in the delivery efficiency (Dizaj, Jafari et al. 2014).

### **1.6.1.3 Gold**

Gold nanoparticles are a promising area of research for gene therapy due to their low toxicity, ease of preparation, inert nature, and multiple surface characterizations (Ding, Jiang et al. 2014, Ramamoorth and Narvekar 2015). Gold nanoparticles work by interacting with the nucleic acid through electrostatic interaction due to the presence of cationic quaternary ammonium groups (McIntosh, Esposito et al. 2001), which in turn protect the DNA from enzymatic degradation. Gold nanoparticles have the ability to absorb near the infra-red light, which can penetrate deeply into the cells to deliver the DNA. The main problem of gold nanoparticles is its chemical stability that results from the accumulation of nanoparticles rather than being dissolved, which could lead to cell death (Ramamoorth and Narvekar 2015, Riley and Vermerris 2017).

### **1.6.2 Organic synthetic/natural materials**

Transfection vectors are based on cationic lipid, cationic polymer, lipid nano emulsion (Nam, Park et al. 2009, Fraga, de Carvalho et al. 2015), solid lipid-based nanoparticles (Ezzati Nazhad Dolatabadi and Omid 2016), and peptide-based (Riley and Vermerris 2017) materials are widely being investigated and all well described in the literature. In this section, given that this thesis emphasizes gelatin-based nanoparticles, we will focus only on cationic lipids and cationic polymers only.

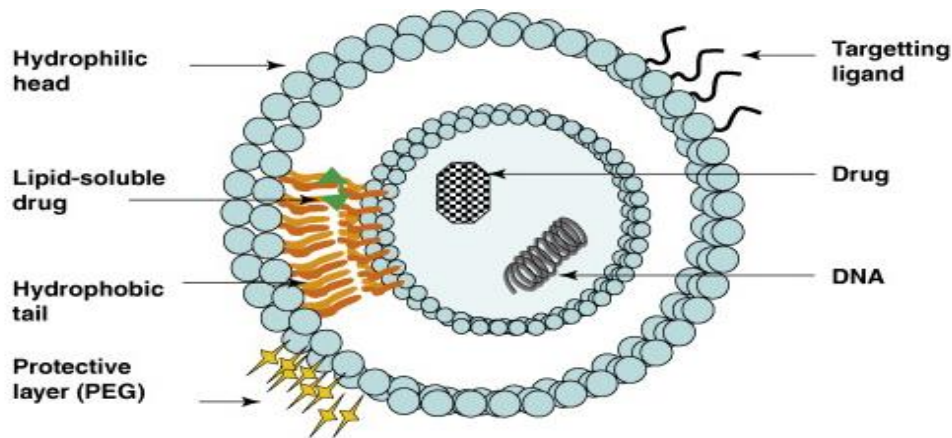
## **1.7 Cationic Lipids as Non-viral Systems for Gene Delivery**

Cationic lipids complexed with DNA (also called lipoplexes - Figure 1.7-1) are considered one of the most efficient non-viral vectors for DNA transfection. Cationic lipids consist of a

hydrophilic head group, typically linked to two hydrophobic hydrocarbon chains that which allow them to assemble into various aggregated structures, including vesicles and bilayers, among many others. The hydrophilic head group of cationic liposomes consists of positively charged functional groups (most commonly primary, secondary, tertiary amines, quaternary ammonium salts, guanidine, imidazole, pyridinium, phosphorus, and arsenic groups) that have the ability to complex with negatively charged naked DNA through electrostatic interaction resulting in complexes called lipoplexes. Cationic liposomes have been examined in safety studies through different routes of administration such as intratumoral, intrapulmonary, intracerebral and intravenous with little or no toxicity reported in any of these studies (Clark and Hersh 1999). Liposomes also have other advantages such as simplicity of preparation, low toxicity, low immunogenicity, ability to produce in large amounts, biocompatibility, and their versatility for use with any size or type of DNA/RNA (Hung, Huang et al. 1999, Pezzoli, Kajaste-Rudnitski et al. 2013, Fisher, Mattern-Schain et al. 2017).

Dioleylpropyltrimethylammonium chloride (DOTMA) and dioleoyltrimethylammonium propane (DOTAP) (Stamatatos, Leventis et al. 1988, Mintzer and Simanek 2008, Yin, Kanasty et al. 2014) are the most commonly used cationic lipids used for gene delivery. In 1987, DOTMA was used by Felgner and colleagues as the first application of cationic liposomes to gene therapy (Felgner, Gadek et al. 1987).





**Figure 1.7-1:** Schematic illustration representing the structure of liposome

Reproduced with permission from (Malam, Loizidou et al. 2009)

The hydrophobic region traps drugs or DNA in the central core when the liposomes are prepared. The outer surface can be functionalized with ligands for active targeting or PEGylated.

### 1.7.1 Helper lipids

Most lipoplex formulations contain “helper” lipids, such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol (Chol), which provides extra stability to the lipoplexes (Kaur, Slavcev et al. 2009). DOPE has exhibited promising transfection efficiencies in both *in vitro* and *in vivo* gene delivery investigations (Farhood, Serbina et al. 1995, Hui, Langner et al. 1996). DOPE is generally thought to improve transfection efficiencies by assisting with the escape of the DNA from the endosome after cellular uptake (Mochizuki, Kanegae et al. 2013). Combination of cationic lipids and DNA in a micellar or liposomal form generally leads to a lamellar organization with DNA molecules sandwiched between lipid bilayers. Some systems, for example, those that contain the neutral lipid DOPE, result in the formation of an inverted

hexagonal structure containing DNA in the center of the inverted hexagonal bilayers (Mochizuki, Kanegae et al. 2013). According to Zhou and Huang (Wang and Huang 1989), under physiological conditions, DOPE is a hexagonal phase-forming lipid (lipoplexes) thought to contribute to its ability to increase the transfection efficiency of DNA-liposome complexes by destabilizing the lamellar structure of the endosomal membrane lipids. Also, DOPE can also help the lipoplexes to escape the endosome through membrane charge density mechanism. In this mechanism, DOPE can trigger lamellar (LC $\alpha$ ) to inverted hexagonal phase (HCII) transitions when the pH drops to acidic condition at late endosome. At the hexagonal phase (HCII), the curvature of DOPE has a negative charge, which results in an elastically frustrated state with the outer positive lipid monolayer of the cationic lipid. This state establishes a driving force for rapid fusion with the cell and endosomal membrane (Lin, Slack et al. 2003, Ewert, Ahmad et al. 2005).

### **1.7.2 Stealth liposomes**

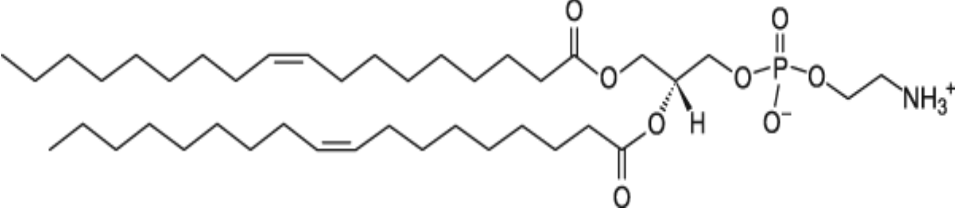
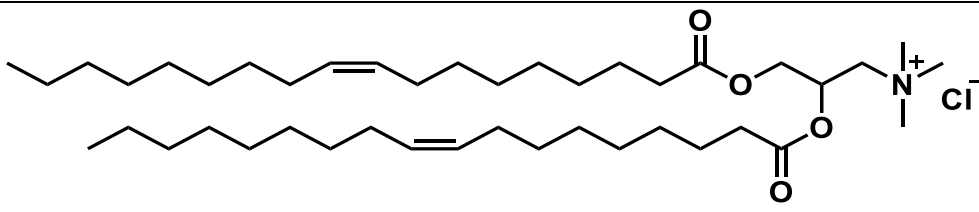
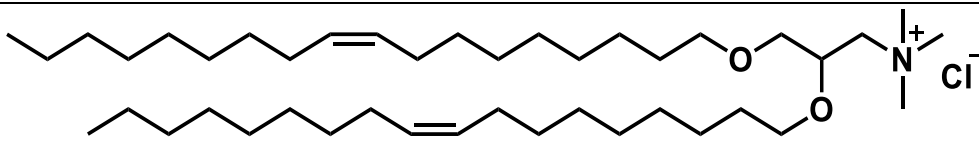
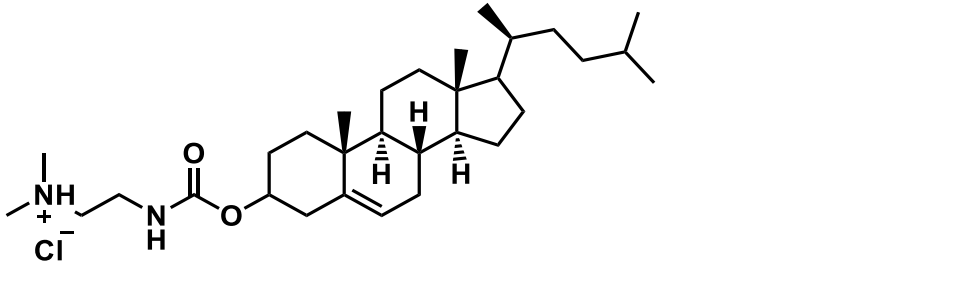
Stealth liposomes are poly-ethylene glycol (PEG)-coated liposomes, which have been recognized to be important for liposomal drug delivery (Immordino, Dosio et al. 2006). PEG is a linear polyether diol that possesses several useful properties, such as biocompatibility, solubility in aqueous and organic media (Davidson 1980), a lack of toxicity, very low immunogenicity (Dreborg and Akerblom 1989), and with good excretion kinetics (Yamaoka, Tabata et al. 1994). The molecular weight and structure of the PEG can be easily modulated for specific purposes, and it is easy and inexpensive to conjugate the polymer to a lipid. It has been demonstrated that grafting of PEG onto liposomes has many biological and

technological advantages. According to Maria et al., the most important properties of PEGylated vesicles are their strongly-reduced mononuclear phagocytic system (MPS) uptake, and their prolonged blood circulation; consequently, they improve distribution in perfused tissues (Immordino, Dosio et al. 2006). In addition, PEG chains on the liposome surface prevent aggregation both with other vesicles and with serum proteins, and thus improving the stability of formulations. Moreover, PEGylation can provide a scaffold on the surface of liposomes for conjugation of different ligands, and have the ability to enhance intracellular delivery, cell-type specific delivery, triggered release, imaging capabilities, tissue localization, and so on (Torchilin 2012). PEGylated liposomal doxorubicin (PLD) was the first, and is still the only, stealth liposome formulation to be approved in the USA and Europe for the treatment of Kaposi's sarcoma (Krown, Northfelt et al. 2004), and recurrent ovarian cancer (Rose 2005).

Lipoplexes also have some drawbacks which include low transfection efficiency and lack of target specificity (Xu, Kumar et al. 1997). In addition, the formation of the lipoplex complex involves interaction among lipid molecules, in addition to that with DNA itself. A major driving force for the complex formation is the release of low-molecular weight counter-ions that makes a large entropic contribution to the free energy of binding (Matulis, Rouzina et al. 2002). The lipids' hydrophobic segments are determinant in the macroscopic characteristics of the ensuing liposomes, particularly their size, shape, and stability in the dispersed state, as well as in their interactions with other lipids, cell membranes, and DNA. This, in turn, affects the transfection efficiency of the resulting lipoplexes. Furthermore, liposomal formulations often require an adjuvant, such as DOPE, for efficient delivery (Hui,

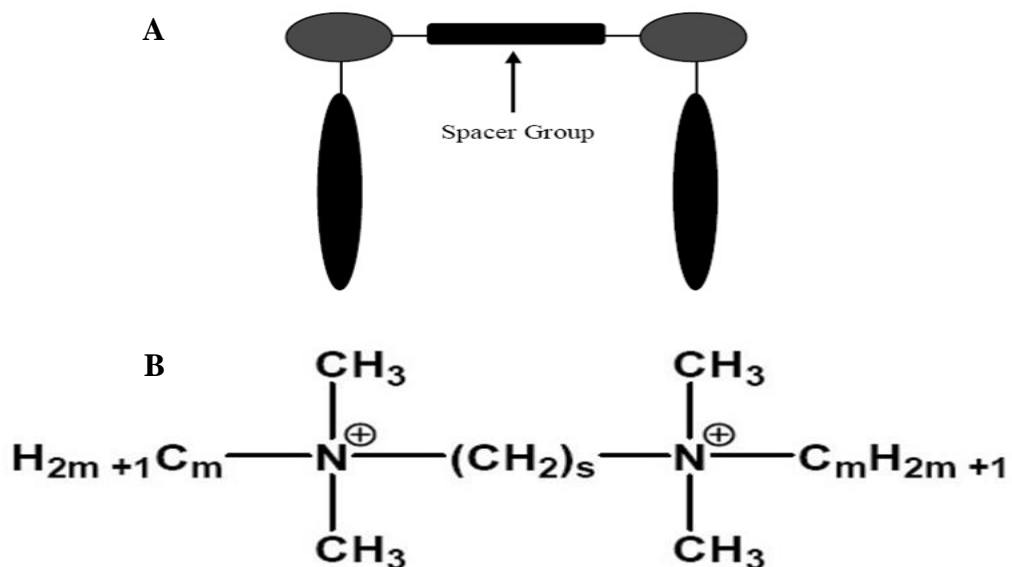
Langner et al. 1996). Liposomes also suffer from the lack of structural integrity, which leads to content leaking and instability during storage (Maurer, Fenske et al. 2001).

**Table 1.7-1:** Chemical structures of most common cationic and helper lipids used for gene delivery

Name of Lipid	Chemical Structure
<b>DOPE</b>	
<b>DOTAP</b>	
<b>DOTMA</b>	
<b>Cholesterol-DC</b>	

### 1.7.3 Gemini surfactants

Gemini surfactants have generated a great deal of research interest, and are considered a suitable replacement for cationic lipids (Ahmed, Kamel et al. 2016, Islam, Shortall et al. 2017). The term “gemini surfactant” was first introduced by Menger in 1991 (Menger and Littau 1991) to mainly describe dimeric bis-surfactants having a rigid spacer linking two surfactant moieties. Gemini surfactants are molecules consisting of two head groups (polar or charged) and two aliphatic chains linked by a rigid or flexible spacer (Menger and Littau 1991) (Figure 1.7-2). They are easily synthesized at a low cost, which is a significant advantage for industrial drug manufacturing. Gemini surfactants exhibit numerous advantageous properties including low critical micelle concentrations (CMC) and high surface activities. A low value of CMC is significant when considering surfactants as transfection vectors.



**Figure 1.7-2:** General structure of gemini surfactant (A), structure of the m-s-m gemini surfactants (B). Adapted from (Wettig, Verrall et al. 2008)

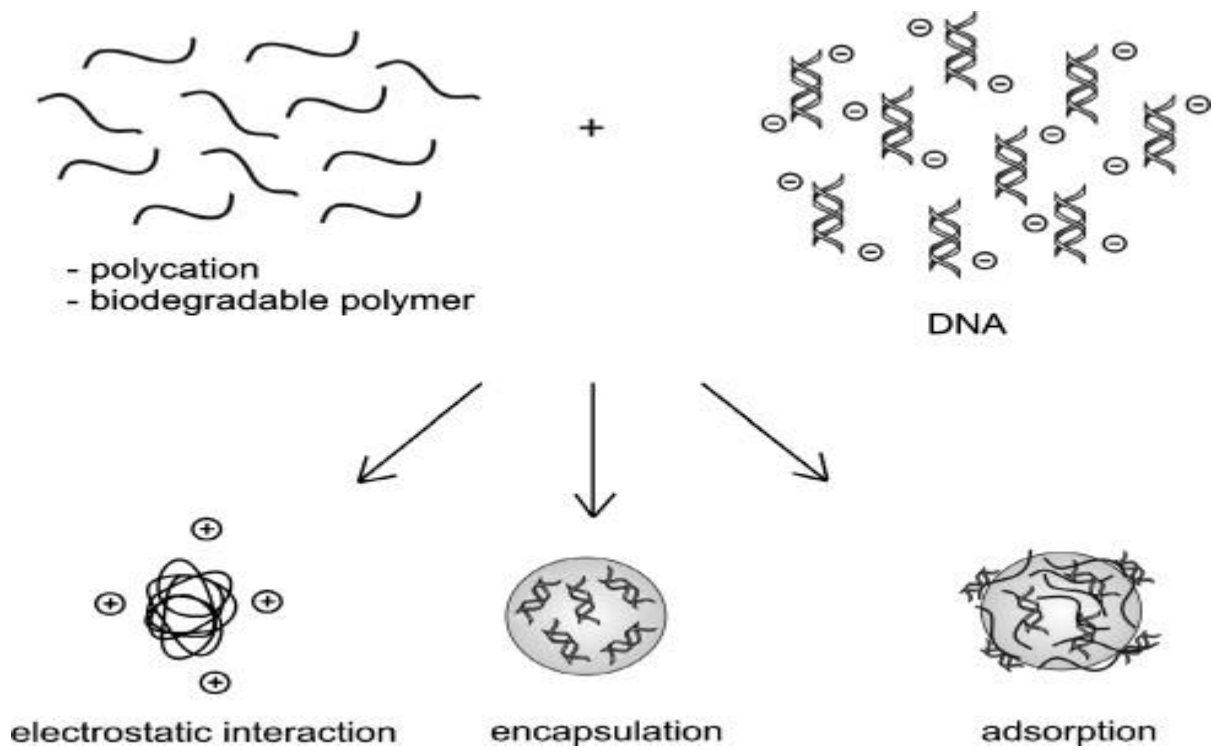
The effect of variations in both the length of alkyl tails and the size of nature of spacer group of gemini surfactants has been demonstrated in many studies. Changes in the length of alkyl tail affect the properties of the surfactant solution in the same way as in traditional monomeric surfactants (Zana and Xia 2003). For example, an increase in the length of alkyl tail of gemini surfactant with a fixed spacer group increases the kraft temperature and decreases the natural logarithm of the CMC linearly (Zana and Xia 2003, Wettig, Verrall et al. 2008). Variations of spacer group of gemini surfactants are more complex because they result from steric, electrostatic, and hydrophobic interactions that serve to give rise to a rather rich array of aggregate structure in the solution (Zana 2002, Wettig, Verrall et al. 2008, Zhi, Zhang et al. 2013, Ahmed, Kamel et al. 2016) .

The cationic *m-s-m* gemini surfactant series is the most commonly studied family of gemini surfactants, where *m* and *s* refer to the alkyl tail length and the number of carbon atoms in the polymethylene spacer respectively. The first appearance of *m-s-m* type of gemini surfactant used for transfection, was in 2001 in a study by Rosenzweig and colleagues, who determined that surfactants having C6 spacer and oleyl (cis-9-octadecene) tails resulted in the highest overall transfection and, in particular, that the addition of the neutral helper lipid DOPE (1,2-dioleoyl-*sn*-glycerin-ophosphatidylethanolamine) diminished transfection efficiency (Rosenzweig, Rakhmanova et al. 2001). Helper lipids work as a stabilizing agent for the DNA-gemini surfactant complexes with C10, C12, and C14 tails (Fisicaro, Compari et al. 2005). Badea has determined that transfection efficiencies are best for spacer group of size  $s \leq 4$  or  $s \geq 12$ . The short spacing (where  $s=2, 3$  and  $4$ , as well as their increased transfection efficiency can be explained in terms of optimizing interaction with

DNA phosphate groups (Badea 2006). The same group also reported that the transfection efficiencies with 16-3-16 increased when the alkyl tail length increased, either in the presence or absence of DOPE. Our group has been focusing on gemini surfactants as gene delivery systems for several years: thus, different modifications were applied to improve the transfection efficiency such as phytanyl substituted gemini surfactant (Wang, Kaur et al. 2013), and pyrenyl gemin surfactant (Al Muslim, Ayyash et al. 2017). Also, we studied the effect of counterions on gemini surfactant 16-2-16 (Islam, Shortall et al. 2017), as well as the effect of the combination of Pluronic block copolymers with gemini surfactant 16-3-16 (Madkhali 2014).

### **1.8 Polymers as Non-viral Systems for Gene Delivery**

Cationic polymers/DNA complexes (also called polyplexes) possess excellent biodegradability, low cytotoxicity, triggered nucleic acid release, and higher transfection efficiency over many other lipoplexed systems (Wang, Su et al. 2012, Zhou, Liu et al. 2012, Li, Yuan et al. 2013). These polymers have the ability to condense, encapsulate, or complex DNA to be used for gene transfection (Wong, Pelet et al. 2007) (Figure 1.8-1). Also, cationic polymer formulations are easier to prepare as aqueous solutions than lipid formulations due to the enhanced solubility of the cationic polymer relative to lipids (due primarily to the long chain, hydrophobic alkyl tails present in the lipid structure). In addition, they are more stable during storage, and they are easy to manipulate through chemical modification to achieve high efficiency or cell targeting without the loss of activity (Tros de Ilarduya, Sun et al. 2010). Consequently, cationic polymers appear to be the most widely investigated group of non-viral vectors, and they are promising to be one of the most successful delivery systems.



**Figure 1.8-1:** Packaging of DNA using polymer. The three main strategies employed to package DNA using polymers are *via* (1) electrostatic interaction, (2) encapsulation within or (3) adsorption onto biodegradable nano- or microspheres. Reproduced with permission from (Wong, Pelet et al. 2007)

Several references in the literature have shown the transfection efficacy of these polymers both *in vitro* and *in vivo* (De Smedt, Demeester et al. 2000, Shi, Dan et al. 2010, Sun and Zhang 2010, Liang, Liu et al. 2012, Bose, Arai et al. 2015, Huang, Zhao et al. 2016, Vaidyanathan, Chen et al. 2016). The most common polymers used for gene delivery are discussed below.



### **1.8.1 Polyethyleneimine (PEI)**

Polyethyleneimine, which is being recognized as a gold standard gene carrier (Boussif, Lezoualc'h et al. 1995, Wang, Niu et al. 2015), has a positive charge and can interact through electrostatic interaction with the negative charge of DNA to form nanocomplexes. PEI, as a delivery system, is very efficient for transfection due to its large buffering capacity, which facilitates endosomal escape using the "proton sponge" mechanism (Varkouhi, Scholte et al. 2011) and thus enhancing the gene delivery to the nucleus. Transfection efficiency of PEI basically depends on molecular weight. PEI with MW higher than 25 kDa demonstrated higher transfection efficiency, whereas PEI with MW lower than (800-2000 Da) revealed low transfection efficiency (Goula, Benoist et al. 1998, Fischer, Bieber et al. 1999, Godbey, Wu et al. 1999, Lee, Cho et al. 2008). The main drawback of PEI is its high toxicity (Lee, Cho et al. 2008).

### **1.8.2 Poly-L-lysine (PLL)**

Poly-L-lysine (PLL) was one of the first cationic polymers used for gene delivery. PLL has a strong positive charge on the lysine amino acids. Although PLL has good biodegradability in the biological system, its use *in vivo* is very limited due to its poor circulatory half-life (~ 3 min). PLL rapidly attaches to the plasma protein once it enters the circulatory system, which is rapidly removed from the circulation, and consequently results in low transfection efficiency (Nishikawa, Takemura et al. 1998, Dash, Read et al. 1999). The combination of DNA and PLL complexes achieves a size less than 100 nm in diameter. These complexes can be covalently attached to the target ligands of PLL, which can be used in different cell lines (Zauner, Ogris et al. 1998, Farrell, Pepin et al. 2007).

### **1.8.3 Chitosan**

Chitosan is a natural, biodegradable, biocompatible, and cationic polysaccharide used for gene delivery. Since chitosan is a polysaccharide, it is water soluble, and its degradation products are non-toxic, non-immunogenic, and non-carcinogenic (Alves and Mano 2008).

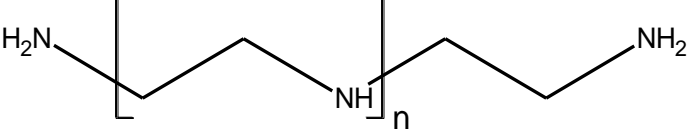
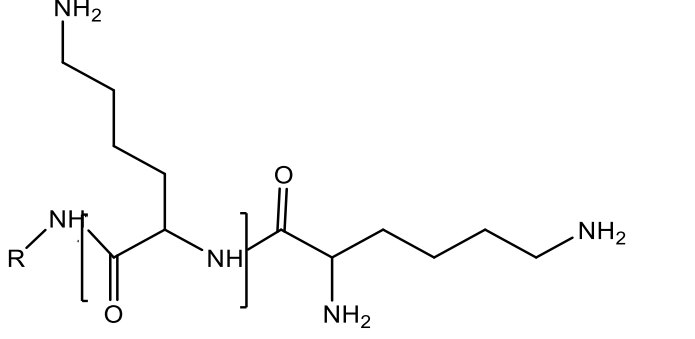
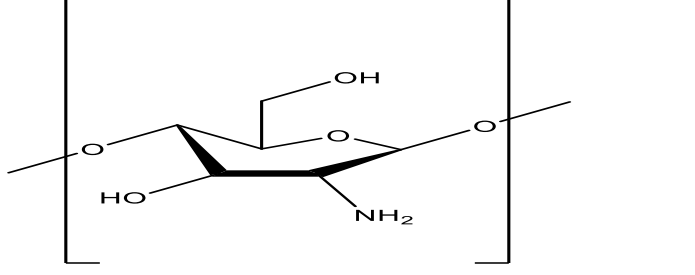
Chitosans are linear polysaccharide containing  $\beta$ -(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine, primary amino groups, and primary and secondary free hydroxyl groups which make it easy to modify chemically (Köping-Höggård, Mel'nikova et al. 2003, Alves and Mano 2008, Mourya and Inamdar 2008). The main problem of chitosan is its limitation in biomedicine because it only dissolves in a mildly acidic solution. However, due to its versatile property, several derivatives have been developed in terms of solubility such as amphiphilic chitosan. Amphiphilic chitosan achieved small particles size (~200 nm), and has been effectively used in gene delivery (Kean, Roth et al. 2005, Dong, Mao et al. 2006).

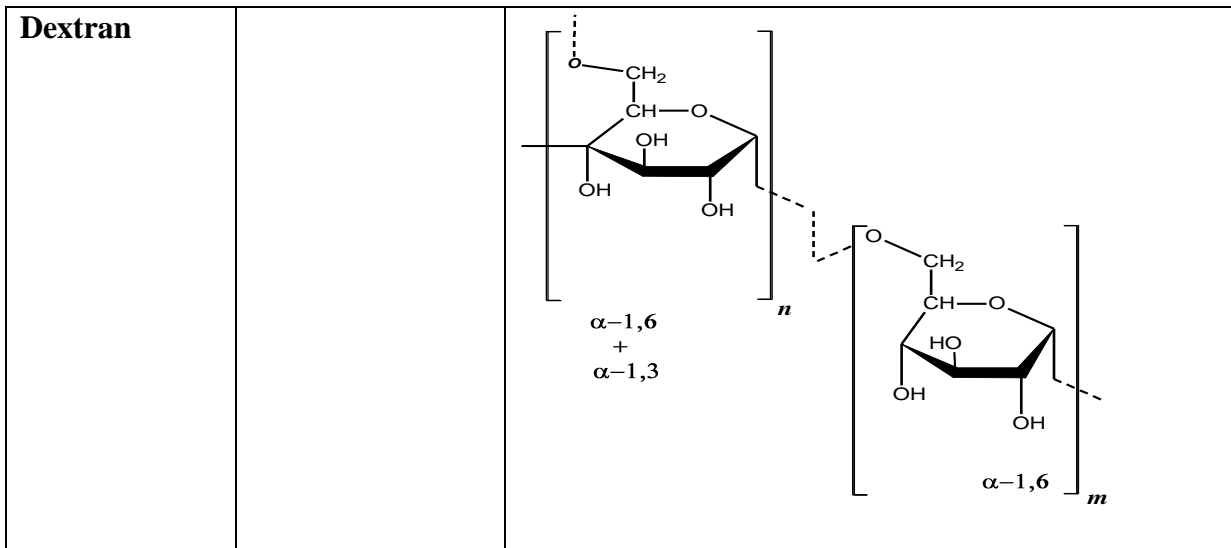
### **1.8.4 Dextran**

Dextran is also a natural and biocompatible polysaccharide made of several glucose molecules. The straight chains of dextran consist of  $\alpha$ -1,6-glycosidic linkage between glucose molecules, while the branches begin from  $\alpha$ -1,3-glycosidic linkage (Purama, Goswami et al. 2009). Dextran can be easily modified by conjugating primary amine groups such as spermine to produce cationic dextran (D-SPM) (Hosseinkhani, Hosseinkhani et al. 2011). D-SPM is water soluble and possesses a positive charge of +42, which is responsible for the nonspecific binding of polyplexes or polymer to plasma protein (Hosseinkhani, Abedini et al. 2015). D-SPM complexes can also be modified by adding PEG in order to increase the circulation time the in the circulatory system. Polyplexes of PEGylated D-SPM revealed an

increase in transgene expression, and are distributed in more organs including liver, lungs, spleen, and kidneys, compared to non-PEGylated polyplexes (Hosseinkhani, Azzam et al. 2004).

**Table 1.8-1:** Chemical structure of the common polymers used in gene therapy

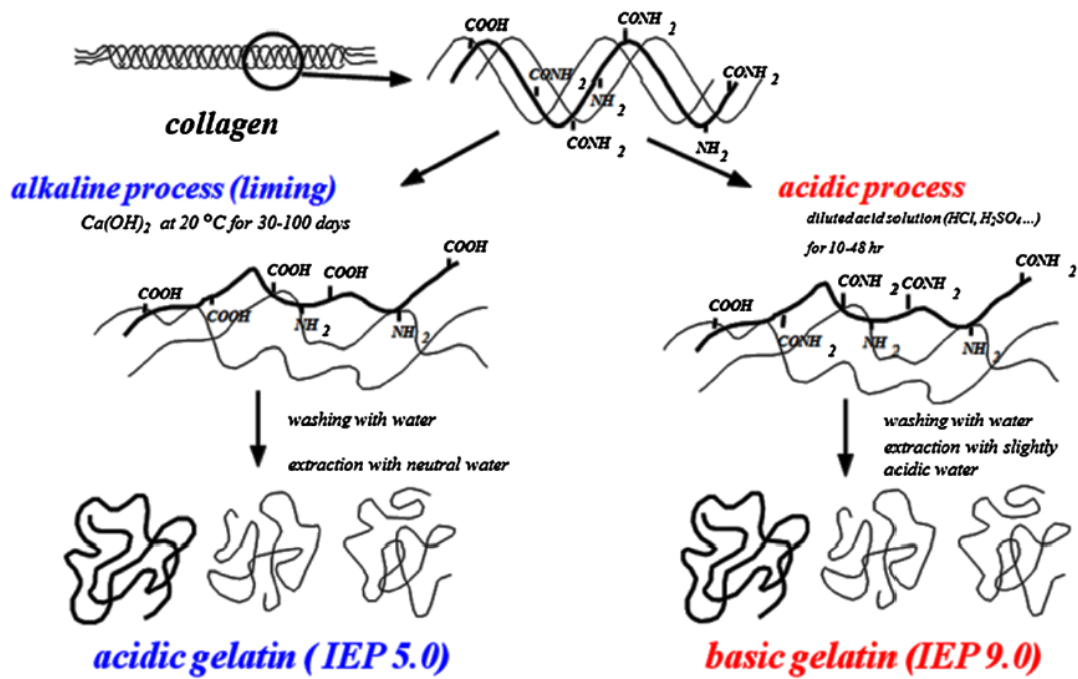
Generic Name	Chemical Name	Chemical Structure
<b>PEI</b>	Polyethyleneimine	
<b>PLL</b>	Poly(L-lysine)	
<b>Chitosan</b>		



## 1.9 Gelatin Nanoparticles for Gene Therapy Applications

### 1.9.1 Introduction to gelatin

Gelatin is a natural polymer that is extracted from animal collagen through either partial acid or alkaline hydrolysis (Figure 1.9-1). There are two types of gelatin: cationic (gelatin type A), and anionic (gelatin type B) (Table 1.9-1) (Wang, Boerman et al. 2012, Elzoghby 2013).



**Figure 1.9-1:** Extraction of gelatin from collagen. The production of type A (cationic) gelatin is shown on the right of the figure; the production of type B (anionic) gelatin is shown on the left of the figure. Reproduced with permission from (Hosseinkhani, Abedini et al. 2015)

**Table 1.9-1:** Typical specification for commercial gelatin

Characteristics	Type A	Type B
Gel strength (bloom)*	50-300	50-250
Isoelectric point (IEP)	7-9	4.8-5
pH	3.8-5.5	5-7.5
Extraction	from pig skin type 1 collagen	from bovine collagen

\*Bloom number: as determined by the Bloom gelometer, it is an indication of the strength of a gel formed from a solution of known concentration. The Bloom unit is a measure of the force (weight) required to depress a given sample area of gel (a distance of 4 mm); the higher the Bloom number, the stronger the gel (GMIA 2013).

Gelatin is distinguished from other synthetic polymers by having amino acid sequences such as Arg-Gly-Asp (RGD) in its structure. These amino acid sequences modulate cell adhesion; consequently, they play a significant role in gelatin's final biological performance in comparison to other synthetic polymers that lack these cell-recognition sites (Wang, Boerman et al. 2012). Gelatin consists of eighteen non-uniformly distributed amino acids with both positive and negative charges (Samal, Dash et al. 2012). Since gelatin is derived from collagen and is found in large amounts in animals, gelatin does not produce harmful byproducts after enzymatic degradation (Elzoghby 2013). As a result, gelatin is considered as GRAS (generally regarded as safe) according to the United States Food and Drug Administration (FDA); and thus, it has been used in pharmaceutical, cosmetics, and food products for decades (Lemieux, Vinogradov et al. 2000, Kommareddy, Shenoy et al. 2005, Elzoghby, Samy et al. 2012). The FDA has also approved gelatin for extravascular

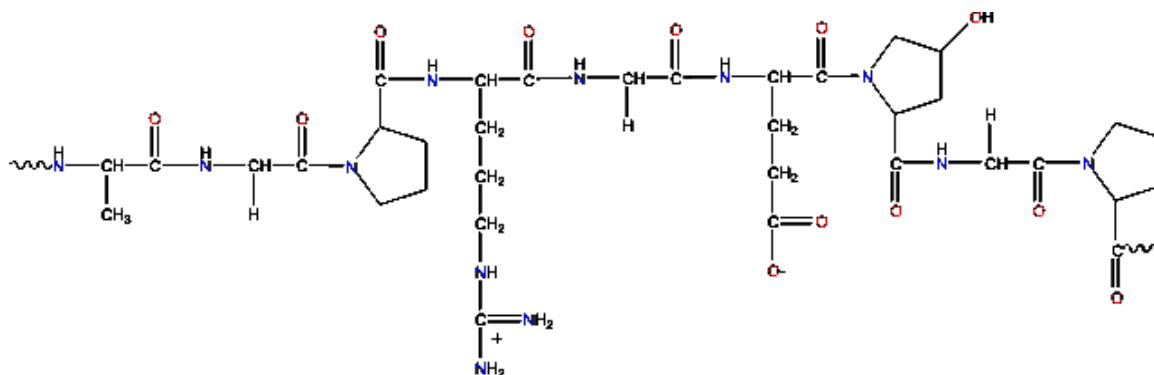
administration, and it is widely used as a stabilizer in vaccines (Zwiorek 2006, Lee, Khan et al. 2011).

Its biodegradability, compatibility, availability and low production cost have attracted many researchers to use gelatin experimentally. Due to its animal origin, gelatin has low antigenicity compared to collagen because gelatin is denatured from collagen during extraction processes, which causes a change in the molecular composition of gelatin's many amino acids (Kommareddy, Shenoy et al. 2005, Elzoghby, Samy et al. 2012). Furthermore, gelatin has a flexible structure that can be easily modified by changing its functional groups with different cross linkers and targeting-ligands. This ability to be easily modified could be very beneficial in improving and developing potential gene and drug delivery systems, with minimal toxic effects on the host cells (Busch, Schwarz et al. 2003, Zhou and Regenstein 2006, Wang, Boerman et al. 2012). Generally, gelatin is a promising gene and drug delivery system due to its multiple advantages and safety features, as noted above.

### **1.9.2 Chemical structure of gelatin**

Gelatin is an amphiphilic polymer having both cationic and anionic charges along with hydrophobic groups present in the approximate ratio 1:1:1, which makes this polypeptide special (Elzoghby 2013). Gelatin has a triple helical structure (Gly-X-Pro), composed mainly of glycine and proline amino acids, with X representing the third amino acids. These amino acids mostly include alanine, lysine, arginine, methionine and valine (Flory and Weaver 1960, Sahoo, Sahoo et al. 2015) (Table 1.9-1). Lysine and arginine represent 13% of gelatin, and both possess a positive charge; 12% of the polymer is negatively charged glutamic and aspartic acid groups. The hydrophobic group consists of leucine, isoleucine, methionine, and

valine representing 11% of gelatin's structure. The remaining chain includes glycine, proline, and hydroxyproline (Elzoghby 2013).



**Figure 1.9-2:** General chemical structure of gelatin

**Table 1.9-2:** Amino acid composition of pigskin gelatin  
Reproduced with permission from (Farris, Song et al. 2009)

Amino acids	Pig skin gelatin (mole %)	Amino acids	Pig skin gelatin (mole %)
alanine	11.05	leucine	2.35
arginine	4.96	lysine	2.65
asparagine	0.60	methionine	0.32
Aspartic acid	4.42	Phenylalanine	1.38
proline	13.10	serine	3.40
glutamic acid	7.10	histidine	0.45
glycine	32.20	threonine	1.80
hydroxyproline	9.80	tyrosine	0.35
hydroxy-leucine	0.75	valine	1.90
isoleucine	1.02		



### **1.9.3 Gelatin nanoparticles for gene therapy**

#### **1.9.3.1 Systemic gene delivery**

Several advantages make gelatin an effective vector for gene delivery. First, as an intrinsic protein structure, gelatin nanoparticles (GNPs) have the ability to conjugate to different moieties that stimulate receptor-mediated endocytosis due to the presence of amino and carboxylic functional groups. They can also encapsulate multiple plasmids, and the transfection efficiency can be improved by protecting the encapsulated plasmids from digestion using long-circulating PEGylated nanoparticles or nucleases (Kaul and Amiji 2005, Kommareddy, Shenoy et al. 2005). The first GNPs as non-viral vectors for gene delivery were developed by Kaul and Amiji in 2002 (Kaul and Amiji 2002), using gelatin type B. They studied the way the GNPs could encapsulate nucleic acid. The first study revealed that the negative charge of gelatin type B could physically encapsulate nucleic acid at neutral pH 7.0; however, the nucleic acid could be condensed electrostatically when it interacted with the positive charge of lipids or polymers. When plasmid DNA exists in a hydrogel-type matrix, it is protected in the systemic circulation and upon cellular transport. Also, the released plasmid DNA forms a supercoiled structure at the nuclear membrane, which is very significant in obtaining an effective transfection because of its minimal size, which improves gene transfer and potential bioavailability (Darquet, Cameron et al. 1997, Magadala and Amiji 2008). Modifying the surface of gelatin with a quaternary amine such as cholamine enables the negative charge of the nucleic acid to be adsorbed on the surface of GNPs, thus increases ionic interactions (Zwiorek, Bourquin et al. 2008). The Amiji group has studied the effectiveness of non-condensing type B gelatin for systemic and oral gene therapy. GNPs

loaded with TMR-dextran as a model hydrophilic drug in BT-20 cells used to study cell trafficking showed that the particles were mainly taken up by endocytosis, and subsequently escaped the endosome through the proton sponge effect (Zhang, Ma et al. 2013); and were also found to be contained in the perinuclear area in the cytoplasm (Kaul and Amiji 2002, Kaul, Lee-Parsons et al. 2003). Another study indicated that most of the GNPs were internalized in NIH-3T3 fibroblast cells within the first six hours of incubation. After 12 hours of incubation, green fluorescent protein expression was observed, which remained stable for approximately 96 hours with 43% transfection efficiency (Kaul and Amiji 2005). GNPs containing LacZ plasmid in the tibialis anterior muscle of 6-week old BALB/c mice showed powerful and sustained gene expression compared to naked DNA and Lipofectamine complexes *in vivo* (Leong, Mao et al. 1998). A successful encapsulation and an effective intracellular delivery of GNPs containing siRNA have also been demonstrated in the literature (Xu, Ganesh et al. 2012). For example, siRNA encapsulated in GNPs revealed high stability, even in an RNase rich environment. HIF-1 $\alpha$  siRNA loaded GNPs showed important downregulation in SKOV3 cells (Leong, Mao et al. 1998, Shah 2010, Xu, Ganesh et al. 2012).

### **1.9.3.2 Oral gene delivery**

Gelatin nanoparticles have also been used to deliver nucleic acid through oral administration. A unique multi-compartmental oral delivery system was developed by Bhavsar and Amiji (Bhavsar, Tiwari et al. 2006, Bhavsar and Amiji 2007, Bhavsar and Amiji 2008). This system is based on encapsulation of DNA-GNPs in poly (epsilon-caprolactone) (PCL) microsphere using a technique termed “double-emulsion-like”. This delivery system was called

“nanoparticles-in-microsphere oral system” or (NiMOS). The role of NiMOS is to protect the DNA during its travel to the stomach. When lipases-enzymes in the intestinal tract degrade the PCL matrix, DNA-GNPs are then internalized by enterocyte, or any other cell in the GI lumen to express the required protein (Bhavsar, Tiwari et al. 2006, Bhavsar and Amiji 2007, Bhavsar and Amiji 2008, Kriegel, Attarwala et al. 2013).

Significant EGFP and Beta-galactoside expression were observed in the small and large intestines after oral administration of NiMOS in fasted Sprague-Dawley rats (Bhavsar and Amiji 2008). In addition, interleukin-10 plasmid DNA and GNPs were encapsulated in PCL for the treatment of inflammatory bowel disease (IBD). The complex was then administered orally in an acute colitis model. IL-10, placed in the large intestine, was able to increase the level of mRNA and proteins, and reduce the levels of proinflammatory cytokines such as IFN- $\gamma$ , IFN- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , and IL-12, in addition to certain chemokines (Bhavsar and Amiji 2008). These studies demonstrated that GNPs are potential and promising delivery vehicles for both systemic and oral gene delivery.

#### **1.9.4 PEGylated gelatin nanoparticles**

GNPs are mainly engulfed by the cells of the reticuloendothelial system (RES) upon systemic administration, which leads to weak transfection and gene expression. However, coating the GNPs with poly (ethylene glycol) (PEG) generates a dense hydrophilic shell of long chains that conserve the core of GNPs from non-specific hydrophobic interaction with serum protein; thus, it significantly reduces the effect of RES (Otsuka, Nagasaki et al. 2003). Another advantage of PEGylation is that it may increase the hydrodynamic size of the particles, which leads to a decrease in their clearance from the kidney, as the renal filtration

is dependent on the molecular mass and volume. These advantages result in an increase in circulation half-life of the particles *in vivo* (Crawford 2002, Kommareddy, Tiwari et al. 2005). In addition, the existence of PEGylation on the surface of the GNPs helps in protecting the particles from digestion by proteolytic enzymes (Xu, Ganesh et al. 2012). Adding PEG to non-condensing type B GNPs has been shown to result in an excellent system to distribute effectively in solid tumor because of the hyperpermeability angiogenic blood vessels in tumors and the enhanced permeability and retention (EPR) effect (Kaul and Amiji 2004, Kaul and Amiji 2005). According to Amiji and Kaul, PEGylated GNPs favorably targeted tumor mass in Lewis lung carcinoma (LLC) bearing female mice, where about 4-5 % of the injected dose remained in the tumor for approximately twelve hours after administration (Kaul and Amiji 2004). Amiji and Kaul also stated that reporter pDNA encoding for  $\beta$ -galactosidase (pCMV- $\beta$ ) was effectively encapsulated in PEGylated GNPs and showed significant expression in LLC tumors with 61% transfection efficiency (Kaul and Amiji 2005). Kushibiki and colleagues (Kushibiki, Matsuoka et al. 2004) studied the long-circulation property of PEGylated gelatin using  $^{125}\text{I}$ -labeled gelatin. They compared between unmodified GNPs and PEGylated  $^{125}\text{I}$ -labeled GNPs after I.V. administration through the tail vein in LLC-bearing mice. They found that PEGylated GNPs have longer circulating properties in the blood and remained in the tumor for up to 24 hours post-administration. In another study thiolated PEGylated GNPs exhibited prolonged circulation and enhanced tumor extravasation *in vivo* in an orthotopic human breast adenocarcinoma xenograft model (Kommareddy and Amiji 2007). Comparing with the non-PEGylated GNPs, the PEGylated nanoparticles showed longer circulation with plasma and tumor half-lives of 15.3 and 37.8

hours respectively. Generally, the advantages of the combination of PEG with GNPs are summarized as follows: increasing the circulation time in the plasma and tumor mass; stabilizing the therapeutic cargo during transportation; preventing RES elimination; and providing a potential for conjugation of targeting moieties.

### **1.9.5 Thiolated gelatin nanoparticles**

The thiol group (SH) has been considered as a potential addition to GNPs due to its ability to respond to the environment changes either inside or outside the cells. The thiol group is similar to alcohol in chemical structure but differs in its chemical properties. Thiols are more nucleophilic, more acidic, and more readily oxidized (Senning 1997). Adding thiol groups to gelatin leads to the formation of disulfide bonds (S-S) in an oxidation reaction within the polymer, which can be beneficial in strengthening the tertiary and quaternary protein structure in the case of gelatin (Bacalocostantis, Mane et al. 2013). Also, disulfide bonds can stabilize the nanoparticles during systemic circulation, and release the encapsulated payload when they are broken inside the cell (Kommareddy and Amiji 2005). Groups of thiols are easily and rapidly crosslinked; therefore, they can be used for the synthesis of polymeric delivery vectors (Bacalocostantis, Mane et al. 2013). Conjugating thiols with PEG has been shown to improve stability and circulation time of the polyplexes *in vivo* (Bacalocostantis, Mane et al. 2013). Glutathione (GSH) is a dipeptide, used as an antioxidant to prevent damage caused by an oxygen species. GSH and peroxide exist in high concentration inside the cells more so than outside (100-fold higher), and their concentration is much higher in the cytoplasm of tumor cells. As a result, Kommareddy and Amiji (Kommareddy and Amiji 2005) introduced a thiol (SH) group into gelatin using 2-iminothilane reagent and prepared

the nanoparticles by desolvation using ethanol under adjusted and controlled condition of pH and temperature. The plasmid DNA was then incorporated into the thiolated gelatin nanoparticles. The thiolated GNP encapsulated DNA showed high transfection efficiency in NIH-3T3 murine fibroblast cells in contrast with unmodified gelatin and lipofectin®-complexed DNA (Kommareddy and Amiji 2005). Six hours after transfection, the expression of green fluorescent protein was observed. These results could be interpreted such that it was the disulfide bonds that increased the stability of the nanoparticles and indicated that thiolated GNPs have a rapid release of their contents into a highly reducing environment inside the cell where the high concentration of GSH (Kommareddy and Amiji 2005, Kommareddy and Amiji 2007). Furthermore, the same group evaluated three modifications of gelatin: PEG-GNPs, thiolated-GNPs, and PEG-modified thiolated GNPs in NIH-3T3 to deliver plasmid DNA. Among all three formulations tested, PEG-thiolated GNPs showed the highest GFP expression even more than the positive control lipofectin-complexed DNA (Kommareddy and Amiji 2007). Generally, both PEG-GNPs and PEG-modified thiolated GNPs demonstrated longer circulation in the blood and higher accumulation in the tumor cells in contrast with unmodified GNPs (Xu, Ganesh et al. 2012). A new tumor-targeted siRNA delivery system using polymerized siRNA (poly-siRNA) and thiol-modified gelatin nanoparticles was developed by Lee et al. (Lee, Yhee et al. 2013). The poly-siRNA was prepared by self-polymerization of thiol group and was encapsulated in the self-assembled thiolated-GNPs using chemical cross-linking. The results revealed that the siRNA was protected from enzymatic degradation, and the siRNA molecules were released effectively in reductive condition. Also, poly-siRNA-thiolated –GNPs demonstrated excellent

accumulation in the tumor cells, induced effective target gene silencing in tumors after intravenous injection, and showed high cell viability (closer to 100%) compared to lipofectamine and non-thiolated siRNA-GNPs (Lee, Yhee et al. 2013). Clearly, the disulfide bonds formed by (thiol) group could play a significant role in the stability of nanoparticles, thereby resulting in effective gene expression and high cell viability.

### **1.9.6 EGFR-targeted gelatin nanoparticles**

One of the greatest challenges for gene delivery is targeting. A delivery vector is required to distinguish the host cells, evade nonspecific binding, and resist degradation in the systemic circulation. After reaching the target cells, the delivery vector should cross the cell membrane, facilitating the escape of the vector from the endosome, and release the nucleic acid from the complex, which can then enter the nucleus to express the required protein (Xu, Gattacceca et al. 2013). Although tumor targeting using PEG surface modified nanoparticles accomplishes some preferential accumulation in the tumor cells and allow for intracellular delivery, there are certain types of cancer that do not have adequate vasculature or the nanoparticles may not be able to penetrate deep into the tumor mass (Xu, Ganesh et al. 2012). Mutation of epidermal growth factor receptor (EGFR) has been shown to be associated with poor prognosis in several types of cancers including ovarian cancer (Ciardiello and Tortora 2001). Between 33 and 75% of EGFR has reported to be overexpressed in ovarian cancer, and has been found in both the growth and progression of this disease (Sewell, Macleod et al. 2002). OVCAR-3 (cell line used in this project) showed a high level of EGFR protein expression (Noske, Schwabe et al. 2011); consequently, targeting

of EGFR using the OVCAR-3 cell line is possible and could improve the transfection efficiency.

EGFR is a member of the ErbB/her family of ligand activated receptor tyrosine kinases (RTKs) that has been recognized as a molecular target. EGFR consists of an extracellular ligand-binding domain similar to any other receptor of tyrosine kinases that is involved in interactions between receptors within the membrane, and a cytoplasmic domain with tyrosine kinase activity (Schlessinger 2002). Consequently, conjugation gelatin with EGFR- targeted peptide has been shown to improve the transfection efficiency in several types of cancer cells. EGFR-targeted GNPs carrying plasmid DNA encoding for EGFP-N1 obtained the highest transfection efficiency in Panc-1 pancreatic adenocarcinoma cells in compared to other controls, particularly 48 hours after transfection (Magadala and Amiji 2008). As another example, transfection of EGFR-targeted GNPs with p53 induced a rapid apoptosis process in Panc-1 cells (Xu and Amiji 2012). Intravenous injection of EGFR-targeted GNPs to a mice bearing Panc-1 pancreatic adenocarcinoma showed nearly double efficiency of targeting in comparison to PEG-GNPs and unmodified GNPs. In addition, it accumulated and was sustained longer in the tumor mass (Xu, Gattacceca et al. 2013). Another study conducted by Xu and Amiji used EGFR-targeted thiolated gelatin nanoparticles to deliver plasmid DNA into Panc-1 pancreatic adenocarcinoma cells (Xu and Amiji 2012). The EGFR improved the targeting, and the thiol group enhanced the stability of GNPs. The results showed that EGFR-targeted thiolated GNPs had nanoparticles that were small in size (150-200 nm) with high GFP expression, even higher than the positive control lipofectin-complexed DNA; they also obtained high cell viability (Xu and Amiji 2012).



Using targeting-ligands with GNPs facilitated the delivery system's recognition of the host cell. As a result, the transfection was improved, and the cytotoxicity was reduced, which is the optimal goal for gene therapy delivery systems.

### **1.9.7 Cationic gelatin and its importance for gene delivery**

As is the case for any polymeric system used as a gene delivery vector, it is necessary for gelatin to carry a positive charge (i.e. be cationic) in order to interact with the negatively charged DNA and cell membrane. According to the literature, positively charged particles are favorable phagocytosed by dendritic cells and macrophage more than the negative or neutral charged particles (Coester, Nayyar et al. 2006). Gelatin is a polyelectrolyte with low charge density; however, the charge density is appreciably changed according to the solution pH. As a result, cationization of gelatin is a significant factor in obtaining an effective gene delivery vector (Zwiorek, Kloeckner et al. 2005). Cationic gelatin is prepared mainly by introducing amine residues to the carboxyl groups of gelatin using polyethyleneimine (Mimi, Ho et al. 2012), cholamine (Fuchs, Klier et al. 2012), ethylenediamine (Xu, Capito et al. 2008, Ishikawa, Nakamura et al. 2012, Xu, Singh et al. 2014), or spermine (Zorzi, Párraga et al. 2011).

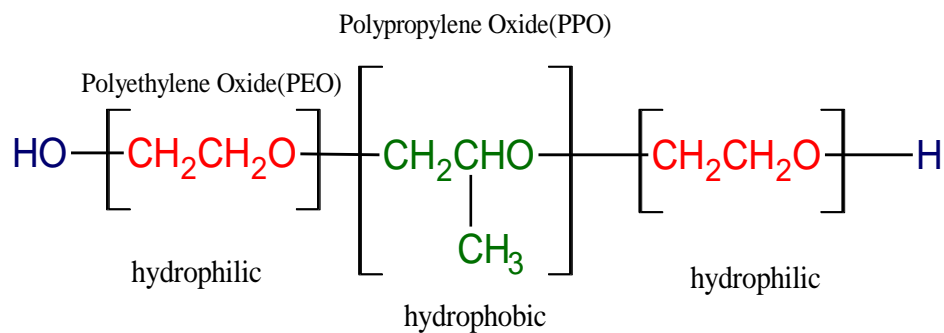
Cationized gelatin containing plasmid DNA expressing insulin-like growth factor (IGF)-1 has shown a five-fold elevation in the amount of IGF in adult articular chondrocyte compared to non-cationized gelatin. Also, chondrocytes treated with pIGF using cationized gelatin were able to maintain stable IGF-1 overexpression when later grown in collagen (type II)-glycosaminoglycan (CG) scaffold for up two weeks and exhibited enhanced biosynthesis (Xu, Capito et al. 2008). Another example is polyethyleneimine (PEI), which has a high

positive charge, was conjugated onto the surface of GNPs to deliver pCMV-Luc and gene presented optimal transfection efficiency with high cell viability (Kuo, Huang et al. 2011). Basically, the existence of positive charge with GNPs will play a major role in improving the transfection efficiency and decreasing the cell toxicity, which is the optimal goal to obtain an effective delivery system for gene therapy.

## 1.10 Pluronic Block Copolymers for Gene Therapy Applications

### 1.10.1 Pluronic block copolymers

Pluronic block copolymers, also called poloxamers, are nonionic polymers consisting of hydrophilic poly (ethylene oxide) (PEO) and hydrophobic poly (propylene oxide) (PPO) blocks arranged in triblock structure:  $EO_x-PO_y-EO_x$  (Kabanov, Lemieux et al. 2002) (Figure 1.10-1).



**Figure 1.10-1:** General chemical structure of Pluronic block copolymers

This arrangement engenders an amphiphilic copolymer in which hydrophobic PO and hydrophilic EO segments can be modified to change the size, hydrophobicity, and hydrophilicity of the Pluronic (Kabanov, Lemieux et al. 2002). Copolymers with various PO

and EO values are characterized by a distinct hydrophilic lipophilic balance (HLB) (Kabanov, Lemieux et al. 2002), which relates to the suitability of a particular polymer for a specific application. Sriadibhatla's studies claim using that copolymers of intermediate hydrophobicity (HLB 9-16) with relatively large hydrophobic blocks (30-69 PO units) were the most effective in gene expression (Sriadibhatla, Yang et al. 2006).

Lack of solubility and stability in the biological fluid was the primary limiting factor of the first generation of polyplexes. The particle size of the polyplexes is very sensitive to the nature of salts that are present in the buffer and serum components (Ogris, Steinlein et al. 1998, Nguyen, Lemieux et al. 2000). As a result, Pluronic block copolymers overcame this barrier by forming micelles that reduce unwanted interactions between cells and/or proteins with a Pluronic-solubilized compound, and increases its circulation time due to the existence of PEO blocks (Kabanov, Lemieux et al. 2002, Kabanov, Zhu et al. 2005)

Due to their amphiphilic character, Pluronic copolymers display surfactant properties, which have the ability in aqueous solution to self-assemble into micelles above the critical micelle concentration (CMC) at fixed temperature, or above the critical micelle temperature (CMT) at a fixed concentration - a process called *micellization* (Kabanov, Lemieux et al. 2002, Xiong, Binkhathlan et al. 2012). The number of block copolymer unimers forming one micelle is known as the *aggregation number*. The hydrophobic PO core of the micelle can serve as a "cargo hold" for incorporating various therapeutic and hydrophobic components (Kabanov, Zhu et al. 2005, Batrakova and Kabanov 2008). In addition, the existence of hydrophobic PO block enables the pluronic unimers to interact with the lipid membrane and translocate inside the cells (Kabanov, Zhu et al. 2005). On the other hand, the hydrophilic EO

shell keeps the micelle in a dispersed state, prevents undesirable interactions with other cells and proteins, and increases its circulation time (Kabanov, Lemieux et al. 2002, Kabanov, Zhu et al. 2005). Polymeric structures often tend to precipitate in water due to a localized hydrophobicity caused by the drug and the hydrophobic portion of the polymeric chain. However, with a core/shell structure, the polymer may remain in water-soluble if the number of monomers in the shell-forming block exceeds the core-forming block (Lavasanifar, Samuel et al. 2002).

Copolymers enhance gene expression using a mechanism that does not condense the DNA. It is possibly because copolymers act as biological adjuvants which enhance transgene expression by changing the response of the cells to the delivered DNA (Kabanov, Sriadibhatla et al. 2010), as discussed in detail in the next section.

### **1.10.2 Pluronic block copolymer for gene therapy applications**

Several studies in the literature review have demonstrated that Pluronic block copolymers significantly increase expression of plasmid DNA in skeletal muscle, spleen, and lymph nodes as well as they stimulate plasmid DNA uptake and expression in antigen presenting cells in mice (Lemieux, Guerin et al. 2000, Alakhov, Lemieux et al. 2001, Yang, Zhu et al. 2005, Sriadibhatla, Yang et al. 2006). Pitard and colleagues have discovered that a formulation containing single pluronic copolymers with plasmid DNA also enhanced the gene transfer in the muscle. In particular, Pluronic L64 improved the level of transfection efficiency more than naked DNA in the skeletal and cardiac muscle (Pitard, Pollard et al. 2002).

Copolymers also increase expression of genes delivered using polycation-DNA complexes, improve transfection with adenovirus and lentivirus vectors both *in vivo* and *in vitro*, and improve gene expression with different delivery routes and different vectors including naked DNA (Kabanov, Zhu et al. 2005). A recent study combined F127 with cationic copolymers including poly(dimethylaminoethyl methacrylate), poly(dimethylaminoethyl methacrylate-tert butyl acrylate), and poly(dimethylaminoethyl methacrylate-acrylic acid). Pluronic F127 was used as a means of reducing the cytotoxicity of the cationic copolymers and for self-assembly. The results demonstrated high gene expression and low cytotoxicity in 293T cells particularly with poly (dimethylaminoethyl methacrylate-acrylic acid) (Huang, Wang et al. 2013). In addition, Chen and colleagues (Chen, Zhang et al. 2014) successfully synthesized a series of F127-PEI-SS/pDNA complex formed by crosslinking disulfide –containing PEI (800 Da). The results demonstrated that in the presence of Pluronic, the complex showed high stability against DNase I or serum and higher transfection efficiency and viability than PEI-25 KDa, both in Bcap and Hela cell line. Among all tested Pluronic (P123, F127, L61, L35, and L64), which were added to the F127-PEI-SS/pDNA complex, L35 with HLB of 19 showed the highest transfection efficiency and noticeable longer blood circulation time than other Pluronic and PEI-25 KDa. Moreover, Pluronic F87 and F108, in combination with cationic gemini surfactant 16-3-16, were able to deliver pDNA in OVCAR-3 cell line with a transfection efficiency (~ 15%) compared to lipofectamine 2000 (33 %) (Madkhali 2014).

Despite the above results, the mechanism of action of gene expression of Pluronic is not completely understood (Kabanov, Batrakova et al. 2002). However, Kabanov et al.

(Kabanov, Batrakova et al. 2002) have speculated some mechanism(s) of action for Pluronics. Also, when Pluronics interact with the cellular membrane, they facilitate cellular uptake of polyplexes (Astafieva, Maksimova et al. 1996) and naked DNA (Lemieux, Guerin et al. 2000). In addition, Pluronics enhanced DNA distribution through the muscle (Lemieux, Guerin et al. 2000), and they increased transport of DNA from the cytoplasm in the nucleus of the muscle cells (Pitard, Pollard et al. 2002). Generally, the mechanisms of action in which Pluronics enhance gene expression are different from those of cationic lipids or polycations (Flotte, Afione et al. 1993).

Given the above, Pluronic block copolymers are promising agents as nonviral vectors for gene therapy applications. Pluronic can modify the biological response during gene therapy which leading to enhancement of gene expression and therapeutic effect of the transgene. Also, Pluronics are able to form novel self-assembling gene delivery vectors that have superior efficacy to currently known systems. In addition, Pluronics block copolymers have shown some promise as formulation agents (Kabanov, Lemieux et al. 2002).

### **1.11 Gelatin-Pluronic-based Nanoparticles as Non-viral Vectors for Gene Therapy**

In addition to its ability to complex DNA, gelatin is capable of forming nano-complexes with different polymers through various mechanisms. These mechanisms include ionic complexation, graft copolymerization, or self-assembly (i.e., micelle-like nanospheres) when combined with hydrophobic polymers (Elzoghby 2013). Gelatin-polyacrylic acid (GEL-PPA) nanoparticles were prepared using a polymerization mechanism without the addition of either organic solvents or additional surfactants (Wang, Zhang et al. 2009, Ding, Zhu et al. 2011). GEL-PPA nanoparticles were formed due to the electrostatic interaction between ionized

carboxyl groups of PPA and protonated amino groups of gelatin, and due to hydrogen bonding between the unionized carboxyl group of PPA and carbonyl groups of gelatin. Subsequently, cisplatin was loaded into this complex using a ligand exchange reaction of platinum (II) from the chloride to the carboxyl group of the nanoparticles. These nanoparticles (GEL-PPA-Cisplatin) exhibited significantly superior anticancer efficacy in hepatic H22 tumor-bearing mice as opposed to cisplatin alone (Ding, Zhu et al. 2011). In another study, different molecular weights of PEI (600, 1.8k, and 10kDa) were added to gelatin nanoparticles prepared using two-step desolvation and loaded with plasmid DNA (Kuo, Huang et al. 2011). Transfection efficacy and cell viability were investigated. The findings demonstrated that low molecular weight of PEI (1.8 kDa) showed high transfection efficiency with low cell toxicity. Gelatin-PEI nanoparticles showed a stable particle size of 200 nm and obtained a positive charge during all ranges of pH, remaining stable in both acidic and basic condition (Kuo, Huang et al. 2011). These studies showed that the combination of gelatin and polymer as non-viral vectors nanoparticles is possible and effective.

### **1.12 Scope of Thesis**

This thesis focuses on gelatin-based non-viral vectors for gene therapy. Gelatin nanoparticles were prepared and modified with a cationic agent to increase the positive charge in order to interact with the DNA and plasma membrane. Pluronic block copolymers were added to gelatin nanoparticles and characterized to study the effectiveness of this complex. This complex was then investigated using the Langmuir monolayer study and tested at cell culture

to evaluate its transfection efficiency and cell viability of delivering plasmid DNA. The thesis is comprised of six chapters:

**Chapter 1:**

**Literature and Introduction to Gene Therapy:** This chapter provides a brief introduction to gene therapy and the methods of delivering DNA using viral and non-viral vectors.

**Chapter 2:**

**Hypothesis and Objectives:** This chapter discusses the hypothesis of this work, as well as the objectives we used to investigate our hypothesis.

**Chapter 3:**

**Characterization of Gelatin Nanoparticles and Cationic Gelatin Nanoparticles:** This chapter describes the methods used throughout the thesis, including the preparation methods for gelatin and modified gelatin nanoparticles, and the characterizations used to test our particles at different conditions.

**Chapter 4:**

**Understanding the Interaction of Gelatin/Pluronic/DNA System with Model membrane: Langmuir Monolayer Study:** This chapter reports on the results of the Langmuir monolayer/BAM studies, and provides new understanding of the interaction of gelatin/Pluronic/DNA complexes with model membranes at the air/water interface.

**Chapter 5:**

**Transfection Efficiency and Cell Viability of Gelatin/Pluronic/DNA System with COS-7:** This chapter reports the ability of gelatin/Pluronic/DNA complexes to transfect DNA *in vitro* using COS-7 cells.



## **Chapter 6:**

**Conclusion and Future Studies:** The chapter summarizes this thesis, and recommends future studies that might be conducted using gelatin/Pluronic/DNA systems to both optimize their transfection efficiency, and to provide further insight into the transfection mechanism(s).

## **Chapter 2 Hypothesis and Objectives**

### **2.1 Aim of the Thesis**

The aim of this thesis is to design an effective non-viral delivery system that is capable of delivering DNA *in vitro*. Gelatin was selected as a cationic agent, which was combined with Pluronic block copolymers in order to protect the DNA from degradation during its journey into the cell.

### **2.2 Hypothesis**

*Nanoparticles prepared from cationic gelatin and pluronic block copolymer can effectively complex DNA and interact electrostatically with the cell membrane, therefore improving the transfection efficiency of DNA.*

### **2.3 Objectives**

1. Preparation of gelatin nanoparticles: gelatin nanoparticles were prepared from type A gelatin using the two-step desolvation method.
2. Preparation of cationic gelatin: cationic gelatin nanoparticles were prepared by introducing a cationic agent (cholamine) to the native gelatin nanoparticles.
3. Preparation, optimization, and characterization of cationic gelatin-Pluronic-DNA transfection complexes: cationic gelatin and Pluronic were prepared, optimized, and characterized in the presence of plasmid DNA by studying the physiochemical properties as follows:
  - a) Measuring of particles size using dynamic light scattering: for all systems including native gelatin, cationic gelatin, cationic gelatin-Pluronic-DNA complex.

- b) Measuring of zeta potential of the particles using Laser Doppler Electrophoresis:  
for all systems including native gelatin, cationic gelatin, cationic gelatin-Pluronic-DNA complex, as well as the effect of pH on both native and cationic gelatin.
  - c) Imaging of nanoparticles using scanning electron microscopy (SEM), and transmission electron microscopy (TEM) were conducted for both native and cationic gelatin.
4. Characterization the interaction (s) of cationic gelatin-Pluronic-DNA on the model membrane using Langmuir monolayer study at the air-water interface: the cationic gelatin-Pluronic-DNA system was investigated at air-water interface in combination with model biological membranes DPPC-CHOL and POPC-CHOL.
  5. *In vitro* transfection efficiency of cationic gelatin-Pluronic nanoparticles containing plasmid DNA: EGFP plasmid DNA was tested in cell line COS-7 to evaluate the transfection efficiency of cationic gelatin, and the cationic gelatin/Pluronic systems.
  6. Cell viability studies of transfected cells to assess the toxicity levels of this complex: the cell toxicity of the transfection system was also tested in COS-7, correspondingly with the transfection experiment, by adding the propidium iodide (PI).

## **Chapter 3 Characterization of Gelatin Nanoparticles and Cationic Gelatin Nanoparticles**

### **3.1 Introduction**

Nanoparticles for drug and gene delivery applications should not produce toxic by-products when biodegradability occurs inside the biological system. As a result, a potential and safe matrix molecule should be achieved for these nanoparticles. Several methods described in the literature in the last few decades used synthetic or natural polymers to prepare biodegradable nanoparticles for gene delivery. Examples of natural polymers include proteins such as albumin, gelatin, and collagen, polysaccharides such as chitosan and cyclodextrin, among many others; while synthetic polymers include cationic dendrimers, polyesters, polymethacrylates and polyethyleneimine (Nezhadi, Choong et al. 2009) (recall Chapter 1). Among these polymers, nanoparticles of proteinaceous origin such as albumin, collagen, and gelatin generate more interest. Gelatin, as mentioned in Chapter 1, is a natural polymer extracted from collagen. Gelatin has several accessible moieties that enable multiple modification opportunities for coupling such as targeting-ligands, crosslinkers, and shielding substances. Preparing gelatin as nanoparticles for gene delivery is important for several reasons. Firstly, nanoparticles are taken easily and efficiently by cells more so than large particles (Panyam and Labhasetwar 2003). Secondly, nanoparticles have the ability to escape rapidly from the endosome and therefore, are protected from degradation (Labhasetwar 2005). In addition, nanoparticles have been demonstrated to improve the transfection efficiency of plasmid DNA into the nucleus (Prabha, Arya et al. 2016). Several methods have been employed to prepare gelatin nanoparticles since the 1970s. Two major methods are:

1. Preparing gelatin nanoparticles using desolvation of the protein by adding non-solvent, salting-out, or focus on isoelectric point (IEP) by adjusting pH level. Examples of this kind of preparations include two-step desolvation, one-step-desolvation, and nanoprecipitation.
2. Preparing gelatin nanoparticles using a biphasic system. This method depends on emulsifying an aqueous solution of gelatin within an oily phase such as microemulsion and solvent evaporation techniques.

Most of the preparation methods using emulsion are not appropriate for gene delivery due to some specific disadvantages. Firstly, the yield obtained from these methods is low comparing to other methods. Secondly, the broad size distribution of nanoparticles may result in aggregation of biological systems. Thirdly, the purification of nanoparticles using these methods are very complicated but are critically important in order to remove some organic phase(s) as well as to maintain the emulsification state of the nanoparticles. The last drawback of these methods includes the energetic methods that are necessary to generate nanoparticles, such as high-speed or high-pressure homogenization, or the use of ultrasound to disperse particles in order to obtain appropriate particle sizes (Zwiorek 2006).

In this project, the two-step desolvation method was selected to prepare gelatin nanoparticles. Small particles size (~ 150 nm) with a low polydispersity index (0.06) can be achieved using this method. Also, a higher yield can be obtained compared to emulsification techniques. In addition, the high stability of nanoparticles (up to 3 months) was obtained using this method (Coester, Kreuter et al. 2000). Finally, the method itself is simple and straightforward

(Coester, Kreuter et al. 2000). One-step desolvation and nanoprecipitation methods were also attempted in this project, and then compared to the two-step desolvation.

In this chapter, we also describe the characterization of modified gelatin. Gelatin nanoparticles were modified by adding a cationic agent (cholamine) in order to increase the positive charge of gelatin. As mentioned earlier in Chapter 1, a positive charge is necessary to interact with the negative charge of DNA and cell membrane.

## **3.2 Materials**

Gelatin type A (Protein content 81% Bloom ~300), (2-aminoethyl) trimethylammonium chloride hydrochloride (cholaminchloride hydrochloride), glutaraldehyde grade I 25%, 1-ethyl-(3dimethylaminopropyl) carbodiimide (EDC), Sucrose (HPLC) 99.5%, and HCL were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). E.Z.N.A. Plasmid Maxi-Prep Kit was purchased from OMEGA Bio-Tek, (Georgia, USA). Acetone (99%) and Ethanol (99%) were purchased from Chem store, University of Waterloo.

## **3.3 Methods**

### **3.3.1 Preparation of gelatin nanoparticles**

#### **3.3.1.1 Two-step desolvation**

Gelatin nanoparticles were prepared using a two-step desolvation method previously described by Coester et al (Coester, Kreuter et al. 2000). In brief, 300 mg of gelatin type A was dissolved in 10 mL of distilled water under constant heating (45-50 °C). 10 mL of acetone was added as a desolvating agent to precipitate the high molecular weight (HMW) fraction of gelatin. After the supernatant was discarded, the HMW gelatin was re-dissolved in

10 mL of distilled water and stirred at 600 rpm under constant heating. Subsequently, the pH was adjusted to 2.5 using HCL. After that, 25 mL of acetone was added dropwise to form nanoparticles. 48  $\mu$ L glutaraldehyde (GA) 25% (v/v) was added as a cross-linking agent and stirred at 600 rpm at room temperature overnight. The next day, the nanoparticles were purified using dialysis membrane before freeze drying. Various concentrations of gelatin were optimized to determine the best nanoparticles in terms of the size and the homogeneity.

### **3.3.1.2 One-step desolvation**

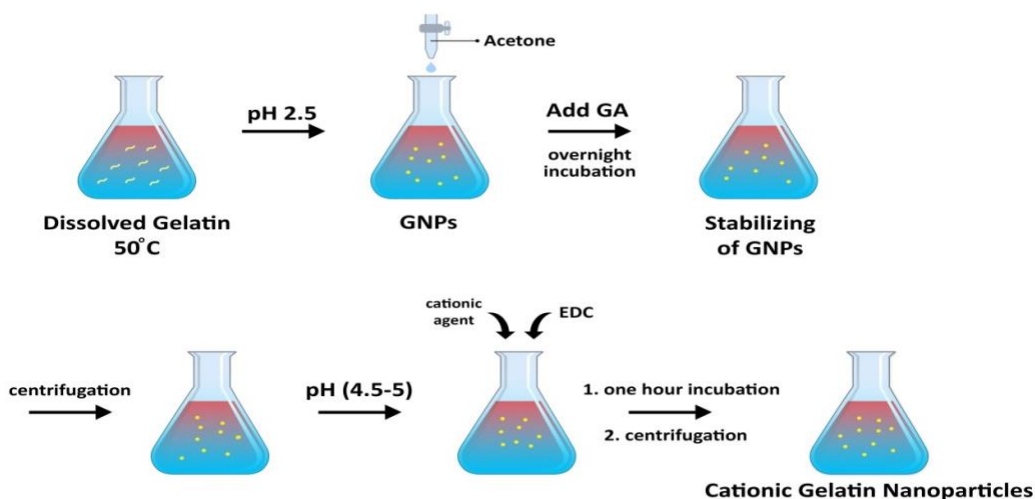
One-step desolvation was prepared as a modification to the standard two-step desolvation method (Ahlers, Coester et al. 2007). In this approach, the first step described above, which precipitates the high molecular weight, was omitted, while the remaining steps were carried exactly out as in the two-step method. This method was shown to be a robust in the preparation of gelatin nanoparticles (Geh, Hubert et al. 2016).

### **3.3.1.3 Nanoprecipitation**

50 mg of gelatin type A was dissolved in 10 mL of distilled water under constant heating (40-45 °C). This was then added drop-wise to 100 mL of ethanol containing 1600 mg Pluronic F127, used as an emulsifying agent to give 1:32 ratio of gelatin to Pluronic. Subsequently, 8  $\mu$ L glutaraldehyde (GA) 25% (v/v) was added as a cross-linking agent and stirred at 600 rpm at room temperature overnight. The next day, the nanoparticles were purified using dialysis membrane before freeze drying.

### 3.3.2 Preparation of cationic gelatin

After preparing GNPs using the two-step desolvation method (section 3.3.1), the nanoparticles were cationized by introducing amino residues (cholamine) to the carboxyl group of gelatin nanoparticles, as follows: The gelatin nanoparticles were dissolved in highly purified water, and then the pH was then adjusted between pH 4.5 and pH 5. 50 mg of cholamine was added and incubated for five minutes. Next, the same amount of EDC (50 mg) was added to the solution and the reaction was left for 1 hour in the dark. Cationic gelatin nanoparticles were then purified using centrifugation or dialysis for two days before lyophilization.



**Figure 3.3-1:** Schematic illustration of the preparation of cationic gelatin nanoparticles using one-step desolvation method.



### 3.3.3 Particle size measurement

The particle size was measured using the Dynamic Light Scattering method (DLS) at 25 °C. This technique relies on the effect of time-dependent light scattering of the random motion of suspended particles (Brownian motion) that depends on particle size. The particles in a liquid move randomly, and their speed of movement was used to determine the size of the particle (Malvern 2013). The distribution of particle size in water was obtained from the light scattered by particles illuminated at a scattering angle of  $\theta = 173^\circ$ . Particle size measurements of gelatin nanoparticles, as well as Pluronic-based transfection mixtures (Chapter 5) were done using a disposable Solvent Resistant Micro Cuvette (ZEN0040) in a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) which is a part of the School of Pharmacy's core facility, while particle size distributions were calculated using the Malvern DTS software. The size measurements were carried out in triplicate, with the average size is reported as  $\pm$  the standard deviation. The Malvern Zetasizer reports both an average size for the particle population (z-average) as well as sizes corresponding to peak intensity for the case of multiple particle populations. In all cases, the z-average size was used throughout this thesis. The polydispersity index (PDI) was measured along with the particle size measurements with an average PDI expressed as  $\pm$  standard deviation. PDI measures the broadness of the size distribution, which was calculated from the cumulants analysis. PDI less than 0.3 is desired in pharmaceutical nanoparticles according to Malvern standard for Zetasizer instrument.

### 3.3.4 Zeta potential measurement

Zeta potential ( $\zeta$ ) is the electrostatic potential that exists at the boundary between two layers of ions, namely, the compact and the diffuse layers, which surround a particle in solution. This is an important property for understanding colloidal and interfacial behaviors. Zeta potential was measured using a combination of the measurement techniques, namely, Electrophoresis and Laser Doppler Velocimetry (sometimes called Laser Doppler Electrophoresis - LDE). This method measures the speed at which a particle moves in a liquid when an electrical field is applied (i.e., its velocity). If all particles of the suspension mixture have a large positive or negative charge, they will repel each other, and there will be no tendency to fluctuate. On the other hand, low zeta potential values of the particles mean there is no force to prevent the particles from coming together, therefore resulting in fluctuation. Practically, particles with zeta potential more positive than +30 mV or more negative than -30 mV are considered to be stable (Wissing, Kayser et al. 2004).

Similar to particle size measurements, zeta potential measurements of the gelatin nanoparticles as well as Pluronic-based transfection mixtures (Chapter 5) were done using a disposable capillary cell (DTS1070) and the Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK); zeta potential distributions were calculated using the Malvern DTS software. Similar to particle size, zeta potentials were automatically measured three times per sample, and are reported as the mean  $\pm$  standard deviation. Also, the zeta potential of both native and cationic gelatin was measured at different pH values from acidic to basic. A pH meter was used to adjust the pH values, and the Zetasizer was used to measure the charge.

### **3.3.5 Scanning electron microscopy (SEM)**

Scanning electron microscopy (SEM) was used to visualize the gelatin nanoparticles and cationic gelatin to investigate their size and morphology. SEM images were taken using a Zeiss MERLIN FESEM 1530 microscope (part of the WATLAB facility at the University of Waterloo) equipped with a field-emission source and an acceleration voltage up to 25 kV, as well as Energy Dispersive X-ray Spectroscopy or (EDS) detector for surface elemental analysis. For sample preparation, gelatin nanoparticle suspensions were diluted ten times with their dispersion medium, after which a drop of the diluted nanoparticle suspension was directly deposited on a polished aluminum sample holder. Samples were dried under vacuum. Samples were then coated in gold using EMITECH K450X sputter coater prior to microscopical analysis.

### **3.3.6 Transmission electron microscopy (TEM)**

Transmission electron microscopy was used to visualize the cationic gelatin in order to investigate the details of crystal structure and the morphology of the modified nanoparticles. TEM images were taken on Philips CM10 located in the Department of Biology, University of Waterloo. For the sample preparation, the gelatin nanoparticle suspensions were diluted twenty times with their dispersion medium, after which a drop was directly deposited on a 400-mesh copper grid, and allowed to dry inside a desiccator for 24 hours prior to measurement.

### **3.3.7 Fourier transform infrared spectroscopy (FT-IR)**

Fourier Transform Infrared Spectroscopy (FT-IR) spectra were recorded on a Bruker TENSOR 27 FTIR spectrometer (Bruker, Germany). The attenuated total reflection (ATR) technique was used in this experiment. Samples of 3–5 mg of both native and cationic gelatin were ground and pressed into a Pike Miracle single-bounce attenuated total reflectance cell equipped with a Zn-Se single crystal. The scanning range was 500 - 4000 wavenumber ( $\text{cm}^{-1}$ ) with a scan speed of 10 kHz.

### **3.3.8 Extraction and purification of plasmid DNA (pTGIFN-GFP)**

The plasmid encoding for interferon (IFN) protein and enhanced green fluorescent protein (pEGFP) was amplified in K-12 strains of *Escherichia coli* (a gram negative, anaerobic, rod-shaped bacterium) (provided from Dr. Roderick Slavcev's lab at the School of Pharmacy, University of Waterloo) in the generation of the recombinant cell constructs, extracted using centrifugation protocol of the E.Z.N.A.® Endo-Free Plasmid DNA Maxi Kit (OMEGA Bio-Tek, Georgia, USA). Before the extraction process of plasmids, a single colony of bacterial strain JM109 [pTGIFN] of *Escherichia coli* was grown overnight (18 – 20 hours) in 5 mL of growth media Luria-Bertani (LB) broth + Kanamycin (kan) antibiotic in a temperature-controlled bench-top shaker (New Brunswick Scientific Excella™ E24, Fisher Scientific, US) at 250 rpm and 37 °C with circulating air supply. A new batch of cells were grown overnight from that last day culture at 1:100 dilution in 500 mL of growth media (within a 2 L Erlenmeyer flask), at the same temperature and rpm. After the overnight treatment, the final culture was removed from the shaker when the A600 was  $\approx 1.5$ , at which point indicated the exponential bacterial growth of the mid logarithmic phase. The extraction was

then completed, followed by the E.Z.N.A.® Endo-Free Plasmid DNA Maxi Kit protocols. Two batches of 1.5 mL aliquot of the extracted plasmid in Milli Q water were prepared. The purity, yield, and integrity of the plasmid were measured and analyzed using UV spectrophotometry (NanoDrop 2000, Fisher Scientific, USA), and by gel electrophoresis (AGE) (Alpha-Imager HP, Alpha Innotech, Cell Biosciences, USA). The size of the pEGFP was 4.7 kb. The extracted plasmid stock was then immediately stored in a freezer at -20 C°, as recommended in the protocol for further use.

### **3.4 Results and Discussion**

#### **3.4.1 Preparation of nanoparticles**

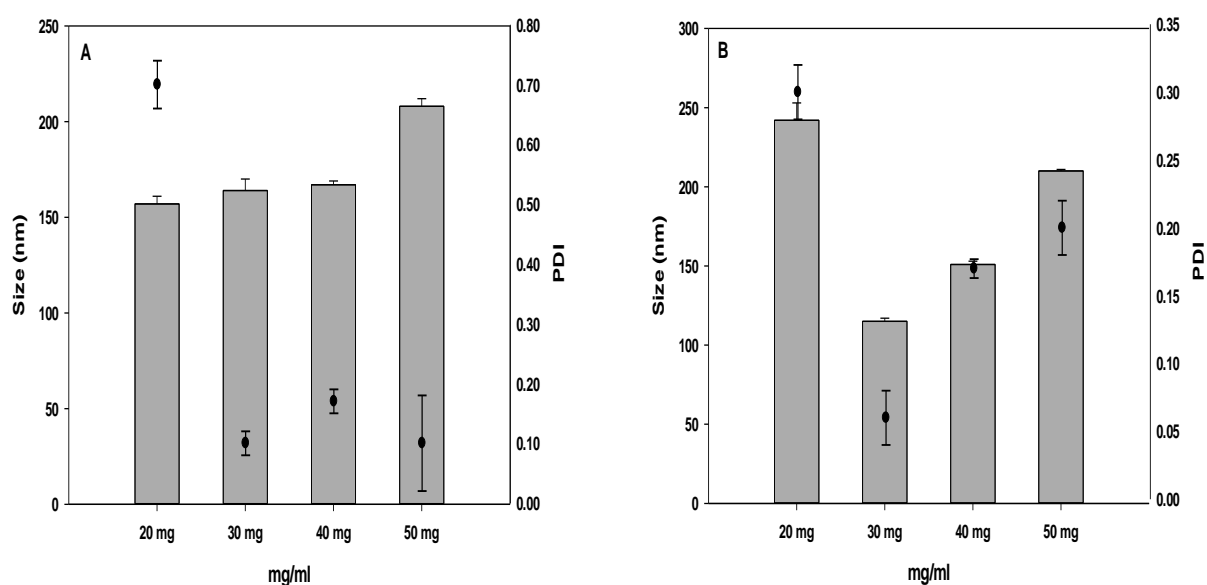
In order to understand our eventual observations of transfection utilizing the gelatin nanoparticles prepared in this work, it is important to understand how the parameters described in section 3.3.1 for the preparation of gelatin nanoparticles that impact the resulting properties of the nanoparticles. Here, we will discuss the effects of choosing the desolvation (or nanoprecipitation) method, and the choice of desolvation agent, temperature, pH, and the choice of crosslinking agent.

##### **3.4.1.1 Desolvation (or nanoprecipitation) method**

###### **3.4.1.1.1 Two-step desolvation method**

As mentioned in the introduction, preparing gelatin nanoparticles using two-step desolvation can achieve homogeneous and stable nanoparticles. The values of the resulting nanoparticles (with respect to particle size and homogeneity) were tested using DLS. Based on the Zetasizer standard, size distribution of nanoparticles achieves a PDI below 0.1 are considered

typical, and DLS gives a monomodal distribution within this range. Four concentrations of gelatin (mg/mL) in water were prepared, and each concentration was measured before and after adding glutaraldehyde (GA) in order to obtain the best size and PDI (Figure 3.4-1). The average mean size and PDI for each concentration are reported, which were calculated using Zetasizer software.



**Figure 3.4-1:** Size (bars) and PDI (dots) of gelatin nanoparticles and reported as mean. A) before adding GA; and B) after adding GA. Samples were measured in triplicate and the average reported - errors are equal to standard deviation. PDI represents polydispersity index. GA represents glutaraldehyde.

From the examination of Figure 3.4-1, it is clear that a nanoparticle concentration of 30 mg/mL gave rise to the “best” nanoparticles in terms of a having both a small average particle size ( $115 \pm 0.5$  nm) and low PDI ( $0.06 \pm 0.02$ ) after crosslinking. The results results are also consistent with a recently published paper using the same type and bloom of gelatin

(type A, 300) (Geh, Hubert et al. 2016). A concentration of 30 mg/mL was used to prepare gelatin nanoparticles for all subsequent experiments in this thesis.

#### **3.4.1.1.2 One-step desolvation method**

In order to obtain an adequate particle size and PDI, the one-step desolvation method was also applied. One-step desolvation was prepared as described in section (3.3.1) and in Figure 3.3-1 using the same weight 30 mg/mL, and following the same standard conditions used for the two-step desolvation method. The one-step desolvation method gave particles with an average size of  $104\pm 0.08$  nm, with a PDI of  $0.08\pm 0.05$ . These results were acceptable compared to the two-step method, although aggregation was very high, particularly after purification. Farrugia & Groves suggested that an increase of HMW and a reduction of low molecular weight (LMW) is significant in order to produce homogeneous and stable nanoparticles (Farrugia and Groves 1999), as achieved above using the two-step desolvation method. Also, Coester et al. (Coester, Kreuter et al. 2000) stated that the reduction of LMW is necessary to prevent aggregation during crosslinking, as well as to prevent secondary aggregation and fluctuation during storage. Zwioerek tried different batches of gelatin and found that gelatin batches containing less than 20% (w/w) of < 65 kDa molecular weight peptide resulted in the successful preparation of homogeneous and stable nanoparticles using the one-step desolvation method. In contrast, the gelatin batches containing molecular weights > 104 kDa failed to achieve homogenous and stable nanoparticles by one-step desolvation. Based on this data, Zwioerek then concluded that it is not important to have HMW in order to prepare homogenous and stable nanoparticles (as stated previously by

Farrugia & Groves), but it is crucial to have a strong reduction of LMW components (< 65 kDa) for the stability and homogeneity of nanoparticles (Zwiorek 2006, Zillies 2007).

#### **3.4.1.1.3 Nanoprecipitation method**

For the preparation of GNP using the nanoprecipitation method, ethanol was used as a non-solvent agent, and Pluronic F127 was used as a surfactant. Gelatin nanoparticles tend to aggregate during the preparation processes, and as a result, the formulation was optimized to obtain stable and homogenous nanoparticles. A range of different concentrations of gelatin and Pluronic F127 were used, giving rise to final conditions of:

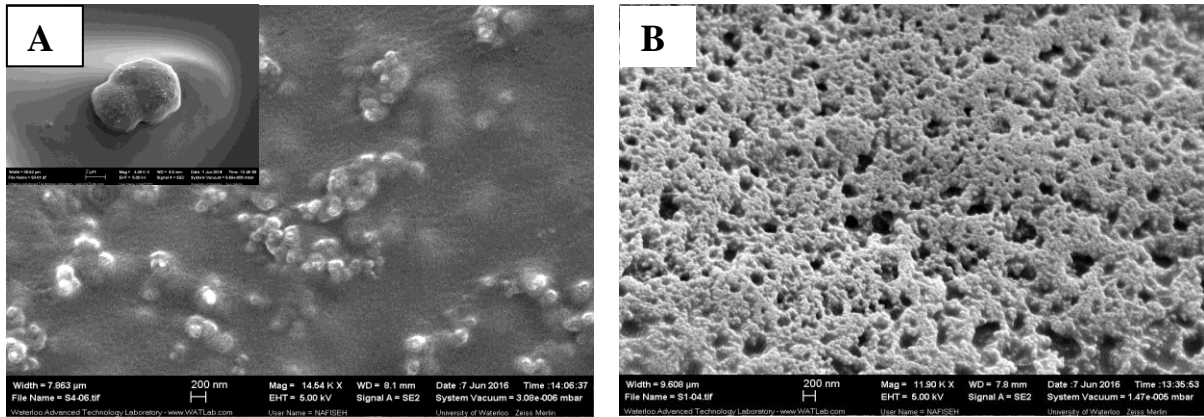
1. The amount of gelatin should be 1:32 to the amount of surfactant
2. The amount of water should be 1:10 to the amount of ethanol.

An average size of ~ 300 nm and a PDI of  $0.05 \pm 0.03$  were obtained, and the solution was cloudy with no visible agglomeration.

All nanoparticles were analyzed using SEM for both two-step and nanoprecipitation methods in order to visualize and investigate the morphology of these nanoparticles (Figure 3.4.2). SEM revealed the particles to be smooth, spheres, and were smaller than DLS. This reduction in size was due to dehydration of the nanoparticles (required for SEM measurements under vacuum conditions). The same observations were also noted in the literature for different polymer-based nanoparticles (Finsy, De Jaeger et al. 1992, Bootz, Vogel et al. 2004).

By examining the three methods of preparing gelatin nanoparticles, we concluded that the two-step desolvation method is the best choice for this project for several reasons.





**Figure 3.4-2:** Scanning-electron-microscopy (SEM) images of the gelatin nanoparticles A) Two-step desolvation, B) Nanoprecipitation. The particle size was determined about 60-100 nm.

First, two-step desolvation has a smaller size than nanoprecipitation. Also, since our project focuses on using Pluronic block copolymers with gelatin, it is not beneficial to use the nanoprecipitation method, since different Pluronics will be compared in our experiments. As a result, the study of the Pluronics effect will not be accurate. In addition, due to the aggregation that might result from using one-step desolvation and the pH range, which has a narrow range, the two-step method was selected.

### 3.4.1.2 Desolvating agent

Gelatin nanoparticles are prepared using desolvating agents such as acetone or ethanol. Gelatin can dissolve in water due to the formation of hydrogen bonds between the water molecules and the hydrophilic amino acids of the gelatin. Adding desolvating agents into gelatin solutions leads to a reduction in water molecules surrounding the gelatin chains; therefore, it results in breakage of the hydrogen bonds and a decrease in hydration. When the hydration becomes very low, the loosely packed gelatin chains precipitate and form

nanoparticles (Arroyo-Maya, Rodiles-López et al. 2012). Both acetone and ethanol have been shown to be effective in the preparation of gelatin nanoparticles (Von Storp, Engel et al. 2012); however, Azarmi et al. found that gelatin nanoparticles prepared using acetone as a desolvating agent are smaller and have lower PDI compared to those prepared using ethanol (Azarmi, Huang et al. 2006). In the nanoprecipitation method, ethanol as a non-solvent agent was found to create smaller particles than acetone; however, the difference was not statistically significant (Khan 2014).

### **3.4.1.3 Temperature**

The temperature of the first and second desolvation in the two-step desolvation method should be between 45 °C and 50 °C. It is well-known from the literature, that gelatin can be easily affected by the temperature due to the occurrence of denaturation or renaturation processes (Farrugia and Groves 1999). Preparing gelatin nanoparticles at room temperature is impossible because gelatin is renatured and forms a viscous gel at this temperature (Sahoo, Sahoo et al. 2015). Temperatures above 56 °C result in reversible denaturation of the higher molecular fractions due to the disruption of hydrogen bonds, which lead to particles clumping (Sahoo, Sahoo et al. 2015). Temperatures between 35 °C and 40 °C are considered the sol-gel transition temperature (Peyrelasse, Lamarque et al. 1996). This means that the range between 40 °C and 55 °C is the ideal temperature to prepare gelatin nanoparticles. In this project, different ranges of temperatures were tested, and we determined that temperature between 45 °C and 50 °C is ideal.

#### **3.4.1.4 Effect of pH on gelatin nanoparticles**

Since gelatin is an amphoteric polymer, it has both positive and negative charge. The size of gelatin nanoparticles is affected by the pH of the medium, which ultimately determines the net charge and charge density of gelatin molecules (Sahoo, Sahoo et al. 2015). Type A gelatin has an IEP in the range of 6.9 to 9.0. Within this pH range, nanoparticle formation is not stable and formed agglomeration due to the electrostatic attraction of the positive charge of  $\text{NH}_3^+$ . At pH ranges between pH 2.3 and pH 3.8, it was possible to produce homogenous and stable nanoparticles with PDI less than 0.1 because of the increase in the ionic strength of the electrostatic repulsion of  $\text{NH}_3^+$  (Zwiorek, Kloeckner et al. 2005, Sahoo, Sahoo et al. 2015). Above pH 4.0, the nanoparticles tend to agglomerate and precipitate because they become closer to IEP of gelatin type A (Azarmi, Huang et al. 2006). In our project, we determined that pH at 2.5 was the optimal value to prepare stable and homogenous nanoparticles.

#### **3.4.1.5 Cross-linking agent**

The addition of crosslinking to gelatin nanoparticles is important to provide stability, to form a spherical shape, and to enhance circulation times *in vivo* (compared to uncrossed-linked particles (Kommareddy, Shenoy et al. 2005, Elzoghby, Samy et al. 2012)). Gelatin nanoparticles tend to aggregate and become unstable when they are prepared without crosslinking (Sahoo, Sahoo et al. 2015). There are several crosslinking agents that were used in the preparation of gelatin nanoparticles, such as glutaraldehyde (Leo, Vandelli et al. 1997, Ofokansi, Winter et al. 2010), genipine (Song, Zhang et al. 2009), and glyceraldehyde (Vandelli, Rivasi et al. 2001). Glutaraldehyde (GA) is used as a non-toxic and effective

cross-linker in the preparation of gelatin nanoparticles as it does not induce aggregation and has been shown to maintain particles stable in solution for more than ten months at temperature between 2 °C and 10 °C (Leo, Vandelli et al. 1997, Azarmi, Huang et al. 2006). Lower amounts of GA are not sufficient to cross-link all particles; however higher amounts are not suitable as this could give rise to a significant size reduction due to the crosslinking of the free amino groups on the surface of gelatin nanoparticles (Azarmi, Huang et al. 2006).

### **3.4.2 Stability of nanoparticles after freeze-drying**

Most nanoparticles tend to aggregate after freeze-drying and become difficult to redisperse due to the lyophilization process, which changes the properties and robustness of the nanoparticles (Fonte, Soares et al. 2014). In order to prevent the aggregation of gelatin nanoparticles, nanoparticles are required to remain spatially separated in the dried cake (Allison, dC Molina et al. 2000). During storage, moisture also plays a major role in the stability of gelatin nanoparticles. As a result, different excipients (cryoprotectants) were used to keep nanoparticles stable for an extended period of time. Examples of freeze-drying excipients used include trehalose, sucrose, and mannitol. Mannitol and mannitol-sucrose formulations have previously been confirmed to have the greatest stability for gelatin nanoparticles, with the nanoparticles staying stable for up to ten weeks (Zillies, Zwioerek et al. 2008). In our experiments, sucrose was chosen to be added to the gelatin nanoparticles as a cryoprotectant (Shilpi, Kushwah et al. 2017). Sucrose was dissolved in 2 mL of highly purified water (HPW) and added directly to gelatin nanoparticles before freeze-drying. Four ratios of sucrose to gelatin were used before freeze-drying (Table 3.4-1). It is apparent from the table that a sucrose concentration of 2.5% resulted in freeze-dried particles having the

smallest size of 116 nm, which is similar to the original size of the gelatin nanoparticles. Nanoparticles remained stable in our lab for approximately three months. This stability was confirmed by dissolving the gelatin in water and measuring the size and PDI after three months. The particles showed stability with no noticeable aggregation as they demonstrated a single peak in DLS data with no significant change in diameter.

**Table 3.4-1:** Optimization of the amount of sucrose added to gelatin nanoparticles before freeze-drying. Samples were measured in triplicate, errors are equal to standard deviation. PDI represents polydispersity index.

Size before sucrose (nm)	PDI	Concentration (w/w%)	Size after sucrose (nm)	PDI
115±0.5	0.06±0.02	1%	127±0.5	0.17±0.01
		2.5%	116±0.3	0.13±0.02
		3.5%	119±0.1	0.13±0.008
		5%	129±2	0.13±0.01

### 3.4.3 Cationic gelatin

As mentioned previously in the introduction, a positive charge is important for gene delivery in order to interact with the negative charge of DNA and the cell membrane. Gelatin nanoparticles are cationized by introducing a quaternary amino group onto the surface of the nanoparticles, as previously described in section (3.3.2). Although there are several amino agents that were used to cationize gelatin, cholamine chloride was chosen in this project based on the previous study conducted by Zwioerek et al., (Zwioerek, Kloeckner et al. 2005). They tried different amino agents and discovered that cholamine was the best choice based on the particle size, PDI, and zeta potential. During cationization, cholamine can interact

with possible functional groups as shown in Figure 3.4-3. The first interaction is with residual aldehyde group derived from mono-functionally bound cross linking glutaraldehyde. The second interaction occurs between the amino groups of cholamine and the activated carboxyl groups of nanoparticles. Unlike other cationic agents, cholamine results in pH-independent positive charges through the quaternary ammonium groups, which remain positive at all pH conditions as will be shown in pH data later in this chapter.

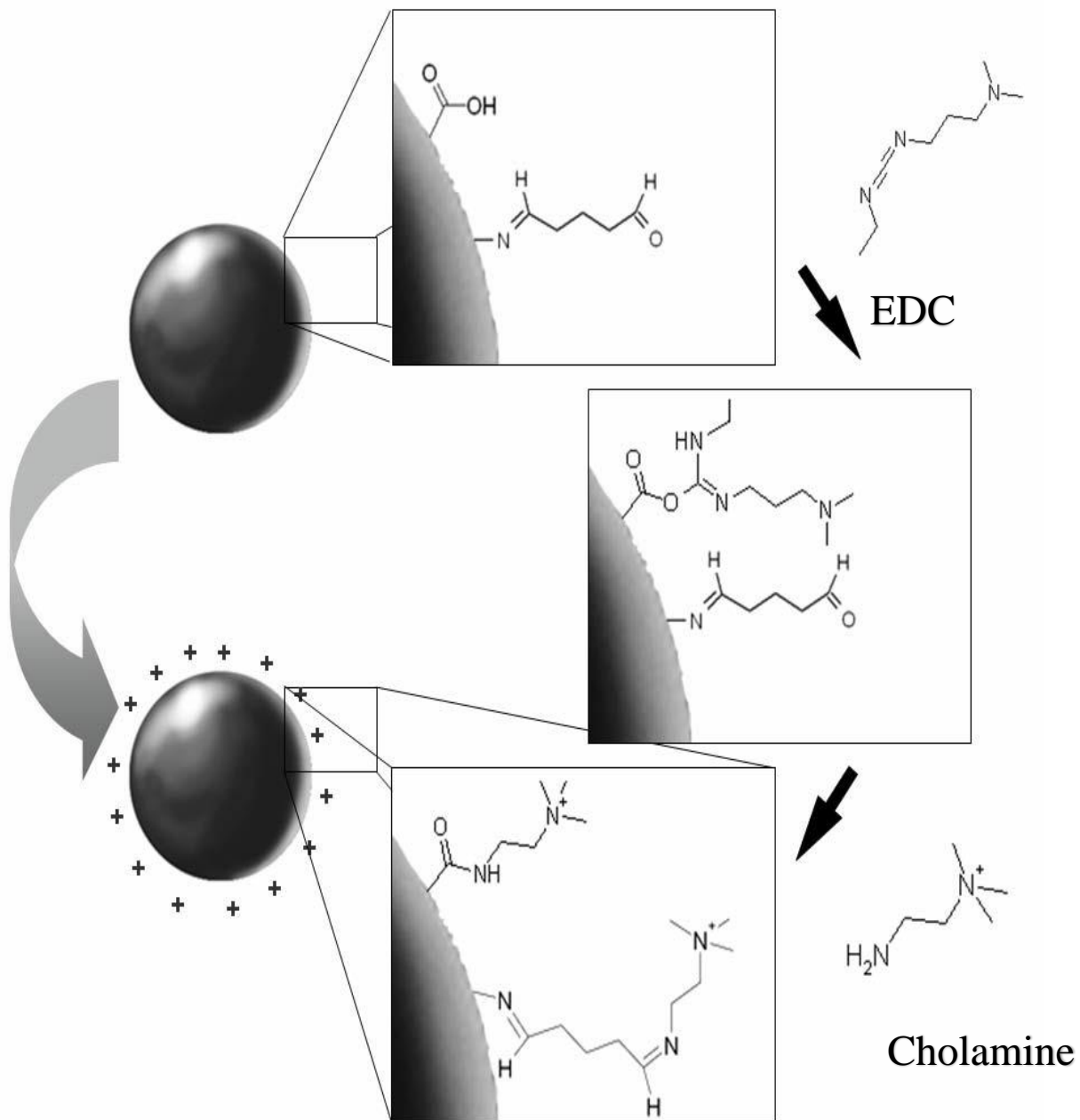
#### **3.4.4 Size and zeta potential of cationic gelatin**

The size of gelatin nanoparticles remains relatively homogenous and stable after modification. It is clear from Table 3.4-2 that the size and PDI of the gelatin nanoparticles increased after cationization from 115 nm to 173 nm, and from 0.06 to 0.2, respectively, and that the zeta potential increased from 11 mV to 32 mV. This increase in size may be the result of interparticulate covalent cross-linking (induced by the presence of EDC) between an activated carboxylic acid group on one particle, and a primary amino group on an adjacent particle. It could also result from a simply increase in the molecular weight of gelatin due to the addition of the cholamine groups.

While the PDI increase is indicative of a decrease in the homogeneity of the cationic gelatin nanoparticles (i.e., a broader distribution of particle sizes), a PDI of 0.2 is still very consistent with examples from other non-viral vector systems (both liposomal or polymeric in nature).

**Table 3.4-2:** Particle size and zeta potential of native and cationized gelatin. Samples were measured in triplicate, errors are equal to standard deviation. PDI represents polydispersity index.

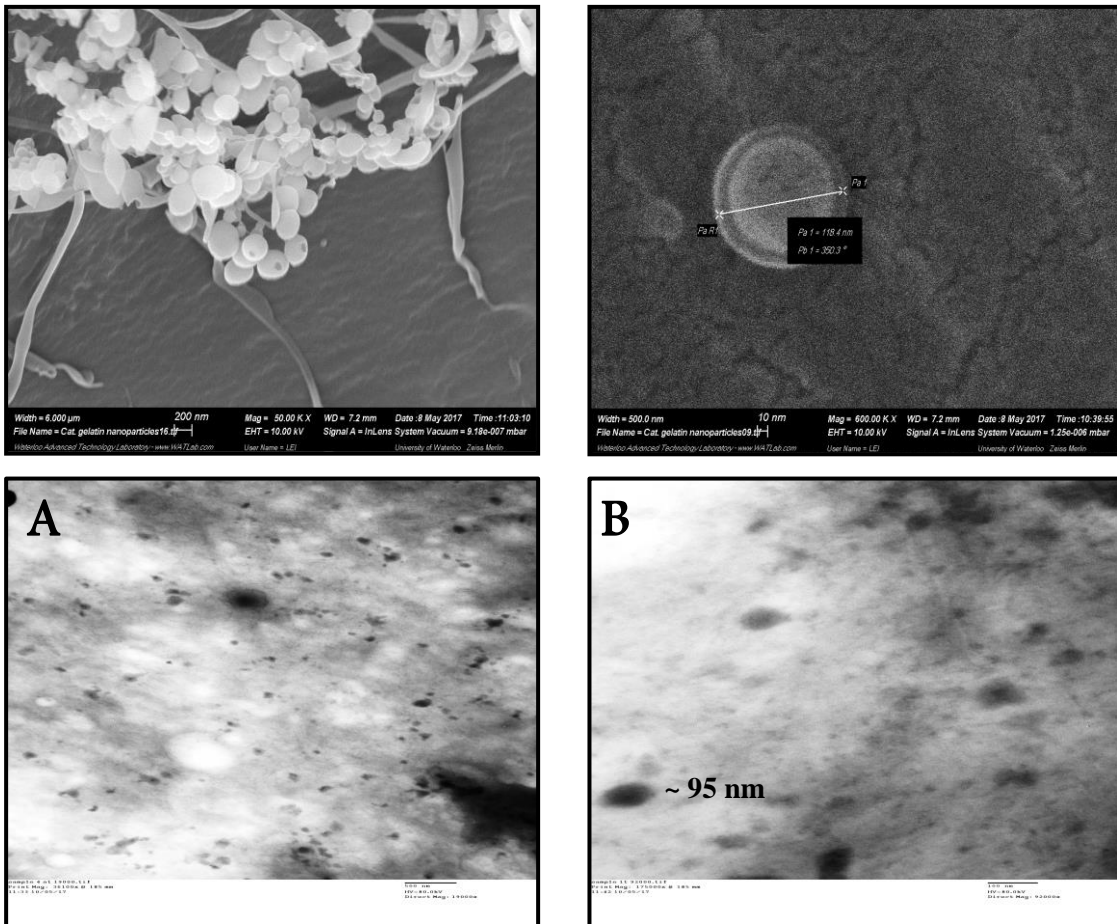
<b>Gelatin Type A</b>	<b>Particle Size (nm)</b>	<b>PDI</b>	<b>ζ-Potential (mV)</b>
<b>Native gelatin</b>	115±0.3	0.06±0.02	11±0.4
<b>Cationic gelatin using cholamine as a cationic agent</b>	173±0.5	0.2±0.008	32±0.2



**Figure 3.4-3:** Schematic illustration of surface cationization of gelatin nanoparticles using cholamine. Reproduced from (Zwiorek 2006)



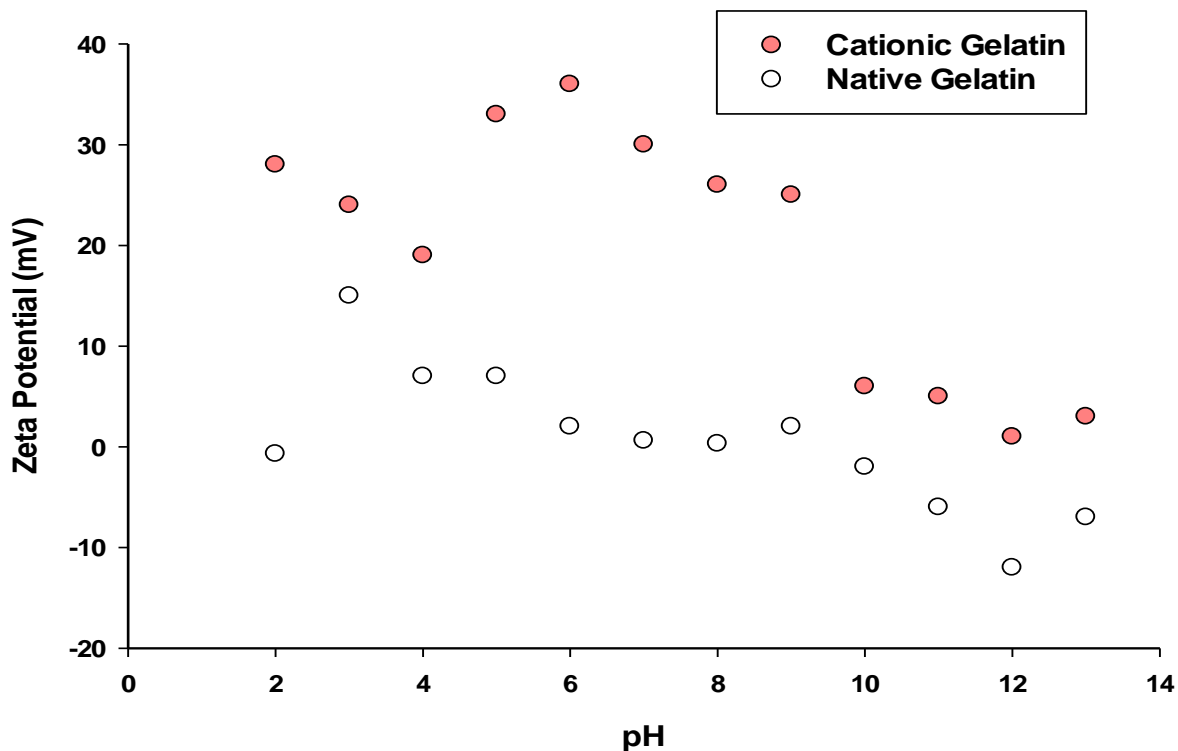
As for native gelatin nanoparticles, the cationic gelatin particles were also visualized using SEM. Figure 3.4-4 reveals that the cationic gelatin nanoparticles is very similar in size and morphology to those obtained with native gelatin. They are spherical in shape and they are attached to each other. The crystalline structure and the size were also investigated and analyzed using TEM through GMS3 software. Similar to what is observed from SEM, the cationic gelatin nanoparticles are approximately 95 nm in diameter and are crystalline with a plane separation of 30 Å (Figure 3.4-4). The decrease in size in TEM images is attributed to the method of preparation. In TEM, the sample is diluted more than in SEM. Also, the grid in TEM is much thinner than the SEM grid due the requirement to view the particles in high resolution. In addition, the drop of particles diffuses through the holes placed in the perforated grid. Then, the small particles are able to cross the streamline of the grid and stay on the carbon film. These results are in a bias to small particles (Baalousha, Ju-Nam et al. 2012, Tuoriniemi, Johnsson et al. 2014). Therefore, the results of TEM are determined to be unreliable because of the relic of sample preparation (Tuoriniemi, Johnsson et al. 2014).



**Figure 3.4-4:** SEM (top) and TEM (bottom) images of cationic gelatin nanoparticles  
 A) at magnification 19000X, B) at magnification 92000X

As we mentioned previously, the addition of cholamine induced a pH-independent positive charge using the quaternary amino groups, which remained positive at all pH conditions.

This is obvious from the Figure 3.4-5; where we tested both native and cationic gelatin at different pH conditions. It is clear from the figure that native gelatin has IEP  $\sim 9$ , while the cationic gelatin remained positively charged over the measured pH range. This means that the pH of the physiological environment should not affect the delivery system.

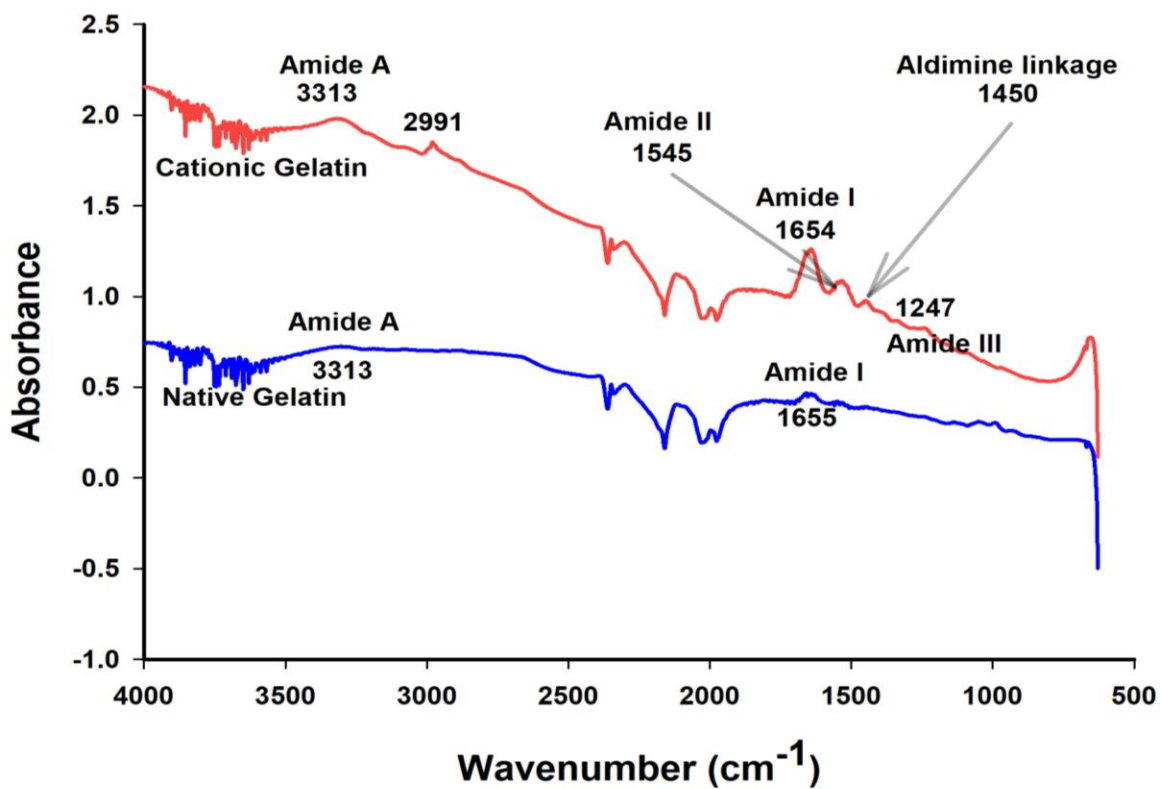


**Figure 3.4-5:** Zeta potential values at different pH conditions for both native and cationic gelatin. Samples were measured in triplicate.

### 3.4.5 FT-IR

In order to confirm the modification of gelatin, FT-IR was carried out for both native and cationic gelatin. The infrared spectrum of both native and cationic gelatin is shown in detail in Figure 3.4-6, with important peak positions listed in Table 3.4-3. Both native and cationic gelatin exhibit an amide I peak (C=O stretch) at  $1655\text{ cm}^{-1}$  and  $1654\text{ cm}^{-1}$ , respectively, but the intensity is stronger in cationic gelatin. An amide A peak (N-H stretching vibrations) at  $3313\text{ cm}^{-1}$  was observed for both types of native and cationic gelatin which, combined with the amide I peak, is characteristic for gelatin. Cationic gelatin also has an amide II peak (N-H

bend and C-H stretch) at  $1545\text{ cm}^{-1}$ , and an amide III peak (C-N stretch plus N-H in phase bending) at  $1247\text{ cm}^{-1}$  due to the presence of cholamine. The FTIR spectrum of cationic gelatin also shows an additional peak at  $2991\text{ cm}^{-1}$ , which might be associated with the C-H stretching from the cholamine groups (Jalaja, Naskar et al. 2015). Finally, a small peak at  $1450\text{ cm}^{-1}$  was also observed for the cationic gelatin, which might be attributed to aldimine absorption between the residual groups of glutaraldehyde and the amino groups of cholamine (Akin and Hasirci 1995). Glutaraldehyde has an aldehyde group (-CHO) that reacts with the free amino group to establish an aldimine linkage ( $\text{CH} = \text{N}$ ) (Bigi, Cojazzi et al. 2001).



**Figure 3.4-6:** ATR-FTIR spectra of native and cationic gelatin

**Table 3.4-3:** IR absorption frequencies of amides groups

Bond	Functional group	Wavenumber (cm <sup>-1</sup> )
N-H- (Stretching)	Amides A	3100 - 3500
C=O (Stretching)	Amides I	1640-1690
N-H- (Bending)	Amides II	1550-1640
C-N stretch plus N-H in phase bending	Amides III	1247

### **3.4.6 Optimization of cationic gelatin and DNA**

Since our project focuses on gene delivery, plasmid DNA is used with cationic gelatin to form polyplexes between the negative charge of DNA and the positive charge of cationic gelatin. Cationic gelatin was dissolved in water and simply mixed with the DNA using w/w ratios listed in Table 3.4-4. It is clear from Table 3.4-4 below that particle size decreases upon increasing the amount of cationic gelatin. This decrease in size due to the increase of positive charge provided by cationic gelatin, which interacts electrostatically with the negative charge of DNA. As increasing amounts of gelatin is added, the DNA is more completely neutralized, resulting in a greater degree of compaction and smaller particle size. As expected, the zeta potential increases with an increase in the amount of cationic gelatin, and switches from negative to positive between w/w ratios of 10:1 and 15:1 CG:DNA). A ratio of 20:1 CG:DNA yielded particles that had the smallest diameter (163 nm with a polydispersity of 0.09) while still having a strong positive zeta potential (+21 mV). Particles containing less gelatin carried less positive charge, while particles containing more gelatin, increased in size. As such, a ratio of 20:1 CG:DNA was selected as the optimal formulation for the nanoparticles. While ideally a zeta potential more positive than 30 mV or more negative than -30 mV is desired, as they are considered stable (Wissing, Kayser et al. 2004), in the case of our system, it appears that cationic gelatin does not have a large enough total positive charge to continue to increase the zeta potential while avoiding aggregation.

**Table 3.4-4:** Particle size and zeta potential of different ratios of plasmid DNA and cationic gelatin (W/W ratios). Samples were measured in triplicate, errors are equal to standard deviation. PDI represents polydispersity index.

Ratio (CG: DNA)	Size (nm)	Zeta (mV)	PDI
<b>DNA alone</b>	469±15	-50±2	0.5±0.03
<b>2.5: 1</b>	193±5	-25±0.5	0.05±0.05
<b>5: 1</b>	186±2	-18±0.2	0.2±0.02
<b>10: 1</b>	182±2	-12±0.3	0.07±0.02
<b>15: 1</b>	172±1	16±0.4	0.1±0.03
<b>20: 1</b>	163±0.4	21±2	0.09±0.02
<b>50: 1</b>	170±0.8	23±0.5	0.2±0.01

### 3.5 Summary

Gelatin nanoparticles were successfully prepared using a two-step desolvation method. In comparison to nanoprecipitation and one-step desolvation methods, the two-step desolvation provided the most homogeneous and stable nanoparticles. Parameters of pH equal to 2.5, acetone as the desolvating agent, temperature between 45 °C and 50 °C, and glutaraldehyde as the crosslinking agent were followed in order to obtain stable and homogenous nanoparticles. The removal of high molecular weight (HMW) fractions in the two-step desolvation method is considered to be an essential step in obtaining homogeneous and stable nanoparticles. Gelatin nanoparticles were then modified with cholamine as a cationic agent in order to increase the positive charge of nanoparticles, which is essential to interact with the negatively charged DNA and the cellular membrane. Nanoparticles became less homogeneous after they were modified with cationic agent (cholamine). The size increased from 116 nm to 173 nm, and the zeta potential also increased from 11 mV to 32 mV. Cationization of the nanoparticles was confirmed by conducting the FT-IR. Cationic gelation

nanoparticles showed a good tolerance and remained positive in all pH values, which is essential when they enter the biological system. Cationic gelatin was then mixed with DNA and it was determined that a ratio 20:1 CG:DNA gave particles having the smallest size (163 nm) while still maintaining a moderately high, positive zeta potential of 21 mV.



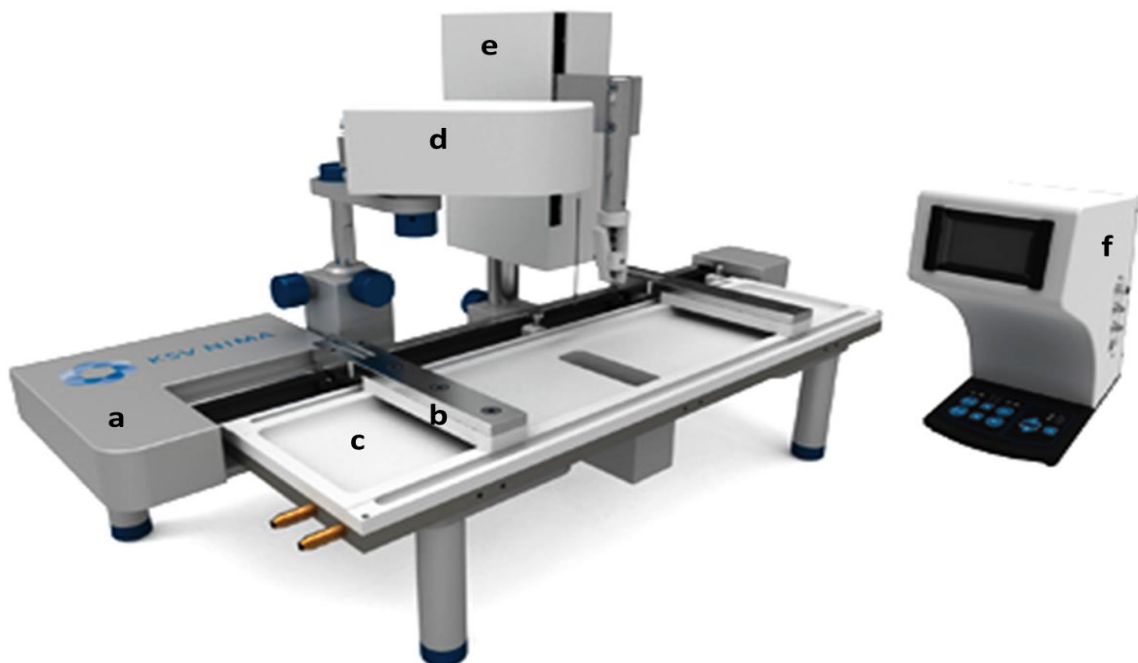
## **Chapter 4: Understanding the Interaction of Gelatin/Pluronic/DNA System with Model Membranes: A Langmuir Monolayer Study**

The main goal of this chapter is to describe the interaction of our complete gene delivery system (gelatin -Pluronic-DNA) with model membranes comprised of DPPC-CHOL and POPC-CHOL. Four Pluronics were used (L44, F87, P103, and F108), covering a range of molecular weights and hydrophobic-lipophilic balances. The interactions of the nanoparticles with the model membranes were characterized using Langmuir monolayers, and measured using Langmuir isotherms; monolayers were also imaged using Brewster's Angle Microscopy (BAM) as a means of visualizing any lipid domain changes that may have occurred. Understanding these interactions can provide important insight into the interaction of these types of nanoparticles with cellular membranes and might provide information that could be used in the optimization of transfection formulations and help in improving transfection efficiency *in vitro*.

### **4.1 Introduction to Langmuir Monolayer Studies**

The Langmuir-Blodgett (LB) monolayer technique, along with Brewster's Angle Microscopy (BAM), provides an understanding of both the interactions between polymers and DNA, as well as how they interact with the biological membrane at the air/water interface. A Langmuir monolayer can be used as a model interface to allow for the study of film chemistry and film structure with changes in film compression, changes in sub-phase composition, and other changes such as pH and temperature (Ulman 2013). This technique

explains the homogeneity of the monolayer and formation of domains, phase transitions and adsorption of

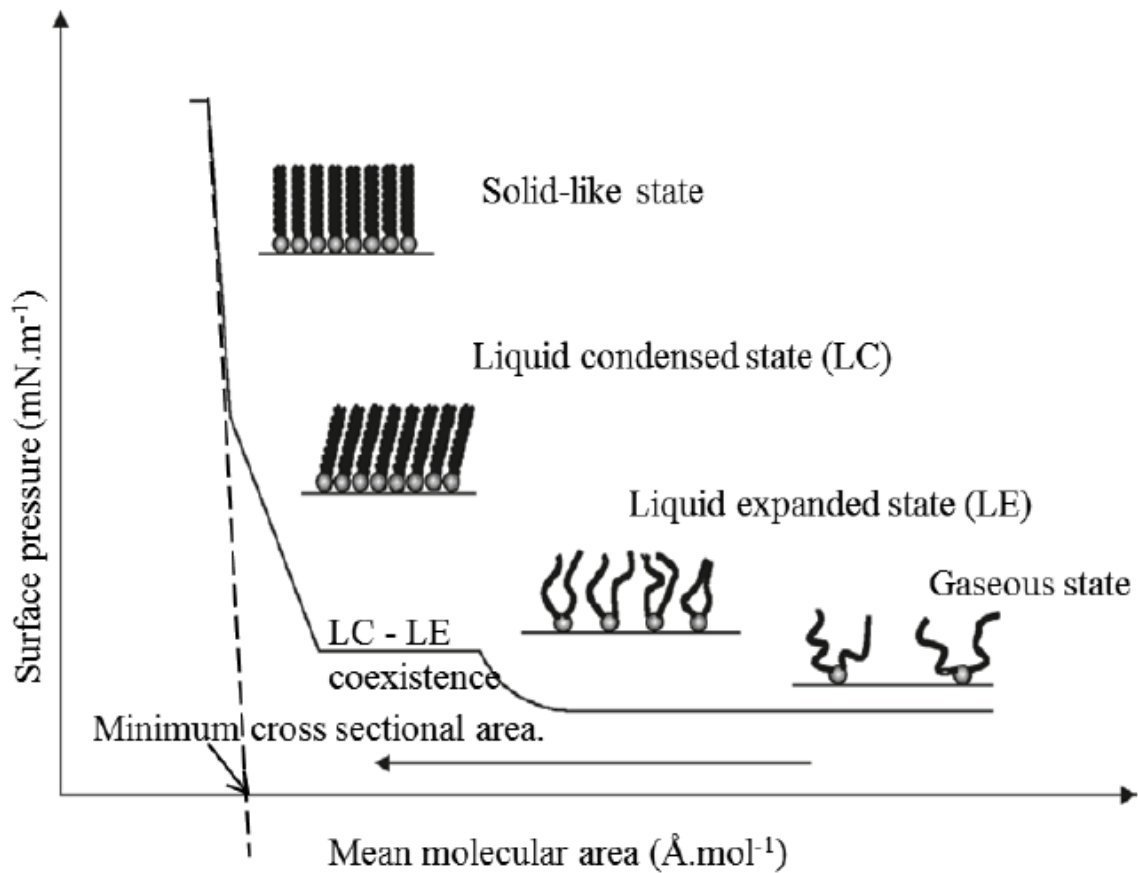


**Figure 4.1-1:** A schematic of the Langmuir trough used to determine Langmuir isotherms; a) frame, b) barriers, c) the trough, d) surface pressure sensor, e) Dipping mechanism (LB option), and f) Interface unit. Reproduced from (<http://www.ksvnama.com>)

materials from the aqueous phase. The LB trough is coupled with Brewster angle microscopy (BAM) to view the domains of the floating monolayers (Figure 4.1-1).

In a typical LB monolayer, compressing the barriers provides an isotherm of surface pressure as a function of mean molecular area ( $\pi - A$ ) of the compounds spread on the surface of the sub-phase (Figure 4.1-2). Surface pressure is the difference between the surface tension of pure sub-phase ( $\gamma_0$ ) and the monolayer covered phase ( $\gamma$ ). At 20°C pure water possesses a surface pressure of approximately -72 mN/m. A schematic of a Langmuir isotherm is shown in Figure 4.1-2 where the surface pressure of the two-dimensional monolayer increases as the

molecules in the monolayer are forced closer together (i.e. smaller molecular areas) and the monolayer shows different phase transitions. The phases which may be observed in Langmuir monolayers include: gaseous (G); liquid expanded state (LE); a coexistence region of LE and liquid condensed (LC) states; liquid condensed (LC) state; and a solid/like (S) state. In the G-phase, surfactant molecules remain far apart; as a result, no interaction is observed, and the surface pressure remains at or near Zero. Increasing the surface pressure (by compressing the barriers) decreases the area per unit molecule, forcing the molecules to begin interacting with one another; this initial interaction is termed the liquid expanded (LE) phase. In this phase, molecular interactions similar to those that would be expected if the material comprising the monolayer were in a true liquid state; although in this case, the interactions would be weaker than those seen in the pure liquid. Additional compression of the monolayer results in a steeper rise in surface pressure at slightly smaller areas, which results in the liquid condensed (LC) phase, where the molecules in the monolayer are now are closely packed (as compared to the LE phase); however, there is still anticipated to be some level of molecular motion. Finally, the monolayer reaches the solid (S) phase, as does the compressibility of the molecules, and therefore further compression of the monolayer causes monolayer collapse. In this region, surface pressure is observed to increase linearly with decreasing molecular area, and the minimum cross-sectional area ( $A_0$ ) for the molecules in the monolayer can be obtained by extrapolating from this region. In practical experimental conditions, monolayers may not show all these phases due to the narrow ranges of temperature and surface pressure that may be available.

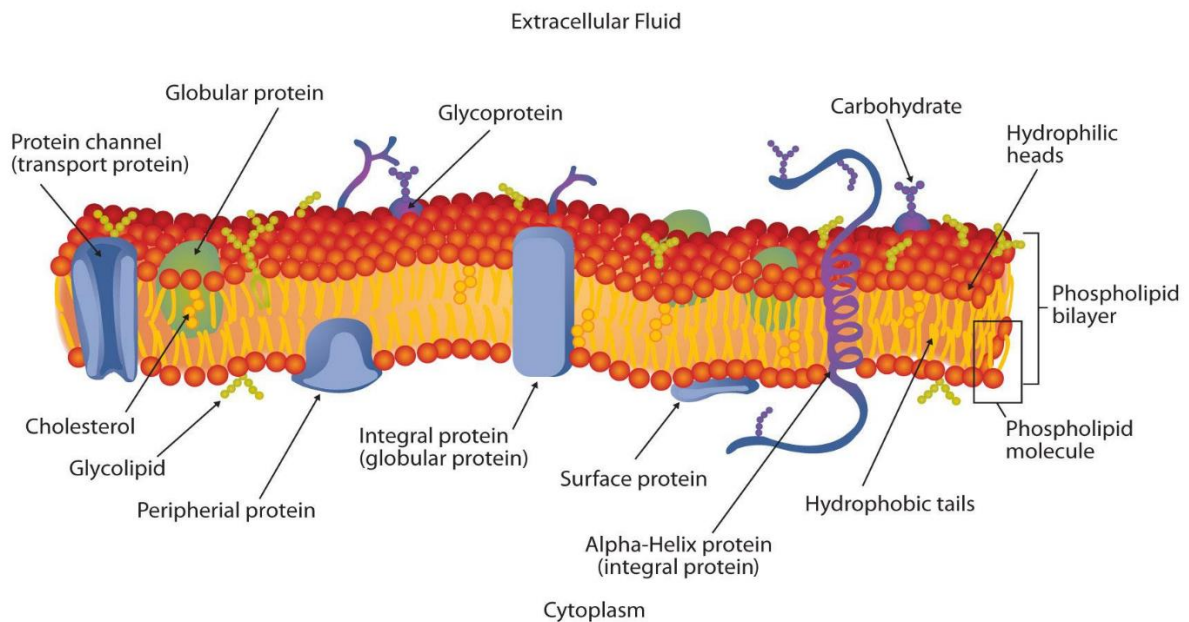


**Figure 4.1-2:** Theoretical  $\pi$  -A isotherm obtained by compressing an insoluble lipid monolayer formed at an air-water interface. Reproduced from (Eeman and Deleu 2010)

## 4.2 Cell Membranes

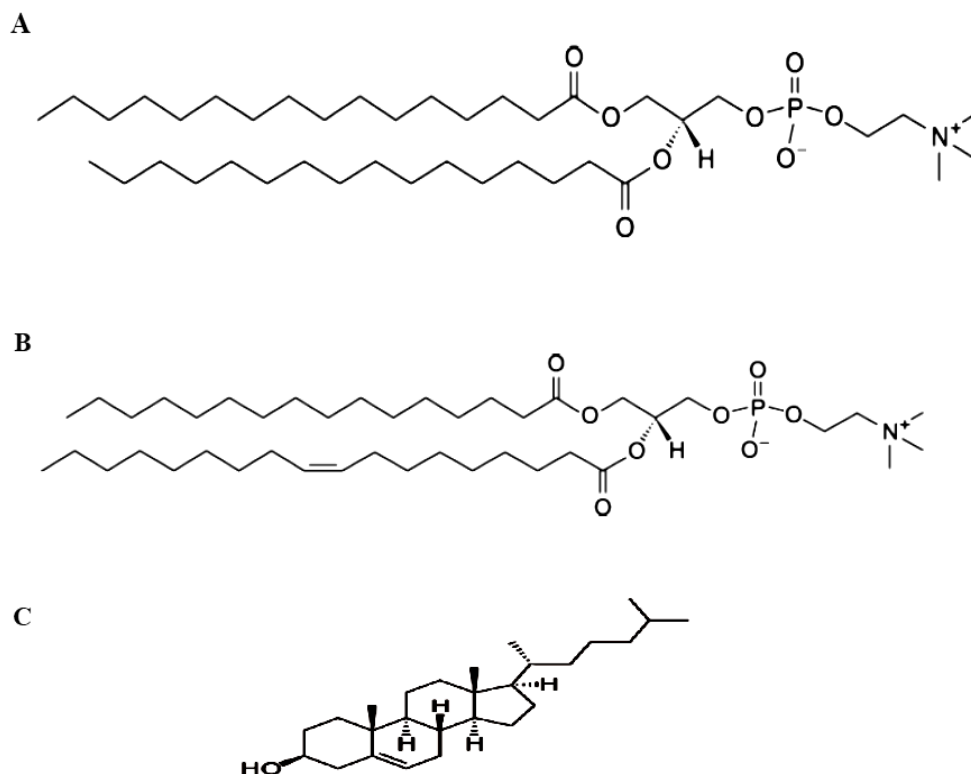
The typical cell membrane is a bilayer membrane consisting of phospholipid molecules with embedded sterols and proteins (Figure 4.2.1). The lipids that make up the membrane can be divided into: glycerol-based lipids (phospholipids), ceramide-based sphingolipids, and cholesterol. According to the nature of their hydrophilic head groups, phospholipids are also divided into phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), among many others (Peetla, Stine et al. 2009). PC, also called

lecithin, is the major component of the cell membrane and pulmonary surfactant. It is more commonly used in the exoplasmic and the outer leaflet of the cell membrane (Wirtz 2006). It also plays a role in membrane mediated cell signaling and phosphatidylcholine transfer protein (PCTP) activation of other enzymes (Kanno, Wu et al. 2007). PE, also called cephalin, is the second most abundant phospholipid after PC, and is present in all eukaryotic and prokaryotic cells. The major role of PE is to assist in membrane fusion and in disassembly of the contractile ring during cytokinesis in cell division (Emoto, Kobayashi et al. 1996). PS is also found in all living cells, but in less amounts than in PC and PE. PS plays an important role in cell cycling signaling, particularly in relation to the early stage of apoptosis (Vance and Tasseva 2013).



**Figure 4.2-1:** Structure of the human cell membrane. Reproduced from (<http://ruleofsix.fieldofscience.com/2013/01/hiv-finds-cellular-door-knob-siglec1.html>)

To provide a simplistic model for a cellular membrane, mixtures of the above lipids can be used to prepare Langmuir monolayers. It is recognized that this is a highly simplified model and completely negates the effects of membrane proteins; however, interactions that serve to disrupt lipid packing can be identified in this approach. 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), dioleoyl phosphatidylcholine (DOPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), in combination with cholesterol, are the most common mixtures used for model membrane studies (see Figure 4.2-2 for structures) (Escribá, González-Ros et al. 2008). It is well known that a healthy membrane has a larger amount of cholesterol and saturated phospholipids, while tumor cell membranes contain higher amounts of unsaturated lipids such as POPC (Klock and Pieprzyk 1979). As a result, a cancer cell membrane is more fluid than a normal membrane (Inbar, Goldman et al. 1977). In this project, a mixture of 80% POPC and 20% cholesterol was used as a model membrane to mimic the cancer cell membrane (Wnętrzak, Łątka et al. 2013). Based upon literature data, it has been shown that endosome membranes (early or late) contain cholesterol in the range of 25% to 30% (Guha, Rajani et al. 2007). As such, a mixture of 75% DPPC and 25% cholesterol was used as a model membrane to mimic the endosomal membrane.



**Figure 4.2-2:** Chemical Structures of A) 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), B) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), C) Cholesterol

### 4.3 Materials

Cationic gelatin was prepared as described in Chapter 3. Chloroform and double-stranded salmon sperm DNA (used without further purification) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Pluronic block copolymers L44, F87, P103, and F108 were a gift from BASF Corporation (Florham Park, NJ) (Table 4.3-1). 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and cholesterol were purchased from Avanti Polar Lipids (Alabaster, USA).

**Table 4.3-1:** Physiochemical Characteristics of Pluronics Block Copolymers (Kabanov, Zhu et al. 2005)

<b>Copolymer</b>	<b>MW<sup>a</sup></b>	<b>Average no. of EO units (N<sub>po</sub>)<sup>b</sup></b>	<b>Average no. of PO units (N<sub>po</sub>)<sup>b</sup></b>	<b>HLB<sup>c</sup></b>	<b>CMC, M<sup>d</sup></b>
<b>L44</b>	<b>2200</b>	<b>20.00</b>	<b>22.67</b>	<b>16</b>	<b>3.6×10<sup>-3</sup></b>
<b>F87</b>	<b>7700</b>	<b>122.50</b>	<b>39.83</b>	<b>24</b>	<b>9.1×10<sup>-5</sup></b>
<b>P103</b>	<b>4950</b>	<b>33.75</b>	<b>59.74</b>	<b>9</b>	<b>6.1×10<sup>-6</sup></b>
<b>F108</b>	<b>14600</b>	<b>265.45</b>	<b>50.34</b>	<b>27</b>	<b>2.2×10<sup>-5</sup></b>

<sup>a</sup>The average molecular weight provided by the manufacturer (BASF Co., Parsippany, NJ).

<sup>b</sup>The average numbers of EO and PO units were calculated using the average molecular weights.

<sup>c</sup>HLB values of the copolymers the cloud points were determined by the manufacturer.

<sup>d</sup>CMC values were determined previously using Pyrene probe

## 4.4 Methods

### 4.4.1 Monolayer formation and surface pressure measurements

CG was dissolved in Millipore-Q water to prepare a stock solution at a concentration of 1 mg/mL. Salmon DNA was also dissolved in Millipore-Q water to prepare a stock solution of 1 mg/6 mL. DPPC-CHOL was prepared at a ratio of 75%-25% (1.875 mM- 0.625 mM) while POPC-CHOL was prepared at a ratio of 80%-20% (2 mM-0.5 mM); both solutions were prepared so that the total lipid concentration was 2.5 mM. All Pluronics were dissolved in chloroform at a concentration equal to 1× their critical micelle concentration (CMC). Monolayers were prepared on a large (14.5 cm by 53 cm) Langmuir-Blodgett trough (KSV Instruments, Helsinki, Finland), using Milli-Q water as the subphase, as follows:



1. The trough was filled with Milli-Q water, to which DNA was added to obtain 0.2  $\mu\text{g}/\text{mL}$  as the final concentration (where required).
2. 160  $\mu\text{L}$  of cationic gelatin stock solution was then added into the subphase. A w/w ratio of 1:40 of DNA to CG was used in this study.
3. A volume of 20  $\mu\text{L}$  of Pluronic was next added to subphase (where required). A glass rod was used to mix CG with DNA or CG/DNA mixture with Pluronic prior to adding the monolayer as a final step.
4. A volume of 20  $\mu\text{L}$  of DPPC-CHOL or POPC-CHOL stock solution was placed onto the surface of the subphase using a micro-syringe (GASTIGHT<sup>®</sup>, Hamilton-Bonaduz, Schweiz, Switzerland).
5. For experiments involving DNA, the monolayer was allowed to equilibrate for 30 minutes. For experiments that did not involve DNA, the sub-phase was given 15 minutes to equilibrate before adding monolayer. The monolayer was then allowed to equilibrate for another 30 minutes on the DNA and the subphase before the isotherms were collected.

Surface pressure was monitored using the Wilhelmy plate method and the monolayers were compressed using barriers at a rate of 15 mm/min to obtain the surface pressure vs. molecular area. Isotherms and BAM images of the following samples with or without DNA were collected: CG and L44, CG and F87, CG and P103, CG and F108 with DPPC-CHOL and POPC-CHOL monolayers.

#### **4.4.2 Imaging the monolayer**

Briefly, the Brewster angle microscope (BAM) (KSV Instruments, Helsinki, Finland) uses polarized light that shines incident to the surface of the water at the Brewster angle (approximately  $53^\circ$ ). When a monolayer is applied onto the sub-phase, this changes the local refractive index (RI); as a result, a small amount of laser (He-Ne laser, with a power of 50 mW, wave length of 658 nm) is reflected on the surface, and the monolayer image is then observed using a CCD camera, and analyzed using KSV NIMA Image software (Version 1.1.2, KSV-NIMA, Finland).

#### **4.4.3 Analysis of the $\pi$ - A isotherms**

A variety of parameters can be obtained from the isotherm. For example, limiting area or minimum cross-sectional area ( $A_\infty$ ) is determined by regression of the linear region of the isotherm corresponding to the stiffest phase of the monolayer, prior to collapse. From the linear equation we can calculate the limiting area at which the surface pressure (i.e.  $y$  in the regression) is equal to zero. The monolayer is more closely packed when the value of limiting area is small (Chen, Zhang et al. 2008). Also, the collapse pressure ( $\pi_C$ ) can be determined from the isotherm, which corresponds to the maximum surface pressure on the  $y$ -axis and before the collapse. The monolayer is stable when the surface pressure at collapse is high (Barnes and Gentle 2011). The molecular area at collapse ( $A_C$ ), corresponds to the molecular area for the lipids in the monolayer, just at the point of collapse. Both  $\pi_C$  and  $A_C$  are read directly from the isotherm.

#### 4.4.4 Compressibility modulus

Interfacial elasticity describes the changes in surface tension regarding the area of the surface associated with a liquid film, which is actually related to the compressibility of the monolayer (Vollhardt and Fainerman 2006). As a result, based on the following equation:

$$C_s^{-1} = -A(\partial\pi/\partial A)_T,$$

the compressibility modulus ( $C_s^{-1}$ ) was determined, where, A is the molecular area at a given surface pressure,  $\pi$ . The molecular area at collapse ( $A_c$ ) and collapse pressure ( $\pi_c$ ) were directly determined from the compression isotherms. Davis and Rideal (Rideal and Davies 1963) stated that the phases of the monolayer are determined by the compressibility modulus, as follows:

- Gaseous phase(G)  $C_s^{-1} < 12.5$  mN/m
- Liquid-expanded phase (LE)  $C_s^{-1} = 12.5\text{--}50$  mN/m,
- Liquid-expanded liquid condensed (LE-LC)  $C_s^{-1} = 50\text{--}100$  mN/m,
- Liquid-condensed (LC)  $C_s^{-1} = 100\text{--}250$  mN/m,
- Solid (S) for  $C_s^{-1} > 250$  mN/m.

The monolayer is less elastic when the  $C_s^{-1}$  is high and vice versa. Since the surface pressure of the real biological membrane ranges between 30 mN/m and 35 mN/m (Seoane, Minones et al. 2000), we will focus on this surface pressure in this study. Strong or weak interactions were interpreted through the change that occurs to the parameters of model membranes, particularly limiting area and compressibility modulus, after the model membranes interact with the delivery system. A large change

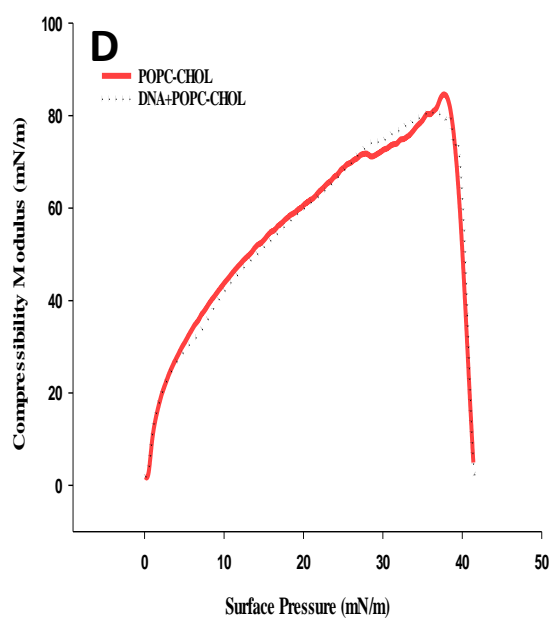
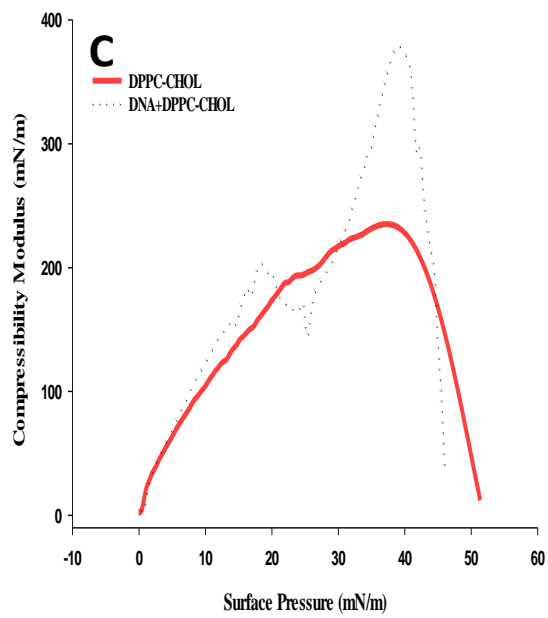
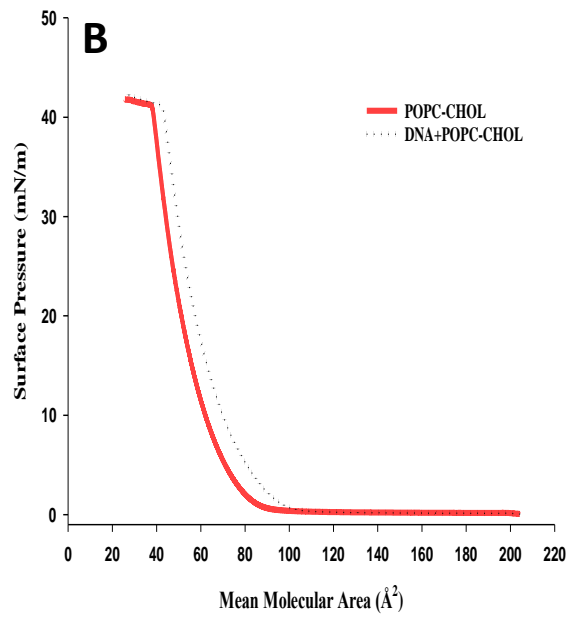
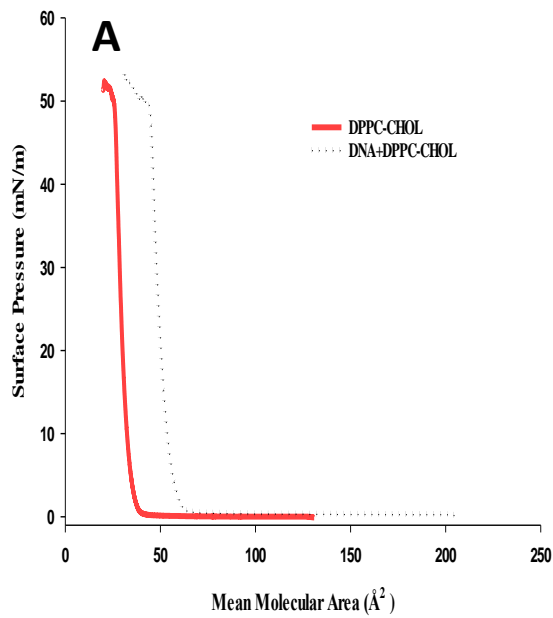
in these parameters indicates strong interaction, while a slight change indicates weak interaction.

## **4.5 Results and Discussion**

Since our monolayers were prepared using 75% DPPC or 80% POPC in addition to cholesterol, it is important to study the individual components of DPPC, POPC, and cholesterol to gain a better understanding of the behaviour of monolayers containing only these single components. Our group previously reported the analysis of isotherms for monolayers of pure DPPC, POPC, and cholesterol (Ahmed 2015). The results demonstrated that cholesterol formed a condensed monolayer in which the molecules were vertically arranged, or they slightly shifted towards the surface. These findings were in agreement with the results found in the literature (Seoane, Minones et al. 2000). DPPC shows a characteristics transition from LE to LC phase starting at surface pressure (5-7 mN/m) (Barnes and Gentle 2011), and the isotherm continues at LC until collapse while POPC shows a liquid-expanded phase with no noticeable transition until collapse.

### **4.5.1 DPPC-CHOL and POPC-CHOL**

The isotherms for the DPPC-CHOL and POPC-CHOL systems are shown in Figure 4.5-1; parameters derived from the isotherms are listed in Table 4.5-1. Our results show that the DPPC-CHOL monolayer is more rigid than the POPC-CHOL monolayer. From the isotherm, the minimal cross sectional areas ( $A_{\infty}$ ) were determined to be  $41 \text{ \AA}^2$  and  $63 \text{ \AA}^2$  for both DPPC-CHOL and POPC-CHOL respectively, which indicates that DPPC-CHOL is more closely packed than POPC-CHOL (Chen, Zhang et al. 2008).



**Figure 4.5-1:** Isotherms (A and B) and compressibility modulus plots (C and D) for DPPC-CHOL (A, C) and POPC-CHOL (B, D) in the presence and absence of DNA.

**Table 4.5-1:** Monolayer properties derived from isotherm and compressibility modulus plots, for untreated DOPC-CHOL or POPC-CHOL monolayers, and for monolayers treated with DNA.

System	$A_c$ ( $\text{\AA}^2$ )	$\pi_c$ (mN/m)	$A_\infty$ ( $\text{\AA}^2$ )	$C_s^{-1}$ (mN/m)		Phase <sup>a</sup>
				30 mN/m	35 mN/m	
DPPC-CHOL	29	52	41	221	229	LC
DPPC-CHOL + DNA	41	50	53	225	325	S
POPC-CHOL	34	41	63	74	78	LE-LC
POPC-CHOL + DNA	35	42	67	76	86	LE-LC

Molecular area at collapse ( $A_c$ ), collapse pressure ( $\pi_c$ ), limiting (or minimum cross-sectional) area ( $A_\infty$ ), compressibility modulus ( $C_s^{-1}$ ), determined from the value of  $C_s^{-1}$  at surface pressure 30 and 35 mN/m, LE = liquid expanded, LC = liquid condensed. <sup>a</sup> Phase of the monolayer based upon the value of  $C_s^{-1}$  as a surface pressure of 35 mN/m

A compressibility modulus ( $C_s^{-1}$ ) value of  $\sim 225$  mN/m also suggests that DPPC-CHOL is in the liquid condensed (LC) phase (see section 4.4.4, above) while POPC-CHOL is in the liquid expanded phase (LE-LC) as indicated by the  $C_s^{-1}$  75 mN/m. These results correspond with the previous data that demonstrated a similar rigidity for the DPPC-CHOL monolayer (Melik-Nubarov, Pomaz et al. 1999, Zhao and Feng 2006, Guzmán, Liggieri et al. 2013). Introducing cholesterol to DPPC or POPC substantially influences molecular packing within the monolayers, resulting in a very different molecular organization as compared to pure DPPC or POPC (Kim, Choi et al. 2013). The interaction of cholesterol with POPC is weaker and less attractive than with DPPC (Silvius 2003). The interpretation of this weak interaction is that the cholesterol's higher affinity of DPPC over that for POPC depends on its alignment with the normal bilayer, with lower tilt angles promoted by cholesterol-cholesterol steric exclusion (De Joannis, Coppock et al. 2011). Also, the presence of double bonds in a cis conformation in POPC molecules results in induction "kink" or bend in the hydrocarbon

chains, rendering some of the POPC molecules less suited to packing among aligned cholesterol, as compared to DPPC (Rog, Murzyn et al. 2004, Wydro, Knapczyk et al. 2011, Jurak 2013). This results in a less condensed monolayer for the POPC-CHOL system.

#### **4.5.2 DPPC-CHOL and POPC-CHOL with DNA**

Isotherms obtained upon mixing DNA with the DPPC-CHOL or POPC-CHOL monolayer are also shown in Figure 4.5-1; the monolayer properties for the DNA/monolayer systems are again listed in Table 4.5-1. The addition of DNA had an impact on the DPPC-CHOL monolayer, where the cross-sectional area ( $A_{\infty}$ ) increased from 41 Å<sup>2</sup> to 53 Å<sup>2</sup>. In contrast, the addition of DNA to the POPC-CHOL monolayer resulted in a slight increase from 63 Å<sup>2</sup> to 67 Å<sup>2</sup>. The compressibility modulus for both monolayers remained essentially unchanged with the addition of DNA.

It has been demonstrated in the literature that DNA can interact with the model membrane and has some effect on cellular processes (Michanek, Kristen et al. 2010). For example, DNA is able to either fluidize or condense the model membrane when it interacts with a positively charged lipid monolayer (Antipina, Schulze et al. 2007, Chen, Kang et al. 2012, Dabkowska, Barlow et al. 2012, Dabkowska, Barlow et al. 2014). In our results, DNA shows a condensation effect on DPPC-CHOL, which is then transformed to a fluidization effect as the surface pressure increases (Figure 4.5-1 C).

DNA can potentially interact with cellular membranes, or in our case with the model membranes, through different types of interactions. Firstly, as a negatively charged polyelectrolyte, DNA can interact through electrostatic interactions with cationic lipids such as DOTAP or DOTMA (Langecker, Arnaut et al. 2014). In the case of DPPC-CHOL or

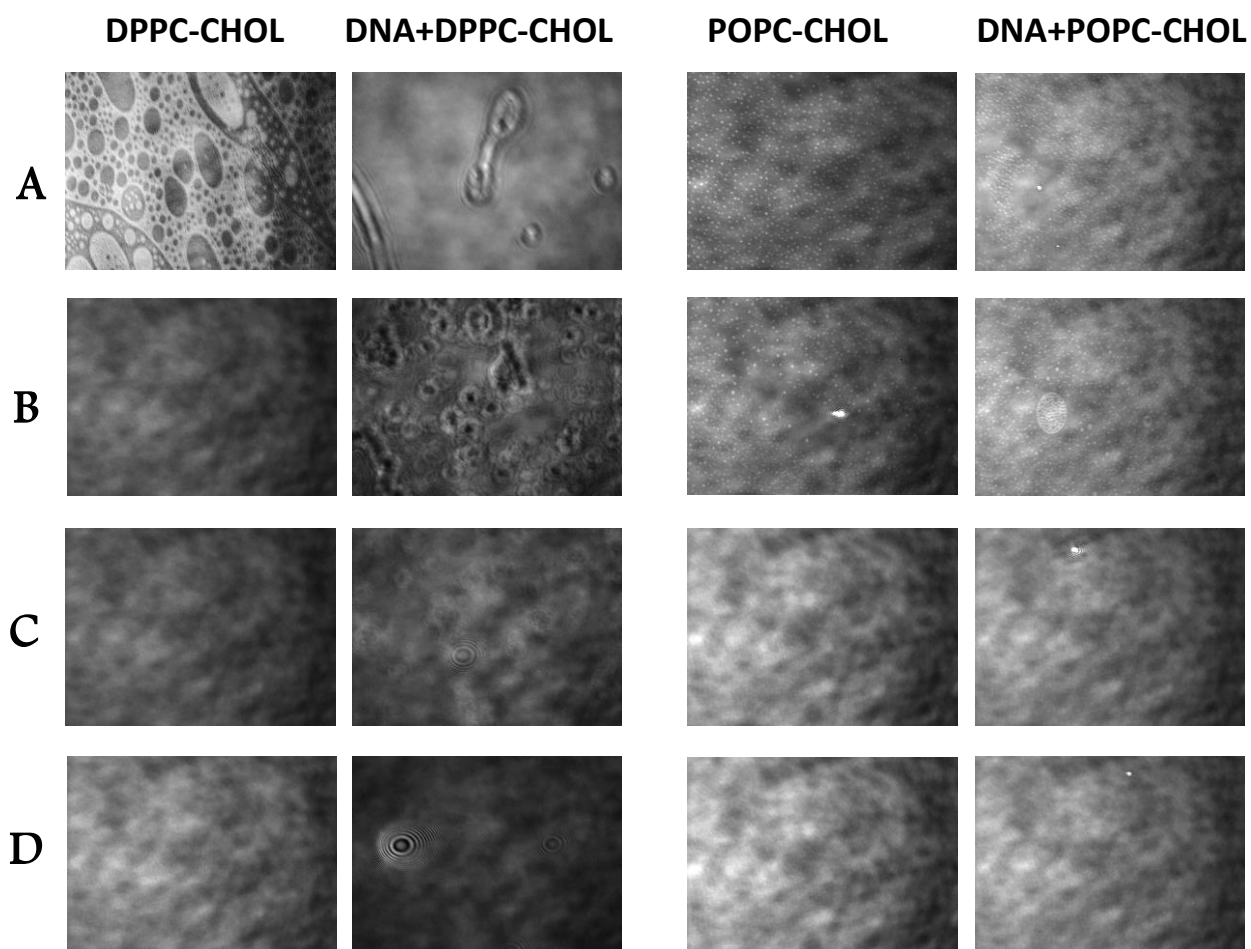
POPC-CHOL (which are zwitterionic), this interaction is expected to be negligible (McLoughlin, Dias et al. 2005, Michanek, Yanez et al. 2012). Secondly, solvation effects (i.e. hydrophobic hydration and/or hydrophobic interaction) may play a role in modulating the interaction(s) between DNA and the lipid monolayer. However, as a charged polyelectrolyte, DNA is highly hydrophilic, and these interactions are also expected to be negligible. Finally, DNA-based nanostructure can be attached to the lipid membrane using DNA conjugates with lipid, such as cholesterol, or other hydrophobic molecules (Langecker, Arnaut et al. 2014). DNA-lipid conjugates can be used for gene transfection to induce vesicle fusion or to cross-link vesicle or even cells (Chan, van Lengerich et al. 2009, Beales, Nam et al. 2011). Even with the addition of multiple cholesterol modification that can be added to DNA, the interaction with the lipid membrane is weak and irreversible (Pfeiffer and Höök 2004). As a result, the interactions of DNA with the model membrane are not perceived to be realistic because there is more of an interaction occurring with the membrane rather than penetration (Cárdenas, Nylander et al. 2005, Castano, Delord et al. 2009, Ahmed 2015). These results confirmed that the DNA is not able to penetrate in the absence of the delivery system.

Interesting BAM images were obtained upon the addition of DPPC-CHOL alone at low surface pressure (1-3 mN/m), where the monolayer was in the G-LE phase transition (Figure 4.5-2). The resulting domains appears to have circular or ovoid shapes. Upon compressing the monolayers, these domains disappear, and a uniform grey background is shown until the monolayer collapsed. The domains of DPPC-CHOL were then disappeared after adding CG to the model membrane. The reason for this disappearance is probably because the CG gets



adsorbed into or it is penetrated into the monolayer, which explains the fluidity of the model membrane (Nasir and Besson 2012).

The addition of DNA to DPPC-CHOL resulted in small circular domains at low surface pressure (1-5 mN/m), which were then changed to a grey background at a high surface pressure (Figure 4.5-2). These domains could be attributed to the interaction between DNA and DPPC-CHOL through either hydrophobic interaction or local polarization (Raudino and Mauzerall 1986, Michanek, Kristen et al. 2010). Upon increasing the surface pressure, the DNA could be excluded from the monolayer and could enter into the subphase, thereby resulting in featureless domains similar to DPPC-CHOL alone, at high surface pressure (Lopes-Costa, Gámez et al. 2011). Similar to POPC-CHOL alone, the addition of DNA to POPC-CHOL did not show any characteristic domain formation (Figure 4.5-2). In terms of POPC-CHOL, the addition of DNA resulted in only small white dots as opposed to larger domains. These small dots likely correspond to 2-dimensional aggregates and were also observed at low surface pressure. This further suggests only weak interactions between the POPC-CHOL monolayer and DNA, possibly due to the unsaturated oleyl tail in the POPC lipid. The presence of the double bond results in a POPC-CHOL more fluidized monolayer, and therefore, the impact of the addition of DNA becomes negligible.



**Figure 4.5-2:** BAM Images of DPPC-CHOL (left) and POPC-CHOL (right) in the presence and absence of DNA at different surface pressure

A)  $<1\text{ mN/m}$ , B)  $5\text{ mN/m}$ , C)  $30\text{ mN/m}$ , D)  $50\text{ mN/m}$  for DPPC-CHOL and  $40\text{ mN/m}$  for POPC-CHOL

### 4.5.3 Interaction of cationic gelatin with model membrane

The addition of CG to the DPPC-CHOL membrane results in fluidization of the membrane, with the compressibility modulus decreasing from 221 mN/m to 182 mN/m at a surface pressure 30 mN/m (Figure 4.5-3 and Table 4.5-2). Upon mixing with CG, the  $A_{\infty}$  for the DPPC-CHOL membrane increased from 41 Å<sup>2</sup> up to 63 Å<sup>2</sup>, providing additional evidence for the fluidization of the membrane after adding CG. Recall, a small limiting area value corresponds to a highly packed monolayer formation (Castano, Delord et al. 2008).

**Table 4.5-2:** Monolayer properties derived from isotherm and compressibility modulus plots, for untreated DOPC-CHOL or POPC-CHOL monolayers, and for monolayers treated with CG in the presence and absence of DNA

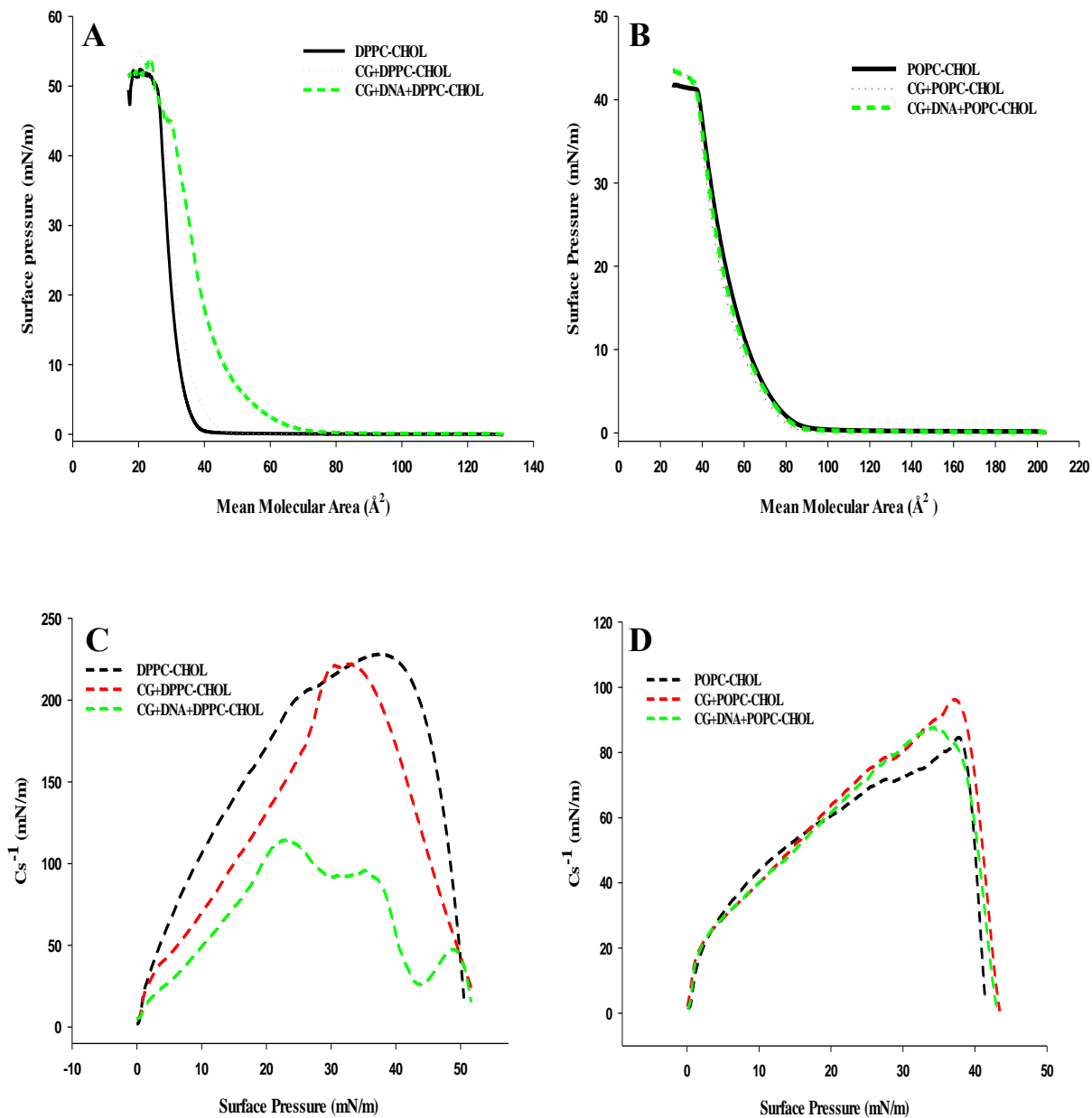
System	$A_c$ (Å <sup>2</sup> )	$\pi_c$ (mN/m)	$A_{\infty}$ (Å <sup>2</sup> )	$C_s^{-1}$ (mN/m)		Phase <sup>a</sup>
				30 mN/m	35 mN/m	
<b>DPPC-CHOL</b>	29	52	41	221	229	LC
<b>CG+DPPC-CHOL</b>	29	50	63	182	195	LC
<b>CG+DNA+DPPC-CHOL</b>	24	54	47	92	90	LE-LC
<b>POPC-CHOL</b>	34	41	63	74	78	LE-LC
<b>CG+POPC-CHOL</b>	36	42	56	84	87	LE-LC
<b>CG+DNA+POPC-CHOL</b>	36	42	59	85	95	LE-LC

<sup>a</sup> phase of the monolayer as determined from the value of  $C_s^{-1}$  at  $\pi = 35$  mN.m

The interaction of gelatin with DPPC-CHOL could arise from several potential sources including hydrophobic interactions, electrostatic interactions (either attractive or repulsive), and hydrogen bonding, among others (Fresta, Ricci et al. 2000). The presence of additional amino groups on the gelatin peptide backbone from the cationization reaction will contribute to an increased electrostatic interaction, specifically a repulsive interaction between the positively charged quaternary ammonium choline head group of the phospholipids, and the positively charged cholamine added to gelatin (Pasenkiewicz-Gierula, Takaoka et al. 1999,

Fresta, Ricci et al. 2000, Fang, Chan et al. 2001). A similar effect on DPPC monolayers was obtained with chitosan (Fang, Chan et al. 2001) or the nonapeptide Leucinostatin A (Fresta, Ricci et al. 2000). The addition of DNA to the CG/DPPC-CHOL complex also induce the fluidization by decreasing the compressibility modulus from 182 mN/m to 92 mN/m, observing a transition from LC to LE-LC phase (Figure 4.5-3 and Table 4.5-2). The electrostatic interaction of DNA with CG might be the reason of this fluidization. H-bonding between cationic polymer and DNA induces the stabilization of polyplexes (Prevette, Lynch et al. 2008), resulted in more effective interaction with the model membrane. These interactions are important to allow the CG to condense the large size of DNA and also to induce the permeability of the cellular membrane.

The POPC-CHOL membrane is less rigid than DPPC-CHOL, which appears to result in weaker interactions with CG or CG and DNA as compared to DPPC-CHOL. It is clear from Table 4.5-2 and Figure 4.5-3 that the addition of CG to POPC-CHOL condensed the monolayer by increasing the compressibility modulus from 74 mN/m to 84 mN/m and decreasing the limiting sectional area from 63 Å<sup>2</sup> to 56 Å<sup>2</sup>. This reveals that the molecules are more closely packed and condensed. This condensation effect might be attributed to the hydrophobic interaction between CG and the unsaturated lipid POPC. Since the interaction between POPC and cholesterol is weaker than with the DPPC, it allows the CG to directly interact with POPC through the hydrophobic chains of POPC, which they bend from the cholesterol due to the presence of the double bond, resulting in more condensed molecules. The addition of DNA did not cause much change in the system with the POPC-CHOL

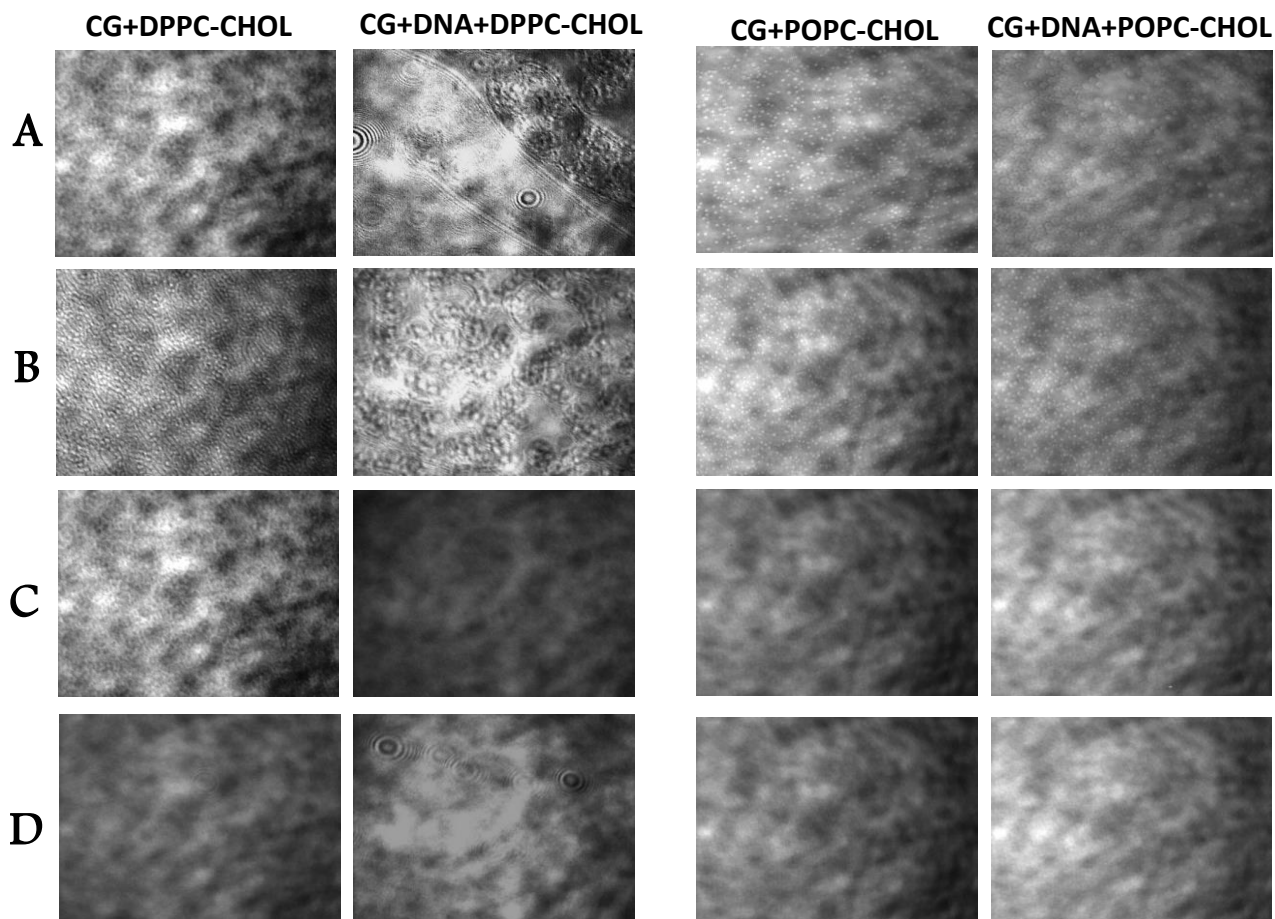


**Figure 4.5-3:** Isotherms (A, B) and compressibility modulus plots (C, D) of the monolayer alone, monolayer and CG, and monolayer, CG, and DNA. DPPC-CHOL (A, C) and POPC-CHOL (B, D).

monolayer. It is apparent that the compressibility modulus and limiting area remained almost the same for both CG/POPC-CHOL system in the presence or absence of DNA.

BAM images of CG combined with either both DPPC-CHOL or POPC-CHOL show a grey background with no distinctive domains at all surface pressures. This means that CG has an impact on the monolayers, resulting in changing the shapes of domains for both DPPC-CHOL and POPC-CHOL (Figure 4.5-4). This impact can be explained through the compressibility moduli and limiting areas (Table 4.5-2), which showed that CG has a fluidization and condensation effect on both DPPC-CHOL and POPC-CHOL respectively. Bead-like domains were observed in the DPPC-CHOL monolayer after the addition of DNA to CG at low surface pressure. These bead-like domains likely resulted from a reorganization of DPPC and CHOL in the lipid monolayer, possibly as means to reduce the solvation energy for the DNA:CG complexes, which due to charge neutralization, are expected to be quite hydrophobic. This reorganization is consistent with the fluidization of the monolayer observed from the isotherm, as described above. The addition of DNA to CG/POPC-CHOL complex did not result in any reorganization similar to what was observed with CG/POPC-CHOL alone. This demonstrates weaker interaction of DNA with POPC-CHOL either in the presence or absence of CG. Upon further compression, a featureless homogeneous grey film with no domains was observed in the whole system in both DPPC-CHOL and POPC-CHOL. From the above, we conclude that CG has a fluidizing effect on DPPC-CHOL, but not on POPC-CHOL. Furthermore, DNA influences the interaction with the membrane through its

strong interaction with CG, which enhances the stability of the delivery system, resulting in more fluidized effect, particularly with DPPC-CHOL.



**Figure 4.5-4:** BAM Images of CG with DPPC-CHOL (left), and POPC-CHOL (right) in the presence and absence of DNA at different surface pressure. A)  $<1\text{ mN/m}$ , B)  $5\text{ mN/m}$ , C)  $30\text{ mN/m}$ , D)  $50\text{ mN/m}$  for DPPC-CHOL and  $40\text{ mN/m}$  POPC-CHOL. CG: cationic gelatin.

#### 4.5.4 DPPC-CHOL and POPC-CHOL with Pluronics, CG, and DNA

Different Pluronics in combination with DPPC-CHOL monolayers have been studied by others, in an attempt to understand the effect of basic surface characteristics based on the number of EO and PO, as well as the molecular weight (Chang, Chang et al. 2008). A

previous study conducted by Chang et al. (Chang, Chang et al. 2008) studied different Pluronics with varying EO and PO units. The results indicated that while the number of EO monomers of the Pluronic increased, the area per molecule also increased especially when the surface pressure increased with significant differences between Pluronics. The same study explained that the area per molecule was larger when the number of PO monomers of Pluronic was also large at fixed EO monomers. Lastly, the study compared Pluronics with different molecular weights (MW), at a constant weight percentage of PEO chains. The authors found that there was a linear relationship between MW and the area per molecules. A larger molecular weight obtained higher area per molecules (Chang, Chang et al. 2008).

Pluronics are amphiphilic structures that contain both hydrophilic (PEO) and hydrophobic (PPO) blocks. It is believed that the interaction of Pluronics with DPPC is influenced by PEO chains. When the compression begins, most of the PEO enters the subphase while the PPO remains on the interface (Chang, Chang et al. 2008). This is due to the "squeezed-out" phenomenon (Weingarten, Magalhaes et al. 1991, Maskarinec, Hannig et al. 2002, Wu, Majewski et al. 2004, Chang, Lin et al. 2005), which was noted earlier by Weingarten et al. in 1991 (Weingarten, Magalhaes et al. 1991). The authors revealed that when Pluronic F68 mixed with the model membrane (DPPC), the Pluronic squeezed out of the lipid monolayer at a high surface pressure ( $\geq 35$  mN/m) (Maskarinec and Lee 2003). Cholesterol is known to change the behaviour of the membrane by reducing passive permeability, increasing the mechanical strength, and manipulating the function of the membrane enzymes (Yeagle 1985, Ohvo-Rekilä, Ramstedt et al. 2002). The addition of cholesterol to DPPC monolayers changed the interaction behaviour of Pluronics with the



membrane (Chang, Chang et al. 2008). The addition of cholesterol to DPPC did not affect the penetration kinetics for the copolymers. Although PPO may have some hydrophobic interaction with cholesterol, this interaction was not strong enough to affect the penetration (Chang, Chang et al. 2008).

At low surface pressure, when the monolayer is not packed, the interaction between cholesterol and DPPC is expected to be weak. Therefore, the penetration of Pluronics into the monolayer is enhanced through hydrogen bonding between cholesterol and the PEO moieties of the Pluronics (Mpofu, Addai-Mensah et al. 2003, Anselmo, Sassonia et al. 2006) . This is obvious from our results, where the addition of Pluronic results in fluidization of the monolayer at low surface pressure, seen from the decrease in the compressibility modulus of DPPC-CHOL from 221 mN/m to less than 200 Nm/m for all Pluronics and increasing the limiting cross-sectional area from  $41 \text{ \AA}^2$  to  $\sim 54 \text{ \AA}^2$  (Table 4.5-3), which indicates another parameter of fluidization. Conversely at high surface pressure, when the monolayer is closely packed and the interaction between cholesterol and DPPC is very strong, the penetration of Pluronics is inhibited. In pure cholesterol monolayers, the hydrogen bond that exists between  $3\beta$ - hydroxyl group of cholesterol and ether oxygens (Lewis base) of PEO chains result in deep penetration of Pluronics especially those with long PEO segments (Chang, Chang et al. 2008).

**Table 4.5-3:** Monolayer properties derived from isotherm and compressibility modulus plots, for untreated DOPC-CHOL or POPC-CHOL monolayers, and for monolayers treated with CG and Pluronics in the presence and absence of DNA.

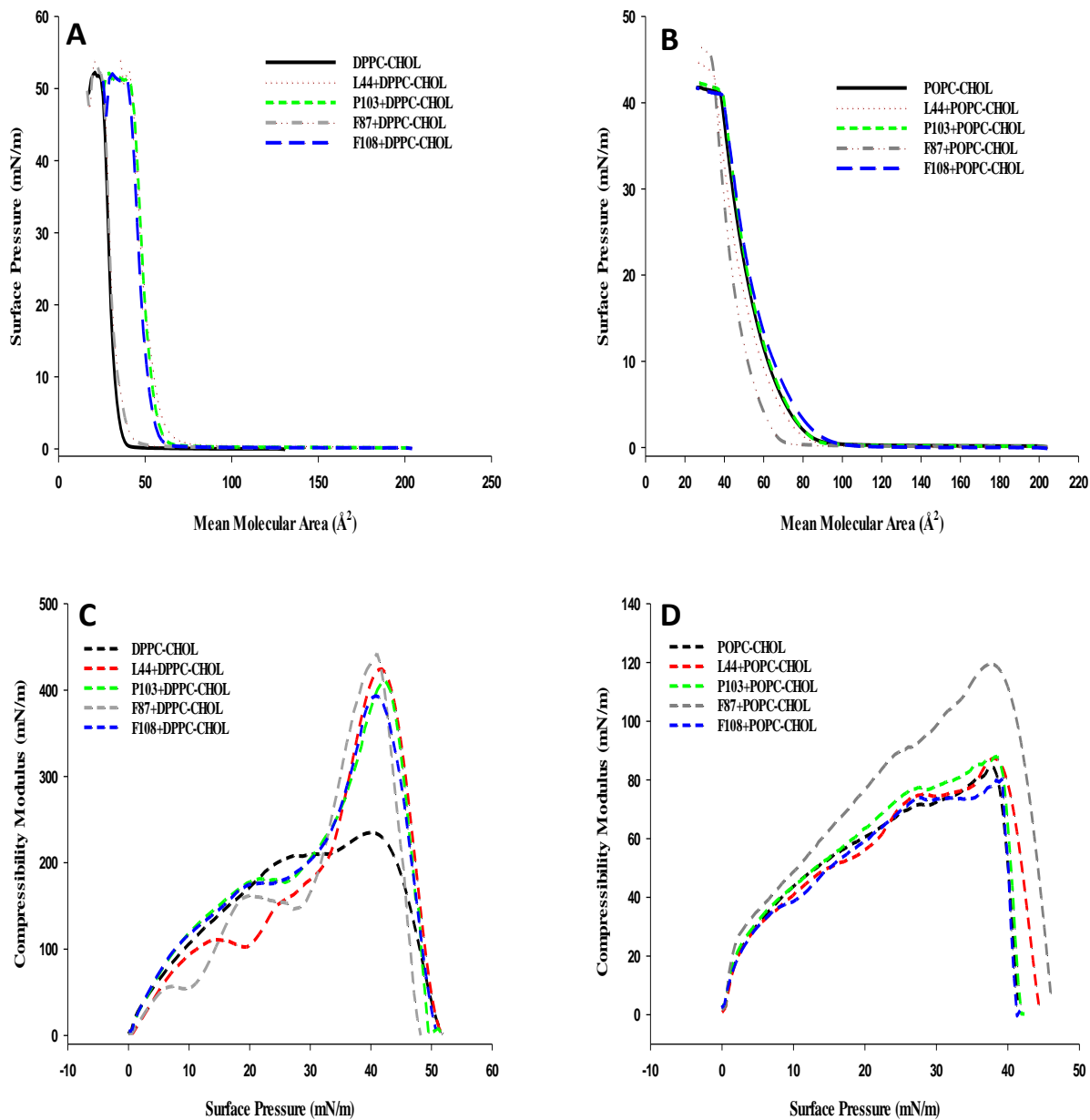
System	$A_c$ ( $\text{\AA}^2$ )	$\pi_c$ (mN/m)	$A_\infty$ ( $\text{\AA}^2$ )	$C_s^{-1}$ (mN/m)		Phase <sup>a</sup>
				30 mN/m	35 mN/m	
<i>DPPC-CHOL Monolayer</i>						
<b>DPPC-CHOL</b>	29	52	41	221	229	LC
<b>L44+DPPC-CHOL</b>	36	52	51	198	264	S
<b>L44+CG+DPPC-CHOL</b>	34	51	65	156	198	LC
<b>L44+CG+DNA+DPPC-CHOL</b>	33	51	55	163	273	S
<b>F87+DPPC-CHOL</b>	20	55	32	179	263	S
<b>F87+CG+DPPC-CHOL</b>	20	54	43	116	109	LC
<b>F87+CG+DNA+DPPC-CHOL</b>	22	53	37	149	227	LC
<b>P103+DPPC-CHOL</b>	39	51	54	195	221	LC
<b>P103+CG+DPPC-CHOL</b>	34	50	69	135	117	LC
<b>P103+CG+DNA+DPPC-CHOL</b>	29	53	62	159	192	LC
<b>F108+DPPC-CHOL</b>	30	52	50	174	250	S
<b>F108+CG+DPPC-CHOL</b>	40	51	66	110	104	LC
<b>F108+CG+DNA+DPPC-CHOL</b>	33	51	63	160	117	LC
<i>POPC-CHOL Monolayer</i>						
<b>POPC-CHOL</b>	34	41	63	74	78	LE-LC
<b>L44+POPC-CHOL</b>	27	45	57	83	76	LE-LC
<b>L44+CG+POPC-CHOL</b>	26	45	60	78	84	LE-LC
<b>L44+CG+DNA+POPC-CHOL</b>	27	44	59	81	87	LE-LC
<b>F87+POPC-CHOL</b>	29	46	50	102	107	LC
<b>F87+CG+POPC-CHOL</b>	30	45	63	82	81	LE-LC
<b>F87+CG+DNA+POPC-CHOL</b>	31	45	55	94	94	LE-LC
<b>P103+POPC-CHOL</b>	30	42	62	76	84	LE-LC
<b>P103+CG+POPC-CHOL</b>	27	42	72	68	50	LE-LC
<b>P103+CG+DNA+POPC-CHOL</b>	25	42	66	74	78	LE-LC
<b>F108+POPC-CHOL</b>	26	41	64	76	77	LE-LC
<b>F108+CG+POPC-CHOL</b>	27	40	76	61	50	LE-LC
<b>F108+CG+DNA+POPC-CHOL</b>	30	42	65	76	78	LE-LC

<sup>a</sup> phase of the monolayer determined from the value of  $C_S^{-1}$  at  $\pi = 35$  mN/m.

In our results, hydrophilic Pluronics F87 and F108 (which have PEO blocks of 122 and 156 EO groups, respectively), resulted in a compressibility modulus at 30 mN/m equal to 179 and 174 mN/m and limiting cross-sectional areas  $32 \text{ \AA}^2$  and  $50 \text{ \AA}^2$  respectively. On the other hand, hydrophobic Pluronics L44 and P103 (with PEO blocks of 20 and 33 EO groups, respectively) resulted in a compressibility modulus of 198 and 195 mN/m and a limiting

cross-sectional area  $51 \text{ \AA}^2$  and  $54 \text{ \AA}^2$  respectively. It is noticed that the limiting cross-sectional areas are very similar for all Pluronics except for Pluronic F87 (likely due to its greater solubility in water). Due to the squeeze out phenomenon, at high surface pressure, there are very few PEO units that remain associated with the monolayer and thus, the DPPC and cholesterol molecules become more closely packed, which leads to penetration inhibition for the Pluronic (Maskarinec and Lee 2003). Our results indicate that there is a deep penetration for Pluronics at low surface pressure, but this penetration is inhibited when the surface pressure has increased.

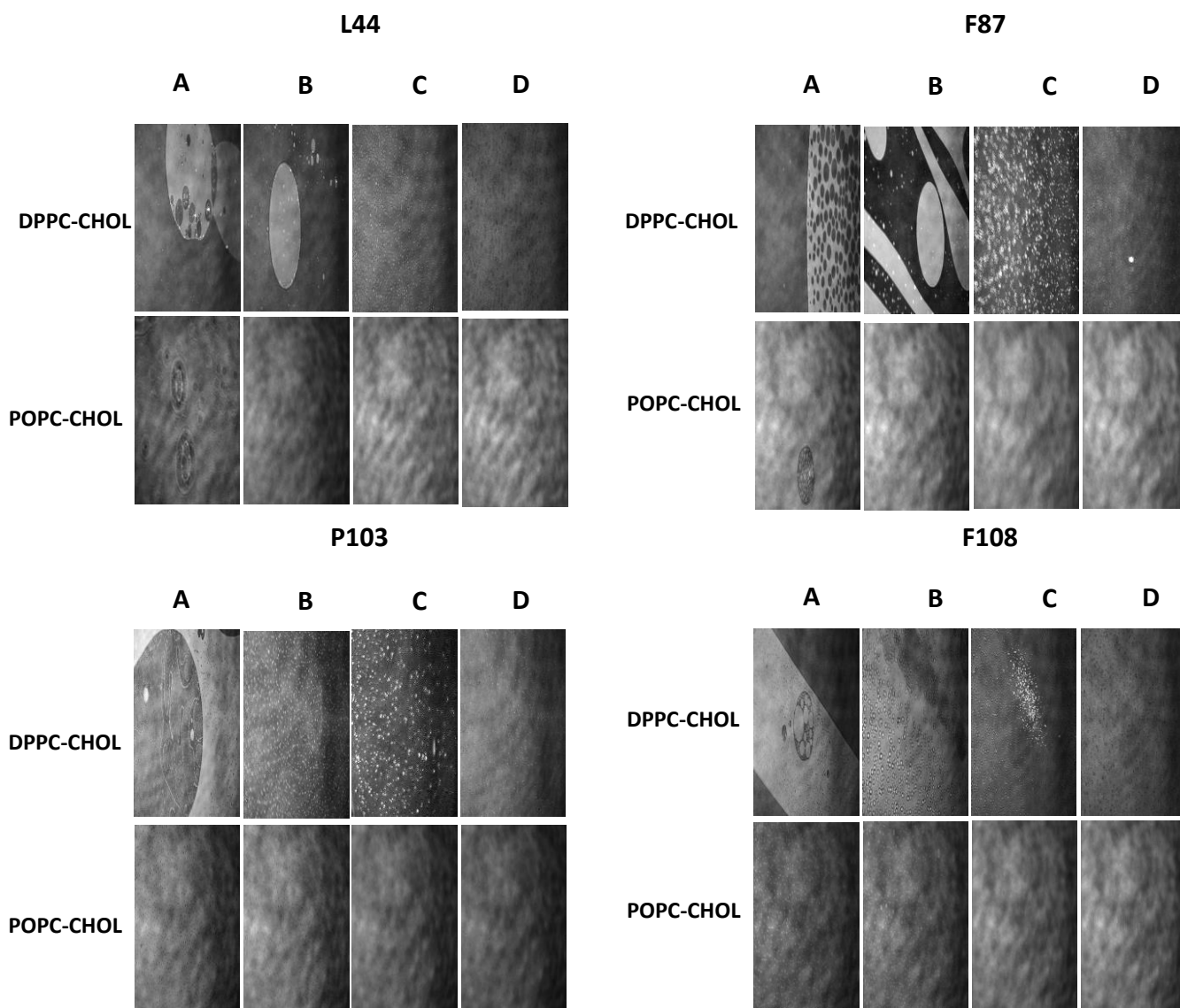
Based upon both the isotherms (see Table 4.5-3 for the area parameters) and the compressibility modulus values, there is no apparent interaction between the POPC-CHOL monolayer on either P103 or F108. On the other hand, L44 and F87 increased the compressibility modulus from  $74 \text{ mN/m}$  to  $83$  and  $102 \text{ mN/m}$  respectively, while the minimal sectional area decreased from  $63 \text{ \AA}^2$  to  $57$  and  $50 \text{ \AA}^2$  for both Pluronics respectively. This suggests that the membrane became more condensed; an effect which mirrors that seen for the addition of CG to the POPC-CHOL monolayer (see section 4.5.3). Nevertheless, the changes in membrane fluidity are not remarkable and in particular do not result in a change in the molecular phase.



**Figure 4.5-5:** Isotherms (A, B) and compressibility modulus plots (C, D) of the monolayer alone, and monolayer and Pluronic. DPPC-CHOL (A, C) and POPC-CHOL (B, D).

BAM images of the DPPC-CHOL monolayer upon the addition of Pluronics shows distinctive domains (Figure 4.5-6). These domains are circular or ovoid in shapes, and they are observed at low surface pressure (1-5 mN/m), where greater interaction between the molecules within the monolayer and the Pluronics appear to occur. Pluronics P103 and F108 (which have the same unit of PPO (~ 50)) do show domains at low surface pressure. However, these domains tend to disappear earlier than in L44 and F87. This might be attributed to PPO blocks, which they can interact with cholesterol through either hydrophobic or hydrogen bonding, or both (Anselmo, Sassonia et al. 2006). Consistent with the isotherm, it is clear from the BAM images that most of the interactions between Pluronics and the model membrane take place at low surface pressure when both PEO and PPO units coexist. Once the surface pressure increases, most of the PEO units enter into the subphase and leaves the PPO units on the surface, resulting in lower interaction and thus, fewer domains.

Images of POPC-CHOL with Pluronics show some domains that were formed at low surface pressure, particularly for Pluronics L44 and F87; although the generally featureless images obtained at most surface pressures regardless of polymer are consistent with a relatively homogeneous LE or LE-LC phase. The fact that the domains are observed for L44 and F87, but not for P103 and F108, is consistent with the discussion above, where L44 and F87 were observed to induce the condensation of the monolayer seen from the increase in the compressibility modulus and decrease in the limiting area (Table 4.5-3 and Figure 4.5-5).



**Figure 4.5-6:** BAM images of all Pluronics with DPPC-CHOL and POPC-CHOL at different surface pressures. A)  $<1$  mN/m; B) 5 mN/m; C) 30 mN/m; D) 50 mN/m for DPPC-CHOL and 40 mN/m for POPC-CHOL.

When CG was added to the Pluronics, and both were added to the model membrane systems, the fluidization of the model membrane was induced, larger than the CG alone, as observed by a decrease in the compressibility modulus at 30 mN/m, independent of which Pluronic is used (Table 4.5-3). Correspondingly, the limiting areas obtained for all CG/Pluronic mixtures increased after adding CG for all Pluronics except for F87 (due to the greater solubility as mentioned previously), a further indication of fluidization. This increase in fluidization is attributed to different interactions occurring between CG and Pluronic with DPPC-CHOL. The interaction between CG and Pluronics is noticeable when the fluidization of the membrane increased after adding CG compared to the Pluronics alone. This interaction between CG and Pluronics is referred to hydrophobic interaction (Schuetze and Mueller-Goymann 1993), which is suggested to be a very strong interaction. The addition of CG/Pluronic complex to the model membrane may have resulted in a greater size, which somehow makes the Pluronic to exclude the PPO block that was inserted into the membrane, resulting in minimizing the water contact. Another interaction which occurs between CG and Pluronics is through the formation of hydrogen bonding. This interaction affects the hydrophilic part of Pluronics, which results in a more fluidized membrane with hydrophilic Pluronics (F87 and F108) compared to hydrophobic Pluronics (L44 and P103).

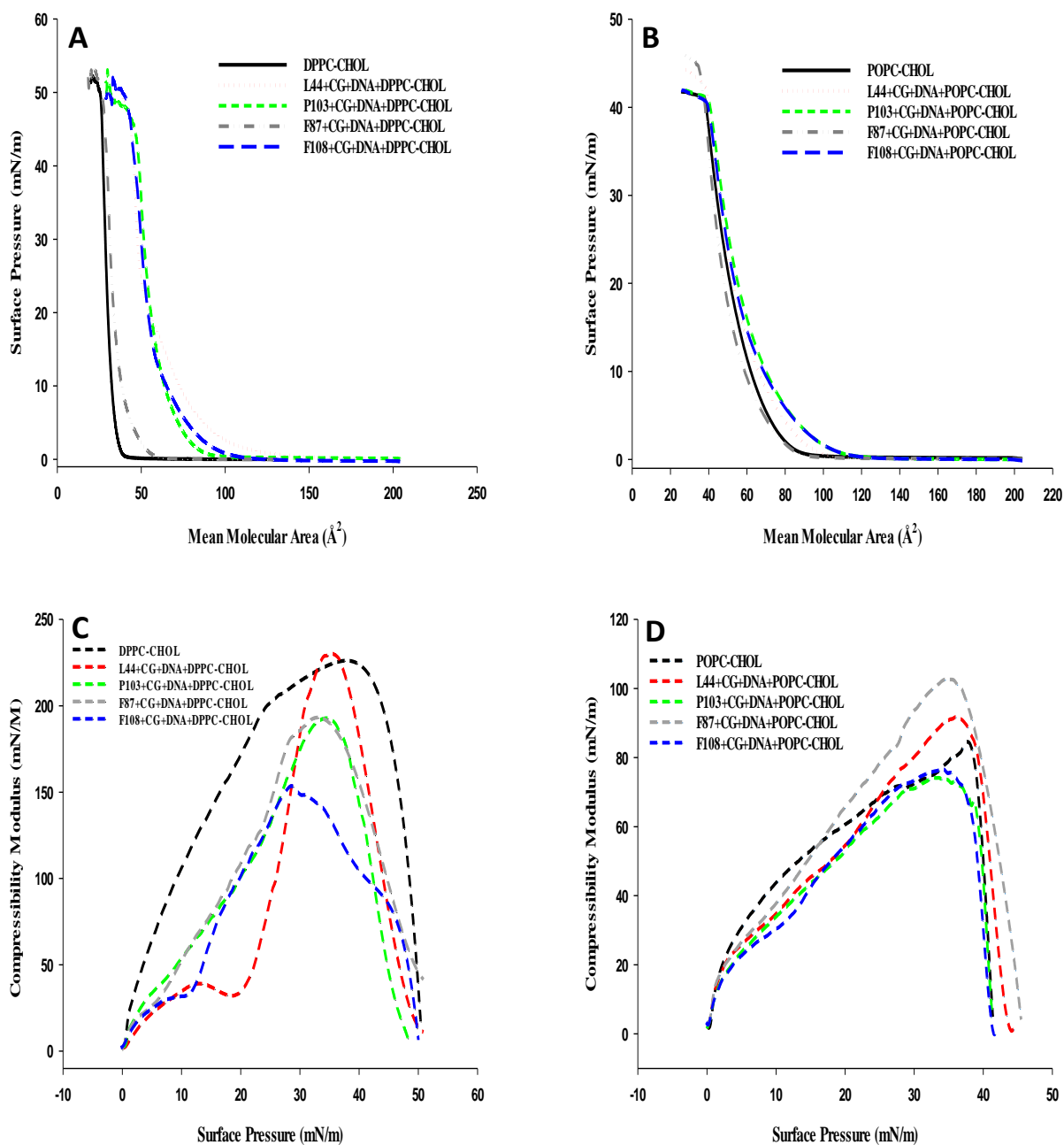
When the DNA was added to Pluronic/CG complex, the compressibility modulus increased with subsequent decreases in limiting areas for all Pluronic subsequently (Table 4.5-3 and Figure 4.5-7). It is apparent that DNA reduces the fluidization of membrane and results in the molecules becoming more closely packed. As mentioned previously, DNA can have either a fluidization or condensation effect, depending upon the nature of the monolayer (Antipina,

Schulze et al. 2007, Chen, Kang et al. 2012, Dabkowska, Barlow et al. 2012, Dabkowska, Barlow et al. 2014). In our project, the DNA has a condensation effect when is added to Pluronic/CG complex.

In terms of POPC-CHOL, CG induces the fluidization of the model membrane by decreasing the compressibility moduli and increasing the limiting areas for all Pluronics. It is clear from Table 4.5-3 that CG induced the fluidity of Pluronics that have longer hydrophobic units (P103 and F108) than Pluronics, which have shorter hydrophobics units (L44 and F87). This explains that hydrophobicity plays an essential role in the fluidity of model membrane POPC-CHOL.

The addition of DNA to the CG/Pluronic complex reduces the fluidization of the monolayers by increasing the compressibility moduli and decreasing the limiting areas (Table 4.5-3, Figure 4.5-7), which makes the molecules closely packed either with POPC-CHOL or DPPC-CHOL. These results confirm the condensation effect of DNA in our system.

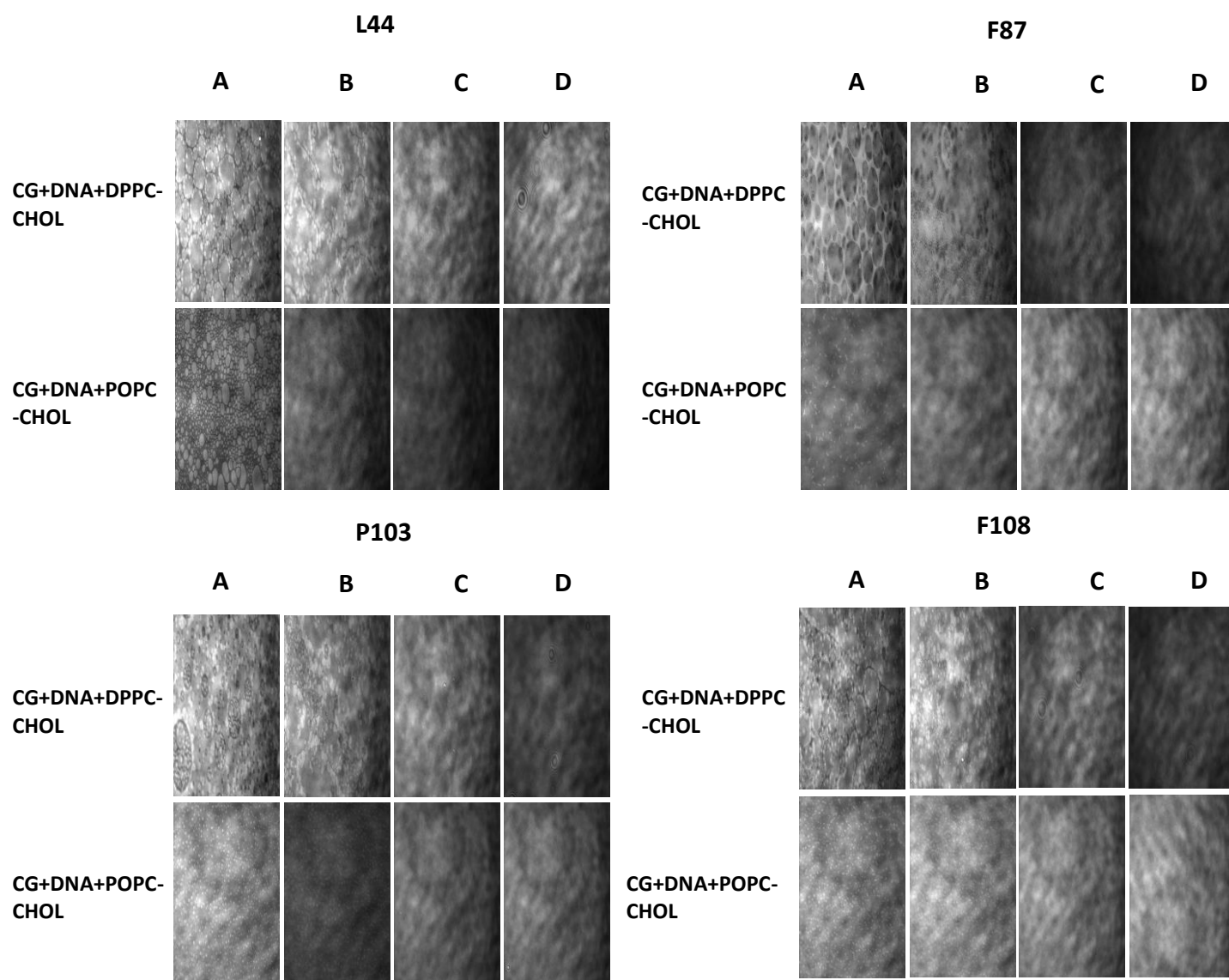




**Figure 4.5-7:** Isotherms (A, B) and compressibility modulus plots (C, D) of the monolayer alone, and monolayer with Pluronics, CG, and DNA. DPPC-CHOL (A, C) and POPC-CHOL (B, D). CG: Cationic gelatin.

Distinctive domains were observed when DPPC-CHOL was mixed with the Pluronic and CG either in the presence or absence of DNA (Figure 4.5-8). These domains are usually seen between the surface pressure 1-5 mN/m and they are different from CG/DNA domains alone, indicating the interaction between CG and Pluronics. The large domains that are observed might be referred to the model membrane or Pluronics, while the small white dots might be referred to CG. This confirms that when the surface pressure increases to 5 mN/m, the large domains disappeared, and what is left are only the white dots (with some lines), particularly in hydrophobic Pluronics L44 and P103. These shapes at surface pressure 5 mN/m might correspond to the hydrophobic interactions between Pluronic and CG, which are previously explained in the isotherm results. As already mentioned above, the domains of DPPC-CHOL are observed in surface pressure between 1-3 mN/m, which confirm that the white dots with lines are related to CG and Pluronics at surface pressure 5 mN/m. DNA did not show distinctive domains when interacting with DPPC-CHOL, indicating that the DNA may have a condensation effect only on the model membrane rather than with any other interaction. Upon the compression of the barriers, the domains gradually vanished until completely disappearing at a surface pressure of around 30 mN/m. These results are in agreement with the results including only Pluronic and model membrane alone, or CG and model membrane alone, indicating that there is a weak interaction between the Pluronics and the model membrane at high surface pressure. These outcomes were also mentioned in the isotherm results above.

Regarding the POPC-CHOL monolayer, featureless images were observed when the monolayer interacted with either Pluronic, CG, or DNA, at low or high surface pressure (Figure 4.5-8). This is due to the fluidization of the POPC-CHOL, which makes the interaction weaker than that of DPPC-CHOL. All systems demonstrated exactly the same domains. The systems showed small domains at low surface pressure, and then, when the surface pressure increased, the small domains disappeared, and were replaced by a grey background, indicating that all systems get fluidized when they interact with POPC-CHOL. L44 was the only Pluronic that showed a slightly greater domain formation. These domains may be formed due to the hydrophobicity, and the short number of EO units, which makes the PO units interact directly with POPC-CHOL at low surface pressure. These results are in agreement with the isotherms of the POPC-CHOL with the delivery system. The change in compressibility modulus, cross-sectional area, and molecular area at collapse is small and not remarkable.



**Figure 4.5-8:** BAM images of all Pluronic with CG and DNA with DPPC-CHOL and POPC-CHOL at different surface pressure. A)  $<1\text{ mN/m}$ , B)  $5\text{ mN/m}$ , C)  $30\text{ mN/m}$ , D)  $50\text{ mN/m}$  for DPPC-CHOL and  $40\text{ mN/m}$  for POPC-CHOL. CG: cationic gelatin

## 4.6 Summary

The Langmuir monolayer study, combined with Brewster's angle microscopy at the air/water interface, were conducted to in order to gain a better understanding of the interaction of our transfection complexes with simple models of a biological membrane. The DPPC-CHOL monolayers were found to be more rigid compared to the POPC-CHOL monolayers, consistent with literature results, as well as expectations based upon molecular structure. The addition of CG, the key component in our proposed transfection formulations, resulted in an increase in the fluidity of the monolayer for the DPPC-CHOL monolayers, and a decrease in fluidity for the POPC-CHOL. This is attributed to the electrostatic and the hydrophobic interaction of CG with the monolayers. The addition of DNA, alone (i.e., in the absence of CG or Pluronic) induced rigidity in both monolayers, likely due to the electrostatic and hydrophobic interactions with the lipid monolayer, however these interactions are expected to be negligible. The combination of CG and DNA again imparted a small degree of fluidity in both monolayers, due to the electrostatic interaction of DNA with CG and model membrane.

Ultimately, the addition of the complete CG/DNA/Pluronic formulation was observed to weakly induce the fluidization of the DPPC-CHOL and POPC-CHOL monolayers compared to CG/DNA alone. The extent to which fluidization was observed was dependent upon the Pluronic's structures, which have different hydrophilic and hydrophobic units. The strong hydrophobic interaction between Pluronic and CG allowed the DNA to induce the condensation of DPPC-CHOL, however this condensation effect was minimal, and did not affect the interaction with the model membrane. The results highlighted the fact that our

formulations interacted effectively and enhanced the permeability of the model membrane DPPC-CHOL, therefore and demonstrating a minimal effect on POPC-CHOL.

## **Chapter 5 Transfection Efficiencies and Cell Viability of Gelatin/Pluronic/DNA System in COS-7 Cells**

### **5.1 Introduction**

A positive charge was introduced onto the surface of gelatin nanoparticles to produce cationic gelatin (CG). Positive charge is very significant to interact with the negative charge of DNA and the cell membrane. Gelatin was selected because it has several advantages, as mentioned in Chapter 1. In addition, the positive results obtained by using gelatin as a delivery system encouraged us to use it in this project. In order to improve the transfection efficiency of CG/DNA complex, Pluronic block copolymers were added to protect the CG/DNA complex from degradation and to prolong the circulation time of the delivery system. Gemini surfactant (GS) 16-3-16 was also used in order to improve the transfection efficiency of CG/DNA complex. CG/DNA complex, along with GS and four different Pluronics (L44, F87, P103, and F108), were tested *in vitro* to evaluate the transfection efficiency and cell viability in COS-7 cells. COS-7 cells (ATCC® CRL165™) are fibroblast-like cells derived from African monkey kidney tissue. COS are adherent cells carrying SV40 genetic material and obtained by immortalizing CV-1 cells (Jensen, Girardi et al. 1964, Gluzman 1981). The combination of fibroblast-like growth and SV40 make COS-7 a great choice for transfection experiments using plasmid DNA and mutation to the SV-40 virus. COS-7 also possesses easily-transfected cells, which is the reason it was selected for our experiments.

## **5.2 Materials**

Gelatin type A Protein content 81% Bloom ~300 (Refer to chapter 3 for the gelatin and cationic gelatin). Gemini surfactant 16-3-16 was synthesized and purified in our lab according to published methods (Wettig and Verrall 2001, Wang and Wettig 2011). Gibco DMEM high glucose, pyruvate, L-glutamine Phenol Red Sodium Pyruvate, and Fetal Bovine Serum (FBS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Dulbecco's Phosphate Buffered Saline (DPBS), and 0.25% Trypsin- EDTA (1X) were purchased from Gibco by Life Technologies. COS-7 (ATCC® CRL-1651™) African green monkey kidney fibroblast-like was purchased from the American Type Culture Collection (ATCC) in Manassas, VA, USA. JetPEI™ cationic polymer transfection reagent was purchased from PolyPlus. Tryple Express reagent (no phenol red) (1X) was purchased from Life-Technology Inc. Nunc Cell Culture Treated multi-dishes, 6-well, BD 5 mL polystyrene round bottom test tube (FACS tube), Propidium iodide (PI) 95%, and Gibco Opti-MEM™ I Reduced Serum Medium (no phenol red) were purchased from Fisher Scientific. Highly-purified Millipore-Q water (18 mΩ cm and 72.6 mN/m at 20°C) was obtained from a Milli-Q filtration system in the School of Pharmacy, University of Waterloo.

## **5.3 Method**

### **5.3.1 Transfection efficiency and cell viability studies**

COS-7 cells (ATCC) were grown to 70-80% confluency in 75-cm<sup>2</sup> tissue culture flasks in DMEM-high glucose medium, supplemented with 10% FBS. Cells from passage number 5 to 15 (0.5 x 10<sup>6</sup> COS-7 cells) per well, in DMEM high glucose media with FBS, were seeded in a 6-well plate, one day before the transfection, in order to allow the cells to adhere to the



bottom of the wells. The DMEM (+FBS) medium was changed to DMEM medium (without FBS) one hour prior to transfection. The cells were then transfected with *pTGINF-GFP* plasmid using jetPEI<sup>®</sup> (Polyplus-transfection<sup>™</sup>) as a positive control. For each well, 2 µg of plasmid was used for all formulations, except for the one with positive control. The transfection method of jetPEI<sup>®</sup> followed the manufacturer's protocol and was optimized for the COS-7 cells. 3 µg plasmid was briefly mixed with 6 µL jetPEI<sup>®</sup> reagent in 100 µL 150 mM NaCl and was incubated at room temperature for 15 to 30 minutes. After incubation, the jetPEI<sup>®</sup>/DNA mixture was added dropwise to the treated cells covered with 2 mL of serum-free medium.

GS and CG were mixed with the plasmid DNA separately in Opti-Mem, and were incubated at room temperature for 15 minutes. The required amount of the Pluronic solution was then added, and the mixture was incubated for a further 30 minutes. The mixture was then added dropwise to the treated cells. After five hours, the transfection mixture in each well was replaced with 2 mL of DMEM (+FBS) medium, and the cells were then incubated for 48 hours in a tissue culture incubator at 37°C with 5% CO<sub>2</sub>. Transfections experiments were conducted using the following conditions (Table 5.3-1).

**Table 5.3-1:** Conditions used for transfection experiments

<b>Condition name</b>	<b>Condition used</b>
Preparation medium	Opti-Mem
Serum	Serum-free
Concentration of DNA	2 $\mu\text{g}$ for cationic gelatin-Pluronic & 3 $\mu\text{g}$ for the positive control
Incubation time after transfection	5 hours
Incubation time after changing the medium to perform FACS	48 hours
Ratios of prepared nanoparticles	From 1: 20-300 of DNA to CG

### **5.3.2 Fluorescence-activated cell sorting (FACS)**

Two days post-transfection, 2 mL of DMEM (+FBS) medium in each well was aspirated, and 1 mL of Tryple Express<sup>®</sup>/Trypsin was added to detach the cells. Then, 3 mL of DMEM (+FBS) medium was added to neutralize the Tryple Express/ Trypsin. Afterward, the cells were collected in 5 mL fluorescence-activated cell sorting (FACS) tubes and centrifuged at 4 °C at 125 ×g for 10 minutes. The GFP expressing cells were then washed twice with a phosphate buffered saline (PBS) after centrifugation and were suspended in 350 µL of PBS for FACS analysis. FACS measurements were carried out using a BD FACSAria Fusion 3-laser instrument in the Department of Biology at the University of Waterloo. Just prior to analysis, the samples were stained using propidium iodide (PI) in order to determine the cell viability, along with the transfection efficiency. Transfection efficiency was presented corresponding to the percentage (%) of cells displaying the EGFP expression out of 10,000 cells. Cell viability was expressed as the percentage (%) of dead cells, determined by PI staining. The transfection formulations were prepared according to Table 5.3-2.

**Table 5.3-2:** Transfection formulations template for each well

<b>Formulation</b>	<b>V<sub>plasmid</sub></b>	<b>V<sub>GS</sub></b>	<b>V<sub>CG</sub></b>	<b>V<sub>Pluronic</sub></b>	<b>V<sub>media</sub></b>	<b>V<sub>JetPEI</sub></b>	<b>V<sub>well</sub></b>
<b>Jet+DNA</b>	16.6 (3 µg)				400	12 µL	200
<b>Plasmid Only</b>	6.6(2.4 µg)				300		250
<b>GS only</b>		24			300		250
<b>Gelatin only</b>			40		300		250
<b>Gelatin+DNA</b>	6.6		40		300		289
<b>GS+DNA (2 µg) 1:10</b>	6.6	24			300		276
<b>GS+L44+DNA (2 µg)</b>	6.6	24		47.4	300		315
<b>GS+F87+DNA (2 µg)</b>	6.6	24		42	300		310.5
<b>GS+F108+DNA (2 µg)</b>	6.6	24		18	300		290.5
<b>GS+P103+DNA (2 µg)</b>	6.6	24		9	300		283
<b>CG+L44+DNA</b>	6.6	24	40	47.4	300		328
<b>CG+F87+DNA</b>	6.6	24	40	42	300		324
<b>CG+F108+DNA</b>	6.6	24	40	18	300		304
<b>CG+P103+DNA</b>	6.6	24	40	9	300		296
<b>GS+CG+L44+DNA</b>	6.6	24	26.88	47.4	300		337.4
<b>GS+CG+F87+DNA</b>	6.6	24	26.88	42	300		333
<b>GS+CG+F108+DNA</b>	6.6	24	26.88	18	300		313
<b>GS+CG+P103+DNA</b>	6.6	24	26.88	9	300		305

NOTE: ALL volumes are in µL; V<sub>plasmid</sub> corresponds to the volume of plasmid stock solution; V<sub>GS</sub> corresponds to the volume of gemini surfactant stock solution; V<sub>CG</sub> corresponds to the volume of cationic gelatin stock solution; V<sub>Pluronic</sub> corresponds to the volume of pluronic stock solution; V<sub>media</sub> corresponds to the volume of media; and V<sub>JetPEI</sub> corresponds to the volume of JetPEI transfection mixture. All components were combined, and wells were then treated with a volume of solution corresponding to V<sub>well</sub>.

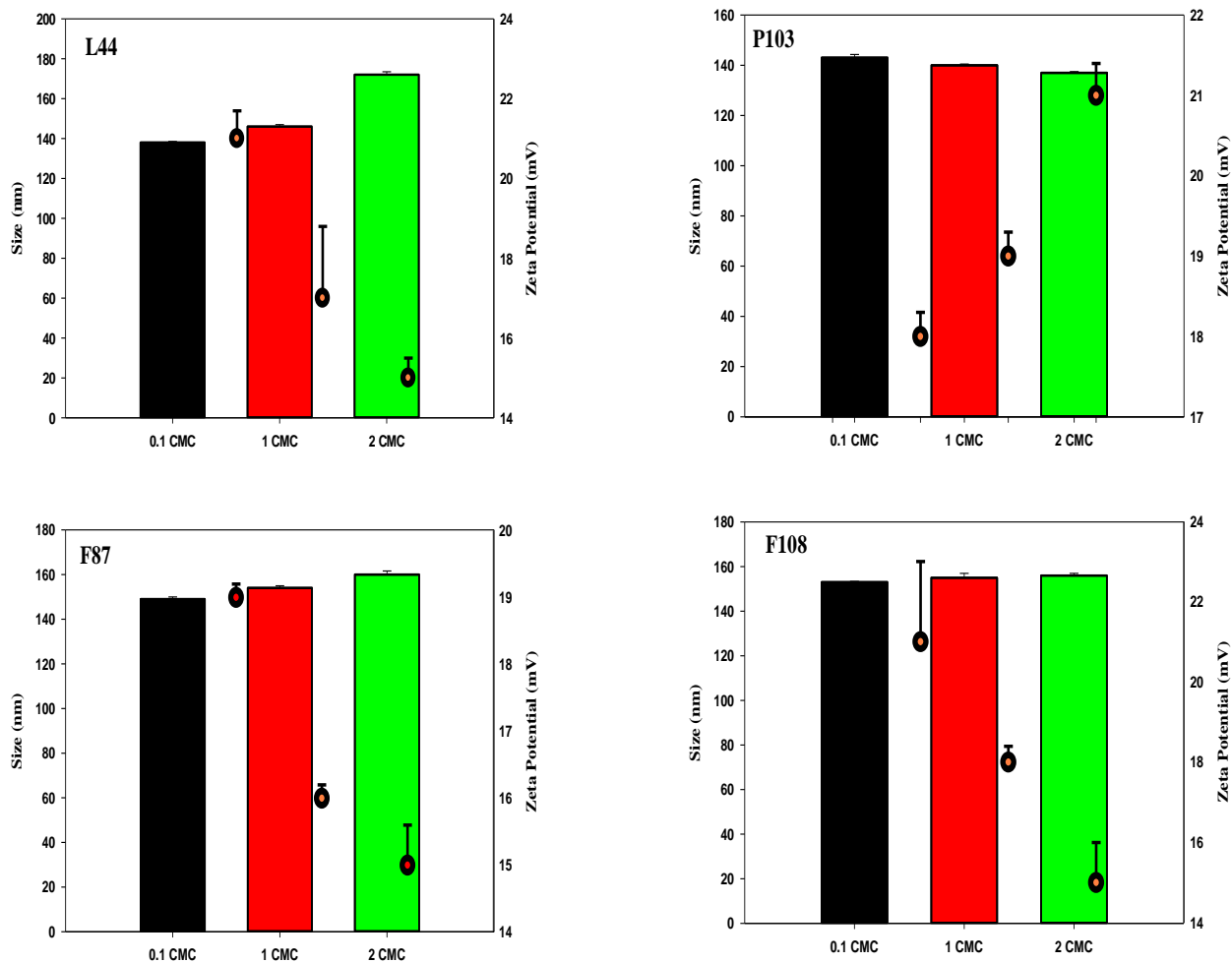
#### 5.4 Statistical Analysis

All the presented data in the *in vitro* transfection studies section correspond to the mean of three determinations; the results were compared using One-Way Analysis of Variance (ANOVA); test significance was accepted at  $P < 0.05$ .

## 5.5 Results and Discussion

### 5.5.1 Particle size and zeta potential

In this project, we were able to design a nanoparticle gene delivery system comprised of CG, DNA, and Pluronic block copolymers. The particle size and zeta potential of these systems were measured and demonstrated small particle size and positive zeta potential values for all formulations (Figure 5.5-1). Nanoparticles were prepared at a ratio of 1:20 (w/w) of DNA to CG and demonstrated the lowest diameter with the greatest positive charge. Adding more CG to the DNA did not change the positive value of the complex and led to larger-sized particles (Chapter 3). The particle size and zeta potential for nanoparticles containing the four Pluronics (L44, F87, P103, and F108) at concentrations corresponding to  $0.1\times\text{CMC}$ ,  $1\times\text{CMC}$ , and  $2\times\text{CMC}$  were also measured (Figure 5.5-1). It is clear from the data that all formulations containing Pluronic demonstrated small particle sizes ( $\sim 140\text{-}170\text{ nm}$ ) and positive zeta potentials. Not surprisingly, the zeta potential decreased with an increased concentration of the Pluronics due to shielding provided by the neutral Pluronic copolymer adsorbed to the surface of the CG/DNA complex. P103 was a clear exception to this behavior, where the zeta potential increased with the increased P103 concentration. While both L44 and P103 are similarly hydrophobic in nature, P103 is much larger with a very low CMC. It is possible that the interactions between the CG/DNA complex and P103 are not sufficiently strong to provide sufficient protection of the hydrophobic PPO segment from water, and that as concentration of P103 increases, there is a preference for it to dissociate from the CG/DNA complex and form its own micellar aggregates that contain only P103. This would have a net result of increasing the zeta potential, as observed in our data.

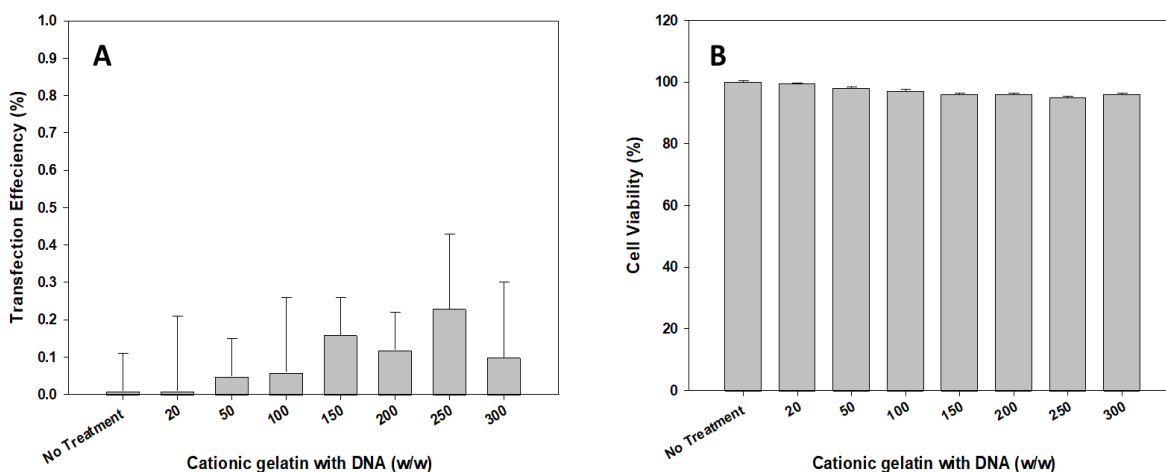


**Figure 5.5-1:** Particle size and zeta potential of nanoparticles containing cationic gelatin, DNA, and the indicated Pluronic at concentrations corresponding to  $0.1 \times \text{CMC}$ ,  $1 \times \text{CMC}$ , and  $2 \times \text{CMC}$ . Bars represent particle size, dots represent zeta potential. Samples were measured in triplicate, errors are equal to standard deviation.

### 5.5.2 Transfection efficiency and cell viability of cationic gelatin/Pluronic/DNA

Initial transfection studies were carried out using nanoparticles prepared using only DNA and CG at a ratio of 1:20 (w/w) DNA to CG. No transfection was observed using this system, in

contrast to the results obtained by Kaul and Amiji (Kaul and Amiji 2005), who found that gelatin nanoparticles were observed around the nucleus six hours after incubation. Our initial

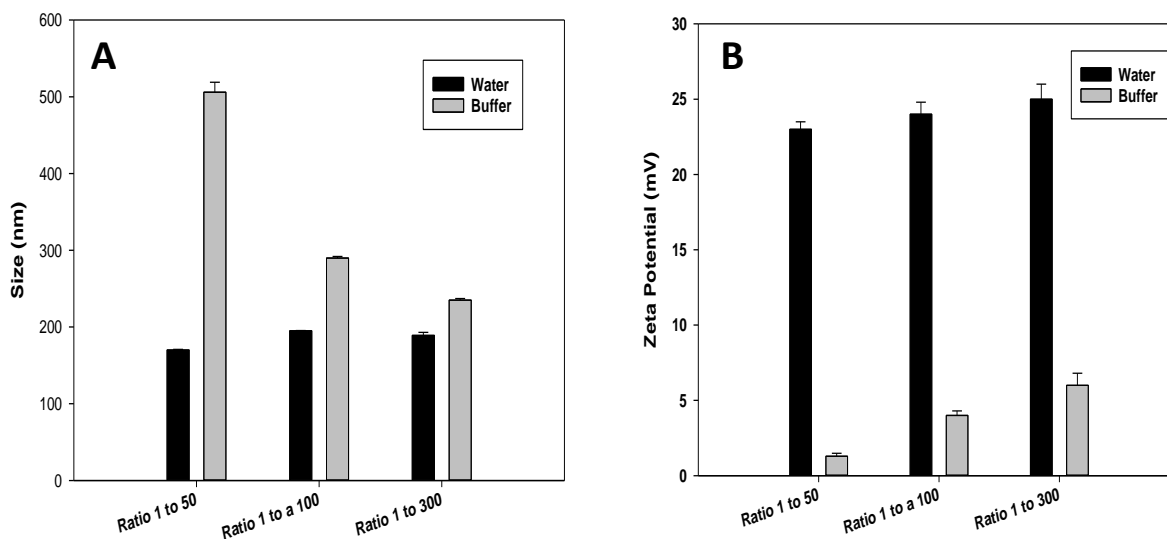


**Figure 5.5-2:** Transfection efficiency in % (expressed in terms of EGFP expression of live cells) (A), and cell viability in % (expressed in terms of dead cells stained by PI) (B) of cationic gelatin and DNA using different w/w ratios in COS-7 cells.

incubation time was five hours; upon increasing it to the six hours used by Kaul and Amiji, the same lack of transfection was observed. The experiment was subsequently run using different ratios of DNA to CG in order to determine if a 1:20 ratio was sufficient. Ratios of 1:20, 1:50, 1:100, 1:150, 1:200, 1:250, and 1:300 were all examined; unfortunately, all these concentrations failed to show any significant gene expression (Figure 5.5-2).

At this point, it was decided to re-examine the particle size and zeta potential of the nanoparticles, this time in buffer so as to determine if buffer had an impact on the formulations. The particle size of the formulations prepared in buffer were both larger and exhibited multiple peaks (corresponding to multiple populations of particle sizes) compared to those prepared in water, indicating aggregation of the nanoparticles in the presence of

buffer (Figure 5.5-3). As a polypeptide, gelatin is very sensitive to pH, due in part to its amino acid composition; this might be the reason for the observed aggregation in the presence of buffer (Misra, Meher et al. 2016). Even though the CG/DNA nanoparticles possessed substantial positive charge in water, this was largely neutralized in buffer, even for nanoparticles containing the highest amount of CG (1:300). It is likely that the lack of transfection can be attributed to aggregation in the presence of buffer; confocal microscopy studies would be useful to confirm this. The one positive result in these initial transfection studies is that even at the high DNA:CG ratios (i.e., 1:300), low cell toxicity was observed; however, with the lack of transfection, this observation is not particularly useful.



**Figure 5.5-3:** Particle size (A) and zeta potential for nanoparticles containing different ratios of DNA to cationic gelatin in water (black) and buffer (grey). Samples were measured in triplicate, errors are equal to standard deviation.



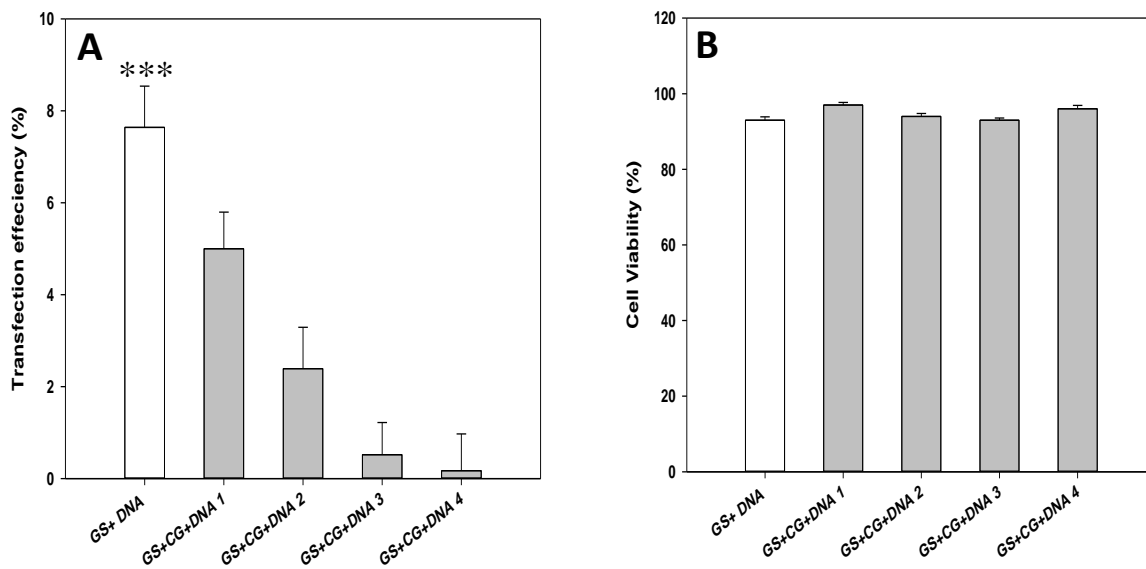
While the above results clearly suggest that aggregation of the DNA/CG complexes was the reason for the lack of transfection, it is also possible that cholamine may not have been the proper agent to cationize gelatin, as it may interact poorly with the DNA, thus resulting in weak complexation. Similar results for gelatin nanoparticles modified with polyamines, such as spermidine and spermine, also failed to efficiently complex pDNA in buffer (Zwiorek 2006). Cationic agents with a stronger positive charge, such as PEI, might be required to complex the DNA and to facilitate the passage of the negatively-charged cellular membrane (Kuo, Huang et al. 2011). The batch of gelatin nanoparticles itself is probably another factor that could affect the transfection efficiency. Zwiorek (Zwiorek 2006) tried different batches of gelatin with different size and zeta that failed to adsorb the DNA, even when the batches were modified with cholamine. Consequently, loading nucleic acids on the surface of CG nanoparticles could not be generalized. What successfully worked with a certain nucleic acid was not necessarily successful with another. Based on these outcomes, we decided to add gemini surfactant (GS) to our formulations.

### **5.5.3 Gemini surfactant and cationic gelatin**

As mentioned in Chapter 1, GSs can form micelles at low critical micelle concentrations (CMC). GS also possesses two positive charges, giving rise to strong electrostatic interactions with plasmid DNA as well as with the cellular membrane, which contributes to their effectiveness as transfection vectors. In this project, it was thought that adding GS would improve the transfection efficiency of CG. GS 16-3-16 was chosen due to the previous results of transfection efficiency obtained in previous studies, both in our group and among

other teams (Foldvari, Badea et al. 2006, Badea, Wettig et al. 2007, Wang, Kaur et al. 2013, Madkhali 2014).

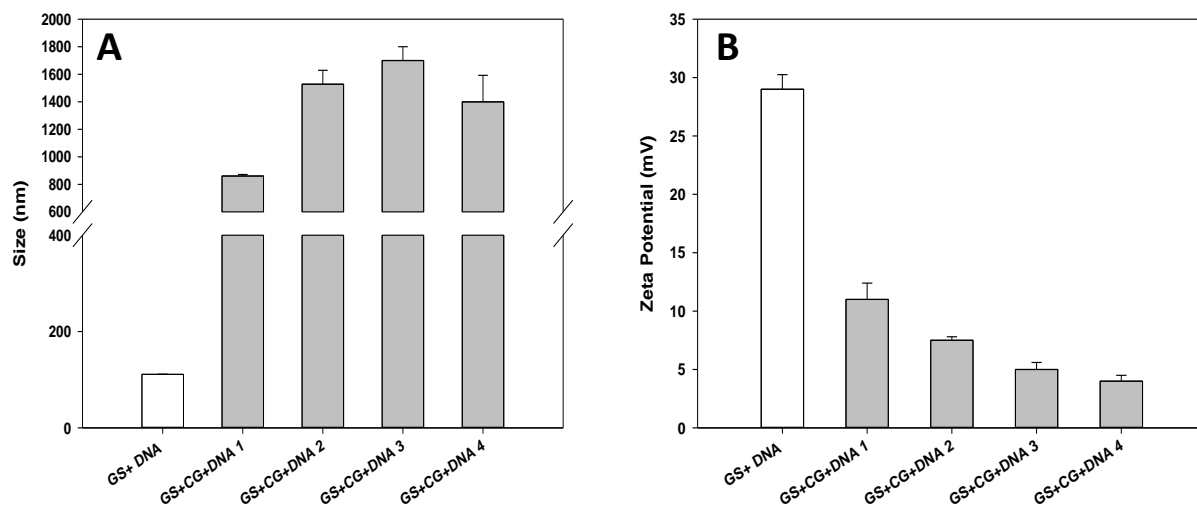
The first detectable gene expression was seen with GS at the ratio of 1:10 DNA to GS. CG was then added to determine if it would increase the transfection efficiency of the 16-3-16 surfactant. Four different w/w ratios of GS to CG were tested (1:1, 1:2, 1:3, and 1:4). The results revealed that as more CG was added, the lower the transfection became, while cell viability increased (Figure 5.5-4). In order to understand these results, examination of particle size and zeta potential were conducted for GS/CG complex in buffer.



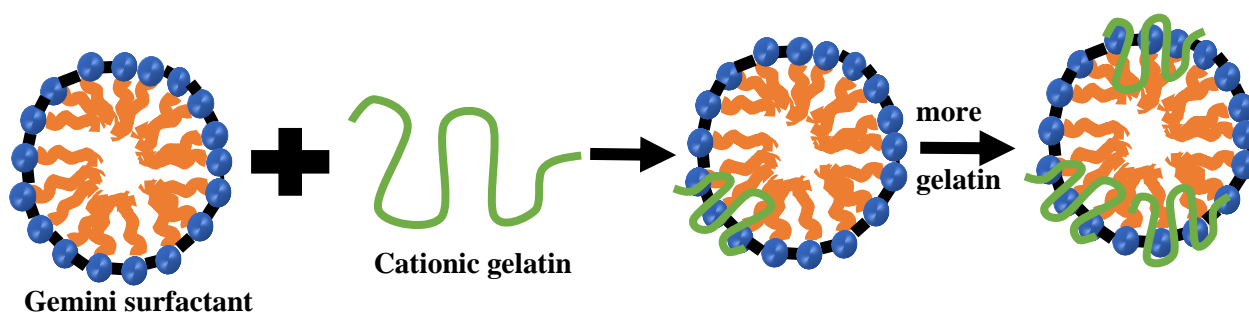
**Figure 5.5-4:** Transfection efficiency in % (expressed in terms of EGFP expression of live cells) (A), and cell viability in % (expressed in terms of dead cells stained by PI) (B) of different w/w ratios of gemini surfactant and cationic gelatin (1:1, 1:2, 1:3, and 1:4) in COS-7 cells. GS is significantly different to CG at all ratios (\*\*\*) $P < 0.0001$ ).

The results showed that adding CG to GS enhanced the aggregation of the complexes.

Particle size increased after adding CG from 110 nm to ~ 1800 nm (Figure 5.5-5), while a corresponding decrease in zeta potential (i.e., a decrease in the positive charge of the complexes) was seen when increasing the amount of CG, which probably led to antagonistic interaction between GS and CG. It would seem that the addition of the CG results in a shielding or partial neutralization of the positive that exists on the surface of the GS micelles, suggesting that the CG is localized at or near the surface of the surfactant aggregates. Once the surface charge is reduced, increased aggregation of the complexes can occur (Figure 5.5-6). It is this aggregation that is the likely source of the low transfection efficiencies observed for the GS/CG/DNA complexes.

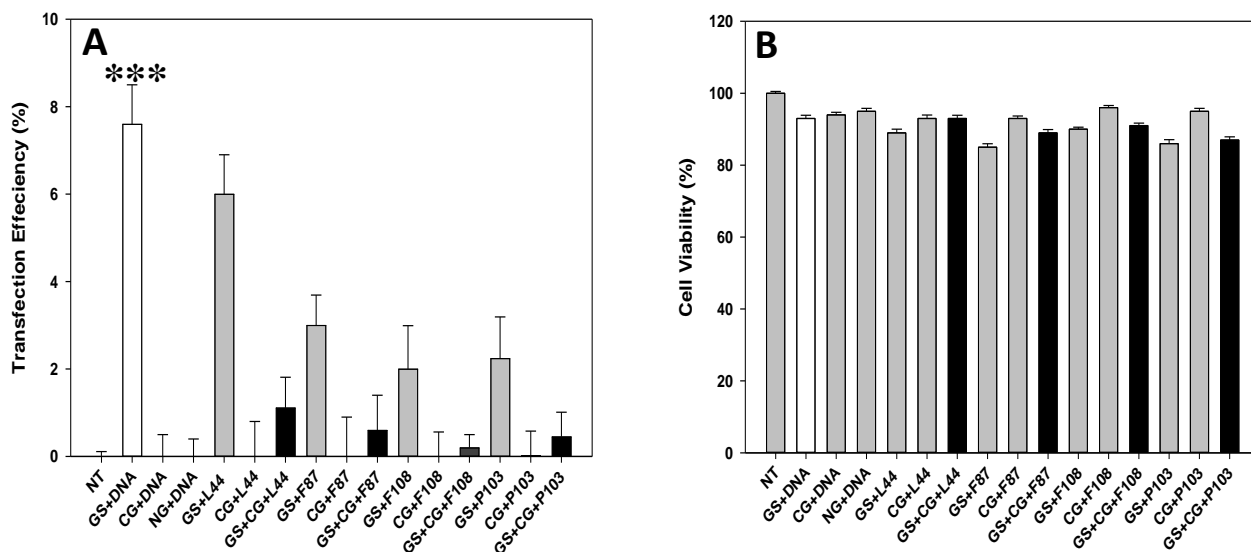


**Figure 5.5-5:** Size (A) and Zeta potential (B) of gemini surfactant and cationic gelatin at different w/w ratios (1:1, 1:2, 1:3, and 1:4). Samples were measured in triplicate, errors are equal to standard deviation.



**Figure 5.5-6:** Schematic illustration of the interaction between gemini surfactant and cationic gelatin. Gelatin forms a shield around the gemini surfactant, which results in aggregation and decreases the positive charge of gemini surfactant.

The last step of the transfection studies was to add Pluronics to the transfection complexes. It was believed that Pluronics could protect the DNA from degradation and increase the circulation time *in vivo*; that was, however, assuming that the CG was able to transfect DNA,



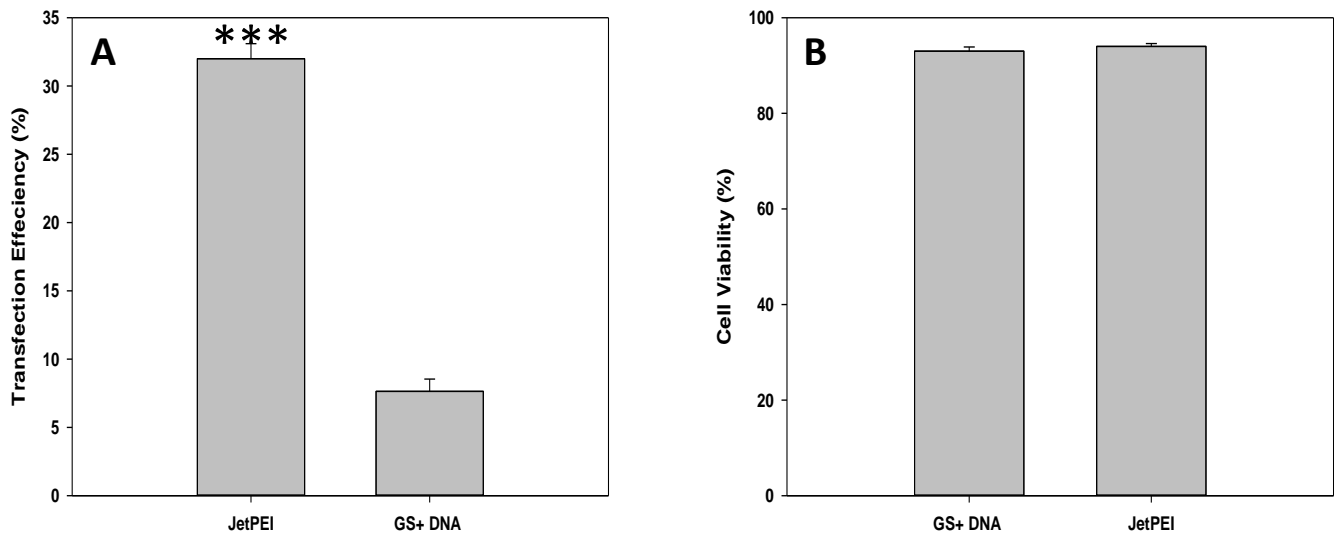
**Figure 5.5-7:** Transfection efficiency in % (expressed in terms of EGFP expression of live cells) (A), and cell viability in % (expressed in terms of dead cells stained by PI) (B) of different combinations of delivery system in COS-7 cells. NG represents native gelatin, CG represents cationic gelatin, and GS represents gemini surfactant. GS alone with DNA is significantly different to all other nanocomplexes containing Pluronics, CG, or both (\*\*\*P<0.0001).

which as discussed above – however, it was not. Figure 5.5-7 shows the combined results of transfection experiments using GS/DNA, CG/DNA, CG/GS/DNA, CG/Pluronic/DNA, and CG/GS/Pluronic/DNA complexes. Not surprisingly, the addition of Pluronics to CG did not result in transfection. Given that Pluronics are neutral polymers, it would not be expected that their addition would improve the transfection of the weak CG.

When added to the GS/DNA complexes, the Pluronics decreased the transfection efficiency of the GS, similar to what was observed by the addition of CG to the GS/DNA complexes (see discussion above). The Pluronic is expected to adsorb at the surface of the GS/CG/DNA complexes (in particular the EO segments), further shielding the positive charge of the complexes, again likely resulting in increased aggregation that then decreases the ability of the complexes to transfect the DNA.

One positive result seen in the transfection studies relates to cell viability. CG showed excellent cell viability in all systems; although this is most likely due to the weak cationic charge that seems to be imparted to the gelatin using our method. As it is known that cytotoxicity increases with increased positive charge (i.e., an increasing number of quaternary ammonium groups) (Vieira, Hartmann et al. 2008, Garcia, Kaczerewska et al. 2016), it is likely that this may not be the case where a more strongly CG is prepared.

Finally, our results were compared to the positive control jetPEI<sup>®</sup>, which showed approximately 32% gene expression compared to the highest transfection obtained by the GS alone (~ 8%) (Figure 5.5-8). We are still far away from the positive control and therefore, further studies are required to improve the transfection efficiency of CG/GS systems prior to introducing the Pluronics.



**Figure 5.5-8:** Transfection efficiency in % (expressed in terms of EGFP expression of live cells) (A), and cell viability in % (expressed in terms of dead cells stained by PI) (B) of the positive control (JetPEI) and GS/DNA complex in COS-7 cells . Positive control is significantly different than the highest transfection obtained with GS and DNA alone (\*\*\*P<0.0001).

## **5.6 Summary**

Transfection efficiency and cell viability of cationic gelatin/Pluronic/DNA were tested in COS-7 cells. The results showed that cationic gelatin is unable to transfect the DNA, either in the presence or absence of Pluronics, in water, or in buffer. From the results, it appears that choline-modified gelatin nanoparticles are not able to transfect DNA, most likely due to aggregation of the complexes upon addition of DNA. It is clear that a different method of introducing cationic groups into the structure of gelatin is required in order to optimize the potential for gelatin as a transfection agent. Alternatively, small peptide sequences could be coupled to the amino acid side-chains to improve the targeting, to enhance the cellular uptake, or to induce the permeability of the cells, and combine such a gelatin polymer with the GS, which was able to transfect DNA. For such a strategy to be successful, a means of blocking the aggregation of the GS/gelatin/DNA complexes would be required, possibly utilizing a layer-by-layer formulation strategy, where the GS/gelatin/DNA complexes are stabilized by an additional component. Ultimately, further studies are required to understand the interaction of the gemini surfactant and the gelatin, which seems to be an antagonistic interaction, based on our results.



## Chapter 6 Summary and Future Studies

### 6.1 Summary

Gelatin, as a natural polymer, is a promising agent for gene delivery due to several advantages that include biodegradability, biocompatibility, low cost, and the relative ease with which it can be functionalized. In this project, we hypothesized that gelatin-modified nanoparticles, in combination with the Pluronic block copolymers, could improve the transfection efficiency of DNA in COS-7 cells *in vitro*. The project started with the preparation of gelatin nanoparticles in Chapter 3 using the two-step desolvation method. The gelatin was then modified by adding a cationic agent (cholamine) in order to increase the positive charge, which is essential to interact with the DNA and the cellular membrane. Gelatin nanoparticles were successfully modified and characterized to confirm to the cationization. Cationic gelatin nanoparticles carry increased positive charge, with an average zeta potential of approximately 30 mV whereas unmodified gelatin nanoparticles have an average zeta potential of approximately 10 mV. SEM and TEM showed that the nanoparticles are spherical in nature, with an average diameter of ~100 nm. We also studied the characterizations of cationic gelatin and DNA in the presence and absence of Pluronics in terms of size and zeta potential in water. The results showed that cationic gelatin is able to complex the DNA by changing the negative charge of DNA to a positive charge at a ratio of 1:20. At the same ratio, the size of DNA (~ 450 nm) decreased to 160 nm after adding CG indicating a successful complexation. Adding Pluronic to CG/DNA complex resulted in a small size (140-170 nm) with a positive charge for all formulations. The size and the charge of CG/DNA/Pluronic varied depending on the type and the concentration of each Pluronic. A

study on the effect of transfection complex CG/Pluronic/DNA on the model membranes was outlined in Chapter 4. The objective of that chapter was to understand the interaction of the transfection complexes, and their components, with the biological membranes. Two types of the model membrane were used: 1) DPPC-CHOL at the ratio of 3:1 (which mimics a healthy biological membrane); and 2) POPC-CHOL at the ratio of 4:1 (which mimics a cancerous cell membrane). DPPC-CHOL formed a more condensed monolayer compared to POPC-CHOL. Interactions between DNA and the model membranes resulted in more condensed monolayers for both the DPPC-CHOL and POPC-CHOL systems, which were attributed to the electrostatic or the hydrophobic interactions. The addition of CG alone was then added to DPPC-CHOL. Due to both the electrostatic and the hydrophobic interactions, CG induced the fluidization of the membrane, which is important to enhance the permeability of a cellular membrane. The same effect was noted with Pluronic, either alone or in combination with CG, but to a lesser degree than CG. In terms of POPC-CHOL, not much change was noticed, neither with CG nor with Pluronics due to the fluidization of POPC-CHOL. Adding DNA induced the condensation of CG/DNA/Pluronic in both DPPC-CHOL and POPC-CHOL. These results indicate that CG/Pluronic/DNA complex was able to interact with the cellular membranes and enhanced their permeability. Further studies are required to fully understand the interaction between the CG, DNA, and the Pluronic.

The transfection efficiency and cell viability of the CG/Pluronic/DNA complexes were evaluated *in vitro* in the COS-7 cell line. Despite the observations from Chapter 4, which suggested successful disruption of biological membranes, the use of CG as a transfection agent failed to show any gene expression, despite being attempted under several conditions

and after several optimizations. The most likely reason is that the cholamine, used to provide the additional cationic character to the gelatin polymer, was not able to provide a sufficient degree of cationic character to be able to both bind and condense DNA, and to passively target the cell membrane. Other possible reasons could include the size of the DNA; however, this can be discounted as the size of the CG/DNA complexes (Chapter 3) are in fact comparable in size to the gemini surfactant/DOPE/DNA complexes previously studied by our group and others (Foldvari, Badea et al. 2006, Wang, Kaur et al. 2013). Other factors could include batch effects in the gelatin (i.e., variation in amino acid sequences that can occur between batches), as well as the method of introducing cationic groups into the structure of gelatin. Transfection with the cationic gelatin based nanoparticles was only achieved upon addition of the 16-3-16 gemini surfactant to our formulation. Transfection levels achieved with gemini surfactant in combination with the cationic gelatin system was at significantly lower levels than observed for the GS/DOPE systems reported elsewhere in the literature, indicating that not only was cationic gelatin insufficient in being able to transfect DNA on its own, but also that its presence in fact likely shielded the overall positive charge of the nanoparticles, ultimately decreasing transfection efficiency. The addition of Pluronics appeared to have no effect, and it may be that *in vivo* studies are required to determine whether or not any benefit was obtained from the inclusion of Pluronics in our nanoparticle formulation.

## 6.2 Future Studies

The first and most important step required before applying the following suggestion is to conduct a cellular trafficking study for the cholamine-modified gelatin nanoparticles. This research will help to understand if cholamine-modified gelatin nanoparticles are able to enter into the nucleus and whether or not DNA is able to dissociate from the nanoparticles, and whether or not it gets degraded; these are all possible alternative reasons for the observed low transfection. Following this study, many other experiments can be conducted in the future in order to design more effective vectors that are capable of delivering DNA into the cells.

Firstly, there are many other cationic agents other than cholamine that can be used for gelatin modification. These include, but are not limited to spermine, spermidine, putrescine, and ethylenediamine. Spermine showed favourable results as a transfection agent in several studies in the literature (Hosseinkhani, Aoyama et al. 2002, Konat Zorzi, Contreras-Ruiz et al. 2011, Zorzi, Parraga et al. 2011, Zorzi, Parraga et al. 2015). Since cholamine was chosen based on previous studies, it is worth trying other cationic agents, as gelatin batches differ from one to another. Short, cationic amino acid sequences, such as polylysine, can also be readily coupled to gelatin to provide an alternative strategy for cationization.

Secondly, gelatin nanoparticles were prepared using a two-step desolvation method. This method is simple and resulted in stable and homogenous nanoparticles. However, mixing DNA with gelatin nanoparticles might not form an effective and robust complex, as the DNA may readily detach from the surface of the nanoparticles, which could then result in the degradation of DNA through enzymatic degradation. Different methods can be used other than desolvation. For example, Zorzi et al. (Zorzi, Párraga et al. 2011) prepared cationic

gelatin nanoparticles using the ionic gelation technique for an ocular surface. In this technique, Zorzi and his colleagues used chondroitin sulfate (CS) and dextran sulfate (DS) to produce cationic gelatin. The cationized gelatin was then added to triphosphate (TPP) containing the plasmid and CS or DS. One advantage of this method is the fact that no organic solvents such as acetone were used, in comparison to two-step desolvation (Zorzi, Parraga et al. 2011).

Thirdly, the addition of PEG is important to protect the DNA from the *reticuloendothelial system* (RES) upon systemic administration. While Pluronics were used in our project to replace the function of PEG, it appears that the hydrophobic-hydrophobic interaction between Pluronic and gelatin decreased the effectiveness of the gelatin, which resulted in lower transfection efficiency. Since the interaction between gelatin and Pluronics is not completely understood, more studies are needed to understand this interaction before applying them to gelatin.

Fourthly, gelatin is a weak transfection agent when used on its own. Since gelatin is easily modified, many targeting ligands can be applied in order to target specific cell types through the receptor-ligand interaction. The most common example of these ligands is the epidermal growth factor receptor (EGFR), which has been used with gelatin nanoparticles and showed higher transfection efficiency in several types of cancer. Another example of ligands includes folate receptor, which can be used alone, with gelatin, or in combination with PEG-gelatin nanoparticles.

Finally, gemini surfactant was used in the transfection experiments, and the results showed that the interaction of cationic gelatin and gemini surfactant resulted in aggregation. As a result, we are interested in further studying this interaction to understand the reasons for this aggregation. Although the results of monolayer study showed that there is no correlation with the transfection results of the cationic gelatin and the Pluronic, it might be helpful to use the monolayer study to understand the interaction between gemini surfactant and cationic gelatin. This understanding will help in developing and improving the effectiveness of either gelatin or gemini surfactant, or both, as the science is about a building of information.

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