CHARACTERIZATION OF LIPOSOMAL DRUG DELIVERY SYSTEMS

UTILIZING CELL CULTURE METHODS

by

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(Department of Pharmacology & Therapeutics, Faculty of Medicine)

We accept this as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 2001

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The University of British Columbia
Vancouver, Canada

Date April 25, 2001
ABSTRACT

This thesis deals with the characterization of liposomal drug delivery systems utilizing cell culture methods. Drug bioavailability from liposomes is believed to be an important factor in determining the therapeutic activity of a drug. Therefore, the development of liposomes that ensure full drug bioavailability or deliver their contents to the cell cytoplasm are under investigation.

To develop liposomes capable of cytoplasmic delivery, it is necessary to have an assay procedure capable of distinguishing between cell surface binding and intracellular delivery. The research presented in this thesis addresses this central problem by developing assays that characterize liposomes specifically designed for cytoplasmic delivery.

One liposomal formulation designed for improved cytoplasmic delivery, termed programmable fusogenic vesicles (PFVs), has shown promising preliminary results in vivo. Initial studies examined the bioavailability of mitoxantrone, an anticancer agent, encapsulated in PFVs and conventional liposomes to assess drug-induced toxicity against a human cell line (HEK 293) and a murine cell line (L1210) in vitro. These studies demonstrated greater mitoxantrone cytotoxicity when the drug was encapsulated in PFVs as compared to conventional liposomes. Subsequent studies utilized a lipid-mixing assay employing the lipid probe Rhodamine-PE (Rh-PE) to characterize fusion of PFVs with cellular membranes. These studies demonstrated that PFVs were capable of fusion with HEK 293 cells.
An assay employing the nucleic acid dye, YOYO-1 iodide, was developed to quantitate cytoplasmic delivery to cultured cells. This novel assay was then used to compare the ability of PFVs to achieve cytoplasmic delivery. The results also demonstrated that cytoplasmic delivery of YOYO was greater when encapsulated within PFVs compared to other liposomal formulations. Moreover, the importance of individual lipid components in the PFV formulation to the cytoplasmic delivery of YOYO was established.

To assess the therapeutic potential of these PFV formulations, studies with antisense oligonucleotides (ASO) were completed. Intracellular delivery of fluorescent-labeled ASO encapsulated in PFVs was quantitated by flow cytometry and confirmed using fluorescence microscopy. Furthermore, an antisense oligonucleotide to the bcl-2 gene encapsulated in PFVs demonstrated down-regulation of mRNA levels by 20% compared to empty PFVs and free antisense in a human melanoma cell line (518A2). The development of PFVs for use in the cytoplasmic delivery of toxic drugs or biologically active nucleotide sequences is discussed.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xiii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xv</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>xvii</td>
</tr>
<tr>
<td>CHAPTER 1 - INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 LIPOSOMES</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1 Classification of Liposomes</td>
<td>3</td>
</tr>
<tr>
<td>1.1.1.1 Multilamellar Vesicles (MLVs)</td>
<td>3</td>
</tr>
<tr>
<td>1.1.1.2 Small Unilamellar Vesicles (SUVs)</td>
<td>5</td>
</tr>
<tr>
<td>1.1.1.3 Large Unilamellar Vesicles (LUVs)</td>
<td>5</td>
</tr>
<tr>
<td>1.1.2 Pharmacokinetics of Liposomes</td>
<td>6</td>
</tr>
<tr>
<td>1.1.2.1 Mononuclear Phagocyte System (MPS)</td>
<td>6</td>
</tr>
<tr>
<td>1.1.2.2 Factors Influencing Liposome Elimination</td>
<td>8</td>
</tr>
<tr>
<td>1.1.3 Liposome Interactions With Cells</td>
<td>9</td>
</tr>
<tr>
<td>1.1.4 Liposomal Formulations</td>
<td>13</td>
</tr>
<tr>
<td>1.1.4.1 Conventional Liposomes</td>
<td>13</td>
</tr>
<tr>
<td>1.1.4.2 Targeted Liposomes</td>
<td>15</td>
</tr>
<tr>
<td>1.1.4.3 Viral Fusogenic Liposomes</td>
<td>16</td>
</tr>
<tr>
<td>1.1.4.4 pH-Sensitive Liposomes</td>
<td>17</td>
</tr>
<tr>
<td>1.1.4.5 Cationic Liposome Complexes</td>
<td>18</td>
</tr>
<tr>
<td>1.1.4.6 Sterically Stabilized or Stealth Liposomes</td>
<td>19</td>
</tr>
<tr>
<td>1.2 PROGRAMMABLE FUSOGENIC VESICLES</td>
<td>20</td>
</tr>
<tr>
<td>1.2.1 Composition of PFVs</td>
<td>21</td>
</tr>
<tr>
<td>1.2.1.1 Non-Bilayer Forming Lipids</td>
<td>21</td>
</tr>
<tr>
<td>1.2.1.2 Cholesterol</td>
<td>24</td>
</tr>
<tr>
<td>1.2.1.3 Cationic Lipids</td>
<td>27</td>
</tr>
<tr>
<td>1.2.1.4 Polyethylene Glycol-Conjugated Lipids: Bilayer Stabilizing Lipid and Exchangeable Lipid Component</td>
<td>28</td>
</tr>
<tr>
<td>1.2.2 Hypothesized Mechanism of PFV Function</td>
<td>30</td>
</tr>
</tbody>
</table>
2.10 ANALYTICAL METHODS
2.10.1 Phospholipid Quantitation
2.10.2 Quantitation of Mitoxantrone Concentration
2.10.3 Quantitation of YOYO-1 Iodide Concentration
2.10.4 Quantitation of Antisense Oligonucleotide Concentration
2.10.5 Vesicle Size Analyses: Quasi-Elastic Light Scattering (QELS)

2.11 DOWN-REGULATION OF bcl-2 mRNA USING FREE ANTISENSE OLIGONUCLEOTIDES, ANTISENSE-LIPOSOME COMPLEXES OR ANTISENSE OLIGONUCLEOTIDES ENCAPSULATED IN PFVS

2.12 STATISTICAL METHODS

CHAPTER 3 - CHARACTERIZATION OF DRUG BIOAVAILABILITY: COMPARISON OF CONVENTIONAL LIPOSOMES AND PROGRAMMABLE FUSOGENIC VESICLES

3.1 INTRODUCTION

3.2 RESULTS
3.2.1 Determination of the Growth Rate of HEK 293 Cells
3.2.2 Determination of Cell Growth in 24-Well Plates and Viability Assay Design and Protocol
3.2.3 Comparison of Mitoxantrone Cytotoxicity Against HEK 293 Cells
3.2.4 Comparison of Mitoxantrone Cytotoxicity Against Murine L1210 Cells

3.3 DISCUSSION

CHAPTER 4 - CHARACTERIZATION OF CONTROLLED FUSION AND INTRACELLULAR DELIVERY BY PROGRAMMABLE FUSOGENIC VESICLES WITH CULTURED CELLS UTILIZING PHASE CONTRAST AND FLUORESCENCE MICROSCOPY

4.1 INTRODUCTION

4.2 RESULTS
4.2.1 Characterization of Controlled PFV Fusion with 293 Cells Utilizing Rh-PE Under Phase Contrast and Fluorescence Microscopy
4.2.2 Characterization of Intracellular Delivery by PFVs to 293 Cells Utilizing YOYO-1 Iodide Under Phase Contrast and Fluorescence Microscopy
4.2.3 Characterization of Intracellular Delivery by PFVs Containing PEG2000-Ceramides of Varying Acyl Chain Length

4.3 DISCUSSION

CHAPTER 5 - CHARACTERIZATION AND DEVELOPMENT OF A QUANTITATIVE CYTOPLASMIC DELIVERY ASSAY UTILIZING YOYO-1 IODIDE ENCAPSULATED IN PFVS

5.1 INTRODUCTION

5.2 RESULTS
   5.2.1 Biophysical Characterization of YOYO-Loaded PFVs
      5.2.1.1 Determination of an Optimal DNA Concentration for Maximum YOYO Fluorescence
      5.2.1.2 Development of a Standard Curve of YOYO Concentration
      5.2.1.3 Determination of YOYO Encapsulation Efficiency and Vesicle Size for PFVs of Varying PEG-Ceramide Acyl Chain Length
      5.2.1.4 Determination of YOYO Leakage from PFVs with PEG-Ceramide Varying Acyl Chain Lengths
   5.2.2 Characterization of Cytoplasmic Delivery to 293 Cells Utilizing YOYO-Loaded PFVs
      5.2.2.1 Influence of PFV Concentration on Cytoplasmic Delivery of YOYO
      5.2.2.2 Influence of “Sink” POPC:Chol Vesicles on the Cytoplasmic Delivery of YOYO Encapsulated in PFVs
      5.2.2.3 Comparison of Cytoplasmic Delivery by YOYO Encapsulated in PFVs and Free YOYO in the Presence of “Empty” PFVs

5.3 DISCUSSION

CHAPTER 6 - CHARACTERIZATION OF CYTOPLASMIC DELIVERY OF YOYO-1 IODIDE USING CONVENTIONAL LIPOSOMES, NON-FUSOGENIC LIPOSOMES AND PFVS TO TUMORIGENIC AND NON-TUMORIGENIC CELLS

6.1 INTRODUCTION

6.2 RESULTS
   6.2.1 Comparison of YOYO Delivery to 293 Cells with PFVs, Non-Fusogenic Cationic Vesicles and Conventional Liposomes
   6.2.2 Influence of DODAC Concentration on Intracellular Delivery of YOYO by PFVs
   6.2.3 Influence of PEG-Ceramide Acyl Composition on YOYO Delivery to 293 Cells
6.2.4 Comparison of YOYO Delivery to Tumorigenic and Non-Tumorigenic Cells Using PFVs

6.3 DISCUSSION

CHAPTER 7 - CHARACTERIZATION OF ANTISENSE OLIGONUCLEOTIDE DELIVERY UTILIZING PROGRAMMABLE FUSOGENIC VESICLES

7.1 INTRODUCTION

7.2 RESULTS
   7.2.1 Comparison in Delivery of Antisense Oligonucleotides Encapsulated in PFVs Against Free Antisense Oligonucleotides to Cells In Vitro
   7.2.2 Influence of PFVs Containing PEG2000-Ceramides of Varying Acyl Composition on Antisense Oligonucleotide Delivery to 293 Cells
   7.2.3 Examination of Antisense Intracellular Distribution Following Delivery by PFVs with PEG2000-Ceramides of Varying Acyl Composition to 293 Cells
   7.2.4 Antisense Effects on bcl-2 mRNA expression

7.3 DISCUSSION

CHAPTER 8 - SUMMARY

REFERENCES
# LIST OF FIGURES

| Figure 1.1: Liposome Classification                  | 4 |
| Figure 1.2: Phagocytosis of Liposomes by Cells of the Mononuclear Phagocyte System (MPS) | 7 |
| Figure 1.3: Liposome-Cell Interactions              | 11 |
| Figure 1.4: Classification of Liposomal Formulations | 14 |
| Figure 1.5: Lipid Components of Programmable Fusogenic Vesicles | 22 |
| Figure 1.6: Hypothesized Mechanism of Drug Delivery by Programmable Fusogenic Vesicles | 32 |
| Figure 1.7: Cell Viability/Cytotoxicity Assays      | 43 |
| Figure 1.8: Fluorescence Microscopy                 | 46 |
| Figure 1.9: Components of a Flow Cytometer and Light Scattering Parameters from a Cell Sample | 50 |
| Figure 1.10: Classes of Therapeutic Antisense Molecules | 55 |
| Figure 2.1: Expression of Flow Cytometric Data for Analysis | 71 |
| Figure 3.1: Structures of MTT and XTT Tetrazolium and Formazan. | 80 |
| Figure 3.2: Growth Rate of 293 Cell Line.          | 84 |
| Figure 3.3: XTT/Formazan Standard Curve of 293 Cells. | 86 |
| Figure 3.4: XTT/Formazan Standard Curve of L1210 Cells. | 87 |
| Figure 3.5: Design and Protocol of XTT-Based Cytotoxicity Assay | 89 |
| Figure 3.6: Comparison of Mitoxantrone Cytotoxicity Against 293 Cell Line. | 91 |
| Figure 3.7: Comparison of Mitoxantrone Cytotoxicity Against L1210 Cell Line. | 94 |
| Figure 4.1: Concept of Rh-PE Fusion Assay          | 101 |
Figure 4.2: Concept of YOYO Cytoplasmic Delivery Assay

Figure 4.3: Controlled Fusion of Rh-PE PFVs Containing of PEG2000-Ceramides of Varying Acyl Chain Lengths at 25μM Phospholipid Concentration

Figure 4.4: Controlled Fusion of Rh-PE PFVs Containing of PEG2000-Ceramides of Varying Acyl Chain Lengths at 50μM Phospholipid Concentration

Figure 4.5: Controlled Fusion of Rh-PE PFVs Containing of PEG2000-Ceramides of Varying Acyl Chain Lengths at 100μM Phospholipid Concentration

Figure 4.6: Intracellular Delivery of YOYO Encapsulated in PFVs

Figure 4.7: Intracellular Delivery of YOYO Encapsulated in PFVs Containing PEG2000-Ceramides of Varying Acyl Chain Lengths

Figure 5.1: Structure of Nucleic Acid Detection Dyes

Figure 5.2: Determination of an Optimal DNA Concentration for Maximum YOYO Fluorescence

Figure 5.3: Standard Curve of Fluorescence Intensity as a Function of YOYO Concentration

Figure 5.4: Determination of YOYO Leakage from PFVs with Varying Acyl Chain Lengths

Figure 5.5: Influence of PFV Concentration on Cytoplasmic Delivery of YOYO

Figure 5.6: Influence of POPC:Chol “Sink” Vesicles on the Cytoplasmic Delivery of YOYO Encapsulated in PFVs

Figure 5.7: Comparison of the YOYO Positive Viable Cell Populations With and Without “Sink” Vesicles

Figure 5.8: Comparison of Cytoplasmic Delivery by YOYO Encapsulated in PFVs Against Empty PFVs and Free YOYO

Figure 6.1: Comparison of YOYO Delivery to 293 Cells with PFVs, Non-Fusogenic Cationic Vesicles and Conventional Liposomes

Figure 6.2: Influence of DODAC Concentration on Intracellular Delivery of YOYO by PFVs

Figure 6.3: Influence of PEG-Ceramide Acyl Composition on YOYO Delivery to 293 Cells
Figure 6.4: Comparison of YOYO Delivery to Tumorigenic and Non-Tumorigenic Cells Using PFVs 149

Figure 7.1: Comparison in Delivery of Antisense Oligonucleotides Encapsulated in PFVs Against Free Antisense Oligonucleotides to Cells In Vitro 157

Figure 7.2: Influence of PFVs Containing PEG2000-Ceramides of Varying Acyl Composition on Antisense Oligonucleotide Delivery to 293 Cells 158

Figure 7.3: Examination of Antisense Intracellular Distribution Following Delivery by PFVs with PEG2000-Ceramides of Varying Acyl Composition to 293 Cells 160

Figure 7.4: Antisense Effects on bcl-2 mRNA Expression in 518A2 Cells Utilizing Cationic Liposome-Antisense Oligonucleotide Complexes 162

Figure 7.5: Antisense Effects on bcl-2 mRNA Expression in 518A2 Cells Utilizing Antisense Encapsulated in PFVs containing PEG2000-C14 Ceramide 164
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>IC_{50} Concentrations of Mitoxantrone Inducing Cytotoxicity on 293 Cells</td>
<td>90</td>
</tr>
<tr>
<td>3.2</td>
<td>IC_{50} Concentrations of Mitoxantrone Inducing Cytotoxicity on L1210 Cells</td>
<td>93</td>
</tr>
<tr>
<td>5.1</td>
<td>Comparison of YOYO-1 to Other Fluorescent Dyes When Bound to DNA</td>
<td>121</td>
</tr>
<tr>
<td>5.2</td>
<td>YOYO Encapsulation Efficiency in PFVs with Varying Acyl Chain Lengths and Their Respective Size Analyses</td>
<td>128</td>
</tr>
<tr>
<td><strong>ABBREVIATIONS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AIDS</td>
<td>acquired immune deficiency syndrome</td>
<td></td>
</tr>
<tr>
<td>BCECF</td>
<td>bis-carboxyethyl-carboxyfluorescein</td>
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</tr>
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<td>CHE</td>
<td>cholesteryl hexadecyl ether</td>
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<td>CHEMS</td>
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</tr>
<tr>
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</tr>
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<td></td>
</tr>
<tr>
<td>DABCO</td>
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</tr>
<tr>
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</tr>
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<td>DDAB</td>
<td>$N,N$-dimethyl-$N,N$-dioctadecylammonium bromide</td>
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</tr>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagles Media</td>
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<td>DMPC</td>
<td>1,2-dimyristoyl-$sn$-glycerol-3-phosphocholine</td>
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<td>DMRIE</td>
<td>dimyristooxypropyl dimethyl hydroxyethyl ammonium bromide</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<td>DNA</td>
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</tr>
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</tr>
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<td></td>
</tr>
<tr>
<td>DOTMA</td>
<td>dioleoyl propyl trimethyl ammonium chloride</td>
<td></td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>DPPC</td>
<td>1,2-dipalmitoyl-$sn$-glycerol-3-phosphocholine</td>
<td></td>
</tr>
<tr>
<td>DSPC</td>
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<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td>epithelial growth factor receptor</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
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<td>frozen and thawed MLVs</td>
<td></td>
</tr>
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</tr>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
<td></td>
</tr>
<tr>
<td>FITC-EGFR</td>
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<td></td>
</tr>
<tr>
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<td>green fluorescent protein</td>
<td></td>
</tr>
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</tr>
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<td>$H_{II}$</td>
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<td></td>
</tr>
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<td></td>
</tr>
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<td>HEPES</td>
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<td></td>
</tr>
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<td>MLVs</td>
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<td>MPS</td>
<td>mononuclear phagocyte system</td>
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<tr>
<td>mRNA</td>
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<td>PEG</td>
<td>poly(ethylene glycol)</td>
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<tr>
<td>PEG2000-Ceramide</td>
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<td></td>
</tr>
<tr>
<td>PEG-C$_{14}$ PFVs</td>
<td>programmable fusogenic vesicles containing poly(ethylene glycol)-C$_{14}$ ceramides</td>
<td></td>
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<td>programmable fusogenic vesicle</td>
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<td>phosphatidylserine</td>
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<td>QELS</td>
<td>quasi-elastic light scattering</td>
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<td>(2,3-bis[2-Methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carboxanilide)</td>
<td></td>
</tr>
<tr>
<td>YOYO-1 iodide; YOYO</td>
<td>1,1'-[1,3-propanediylbis[(dimethyliminio)-3,1-propanediyl]]bis[4-[(3-methyl-2(3H)-benzoxazolylidene) methyl]], tetraiodide</td>
<td></td>
</tr>
</tbody>
</table>
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DEDICATION

THIS ONE’S FOR MY FAMILY AND FRIENDS WHO WERE WONDERING WHAT I WAS DOING WITH MY TIME BUT I COULD NEVER EXPLAIN PROPERLY
CHAPTER 1
INTRODUCTION

Liposomes have been widely utilized as carriers of therapeutic drugs for a host of medical conditions. Although such carrier systems can greatly enhance drug delivery to disease sites, the subsequent bioavailability of the encapsulated drug is believed to be an important, if not the most important, factor in determining therapeutic activity. Therefore, the development of liposomal systems that ensure full drug bioavailability, or preferably, that deliver their contents directly into the cytoplasm of a target cell population has been the aim of many studies. However, it has been difficult to demonstrate, and then optimize, cytoplasmic delivery. Specifically, it is important to distinguish between intracellular delivery (which could include delivery to endosomes or phagosomes) and true cytoplasmic delivery. In addition, such investigations must be developed in the context of delivery systems that are viable pharmaceutical products, particularly with respect to applications, which involve intravenous administration of the associated drug.

The research presented in this thesis addresses the central problem of cytoplasmic delivery of a liposomally encapsulated therapeutic agent. Utilizing established techniques to characterize liposome-cell interactions, a new assay to selectively probe cytoplasmic delivery was developed to characterize an exciting liposome delivery technology termed programmable fusogenic vesicles (PFVs).

In an effort to place this research in context, this chapter describes liposomal drug delivery systems and previous cell culture studies utilized to investigate liposome-cell
interactions. This information, in turn, will lead to a focused discussion on PFV technology and the objectives of the research, which lead to this thesis.

1.1 LIPOSOMES

In the 1960's, Dr. Alec D. Bangham and his co-workers discovered that phospholipids, when combined with aqueous solutions, spontaneously formed vesicular structures (Bangham et al., 1965). These vesicles were subsequently termed liposomes and consist of one or more lipid bilayers surrounding an aqueous core. It was also shown that the addition of solute in the aqueous medium used to hydrate the phospholipids would result in encapsulation of the solute into the aqueous core (Bangham et al., 1965). Due to their ability to retain solutes within their aqueous core, liposomes have been used for studies as model membranes evaluating solute permeability (Bangham, 1968). Studies also involving liposomes have examined lipid properties, membrane function and protein-membrane interaction (Bangham, 1978).

In the 1970's, the focus on liposomes as carriers of therapeutic agents for \textit{in vivo} and \textit{in vitro} studies became widespread and is still ongoing. This was due to the observation that liposomes could preferentially accumulate at sites of inflammation, infection and some solid tumors (Proffitt \textit{et al.}, 1983; Patel \textit{et al.}, 1985; Ostro and Cullis, 1989). In addition, studies have shown that liposomes have the potential to decrease the toxicity and improve the therapeutic index of some drugs (Forssen and Tokes, 1983; Szoka, 1991; Gates and Pinney, 1993).

The first section of the Introduction will describe how liposomes are characterized, their fates when injected systemically, the mechanisms involved in
liposome interactions with cells, various formulations of liposomes and the limitations in their design. This will lead into Section 1.2, which describes the design of a novel liposomal system termed programmable fusogenic vesicles (PFVs).

1.1.1 Classification of Liposomes

There are three main classes of vesicles: multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs), as shown in Figure 1.1. These classes of liposomes can be distinguished based on the number of lamellae (or bilayers) present, size and method of preparation. These classifications are important because different classes of liposomes have very different suitability for drug delivery purposes, which will be discussed further in the following sections.

1.1.1.1 Multilamellar Vesicles (MLVs)

Multilamellar vesicles contain two or more concentric lamellae and range in size from 0.2-10µm. The advantages of MLV systems are that they are extremely easy to prepare. The preparation simply involves the addition of an aqueous solution to a dry lipid film followed by mechanical agitation. MLVs have also been shown to encapsulate a variety of molecules (Cullis et al., 1989). One disadvantage of MLVs, however, is that due to their large size they are very rapidly eliminated from the circulation following intravenous (i.v.) administration (Senior et al., 1985). The reason for this will be explained in Section 1.1.2.2.
Figure 1.1: Liposome Classification.

Schematic illustrations of (A) MLVs, (C) LUVs and (E) SUVs, as well as their respective freeze-fracture electron micrographs in (B), (D) and (F). The bar in the freeze-fracture electron micrographs represents 200 nm with the arrow indicating the direction of shadowing (adapted from Madden, 1997).
1.1.1.2 Small Unilamellar Vesicles (SUVs)

In contrast to MLVs, SUVs have a single bilayer and commonly range in size from 0.02-0.05μm. These vesicles are formed by sonication of a solution containing MLVs, which re-forms the MLV systems into single bilayer vesicles (Papahadjopoulos and Miller, 1967). Otherwise, SUVs can be formed by detergent dialysis (Allen et al., 1980; Lichtenberg et al., 1983). SUVs have low encapsulation efficiency due to their size and are eliminated from the circulation more rapidly than LUVs (Allen et al., 1991a). Due to the high radius of curvature in these vesicles, there is an asymmetric distribution of lipids in the outer and inner monolayer that can result in rapid destabilization of the vesicles (review by Pagano and Weinstein, 1978). Therefore, SUVs have not been commonly used as drug delivery systems.

1.1.1.3 Large Unilamellar Vesicles (LUVs)

LUVs also consist of a single bilayer and typically range in size from 0.05-0.4μm. These systems can be prepared from MLV preparations by a well-established extrusion procedure. This involves forcing the MLVs through polycarbonate filters of a defined pore size under nitrogen gas pressure (Hope et al., 1985; Mayer et al., 1986). In vivo studies have suggested that vesicles in the size range of 0.1μm-0.4μm have prolonged half-lives in circulation compared with larger vesicles and have exhibited optimal tumor localization and retention (Fidler et al., 1980; Allen et al., 1989; Gabizon et al., 1990). An explanation for these results will be described in the following section. A large majority of liposomal formulations intended for in vivo drug delivery applications utilize LUVs because they are more stable and are eliminated more slowly from the circulation.
than SUVs and MLVs. However, as indicated in the following section, the elimination behaviour of LUVs is highly dependent on liposomal lipid composition.

1.1.2 Pharmacokinetics of Liposomes

Studies have examined the pharmacokinetics of liposomes following intravenous injection, including the biodistribution of vesicles and the factors involved in elimination from the plasma compartment (Juliano and Stamp, 1975; Senior, 1987). The following sections will elaborate on the findings from these studies.

1.1.2.1 Mononuclear Phagocyte System (MPS)

The major organs of the mononuclear phagocyte system (MPS) are the liver, lungs, spleen and bone marrow. This system was formerly referred to as the reticuloendothelial system (RES), but the involvement of monocytes in the mechanism of liposome clearance has since been recognized (Senior, 1987; Allen and Hansen, 1991).

The MPS has a role as an important host defense system. Cells of the MPS, such as monocytes and macrophages, are responsible for removal of dead, senescent, foreign or altered cells, microorganisms and neoplastic cells. When liposomes enter the bloodstream, they can be bound by opsonins (Figure 1.2). Opsonins are a diverse group of plasma proteins (complement component C3b, immunoglobulins, "non-specific" polypeptides such as fibronectin or lysozyme) that are recognized by phagocytic macrophages (Bradfield, 1977; Kao and Juliano, 1981). The liposomes bound with opsonins are recognized as targets for removal by macrophages. Kupffer cells, abundant macrophages located in the liver, are primarily responsible for the removal of liposomes.
Figure 1.2: Phagocytosis of Liposomes By Cells of the Mononuclear Phagocyte System (MPS)

Generalized illustration of opsonins binding to a liposome resulting in the subsequent recognition of the liposome by a macrophage. Consequently, the liposome is phagocytosed, or engulfed, by the macrophage and cleared from the circulation into the organs of the MPS.
1.1.2.2 Factors Influencing Liposome Elimination

The attributes of liposomes that influence their elimination rate are vesicle size, lipid composition and lipid dose. By altering these parameters, liposomes can be designed so that their elimination rates vary dramatically. One primary goal when designing liposomes for in vivo use is to enhance their duration in the bloodstream providing increased opportunities for accumulation at disease sites.

As mentioned in Section 1.1.1., liposomes can be produced in various size distributions by techniques such as sonication, detergent dialysis and extrusion. Controlled size distributions can allow for enhancement of circulation times and extravasation (Papahadjopoulos, 1999). In general, as the diameter of the liposome increases, the elimination rate increases due to increased serum opsonin binding (Allen and Everest, 1983; Liu et al., 1995).

Lipid composition is another major variable affecting the elimination of liposomes from the plasma compartment. Vesicle charge and bilayer fluidity, or rigidity, are two factors dependent on the lipid composition that need to be considered. Some studies show that neutral liposomes exhibit longer circulation lifetimes than liposomes with a net surface charge (Juliano and Stamp, 1975; Miller et al., 1998). This observation might be related to the greater affinity of the charged vesicles for serum proteins (Juliano and Lin, 1980; Cullis et al., 1989). Liposomes composed of saturated lipids and cholesterol have very rigid bilayers which reduces opsonin binding and hence elimination rate (Moghimi and Patel, 1989). Lipid conjugates such as polyethylene glycol conjugated to phosphatidylethanolamines (PEG-PEs) have been used as surface modifying agents on liposomes to decrease liposome elimination rates. The PEG-lipid
conjugates act as a steric barrier to protein binding and thus reduce macrophage uptake \textit{in vivo} (Allen and Chonn, 1987; Allen \textit{et al.}, 1991; Allen and Hansen, 1991; Papahadjopoulos \textit{et al.}, 1991). These PEG-lipid conjugates will be discussed in greater detail in the following sections.

The final factor influencing liposome pharmacokinetics is the lipid dose administered. Administration of increasing numbers of liposomes has been shown to result in decreased percentages of liposomes in the liver and spleen with increased amounts of liposomes in the blood (Allen and Hansen, 1991). It has been suggested that this is a saturation phenomenon that results from a depletion of plasma opsonins (Oja \textit{et al.}, 1996) and macrophage saturation in the tissues (Mauk and Gamble, 1979). Furthermore, it is important to point out that "conventional" liposomes, which do not possess surface modifying lipids, exhibit dose-dependent biphasic elimination characteristics. Conversely, "sterically stabilized" liposomes, which possess surface modifying lipids, exhibit dose-independent linear elimination characteristics (shown \textit{in vivo} in mice) (Allen and Hansen, 1991; Papahadjopoulos \textit{et al.}, 1991). "Conventional" and "sterically stabilized" liposomes will be more clearly defined in Section 1.1.4.

1.1.3 Liposome Interactions With Cells

The exact mechanisms whereby liposomes and their encapsulated contents can be delivered to cells have not yet been determined. Four processes that are thought to explain how liposomes interact with cells are: stable adsorption, endocytosis, lipid exchange and/or fusion (Figure 1.3). These processes are also believed not to be mutually exclusive, but possibly occur simultaneously.
Stable adsorption involves the binding of intact liposomes to the surface of the cell membrane. Following adsorption, the contents of the liposome are not released directly into the cell, rather they gradually leak from the liposome into the extracellular fluid surrounding the cell and may gain entry to the cell by diffusion across the cell membrane (reviewed by Pagano and Weinstein, 1978). While studies have shown that this process occurs, delivery of large macromolecules to cells by liposomes cannot be explained by adsorption. Rather, the mechanisms of endocytosis and/or membrane-membrane fusion should allow for the efficient uptake of liposome contents (Straubinger and Papahadjopoulous, 1983).

According to literature, endocytosis is commonly believed to be the mechanism of liposome entry and liposome contents delivery into cells (Truneh et al., 1983; Szoka, 1987; Stirk and Baldeschwieler, 1987). There are two types of endocytosis: Pinocytosis involves ingesting small molecules and/or fluids surrounding the cell in a process known as fluid-phase endocytosis; phagocytosis involves the ingestion of large structures, such as microorganisms or cell debris using large vesicles, or vacuoles. This process involves the invagination of the cell plasma membrane around liposomes, eventual engulfment of the liposome, and the subsequent formation of endocytotic vesicles. These vesicles are commonly referred to as endosomes, which end up within the cytoplasm of the cell (Pagano and Weinstein, 1978; Pagano et al., 1981b). Endosomes may then fuse with lysosomes that contain enzymes, which might degrade the liposome and the contents entrapped within the liposome (Dijkstra et al., 1984; Raven and Johnson, 1989). Lipids of the liposome may be re-utilized by the cell and the encapsulated contents are released into the cytoplasm (Pagano et al., 1983; Lipsky and Pagano, 1983; Straubinger and
Figure 1.3: Liposome-Cell Interactions

Illustrations of the possible mechanisms of liposome-cell interactions which include stable adsorption, endocytosis, lipid transfer and/or fusion. Stable adsorption and endocytosis are believed to be the most common of the four mechanisms; however, these events are not mutually exclusive. Detailed definitions of these mechanisms are provided in the text.
Papahadjopoulos, 1983; Rao et al., 1997). However, the encapsulated contents need to be resistant to the lysosomal enzymes to remain intact and functional. Studies characterizing the process of liposomal endocytosis will be discussed further in Section 1.3.

Lipid transfer is defined as the transfer of individual lipid molecules from the liposomal membrane to the cell plasma membrane without the mixing of aqueous contents between the liposome and the cell (Sandra and Pagano, 1979; Struck and Pagano, 1980). Lipids exchanged from the liposome to the cell can either stay at the cell surface or be internalized and redistributed into intracellular membranes. Studies illustrating lipid exchange will also be discussed in Section 1.3.

While these processes have been somewhat elucidated in previous research, no conclusive evidence has demonstrated the extent of fusion that occurs between the liposomal membrane and the cell plasma membrane. Fusion, in this case, is defined as the intercalation of the outer membrane of the liposome into the cell membrane with concomitant release of the aqueous contents of the liposome directly into the cell cytoplasm (Pagano and Weinstein, 1978).

Some recent studies suggest that the process of fusion with the cell plasma membrane is an uncommon occurrence (Szoka, 1991), while older studies proposed that it was a frequent occurrence (Pagano and Weinstein, 1978; Straubinger and Papahadjopoulos, 1983). These observations are, however, dependent on the physical and chemical attributes of the liposome used. In certain delivery applications, fusion with the plasma membrane would be the ideal process of liposomal drug delivery because therapeutically active molecules would be delivered directly to the cell cytoplasm and
avoid degradation by lysosomal enzymes. It is now believed that some liposomes fuse with endosomal membranes following endocytosis releasing their contents into the cell cytoplasm (Connor and Huang, 1985; Wrobel and Collins, 1995).

While experiments to investigate these four proposed processes of liposome-cell interaction might seem straightforward, it is often difficult to obtain conclusive evidence for any single mechanism. In general, a single experiment is not sufficient to demonstrate the exact mechanism that occurs when using a specific liposomal delivery system. Multiple experiments must be performed and/or multiple conditions must be met to elucidate liposome-cell interactions.

1.1.4 Liposomal Formulations

Liposomal formulations have been designed to address specific obstacles and functions as drug delivery systems. These formulations often vary in lipid composition and may include surface modifying groups. For convenience, liposomes can been classified into six main classes: conventional liposomes, targeted liposomes, viral fusogenic liposomes, pH-sensitive liposomes, cationic complexes and sterically stabilized liposomes (Figure 1.4).

1.1.4.1 Conventional Liposomes

Conventional liposomes are vesicles that consist primarily of un-derivatized membrane bilayers composed of natural or synthetic lipids. Typically, they are composed of neutral lipids such as phosphatidylcholine or sphingomyelin (SM) and cholesterol (Chol). As well, they were used for early studies on the *in vivo* disposition of liposomes (Senior, 1987; Gregoriadis, 1988). Of the various conventional liposomes,
Figure 1.4: Classification of Liposomal Formulations

Generalized representations of six liposomal formulations which include conventional liposomes, targeted liposomes, viral fusogenic liposomes, pH-sensitive liposomes, cationic liposome complexes and sterically stabilized liposomes.
distearoylphosphatidylcholine (DSPC):Chol and SM:Chol have the longest circulation residence times (Hwang et al., 1980; Senior and Gregoriadis, 1982; Allen and Everest, 1983; Papahadjopoulos et al., 1991). This is because DSPC and SM, along with cholesterol, produce liposomes with rigid bilayers.

As mentioned previously, neutral liposomes of small-diameter, prepared with lipids that create rigid bilayers, reside in the circulation for longer periods of time compared to large-diameter charged vesicles with non-rigid bilayers. Therefore, for conventional liposomes, a general rule would be to use small diameter liposomes (between 80nm and 200nm) consisting of DSPC:Chol or SM:Chol for drug delivery purposes.

While these liposomal formulations with rigid bilayers can improve circulation lifetimes, a study examining mitoxantrone encapsulated in DSPC:cholesterol liposomes demonstrated that using conventional liposomes does not necessarily result in drug release and subsequent improvement in drug efficacy (Lim et al., 1997). Therefore, liposomes with rigid bilayers may be good for drug retention and decreased elimination rates, but they may also compromise drug bioavailability. Consequently, new formulations have been investigated to improve drug delivery characteristics.

1.1.4.2 Targeted Liposomes

Targeted liposomes are vesicles with antibodies (immunoliposomes), peptides, sugars, vitamins and/or other ligands attached to their surface with the aim of improved cell specific delivery (Allen, 1994; Maruyama et al., 1997; Shimada et al., 1997; Lopes de Menezes, et al., 1998). This type of targeting is referred to as active targeting, in
contrast to passive targeting where only the structural characteristics of the liposome are changed to improve liposome accumulation at a disease site. The attachment of these moieties would ideally lead to enhanced binding to cell receptors and uptake by target-specific cells.

While targeted liposomes have demonstrated positive effects, there are limitations to their use. Previous studies have shown that the attachment of whole antibodies to the surface of conventional liposomes increases their elimination rates from the circulation (Aragnol and Leserman, 1986; Debs et al., 1987). This also applies to some lipid species such as lactosylceramide and other attached ligands (reviewed by Hug and Sleight, 1991). This is most likely due to the recognition of foreign molecules present on the liposome surface by the host’s immune system. Thus, targeted liposomes can result in dramatic increases in liposome size, enhanced immunogenicity, and increased plasma elimination (Tardi et al., 1998). In addition, a study examining immunoliposome targeting to pulmonary endothelium suggests that although such liposomes could accumulate at target sites, the encapsulated drug was limited to release by diffusion from the liposome (Lui and Huang, 1993). Drug delivery would be improved if there were destabilization of liposomes or intracellular delivery of encapsulated drug. Therefore, a liposomal formulation with a “triggered release” mechanism, a stimulus that would facilitate drug release from liposomes, or a mechanism that would facilitate fusion with cells, is still desired.

1.1.4.3 Viral Fusogenic Liposomes

Viral vectors such as retroviruses and adenoviruses are the most frequently used systems to deliver genetic material (Lasic, 1997). They are capable of direct fusion with
eukaryotic plasma membranes, which might improve the delivery of therapeutic agents. To mimic viral systems, liposomes containing viral components have been designed to fuse with plasma membranes. Some liposomal formulations have fusogenic viral lipids incorporated in their membranes or viral proteins (or peptides) attached to their surface to bind to receptors on the surface of cells (Hug and Sleight, 1991; Murata et al., 1993; Puyal et al., 1994). The combination of virus particles and liposomes are often referred to as virosomes. While these delivery systems have shown positive results, there are still concerns of immune responses to these viral-based fusogenic liposomes because they still possess foreign viral molecules. Therefore, non-viral fusogenic liposomes have been investigated as a safer alternative.

1.1.4.4 pH-Sensitive Liposomes

The pH-sensitive liposome was designed for the purpose of controlled destabilization of liposomes resulting in controlled delivery of encapsulated agents (Yatvin et al., 1980; Straubinger et al., 1985; Szoka, 1991; Slepushkin et al., 1997). Essentially, these liposomes are composed of a non-bilayer forming lipid such as dioleoylphosphatidylethanolamine (DOPE) stabilized in a bilayer conformation using a lipid whose charge is dependent on the pH of the surrounding environment. A commonly used lipid is cholesteryl hemisuccinate (CHEMS), which is unprotonated at neutral pH and protonated at an acidic pH (Chu et al., 1990; Kirpotin et al., 1996; Slepushkin et al., 1997). In its unprotonated form, CHEMS is capable of stabilizing DOPE in a bilayer conformation; however, the formulation is destabilized at acidic pH. The rationale behind such pH-sensitive liposomes is that once the vesicle is endocytosed, the endosomal environment becomes acidic and CHEMS becomes protonated. This results
in destabilization of the vesicles from a bilayer conformation to a non-bilayer conformation, fusion with the endosomal membrane, and the subsequent release of vesicle-encapsulated contents into the cell cytoplasm. These vesicles, however, have been reported to be unstable in serum-containing solutions and aggregate, making them undesirable for in vivo delivery (Connor et al., 1986).

1.1.4.5 Cationic Liposome Complexes

In 1987, a study employing a lipid-mediated DNA-transfection procedure using cationic liposome-DNA complexes sparked considerable interest (Felgner et al., 1987). Since that time, many investigators have employed cationic liposome complexes for delivery studies involving genetic material (Scheule and Cheng, 1996; Cooper, 1999). Pre-formed cationic liposomes, composed of various cationic lipids and DOPE, are complexed with anionic plasmid DNA or antisense oligonucleotides via ionic bonding. These complexes are now widely used for in vitro DNA transfection and antisense oligonucleotide down-regulation studies (Lasic, 1997). For in vivo delivery, injectable preparations composed of cationic liposomes and either DNA or RNA have also been utilized.

Despite their relative usefulness and ease of preparation, there are disadvantages to these complexes as delivery systems. They are often unstable, provide lower levels of transfection compared with viral vectors, exhibit extremely short circulation lifetimes and are generally trapped in the capillary beds of organs involved in first pass clearance (Lasic, 1997). Therefore, an improved formulation to deliver genetic materials in vivo is still being sought.
1.1.4.6 Sterically Stabilized or Stealth Liposomes

Sterically stabilized or Stealth liposomes were developed to increase the circulation longevity of liposomes (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Vertut-Doi et al., 1996; Basanez et al., 1997; Du et al., 1997). A number of studies show that these liposomes exhibit improved pharmacokinetics and, in some instances, improved therapeutic efficacy over conventional liposomes (Papahadjopoulos et al., 1991; Northfelt et al., 1998). This is due to their extended duration in the circulation that allows for greater accumulation at disease sites following extravasation.

As mentioned in Section 1.1.2., Stealth liposomes differ from conventional liposomes because they have a surface-modifying moiety that acts to inhibit binding of serum opsonins that can be recognized by macrophages of the MPS. They are termed sterically stabilized, because attached substituents act to prevent surface-to-surface interactions, or Stealth liposomes, because of decreased detection by the MPS (Senior et al., 1991). As a result, these liposomes are eliminated from the circulation at a slower rate. Initial studies utilized either phosphatidylinositol (PI) or monosialoganglioside (G_{Mi}), to act as the surface-modifying component (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen et al., 1989). More recently, however, polyethylene glycol (PEG)-conjugated lipids have been adopted for the majority of studies involving sterically stabilized liposomes.
1.2 PROGRAMMABLE FUSOGENIC VESICLES

Of the liposomes described in Section 1.1.4., conventional liposomes have been most commonly utilized as drug delivery systems in vivo; however, difficulties with drug release from these systems have been reported. As a result, viral fusogenic liposomes, pH-sensitive liposomes and cationic liposome complexes were designed as intracellular drug delivery carriers to improve the efficiency of drug delivery and drug release into cells. Unfortunately, these liposomes have been shown to readily destabilize in physiological solutions or are rapidly cleared from the circulation, as mentioned previously. Consequently, improved intracellular drug delivery formulations are still being sought. Such formulations should maximize drug efficacy while minimizing non-specific toxicity.

The ideal liposomal delivery system would have good drug retention and stability properties in the circulation until it reaches its site of action. It would initially possess qualities similar to conventional liposomes or sterically stabilized liposomes. Upon reaching the target cell, the liposomal carrier would then alter its characteristics becoming unstable (Adlakha-Hutcheon et al., 1999). The liposomal system could then fuse with cellular membranes it comes in contact with, such as the plasma membrane or endosomal membrane, thereby releasing an encapsulated drug directly into the cytoplasm.

In order to design a liposomal carrier that would act in this fashion, a novel system was formulated combining lipids commonly used with conventional liposomes, cationic liposome complexes and sterically stabilized liposomes. Programmable fusogenic vesicles (PFVs) are composed of a non-bilayer forming lipid DOPE,
cholesterol, the cationic lipid DODAC, and a bilayer stabilizing lipid, PEG-PE or PEG-ceramide (Figure 1.5). In addition to stabilizing the lipids in a bilayer conformation, the PEG-lipids also act to sterically stabilize these liposomes. Similar to Stealth liposomes, the inclusion of PEG allows for longer circulation residence times. Each lipid component has a specific function and these will be described in the next sections, in order to explain the design of PFVs.

1.2.1 Composition of PFVs

1.2.1.1 Non-Bilayer Forming Lipids

When an aqueous solution is added to amphipathic phospholipids, the lipids aggregate and are capable of forming a variety of phases. Two of the possible lipid/water phases are termed $L_\alpha$, for lamellar, liquid-crystalline phase, and $H_{II}$, for inverted hexagonal phase. Lipids in the $L_\alpha$ phase are arranged in extended bilayer sheets that are separated from one another by layers of water. The $H_{II}$ phase describes a structure in which long cylindrical cores of water, bound by polar lipid head groups, are set in a hexagonal hydrophobic lattice composed of the lipid hydrocarbon chains. In physiological solutions, lipids that adopt the $L_\alpha$ phase generally form bilayer vesicular structures and lipids that adopt the $H_{II}$ phase do not. This is an important observation because drugs can be encapsulated within bilayer vesicular structures, while they cannot be encapsulated within hexagonal structures.

Some lipids or lipid mixtures can undergo a phase transition between the $L_\alpha$ phase and the $H_{II}$ phase by varying conditions such as temperature, pH or ionic strength. A decrease in the effective lipid headgroup area, introduction of cis double bonds in the
Figure 1.5: Lipid Components of Programmable Fusogenic Vesicles

Structures of the four components of programmable fusogenic vesicles are diagrammed. The four components are dioleoylphosphatidylethanolamine (DOPE), cholesterol, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC) and polyethylene glycol (PEG)-lipids. Shown here are two types of PEG-lipids, the PEG-phosphatidylethanolamine (PEG-PE) and the PEG-ceramide both with the acyl chain lengths of fourteen carbons.
acyl chains and increasing the length of the hydrocarbon chains favor the H$_{II}$ phase (review by Cullis and De Kruijff, 1979; Seddon et al., 1983). Headgroup ionization, which favors the L$_{a}$ phase, can be reduced by salts, divalent cations or a reduction in pH (Litzinger and Huang, 1992). The H$_{II}$ phase is usually favored at higher temperatures than the L$_{a}$ phase (Gruner et al., 1985).

The overall dynamic molecular shape of a lipid determines its phase preference (Cullis et al., 1991). Lipids such as unsaturated phosphatidylethanolamines (PE) have a small headgroup compared to the cross-sectional area occupied by the hydrocarbon chains and an inverted cone-like structure. This lipid tends to adopt the H$_{II}$ phase because lipid packing is better facilitated and the hydrophobic headgroups are oriented such that their interaction with water is minimized.

When PEs are included in liposomal formulations, they can be stabilized into a L$_{a}$ bilayer phase using lipids such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylglycerol (PG) at 20 mol% or greater. Other components, such as fatty acids, detergents, gangliosides, diacylphospholipids, lysolipids, haptenated lipids, diacylsuccinylglycerols, cholesterol, cholesterol derivatives, membrane glycoproteins, palmitylated amino acids and palmitylated antibody can also be used (Litzinger and Huang, 1992). These combinations, however, often produce vesicles that become leaky or destabilize, making it difficult to assess their delivery capabilities (Düzgünès et al., 1989; Bailey and Cullis, 1997). Nevertheless, such vesicles are being investigated as drug delivery formulations to improve the bioavailability of encapsulated drugs. DOPE, a commonly utilized unsaturated PE for liposomal formulations, is also a major lipid component in PFVs (Adlakha-Hutcheon et al., 1999; Holland et al., 2001). It
is an important component in this formulation because of its polymorphic behavior, which allows for a transition from a bilayer configuration back to a hexagonal structure. This polymorphic phase transition may also allow drug encapsulated in a stabilized bilayer system to be released. In addition, DOPE is often referred to as a "fusogenic" lipid or fusion-facilitating lipid because its preference to adopt the H\text{II} phase can trigger fusion with other vesicles or a cell plasma membrane (Allen et al., 1990).

1.2.1.2 Cholesterol

Cholesterol is the major sterol in mammalian membranes. It is amphipathic with a polar head group (the hydroxyl group at C-3) and a nonpolar hydrocarbon body (the steroid nucleus and the hydrocarbon side chain at C-17) and is similar in length to a 16-carbon fatty acid in its extended form (Lehninger et al., 1993). In addition to the fluid liquid-crystalline state (L\text{a}), membrane lipids can also exist in a frozen gel, crystalline state (L\text{b}) depending on temperature (Oldfield and Chapman, 1972; Silvius, 1982). Cholesterol reduces the enthalpy of the phase transition, from the L\text{b} to the L\text{a}, and the lipid phase transition is effectively eliminated at greater than 30 mol\% cholesterol when mixed with dipalmitoylphosphatidylcholine (DPPC) (Demel and De Kruyff, 1976; Cullis and Hope, 1991).

Cholesterol has been shown to exhibit a condensing effect on lipids in the liquid crystalline state and a liquefying effect on lipids in the crystalline state, leading to an intermediate gel state (Demel and De Kruyff, 1976). In other words, cholesterol might have a dual role of preventing formation of crystalline gel areas in some membranes whilst also inhibiting the motion of hydrocarbon chains in more fluid, liquid crystalline regions (Oldfield and Chapman, 1972). Interestingly, when added to mixed unsaturated
DOPC-DOPE vesicles, cholesterol can induce hexagonal (H_{II}) phase organization (Tilcock et al., 1982).

The exact role of cholesterol in membrane systems has yet to be determined. Cholesterol is believed to modulate the "fluidity" of the lipid environment, thereby affecting membrane function (Tilcock et al., 1982). The term "fluid" refers to a state characterized by rapid axially symmetric re-orientation of the phospholipids and lateral diffusion rates comparable to those found in the L_{alpha} phase (Rubenstein et al., 1979). Furthermore, the effect of cholesterol in phospholipid bilayers is to increase the thickness and "strength" of the bilayer while maintaining the "fluid" environment conducive to high lateral mobility (Vist and Davis, 1990). The role of cholesterol can therefore be considered a stabilizing or "dampening" mechanism, inhibiting structural changes in the membrane due to thermal, mechanical and other stresses (Papahadjopoulos et al., 1973). Commonly, cholesterol's effects are temperature- and concentration-dependent. Above the transition temperature of the phospholipid, cholesterol decreases the fluidity whereas below the transition temperature it increases the fluidity (LaBelle and Racker, 1977). In addition, it is now postulated that at low cholesterol concentrations, the solid-ordered (gel) and liquid-disordered (fluid) phases are equally favored by cholesterol.

At high concentrations, cholesterol induces conformational order in the fluid state and destroys the crystalline structure in the low temperature phase. This results in the liquid-ordered phase in which the conformational and the positional degrees of freedom are decoupled. Since this is the only phase at high cholesterol concentrations, there are no interfaces and a very low permeability is expected relative to the permeability of the pure lipid bilayer (Corvera et al., 1992). Therefore, cholesterol has a dual function on
lipid bilayers that results in the permeability of the membrane having a dependence on temperature and the cholesterol concentration.

In general, cholesterol has been shown to decrease the permeability of compounds through phospholipid membranes (De Gier et al., 1968; Demel et al., 1968; Kroes and Ostwald, 1971). The initial rate of permeability of liposomes derived from saturated and unsaturated phosphatidylcholines (PCs) above their crystalline-to-liquid-crystalline transition temperature decreased with increasing cholesterol concentration. However, cholesterol enhanced the rate of permeability of liposomes derived from saturated lecithins below their transition temperature (Demel and De Kruyff, 1976). It is commonly believed that cholesterol could regulate the permeability of biological membranes by affecting the internal viscosity and molecular motion of the lipids within the membrane (De Gier et al., 1968). Furthermore, a study describing the influence of cholesterol on the interaction of several proteins and phospholipid membranes indicated that the presence of cholesterol generally inhibits the ability of proteins to increase the permeability of phospholipid vesicles and "expand" or "penetrate" phospholipid monolayers (Papahadjopoulos et al., 1973). In this manner, cholesterol in vesicles could function as a protective component and improve stability in biological fluids.

With respect to liposomal drug delivery systems, cholesterol is included in the PFV formulation at a high molar percentage because it helps to improve the mechanical stability of the vesicles while at the same time enhancing the retention of encapsulated contents.
1.2.1.3 Cationic Lipids

Naturally occurring cationic lipids are extremely rare, with sphingosine and some lipids in primitive life forms being the only such lipids identified (Lasic, 1997). Cationic lipids have therefore been synthesized for studies involving the incorporation of positively charged lipids into liposomes. The first two synthetic cationic lipids produced were dioctadecyl dimethyl ammonium bromide (DODAB) and dioleoyloxy-3-(trimethylammonio) propane (DOTAP) (Kunitake and Okahata, 1977; Eibl and Wooley; 1979). Early studies using cationic lipids in liposomes showed that cationic liposomes were cleared more rapidly from the circulation than neutral liposomes; this was likely due to increased binding of plasma proteins. Furthermore, there was also evidence that cationic liposomes were toxic to cells or tissues and, as a result, research using these systems lagged. However, in the late 1980’s, cationic liposome-DNA complexes showed positive transfection results with cells in vitro using liposomes containing the cationic lipid, dioleoxy propyl trimethyl ammonium chloride (DOTMA), together with DOPE (Felgner et al., 1987). DOTMA is similar to the DOTAP except that the acyl chains are linked to the propyl backbone via ether and not ester bonds. Since then, there has been an explosion of studies looking at the synthesis of less toxic cationic lipids and improved delivery of genetic material using cationic liposomes.

Other forms of cationic lipids that have been developed are: dimyristooyxpropyl dimethyl hydroxyethyl ammonium bromide (DMRIE); N,N-dioleyl-N,N-dimethylammonium chloride (DODAC); polylysine attached to acyl chains; spermine and spermidine coupled to fatty acids; as well as 3β[N-(n’,N’-dimethylaminoethane)-carbamoyl]cholesterol, dioleoyl) (DC-Chol), which has a positive charge on the sterol
backbone of cholesterol. These lipids are commonly associated with the neutral lipids DOPC, DOPE or cholesterol for gene transfection studies (Wrobel and Collins, 1995; Harvie et al., 1998)

Positively charged lipids are used in liposomal formulations for two purposes. Firstly, cationic liposomes are attracted to cell plasma membranes, which possess negatively charged residues. Secondly, genetic materials such as plasmid DNA, RNA and antisense oligonucleotides have negatively charged phosphate backbones. These molecules are often combined with cationic liposomes to form complexes that are used as carriers for gene therapy and antisense therapy studies. For these reasons, DODAC is incorporated in the design of the PFVs.

1.2.1.4 Polyethylene Glycol-Conjugated Lipids: Bilayer Stabilizing Lipid and Exchangeable Lipid Component

As mentioned in Section 1.2.2.1, DOPE cannot readily form bilayers without being stabilized with an additional lipid component. Liposomes can be formed using DOPE, cholesterol and DODAC. However, these vesicles are inherently unstable in physiological salt and serum-containing solutions and are therefore prone to fuse with each other, other vesicles or erythrocytes (Holland et al., 1996b; Holland et al., 2001). To remedy this problem, PEG-lipid is employed to stabilize the lipid components of the PFVs in a bilayer organization.

As mentioned earlier, PEG-lipids can provide a steric barrier to plasma proteins resulting in long circulation residence times. Numerous studies have verified the use of PEG-lipids to improve circulation lifetimes of liposomal drug delivery systems over conventional liposome systems (Papahadjopoulos, 1999). An additional characteristic of
PEG-lipids that is exploited in the PFV design concerns the ability of this component to exchange out of the vesicle. Essentially, the acyl chains of the lipid attached to the PEG are embedded or "anchored" within the vesicle bilayer. Depending on the acyl chain length of the lipids, the PEG-lipid is lost from the PFV over a predictable timecourse. As the PEG-lipid is gradually lost, the PFV destabilizes or becomes leaky releasing its encapsulated contents. Therefore, the PEG-lipid component has three-fold functionality for steric stability of the liposome, bilayer stabilization and as an exchangeable lipid component.

Rates of spontaneous phospholipid transfer and the influence of acyl chain composition have been extensively researched (Nichols, 1985; Ferrell et al., 1985; Homan and Pownall, 1988; Silvius and Leventis, 1993). PEG-lipid exchange from PFVs is also caused by spontaneous transfer to acceptor sites such as other vesicles and cellular membranes (Holland et al., 1996b; Holland et al., 2001). Therefore, it does not require an external "trigger" or stimulus to allow for drug release as with other liposomal formulations. Consequently, PFVs are capable of longer circulation times which allows them to reach distal disease sites before the PEG-lipid exchange is sufficient to result in vesicle destabilization and subsequent drug release. Furthermore, as a result of slower vesicle destabilization, increased circulation time and improved activity of mitoxantrone encapsulated in PFVs containing PEG-PE have been demonstrated (Adlakha-Hutcheon et al., 1999). By simply varying the acyl chain length and hence the rate of PEG-lipid exchange, the duration of PFV stability can be modified.

The two forms of PEG-lipids that will be discussed in this thesis are PEG-PE and PEG-ceramides. PEG-PE is a commonly incorporated surface-modifying lipid that has a
negative surface charge, whereas PEG-ceramides are neutral. A study suggests that using PEG-PE may allow vesicle leakage of cationic drugs to a much greater extent than when using neutral PEG-ceramides (Webb et al., 1998). Researchers examining the effect of liposome surface charge on the binding and endocytosis of conventional and sterically stabilized liposomes suggest that cationic liposomes are preferentially taken up in HeLa cells over neutral or anionic liposomes (Miller et al., 1998). The use of PEG-PE introduces negative charges that might neutralize the positive charge presented by the DODAC in the PFVs. Consequently, PEG-ceramides have been used for a majority of the studies mentioned in this thesis.

The combination of DOPE, cholesterol, DODAC and PEG-lipids allows for the potential design of a liposomal carrier system that is initially very stable and has good drug retention properties. Then, the PEG lipids, which also allow for extended residence in the circulation, exchange off from the PFV. The positive charge from DODAC becomes exposed and allows binding to and/or uptake into cells. The PFV destabilizes and releases its contents into the cell cytoplasm either by fusing with the cell plasma membrane directly or destabilizing the endosomal membrane, if it is endocytosed. Alternatively, drug leakage may occur without the occurrence of fusion mechanisms such that the destabilized PFVs become leaky allowing drug release and subsequent uptake of free drug by target cells. PFVs, therefore, should ensure improved drug availability, but intracellular drug delivery would be preferred.

1.2.2 Hypothesized Mechanism of PFV Function

To summarize the previous sections, I will re-iterate the hypothesized mechanism of PFV function. In general, PFVs are inherently fusogenic because they consist of lipids
that do not normally maintain a bilayer conformation. The addition of a bilayer-stabilizing lipid, such as PEG-conjugated lipids, PE or ceramide, allows the PFV to maintain its bilayer structure for a designated and predictable time-course (Holland et al., 1996a; Holland et al., 1996b) (Figure 1.6). The PEG-lipid conjugates are an exchangeable component. Their acyl chains are "anchored" within the bilayer of the vesicle and the bilayer structure is maintained until the PEG-lipid exchanges out of the vesicle. Accordingly, the shorter the acyl chain length, the more rapidly the PEG-lipid exchanges out of the liposome; the longer the acyl chain length, the slower the rate of exchange. Once the PEG-lipid exchanges out of the PFV, the PFV regains its fusogenic activity, which results in destabilization of the vesicle. During the destabilization process, the PFV is thought to fuse with the plasma membrane of the target cell and the encapsulated contents of the vesicle are released directly into the cytoplasm of the cell. Ultimately, the encapsulated contents diffuse to their respective sites of action within the cell and, hopefully, the agent elicits an effect on cellular functions.

1.3 CELL CULTURE METHODS AND TECHNIQUES

Numerous cell culture studies have been performed in order to examine liposome characteristics and interactions with cells (reviewed by Pagano and Weinstein, 1978). The potential benefits of cell culture studies include decreased costs, decreased experimental variability, improved examination of cellular mechanisms involved in drug action and improvement in the design of in vivo studies. In terms of cost, cell lines are less expensive than animals. They also require lower amounts of drug and lower maintenance. Experimental variability is reduced because of the homogeneity of the
Figure 1.6: Hypothesized Mechanism of Drug Delivery by Programmable Fusogenic Vesicles

A diagrammatic illustration of intracellular drug delivery by a programmable fusogenic vesicle (PFV) to the cytoplasm of a cell. (A) Ideally, the PFV remains intact for a predictable length of time exhibiting good drug retention. (B) Once a proportion of PEG-lipid gradually exchanges from the PFV, the cationic lipids of the PFV interact with the anionic surface charges of the cell plasma membrane. In addition, the PFV begins to destabilize upon regaining its inherent fusogenic activity. (C) The lipid bilayer of the PFV then fuses with the cell plasma membrane resulting in the release of the drug contents directly into the cell cytoplasm.
samples and the environmental factors—such as pH, temperature, O₂, CO₂ levels, etc.—can be controlled.

More importantly, cell culture techniques allow for direct examination of mechanisms of drug action, which is difficult to perform in whole animals. For instance, in vitro cell studies can easily examine mechanisms of toxicity such as DNA damage, membrane integrity and enzyme function. In addition, cell culture studies can lead the way for improving the design of whole animal studies by acting as an initial screen for potentially beneficial drugs and by determining the appropriate dose ranges of these drugs. Likewise, improvements to the design of liposomal drug delivery systems can be made utilizing cell culture models.

Although there are a number of benefits of cell culture models, there are some disadvantages as well. For example, cells are removed from their normal heterogeneous cellular milieu, homeostatic mechanisms are not present and cells are very delicate and sensitive. In many instances, cells of different types are known to act in conjunction with one another. Isolating a cell type from the presence of other cells may alter its functions and characteristics so that the cell is not the same as in its natural environment. There are also countless homeostatic mechanisms in the circulation that alter cell function and growth. In the absence of these mechanisms, the cells also may not behave as they normally would in vivo. Furthermore, some specific cell lines are extremely delicate and sensitive. This makes them difficult to work with and constant monitoring of their conditions may be required.

Taking the benefits and potential problems of cell culture models into account, the following sections will describe cell culture studies that have been performed to examine
liposomes and the limitations of those studies. As well, the techniques employed in performing these studies will be discussed.

1.3.1 Cell Culture Studies Involving Liposomes

Studies with liposomes utilizing cell cultures can be divided into five groups: lipid transfer studies, liposome-cell fusion, intracellular delivery, DNA delivery and phagocytosis. These studies have been performed to investigate the mechanisms of liposome-cell interactions. This section will focus on research that directly relates to liposomes and relevant background material for the studies described in this thesis.

1.3.1.1 Lipid Transfer Studies

While numerous lipid transfer studies have been performed between vesicle-vesicle populations, relatively few studies have looked at vesicle-cell lipid transfer. In early work, the behavior of lipid transfer from a vesicle to the plasma membrane of a cell, and vice versa, was investigated using radiolabeled or fluorescent lipid derivatives (Struck and Pagano, 1980; Struck et al., 1981; Uster and Pagano, 1986). Phospholipid exchange between SUVs and cells was first observed using $^{14}$Cdioleoylphosphatidylcholine vesicles which contained the aqueous space marker $^3$Hinulin (Pagano and Huang, 1975). Studies show that at 2°C, only radiolabeled lipid was transferred to V79 fibroblasts and $^3$Hinulin did not become cell-associated under these conditions. This study is important because it demonstrates the spontaneous exchange of lipid monomers between a vesicle bilayer and the cell plasma membrane.

Another study looked at N-4-nitrobenzo-2-oxa-1,3-diazone aminocaproic acid (NBD) fluorescent lipid derivatives incorporated into SUVs incubated with cultured
Chinese hamster fibroblasts (Struck and Pagano, 1980). This study demonstrated that, at low temperatures (2°C) lipid exchange occurred, which was primarily restricted to the plasma membrane resulting in a bright peripheral ring of fluorescence under fluorescence microscopy. At physiological temperature (37°C), a substantial amount of cell-associated fluorescent lipid was internalized, although a ring of peripheral fluorescence was clearly visible. Therefore, the cellular localization of the lipid transferred was dependent on temperature.

An additional study looking at $^3$H-DOPE or $^3$H-DOPC vesicles incubated with Chinese hamster fibroblasts suggests that for every molecule of DOPE which is transferred to the cell from vesicles, one molecule of endogenous PE is lost from the cell surface. This then suggests that the stoichiometry of the transfer process is one-for-one (Sandra and Pagano, 1979). This study also suggested that vesicle-cell lipid transfer was phospholipid specific. In order for lipid exchange to occur, the same type of phospholipid species must be present in both the donor vesicle and the plasma membrane of the recipient cell. Therefore, if PE exchanges from a vesicle to a cell’s plasma membrane, there must also be PE present in the plasma membrane. These studies put a focus on liposome membrane interactions with cell plasma membranes. However, a greater emphasis was placed on liposomes as model membranes as opposed to liposomes as drug delivery systems. The next step would be to investigate fusion processes, which are a common occurrence with cellular membranes, and how they relate to liposomes.

1.3.1.2 Liposome-Cell Fusion Studies

Fusion between liposomes and cells was investigated to identify the mechanisms involved and determine potential ways to induce fusion utilizing various liposomal
systems; for example, calcium induced fusion of PS vesicles or the use of unsaturated PE-containing systems, discussed previously. For drug delivery purposes, liposome-cell fusion with the plasma membrane would be the most efficient delivery mechanism to ensure maximum intracellular drug delivery. As mentioned before, it would also result in drug delivery directly to the cytoplasm avoiding degradation by lysosomal enzymes as in the case of endocytosis. The primary evidence for liposome-cell fusion has been the transfer of encapsulated liposome contents to the cell cytoplasm (Pagano et al., 1981b). However, studies mentioned in the following section on intracellular delivery indicate that endocytosis may account for the accumulation of molecules in the cell cytoplasm as opposed to fusion with the plasma membrane (Wilschut, 1989). Fusion and endocytosis have been distinguished largely on the basis of inhibitor studies (reviewed by Pagano and Weinstein, 1978). Inhibitors of endocytosis include low temperatures or the use of chemical inhibitors such as sodium azide and 2-deoxyglucose (Wrobel and Collins, 1995). These compounds are known to inhibit endocytosis by depleting cellular ATP (Silverstein et al., 1977; Sandvig and Olsnes, 1982).

In addition to delivery of liposome contents, stronger evidence for fusion is the transfer of liposomal lipid into the plasma membranes of cells. This must be distinguished from adsorption of liposomes to the cell surface. Adsorption would only involve the intact vesicle resting on the cell membrane and no lipid mixing. Fluorescent lipids have been employed for these studies; nevertheless, it is still difficult to distinguish between vesicle adsorption, lipid incorporation into the plasma membrane resulting from monomer transfer and lipid transfer that has occurred as a consequence of fusion.
Despite the difficulties, lipid incorporation has been shown using red blood cells and distinguished from adsorption of vesicle lipid (Martin and Macdonald, 1976).

Another study has shown fusion between phosphatidylcholine-phosphatidylserine (1:1) vesicles and cultured fibroblasts using resonance energy transfer (Struck et al., 1981). Currently, studies suggest that the primary mechanism of intracellular delivery is endocytosis as opposed to the fusion of liposomes with cell plasma membranes. A study by Wrobel and Collins (1995) suggests that binding to the cell surface is insufficient for cationic liposome-cell fusion and that uptake into the endocytic pathway is required for fusion to occur. The data exclude a major role for the plasma membrane in the fusion event, but cannot explain why endocytosis is required for fusion since the endosomal membrane and the composition of the plasma membrane (as well as vesicles derived from that membrane) should be quite similar. Therefore, this study suggests that liposomes are taken up into an endosome and that fusion does not occur with the plasma membrane, but rather the endosomal membrane. Combining and comparing liposome content delivery and lipid mixing data in the same study should reinforce the evidence for the occurrence of liposome-cell fusion. Unfortunately, the exact mechanisms for fusion between liposomes and cells have yet to be determined.

1.3.1.3 Intracellular Delivery Studies

The aim of many studies utilizing liposomes as drug delivery systems is the direct intracellular delivery of therapeutic agents to the cell cytoplasm (Straubinger and Papahadjopoulos, 1983; Lasic, 1997). Intracellular delivery using liposomes is a means to allow large macromolecules—such as antisense oligonucleotides and plasmid DNA—entry into the cell; these agents would not otherwise be able to cross the plasma
membrane. Intracellular delivery formulations such as viral fusogenic liposomes, pH-sensitive liposomes and cationic liposome complexes have been designed to improve delivery of encapsulated or complexed molecules. The liposomal formulations share a common attribute in their design: the potential to induce fusion, either when in contact with cells or after being endocytosed within cells.

To examine intracellular delivery, many studies have used fluorescent dyes such as carboxyfluorescein and calcein (Weinstein et al., 1977; Straubinger et al., 1985; Connor and Huang, 1985; Slepushkin et al., 1997). In addition, studies have utilized fluorescent-labeled compounds, such as fluoresceinated dextran and Texas Red-conjugated protein, encapsulated in liposomes (Straubinger et al., 1985; Rao et al., 1997). Carboxyfluorescein and calcein are commonly used fluorescein derivatives that are self-quenching at high concentrations (e.g. at concentrations >100mM) and intracellular delivery is indicated when the dye dequenches after dilution, which occurs following distribution to the cytoplasm (Ralston et al. 1981; Allen, 1984).

Although these dyes are commonly used as a tool to evaluate delivery of liposomes, there are limitations to their use. One major limitation of these studies is that dequenching due to dye leakage from unstable or destabilized vesicles may be an inaccurate measure of fusion. As well, previous attempts to use liposome-liposome contents mixing assays with cationic liposome systems have failed due to leakage of vesicle contents (Düzgünès et al., 1989; Bailey and Cullis, 1997). According to these studies, the available contents mixing probes are all multivalent anions that destabilize the cationic liposomes at millimolar concentrations in physiological salt solutions (Bailey and Cullis, 1997). Consequently, it is extremely important to ascertain the reliability of
the methods involved in assaying liposomal uptake into cells. Doing so will improve the chances of elucidating the mechanisms involved in liposomal uptake, as well as aid in the improvement of these delivery systems.

1.3.1.4 Liposome-Mediated DNA Delivery Studies

There are several somatic gene therapy studies that have examined plasmid DNA delivery utilizing polycationic liposomal systems (detailed in Lasic, 1997). The aim of these studies is to provide specific cells of a patient with the genetic information necessary to produce therapeutic proteins for correction or modulation of diseases (review by Mahato et al., 1997).

Since the introduction of the transfection agent Lipofectin (a 1:1 w/w mixture of the cationic lipid DOTMA and DOPE) by Feigner and his co-workers (1987), many cationic liposome formulations complexed with plasmid DNA have been tested \textit{in vitro} and \textit{in vivo} for improved transfection results and decreased toxicity. Lipofectin has paved the way for a whole set of new DNA delivery studies using liposomes.

The problem in the past with the delivery of free plasmids was the net negative surface charge that prevented them from crossing biological membranes efficiently. Using cationic liposomes to neutralize the negative surface charge improved the efficiency of DNA delivery. In addition, the optimal ratio of cationic lipid to DNA occurs when the number of positive charges incorporated in the cationic liposome is greater than the number of negative charges on the DNA. This promotes the association of the liposome-DNA aggregates with the negatively charged surface of the cell (Reimer et al., 1997).
Compared to viral vectors, the transfection efficiency of these liposome-DNA delivery systems is lower. However, they provide greater flexibility in their design in terms of liposome composition, ease of preparation, non-immunogenicity and minimal systemic toxicities. Such systems have also exhibited potentially beneficial results in animals via systemic and local delivery (Lasic and Templeton, 1996) and in human clinical trials via direct intratumoral injections (Nabel et al., 1993).

Unfortunately, a limitation of plasmid/lipid complexes is that they may interact with serum proteins, lipoproteins, heparin and glycosaminoglycans in the extracellular matrix, leading to aggregation or release of DNA from the complexes even before reaching the target cells (Mahato et al., 1997). In addition, the complement system is activated by cationic lipids, which leads to the opsonization of intravenously administered plasmid/lipid complexes by C3b/C4b components (Plank, et al., 1996). Therefore, it appears that aggregation and complement activation may affect the in vivo utility of these complexes by preventing DNA and cationic liposomes from reaching their target sites.

1.3.1.5. Complement-Dependent Phagocytosis Studies

The final area of research where liposomes and cultured cells have been studied extensively is the uptake of liposomes by phagocytic cells of the MPS. Upon exposure to blood, liposomes can become coated (opsonized) with plasma proteins that can mediate their uptake. Recognition and ingestion of opsonized liposomes by phagocytic cells is mediated via binding of liposomal opsonins to specific receptors on these cells. The opsonization with IgG and subsequent Fc-mediated phagocytosis or endocytosis is believed to be the major mechanism for liposome uptake (Hsu and Juliano, 1982). The
complement (C) proteins are another important class of blood proteins that are responsible for the opsonization of liposomes (Wassef and Alving, 1993). However, they have not been studied as thoroughly as the IgG antibody.

It is well known that stealth liposomes containing lipids such as PEG-conjugate lipids, ganglioside G\textsubscript{M1} or phosphatidylinositol (PI) suppress complement-dependent phagocytosis; therefore, they have prolonged circulation lifetimes. Additional factors associated with suppression of complement-dependent phagocytosis include liposomes with negatively charged phospholipids and liposomes with other negatively charged compounds such as prostaglandin E\textsubscript{2} or thromboxane B\textsubscript{2} (Wassef and Alving, 1993). Suppression was proportional to the amount of negative charge on the liposomes, but independent of the chemical nature of the anionic lipid.

In past studies, designing liposomes with long circulation lifetimes has been beneficial as this prevents the activation of complement-dependent phagocytosis. Thus, these systems avoid rapid clearance from the circulation and allow accumulation of liposome encapsulated drugs at disease sites. More recently, however, a converse utility of liposomes has been considered which involves the use of liposomal vaccines to activate the immune system. It is well established that liposomes serve as an efficient delivery system for entry of exogenous protein and peptide antigens into the class I pathway and are very efficient inducers of cytotoxic T-lymphocytes (CTLs) (Rao et al., 1997). Therefore, liposomes have potential to be utilized as immunological adjuvants for vaccine preparations (Parmar et al., 1998). However, these formulations are still in the early stages of development.
1.3.2 Cell Culture Techniques

1.3.2.1 Cell Viability/Cytotoxicity Assays

To assess parameters such as efficacy and potency of therapeutic drug preparations, cell viability or cytotoxicity assays have been employed. These assays give an initial indication of an effective drug concentration range. Consequently, drug activity can be characterized prior to initiation of in vivo studies and the establishment of drug pharmacodynamic behaviour. Changes in cell membrane permeability resulting in leakage of cytoplasmic enzymes or uptake of dyes can be utilized to detect changes in cell viability (Mitchell et al., 1980). A loss of membrane integrity is typically a characteristic of a dead or dying cell (Nieminen et al., 1992).

Many assays look at changes in the membrane integrity or permeability properties of the cell plasma membrane. For example, dye exclusion and inclusion assays shown in Figure 1.7., (as with trypan blue or carboxyfluorescein) examine whether or not cells are viable by the amount of dye taken in through the plasma membrane or released from the plasma membrane, respectively (Bhuyan et al., 1976; Bruning et al., 1980). However, loss of membrane integrity does not necessarily mean that the cell is not viable.

Another way to assay cell viability is through the functioning of cellular enzymes or processes. These assays provide substrates that can be converted, by cellular enzymes, to spectrophotometrically detectable products (Figure 1.7). For example, tetrazolium salts can be reduced to a formazan product by mitochondrial dehydrogenases (Heo et al., 1990). A decrease in cell viability due to drug exposure can decrease specific enzyme levels, such as levels of mitochondrial dehydrogenases, or alter enzyme function (Roehm et al., 1991). Consequently, lower levels of formazan product would be generated from
Illustrations of commonly used assays to measure cell viability, or cytotoxicity as a result of exposure to drugs. (Top) Dye exclusion and inclusion assays indicate which cells may have compromised cell membranes resulting in either uptake or release of dye, respectively. (Bottom) Also shown are assays involving enzymatic processes that convert substrates to spectrophotometrically detectable products. In this diagram, there are three steps. First, the membrane permeable substrate is taken up into viable cells. Secondly, organelles such as the mitochondria contain enzymes that convert the substrates into products. Finally, the products diffuse from the cell into the media to be collected for determination of products. Non-viable cells should not produce products.
the tetrazolium salt substrate. By varying drug concentrations and examining the amounts of formazan generated as an indicator of cell viability, a dose-response profile for a drug may be produced. Subsequently, parameters such as the EC$_{50}$ (effective dose resulting in 50% loss in cell viability) or IC$_{50}$ (the dose of drug which inhibits cell proliferation or induces cell toxicity by 50%) of a drug may be determined to form a basis for additional studies.

Clonogenic assays for in vitro chemosensitivity testing are considered the most accurate method of measuring the effectiveness of cytotoxic drugs. In general, clonogenic assays are based on measuring reductions in mammalian cell colony formation under various plating conditions (Puck et al., 1956). Essentially, it was proposed that cells from most spontaneous human tumors could form colonies in soft agar and that these clonogenic cells are representative of tumor stem cells, which are responsible for sustained malignant growth in vivo (Sikic and Taber, 1981). Human tumor biopsy specimens can then be examined for inhibition of in vitro colony formation as a result of drug exposure. As a result, clinical care of patients might potentially be improved because the selection of chemotherapy could be optimized for individual patients. However, some technical problems with clonogenic assays have been detailed in a review by Weisenthal and Lippman (1985). Most notable are poor cloning efficiencies and the presence of clump artifacts. Studies suggest that poor cloning efficiencies do not fully represent the true percentage of stem cells in the tumor. Ultimately, the techniques may prove to be of major benefit to only a small fraction of cancer patients (Sikic and Taber, 1981).
As an alternative to clonogenic assays, non-clonogenic assays such as short-term cultures of cell suspensions and cell monolayers may be as viable as indicators for \textit{in vitro} chemosensitivity testing. In a study comparing three assays (short-term cell suspensions, monolayer and clonogenic) used for \textit{in vitro} chemosensitivity testing of human tumors, it was shown that the assays were equally valid when used for predictive testing (Wilson \textit{et al}., 1984). While the three assays did not produce identical dose-response curves, they were deemed equally valid because of the selection of cut-off points, which were based on retrospective correlations between \textit{in vitro} sensitivity data and response data adjusted for differences in sensitivity between assays. Consequently, for the purposes of liposomal drug delivery and ease of assay preparation, non-clonogenic assays were used to assess the cytotoxicity of formulations developed.

1.3.2.2 Visualization of Cell Fluorescence Utilizing Fluorescence Microscopy

For therapeutic agents to be effective, they often must reach intracellular target sites within the cytoplasm and nucleus. To determine the cellular localization of liposomal carriers and their entrapped contents, fluorescent lipid probes and fluorescent contents markers have been widely used in association with fluorescence microscopy techniques (Struck and Pagano, 1980; Struck \textit{et al}., 1981; Straubinger \textit{et al}., 1985; Rost, 1992) (Figure 1.8). Fluorescence microscopy can also be used to visualize fluorescently labeled molecules (DNA, proteins, peptides) delivered to cells (Rao \textit{et al}., 1997; Waelti and Glück, 1998; Lutwyche, \textit{et al}., 1998). Examining cellular distribution of fluorescent lipids and contents markers can help to determine mechanisms of intracellular delivery to cells. Likewise, fluorescence intensity measurements using quantitative fluorescence
Figure 1.8: Fluorescence Microscopy

Illustration of cells exposed to fluorescent molecules and how they would appear as seen through a fluorescent microscope under phase contrast and fluorescent light.
microscopy have been applied to determine the extent of delivery. Microfluorometry (a branch of fluorescence microscopy) is a technique where the intensity of fluorescence is measured from a given area of a specimen. By measuring the amount of fluorophore present, it is possible to estimate the concentration present in that region of the specimen (Rost, 1990). The measured intensity is compared to the intensity of a standard containing a known amount of the fluorophore. From the standard, the amount of fluorophore can be estimated.

With respect to liposomes and their interactions with cells, fluorescent lipid probes such as 7-nitro-2,1,3-benzoazadiazol-4-yl conjugated to PE (NBD-PE) and 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-N-(Lissamine rhodamine B sulfonyl) (N-Rh-PE) have been commonly incorporated into liposomal bilayers (Struck and Pagano, 1980; Struck et al., 1981; Ellens et al., 1989; Murata et al., 1993; Zelphati et al., 1998). In addition, calcein, carboxyfluorescein and fluorescein-labeled proteins, peptides or sugars encapsulated within the core of liposomes have been used for intracellular delivery studies (Szoka et al., 1980; Straubinger et al., 1985; Brown and Silvius, 1990; Slepushkin et al., 1997). Using microfluorometry to examine the probes allows researchers to qualitatively measure the amount of fluorescence present within the cell, as well as the intracellular location of fluorescence.

Limitations of fluorescence microscopy include photobleaching, or fading, of probes and blur or reduction of contrast by fluorescence from out-of-focus planes. Photobleaching (the decrease in fluorescence of a probe upon exposure to light) can be very rapid (Lansing Taylor and Salmon, 1989). Studies suggest that a proportion of the excited molecules, instead of fluorescing, undergo a photochemical reaction with the
production of a new molecule that may be non-fluorescent, or at least non-absorbent at
the excitation wavelength (Rost, 1991). Studies also suggest that photobleaching is due
to a reaction between the excited fluorophore and oxygen to form a non-fluorescent
product (Rost, 1991). This sometimes makes cell samples difficult to work with
requiring the sample to be protected from exposure to external light sources and
examined rapidly under illumination. Fortunately, newer probes have been designed to
be less susceptible to photobleaching. Reagents such as n-propyl gallate, ascorbic acid
and 1,4-diazobicyclo(2,2,2)-octane (DABCO) have also been designed to help maintain
the fluorescent levels of probes (Giloh and Sedat, 1982; Johnson et al., 1982).

Interference of fluorescence from out-of-focus planes results when two cells are
in different planes (for example, one cell on top of another) making it difficult to discern
which cell is fluorescent or where the fluorescence is distributed. Fortunately, to
overcome this limitation confocal fluorescence microscopy has been developed. The
resolution of a confocal microscope is at least 14 times greater than that of a conventional
fluorescent microscope (Rost, 1992). Confocal microscopy greatly increases resolution
of fluorescent samples by imaging out-of-focus planes with less intensity than the in-
focus plane. Consequently, background fluorescence is greatly reduced and it is possible
to obtain three-dimensional data by combining a series of optical sections (Rost, 1992).
Examples of confocal microscopy used to examine liposome drug delivery systems
include intracellular delivery of liposomal gentamicin with DOPE-containing liposomes,
peptide nucleic acids combined with DOPE/DDAB liposomes and cationic liposome-
oligonucleotide complexes (Lutwyche et al., 1998; Zelphati et al., 1998; Ljungstrøm et
al., 1999).
1.3.2.3 Quantitation of Cell Fluorescence Using Flow Cytometry

Cytometry refers to the measurement of physical and/or chemical characteristics of cells or, by extension, of other biological particles such as cellular organelles. Flow cytometry is a process in which such measurements are made while the cells or particles pass, preferably in single file, through the measuring apparatus in a fluid stream (Shapiro, 1988). This technique allows for the quantitation of cells as well as the multiparameter acquisition and analysis of cell characteristics. The first commercial flow system available to count cells was the Coulter Counter that measured changes in impedance as cells passed through a narrow capillary orifice (Coulter, 1956). In the late 1960s and early 1970s, research performed by Herzenberg and his co-workers at Stanford University resulted in major developments in fluorescence activated cell sorting (Watson, 1991). Since that time, flow cytometry has been used in a wide variety of areas such as oncology, pathology, haematology, immunology, cell biology, pharmacology and toxicology to count cells as well as separate them according to specific characteristics.

Flow cytometry data are based on light scattering and fluorescence from cells (Figure 1.9). The parameters from which data can be collected include light scattering parameters (forward scatter and side scatter) and the fluorescence parameters that result from the detection of fluorescently labeled molecules. Forward scatter and side scatter result from the incident light that is reflected from the cell specimen. Forward scatter determines the size of the cell while side scatter determines the complexity (cellular organelle content, granularity, etc.) of the cell.

With respect to liposomal drug delivery studies, flow cytometry has been utilized to measure endocytosis of carboxyfluorescein encapsulated in protein A-bearing
Figure 1.9: Components of a Flow Cytometer and Light Scattering Parameters from a Cell Sample

(A) Diagram of the components of a flow cytometer from the sample of cells in a single file through the laser light source, to capturing of the light by the detectors, to the conversion of data from the detector into electronic signals for analysis by the computer. (B) Light scattering parameters that reflect from a cell as it passes through the laser. The parameters include side scatter, forward scatter and light emission from fluorescent probes.
liposomes targeted to defined cells through use of a cell surface-specific antibody (Truneh et al., 1983). The interaction between phosphatidylcholine vesicles and isolated rat liver nuclei has been examined using flow cytometric analysis by studying the uptake of carboxyfluorescein (Caramelli et al., 1989). In addition, flow cytometry was used to examine the delivery of fluorescein isothiocyanate (FITC)-conjugated antibodies, fluorescent label dextran, plasmid DNA complexed with acridine orange, or ethidium monoazide encapsulated in liposomes (Eriksson et al., 1984; Cudd and Nicolau, 1986; Tseng et al., 1997; Skalko et al., 1998). More recently, studies quantitating the liposomal transfer and expression of plasmid DNA encoding for green fluorescent protein (GFP) in cultured cells has been performed using flow cytometry (Tseng et al., 1997; Gubin et al., 1999). Hence, flow cytometry has numerous applications for the examination and quantitation of fluorescent probes or molecules encapsulated within liposomes. Therefore, flow cytometry will be used for studies involved in this thesis because of its powerful ability to gather an immense amount of data from cell samples.

1.4 LIPOSOMES FOR DELIVERY OF THERAPEUTIC AGENTS

There are several areas of liposome research, which include therapy for retroviral infections, pulmonary disorders, inflammation, and cancer (Gregoriadis et al., 1993). In addition, liposomes can be used as immunological adjuvants for vaccines, as blood surrogates, as well as topical treatments for dermal and transdermal drug delivery (Gregoriadis et al., 1993). While this research has been extensive, only a small number
of liposomal drug formulations have succeeded in reaching clinical trials or commercialization.

This next section will focus on liposomal formulations that are commercially available or in clinical trials, primarily for cancer treatment.

1.4.1 Clinically-Approved Liposomal Formulations

It has taken three decades, but liposomal drug formulations have now been approved for clinical use in a number of countries around the world. These drug preparations include liposomal formulations of the antifungal drug amphotericin B as well as liposomal formulations of the anticancer drugs daunorubicin and doxorubicin (Allen, 1997). Ambisome (Vestar), one liposome formulation of the antifungal agent amphotericin B, is licensed and marketed in the U.K. (Gregoriadis et al., 1993). The liposomal formulation of daunorubicin, DaunoXome, is approved for clinical use in the U.S.A., Canada and Europe. In addition, the liposomal formulation of doxorubicin, Doxil (also called Caelyx), has been approved for clinical use in the U.S.A. and Europe.

Along with these preparations, many potential liposomal drug formulations are currently being tested; however, there has been a great deal of emphasis on the use of liposomal anticancer drugs. And while there are a number of liposomal anticancer drugs available, the next section will focus specifically on applications of liposomes for delivery of anthracyclines and antisense molecules for the purposes of this thesis.

1.4.2 Liposomal Anthracyclines

Anthracyclines are among the most commonly used drugs for treatment of solid tumors. Daunorubicin and doxorubicin, the first identified anthracyclines, were isolated
from pigment producing *Streptomyces* spp. in the early 1960s and remain in widespread clinical use (Hortobagyi, 1997). In terms of pharmacological activity, their major mechanism of action appears to involve DNA intercalation. Intercalation causes deformation of the DNA, stabilizing the normally reversible topoisomerase II-DNA complex. This results in double-stranded DNA breaks that cause cell death or inhibit their growth (Rang *et al.*, 1995).

Another possible mechanism of action is the formation of free radicals. This is thought to be associated with cardiotoxicity, the dose-limiting toxicity of anthracyclines (review by Smith, 1983; Calabresi and Chabner, 1990). It has been suggested that tissues contain scavenging enzymes, which detoxify free radicals, prevent tissue damage; myocardial tissue, however, appears to be relatively deficient in these scavenging enzymes. In order to reduce the cardiotoxicity of anthracycline, new derivatives were synthesized, such as idarubicin and epirubicin. In addition, structural relatives of anthracyclines such as the anthracenedione derivative, mitoxantrone, have been developed as effective, but less toxic, anticancer agents. Liposomal preparations of doxorubicin have resulted in a significant reduction in drug levels within the heart, and this is associated with a corresponding reduction in cardiotoxicity (Rahman *et al.*, 1980; Gabizon *et al.*, 1982; Mayer *et al.*, 1989; Northfelt *et al.*, 1998; Toma *et al.*, 2000).

Currently, the most recent studies have been focused on polyethylene glycol-coated (pegylated) liposomal doxorubicin to solid tumors. Research suggests that these liposomes are taken up to a lesser degree by the reticuloendothelial system compared with conventional liposomes and have a reduced tendency for drug leakage while in circulation (Gabizon and Martin, 1997). With this formulation, it was demonstrated that
the plasma elimination of doxorubicin could be progressively decreased as compared to drug administered in conventional liposomes or in free drug form. This is reflected in pharmacokinetics parameters such as increased circulating half-life, decreased plasma clearance and a reduced volume of distribution. Animal studies have also shown an improvement in antitumor activity. Importantly, little or no cardiotoxicity has been observed in patients with AIDS-related Kaposi's sarcoma who received pegylated liposomal doxorubicin in high cumulative doses in clinical trials (Gabizon and Martin, 1997).

In addition to doxorubicin, mitoxantrone may be an improved candidate for encapsulation in liposomes. It has shown antineoplastic activity against breast cancer, leukemia and lymphoma and it appears to exhibit less cardiotoxicity than free doxorubicin (Smith, 1983; Shenkenberg and Von Hoff, 1986). Furthermore, results of studies with liposomal formulations of mitoxantrone have indicated lower toxicity and greater efficacy than the free drug when tested in murine tumor models (Chang et al., 1997; Lim et al., 1997).

1.4.3 Liposomal Antisense Therapy

The aim of antisense therapy is to disrupt gene expression by blocking transcription of DNA, or destruction of mRNA, and blocking translation or production of specific cellular proteins. Like gene therapy, antisense therapy aims to introduce genetic material, DNA or RNA, into cells evoking a therapeutic effect. There are three major classes of antisense agents: antigene sequences, ribozymes and antisense sequences (Figure 1.10). These agents are classified on the basis of their mechanism of action.
Mechanisms of action of antisense molecules: (A) Antigene sequences recognize and bind to nuclear DNA to inhibit mRNA synthesis and subsequently inhibit protein synthesis. (B) Ribozymes recognize and bind to mRNA and catalyze its cleavage, inhibiting protein synthesis. (C) Antisense oligonucleotides recognize and bind to mRNA to inhibit protein synthesis.
Antigene sequences target nuclear DNA directly to inhibit transcription of RNA. Nuclear DNA exists in the form of a double-stranded helix. Antigene sequences hybridize with nuclear DNA forming triple helicities that inhibit transcription either by preventing the binding of sequence-specific DNA proteins required for transcription of mRNA or by directly preventing the transcription of mRNA (Putnam, 1996). Unfortunately, this approach is limited by the decreased, sequence-specific binding of antigene constructs compared with antisense oligonucleotides.

Ribozymes, pieces of ribonucleic acid (RNA), are capable of enzymatically catalyzing the cleavage of specific mRNA sequences. Instead of inhibiting protein synthesis by simply binding to a single targeted mRNA, ribozymes combine enzymatic processes with the specificity of antisense base pairing, creating a molecule capable of incapacitating numerous targeted mRNAs (Uhlenbeck, 1987). The three-dimensional structure of ribozymes is extremely important to their functioning. Consequently, they can be rendered inactive if their catalytic motif is disrupted.

Antisense sequences, also termed antisense oligonucleotides, are site-specific nucleic acid sequences that bind to mRNA sense strands through hydrogen bonding to complementary nucleic acid bases. Normally, nuclear DNA passes on genetic information by transcribing single-stranded sense mRNA, which then translocates to cytosolic ribosomes, where the ribosomes translate the information into specific cellular proteins. A requirement of protein synthesis is that the sense mRNA stays single-stranded. Antisense oligonucleotides are designed to bind to the specified sense mRNA forming a double-stranded hybrid so that the translation of cellular proteins cannot be initiated. In addition, the formation of a double-stranded hybrid is a substrate for
ribonuclease (RNase) H, an enzyme that cleaves duplex RNA strands (Walder and Walder, 1988).

In terms of treatments for cancer, antisense oligonucleotides have been employed to inhibit protein synthesis regulated by specific proto-oncogenes. To date, the main proto-oncogenes studied are the myc family (including c-myc and N-myc), c-myb, c-fos, the ras family (including N-ras and c-H-ras), bcl-2, c-raf-1, cdc-2 and c-mos (Tidd, 1990; Calabretta, 1991; Carter and Lemoine, 1993; Neckers and Whitesell, 1993; Putnam, 1996). Proto-oncogenes are naturally occurring gene sequences in the human genome that are implicated in certain malignancies. Malignant tumors tend to overexpress these proto-oncogenes which results in continued cell division and poor cell differentiation (Putnam, 1996). If overexpression of a proto-oncogene could be blocked or down-regulated, then the tumor might cease to divide or may become more sensitive to other forms of cancer treatment like chemotherapy and radiation. Therefore, several studies have tried to identify specific proto-oncogenes related to malignances and designing antisense oligonucleotides specific for their mRNA.

Limitations to antisense oligonucleotides are their low permeability across cell membranes and susceptibility to degradation by nucleases. To be effective, it is necessary that these compounds enter the cytoplasm or nucleus of cells. Liposomal preparations have been designed as carriers that could improve the delivery of antisense oligonucleotides into cells, as well as confer protection against degradation by nucleases. Liposomes have shown positive in vitro and in vivo results as drug delivery systems for antisense oligonucleotides (Akhtar et al., 1991; Tari et al., 1994; Aoki et al., 1997; Soni et al., 1998; Gutierrez-Puente et al., 1999; Yu et al., 1999; Matsuno et al., 2000; Tari,
2000). However, novel formulations such as PFVs may improve the bioavailability of oligonucleotides as well as enhance their delivery to the cytoplasm of target cell populations.

By designing novel cell culture assays and utilizing pre-existing cell culture assays, liposomal drug delivery systems, in particular programmable fusogenic vesicles, will be characterized. The following research will assess whether this liposomal drug delivery system is a prime candidate for delivery of anticancer drugs such as mitoxantrone and genetic material such as antisense oligonucleotides. In addition, if PFVs show promise as an intracellular delivery system for these anticancer therapeutic agents, this might also indicate their potential for intracellular delivery of a variety of other drug classes.
1.5 RESEARCH HYPOTHESIS

In the field of drug delivery, the development of a drug carrier system that can demonstrate intracellular delivery (fusion and cytoplasmic delivery) with target cells is a major topic of investigation. A carrier system that could achieve this would greatly improve the delivery efficiency of therapeutic agents potentially resulting in improved therapeutic activity. Liposomes such as programmable fusogenic vesicles (PFVs) are potential candidates for intracellular drug delivery. To date, the investigation of delivery mechanisms between liposomes and cells has been limited by the lack of specific assays to characterize the events involved in the process. It is proposed that cell culture methods can be used together with the development of specific assays for cytoplasmic delivery to characterize the interaction of liposomal systems with cells. These studies in turn will allow the design of improved liposomal systems for cytoplasmic delivery of therapeutic agents.
1.6 SUMMARY OF RESEARCH OBJECTIVES

The overall thesis objective is to develop novel cell culture models to characterize the delivery properties of liposomal carriers, in particular programmable fusogenic vesicles (PFVs).

The specific objectives are listed below:

1. To compare bioavailability of an anticancer agent encapsulated in conventional liposomes and PFVs utilizing an *in vitro* cell viability assay.
2. To examine lipid interaction (fusion) between cultured cell plasma membranes and bilayer vesicle membranes of PFVs.
3. To develop an assay that allows quantitative determination of cytoplasmic delivery.
4. To compare cytoplasmic delivery by PFVs and conventional liposomal delivery systems.
5. To examine the cytoplasmic delivery of large polar compounds, antisense oligonucleotides, using PFV delivery systems.

The specific objectives 1 to 5 are presented in thesis chapters 3 through 7, respectively.
CHAPTER 2
MATERIALS AND METHODS

2.1 LIPIDS, CHEMICALS AND CELL LINES

1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (MW 744.04), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (MW 786.12), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (MW 790.15), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) (MW 760.08), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[Poly(ethylene glycol) 2000] (PEG2000-DSPE) (MW 2748.07), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[Poly(ethylene glycol) 2000] (PEG2000-DMPE) (MW 2635.86) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (Rh-PE) (MW 1285.11) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Antisense phosphorothioate oligonucleotides complementary to the first six translation initiation codon of the human bcl-2 mRNA (product number: G3139, AS) (base sequence: 5'-TCT CCC AGC GTG CGC CAT-3') and reverse polarity (RP) control oligonucleotide (product number: G3622, RP) (base sequence: 5'-TAC CGC GTG CGA CCC TCT-3') were originally synthesized by Genta Inc. (Lexington, MA, USA). Fluorescein isothiocyanate (FITC)-labeled antisense oligonucleotides to the gene stop codon of epithelial growth factor receptor (FITC-EGFR) (5'-CCG TGG TCA TGC TCC-3'), N,N-dioleyl-N,N-dimethylammonium chloride (DODAC) (MW 582.5), DNA (pACN 53) and Poly(ethylene glycol) 2000 succinate-(C8:0, C14:0 and C20:0) ceramides (PEG-C_n ceramides) (MW 2507, 2591 and 2675, respectively) were obtained from Inex Pharmaceuticals (Burnaby, BC, Canada).
[\textsuperscript{3}H]-Cholesteryl hexadecyl ether (\textsuperscript{3}H-CHE) was purchased from Amersham (Oakville, ON, Canada). YOYO-1 iodide, 1,1'-[1,3-propanediylbis[(dimethyliminio) -3,1-propanediyl]]bis[4-[(3- methyl-2(3H)-benzoxazolylidene) methyl]]-1, tetraiodide (YOYO) (MW 1270.65), also known as oxazole yellow homodimer, was bought from Molecular Probes (Eugene, OR, USA). Packard Ultima Gold liquid scintillation cocktail was obtained from Packard Instrument Company (Meriden, CT, USA). Novantrone\textsuperscript{®} (mitoxantrone hydrochloride) was acquired from the British Columbia Cancer Agency and is a product of Wyeth-Ayerst (Montreal, PQ, Canada). Polycarbonate filters were purchased from Costar (Cambridge, MA, USA). Dulbecco's Modified Eagles Media (DMEM), Dulbecco's phosphate buffered saline (D-PBS), heat-inactivated fetal bovine serum (FBS) and trypsin-EDTA (1X), TRIZOL\textsuperscript{®} reagent, M-MLV reverse transcriptase, random hexamers and Taq DNA polymerase were supplied by Gibco BRL-Life Technologies (Grand Island, NY, USA). Propidium iodide (PI) (MW 668.4), cholesterol (Chol) (MW 386.7), (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES) (MW 238.3), Sephadex G-50, DEAE-Sepharose CL-6B, (2,3-bis[2-Methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carboxanilide) (XTT) (MW 673.5) and (N-Methyldibenzopyrazine methyl sulfate salt), phenazine methosulfate (PMS) (MW 306.3) were obtained from Sigma (St. Louis, MO, USA). Sodium chloride (NaCl) (MW 58.44), Triton X-100 and sodium thiocyanate (NaSCN) (MW 81.07) were purchased from BDH Incorporated (Toronto, ON, Canada). RPMI-1640 media was supplied by Stem Cell Technologies (Vancouver, BC, Canada). Cell lines (293, B16, BHK, L1210, J774) were purchased through the American Type Culture Collection (ATCC) (Manassas, VA, USA). Human melanoma 518A2 cells were obtained from the British Columbia Cancer
Agency. As well, all cell culture plasticware required was obtained through VWR CanLab (Mississauga, ON, Canada).

2.2 PREPARATION OF MITOXANTRONE ENCAPSULATED LIPOSOMES

2.2.1 Preparation of Liposomes

Conventional liposomes, DSPC:Chol (55:45 mol%), and two types of PFVs containing PEG-PEs of varying acyl chain lengths, DOPE:cholesterol:DODAC:PEG2000-DMPE (30:45:15:10 mol %) and DOPE:cholesterol:DODAC:PEG2000-DSPE (30:45:15:10 mol %), were made. Lipids were weighed out in appropriate amounts and co-solubilized in benzene:methanol (95:5 v/v). The solutions were then combined together accordingly, frozen in liquid nitrogen and lyophilized (Virtis, Gardiner, NY, USA) under high vacuum (30-60 mtorr) for a minimum of five hours.

The dried lipid samples were hydrated with 300mM citrate, pH 4.0 buffer and vortexed well to form MLVs. The hydrated lipid samples were then frozen in liquid nitrogen for 5 minutes, then thawed in a 37°C water bath for 5 minutes and this cycle was repeated five times to equilibrate the solute distribution (Mayer et al., 1985). LUVs were then formed by extruding the MLVs ten times through two stacked polycarbonate filters (Hope et al., 1985) using an Extruder (Lipex Biomembranes, Vancouver, BC, Canada) under nitrogen pressures of 200-500 psi at 37°C.

2.2.2 Uptake of Mitoxantrone into Liposomes

Mitoxantrone was encapsulated into liposomal formulations using a previously described method (Chang et al., 1997). In brief, a transmembrane pH gradient was
established by passing the LUV suspensions down a 1.0 cm x 15.0 cm Sephadex G-50 column buffered with 150mM NaCl, 20mM HEPES (HEPES buffered saline, HBS), pH 7.4. Fractions of 0.5 ml were collected and the most concentrated fractions were combined.

Free mitoxantrone (2.0 mg/ml) was titrated to pH 7.4 using 0.1 M sodium hydroxide and diluted to a desired volume using HBS. It was then combined with an appropriate volume of LUV suspensions, exhibiting a transmembrane pH gradient, to achieve a drug to lipid molar ratio of 0.1:1. The samples were then incubated at 37°C for 60 minutes to allow for uptake of mitoxantrone into the liposomes. To determine mitoxantrone uptake efficiency, 100μl aliquots were taken at various timepoints and unencapsulated mitoxantrone was separated from mitoxantrone encapsulated in liposomes by passage of LUVs through 1ml Sephadex G-50 minicolumns that were centrifuged at 2000 rpm for two minutes, as previously described (Pick, 1981). Mitoxantrone uptake into vesicles was determined to be greater than 98% using a spectrophotometric assay described in Section 2.10.2.

2.3 PREPARATION OF RHODAMINE-PE INCORPORATED IN PFVS

To examine lipid mixing with the plasma membranes of cultured cells, the fluorescent labeled lipid, Rh-PE, was incorporated into the bilayers of the PFVs. Lipids were weighed out, co-solubilized in benzene:methanol (95:5 v/v) and combined together. Rh-PE was also solubilized in benzene:methanol (95:5 v/v) and added to the lipid mixtures resulting in a final concentration of 1 mol %. The solutions were then vortexed well, frozen in liquid nitrogen and lyophilized (Virtis, Gardiner, NY, USA) under high vacuum (30-60 mtorr) for a minimum of five hours. The lipid composition of the
vesicles for these studies were DOPE:cholesterol:DODAC: PEG2000-Cn Ceramides:Rh-PE (35:45:15:5:1 mol%), where the acyl chain lengths of the PEG ceramides were C₈, C₁₄ and C₂₀.

The dried lipid mixtures were hydrated with HBS, pH 7.4 and vortexed well to form MLVs. The hydrated lipid mixtures were then frozen in liquid nitrogen for 5 minutes, then thawed in a 37°C water bath for 5 minutes and this cycle was repeated five times to equilibrate the solute distribution (Mayer et al., 1985). LUVs were then formed by extruding the MLVs ten times through two stacked polycarbonate filters (Hope et al., 1985) using an Extruder (Lipex Biomembranes, Vancouver, BC, Canada) under nitrogen pressures of 200-500 psi at 37°C.

2.4 PREPARATION OF YOYO-1 IODIDE ENCAPSULATED LIPOSOMES

Lipids were weighed out, co-solubilized in benzene:methanol (95:5 v/v) and combined together. The solutions were then combined together accordingly, frozen in liquid nitrogen and lyophilized (Virtis, Gardiner, NY, USA) under high vacuum (30-60 mtorr) for a minimum of five hours.

The dried lipid mixtures were hydrated with HBS, pH 7.4 and vortexed well to form MLVs. YOYO-1 iodide (50μM), used as a fluorescent probe to assess cytoplasmic delivery to cultured cells, was added to the hydrated lipid mixture. These solutions were then frozen in liquid nitrogen for 5 minutes, then thawed in a 37°C water bath for 5 minutes, and this cycle was repeated five times to equilibrate the solute distribution (Mayer et al., 1985). LUVs were then formed by extruding the MLVs ten times through two stacked polycarbonate filters (Hope et al., 1985) using an Extruder (Lipex
Biomembranes, Vancouver, BC, Canada) under nitrogen pressures of 500-700 psi at 37°C.

To remove external YOYO-1 iodide from YOYO-1 iodide encapsulated liposomes, LUV suspensions were passed down a 1.0 cm x 15.0 cm Sephadex G-50 column buffered with HBS, pH 7.4. Fractions of 0.5 ml were collected and the most concentrated fractions were combined.

2.5 PREPARATION OF ANTISENSE OLIGONUCLEOTIDE ENCAPSULATED IN PFVS

DOPE, Chol, DODAC and PEG-ceramides (C₈, C₁₄ or C₂₀) in stock solutions of 100% ethanol were combined together at molar ratios of 35:45:15:5. The lipid mixture was made up to a final volume of 1ml using 100% ethanol containing [³H]-CHE (1μCi/μl) as a lipid marker. The lipid mixture (50mM) was added to an aqueous solution of antisense (1mg/ml) in 200mM NaCl buffered with 20mM HEPES, pH 7.4 (HBS), such that the final antisense:lipid ratio was 0.07:1 (w/w) and the final ethanol concentration was 30%.

To produce LUVs, these samples were extruded ten times through 2 x 80nm stacked polycarbonate filters under nitrogen pressures of 300-500 psi at 37°C. Residual ethanol was removed by dialyzing the samples overnight in HBS, pH 7.4, using Spectra/Por Membranes 12-14K molecular weight cutoff dialysis membrane tubing, (Spectrum Laboratories, Inc., Laguna Hills, CA, USA). In addition, free and externally bound antisense was removed by dialysis in 150mM NaCl, 50mM NaSCN, 20mM HEPES, pH 7.4 for 4-5 hours followed by ion exchange chromatography using 2cc DEAE-Sepharose CL-6B minicolumns. Fractions of 0.5 ml were collected and the most
concentrated fractions were combined. The final antisense:lipid ratio in loaded PFV was approximately 0.03-0.04:1 (w/w).

2.6 CELL CULTURE AND PASSAGING

Typically, adherent cell lines were grown in 25cm² or 75cm² cell culture flasks, fed Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and incubated at 37°C, 5% CO₂. When passaging was required, the media was removed, the cells were washed with phosphate buffered saline (PBS) and then detached by incubating cells with 0.5% trypsin-ethylenediamine-tetraacetato (EDTA) for 30 seconds ("trypsinized" for short). Fresh media with serum was then added to the cells to halt the trypsinization and cells were transferred from the flask to a conical tube to be centrifuged at 2000 rpm for 2 minutes into a pellet. The trypsin containing media was removed, the cells were then resuspended in fresh media once again and divided to 1/10 or 1/4 into a fresh flask. Non-adherent cell lines were cultured and passaged under the same conditions except that the trypsinization step was not required.

2.7 XTT/FORMAZAN CELL CYTOTOXICITY/VIABILITY ASSAY

The growth medium for the HEK 293 cell line was DMEM supplemented with 10% heat-inactivated FBS, while the growth medium for L1210 cells was RPMI-1640 supplemented with 10% heat-inactivated FBS. The XTT assay reagent was a combination of the tetrazolium salt, XTT, and phenazine methosulfate (PMS; 1% by weight) as a reducing agent, made up to 1mg/ml in fresh cell culture media. Following 24, 48, 72 and 96 hours of incubation with mitoxantrone, 80μl of the XTT viability
reagent was added to the wells which was equivalent to 20% of the total well volume. The reagent was allowed to incubate in the cell media for 4 hours then the media was transferred to fresh test tubes containing 1440μl 1% Triton X-100 in HBS, a detergent that would disperse all liposomes and detached cells in the media, at a ratio of 3:1 (Triton X-100 to cell media containing formazan product by volume). The samples were transferred to plastic cuvettes and their absorbances were then read on a spectrophotometer at 450nm. This assay was performed in a similar manner for both L1210 cells and HEK 293 cells.

2.8 FLUORESCENCE MICROSCOPY AND PHOTOGRAPHY

Cell lines were grown in 25cm² flasks, fed DMEM supplemented with 10% heat-inactivated FBS and incubated, at 37°C, 5% CO₂, with various liposome samples for predetermined lengths of time. Photographs were taken at various time-points under the Zeiss Axiovert S100 inverted microscope with an epi-fluorescence attachment using either Kodak Ektachrome P1600 or Fuji 1600 Provia film. The camera attachment was set on auto-exposure. In some experiments, the cells were washed with Dulbecco's phosphate buffered saline (D-PBS) prior to taking photographs. Control cells were also incubated with and without PFV added to the media. Photographs also were digitally scanned and enhanced using Adobe Photoshop (Version 5.0) software.

2.9 FLOW CYTOMETRY AND DATA ANALYSIS

Cell lines were grown in 25cm² flasks, fed DMEM supplemented with 10% heat-inactivated FBS, and incubated at 37°C and 5% CO₂ with YOYO-loaded vesicles for predetermined lengths of time. Following the incubation period, the media from each flask
was transferred to 15ml conical tubes. When required, the adherent cell lines were trypsinized and added to their respective conical tubes. The conical tubes were then centrifuged for three minutes at 2000 rpm, the supernatant was aspirated, and the cells were then washed with fresh HBS. This was repeated for two washes in HBS. Subsequently, the supernatant was aspirated, the cells were resuspended in 500µl of fresh HBS and transferred to 6ml plastic tubes for flow cytometric analyses. Just prior to flow cytometric analysis, 10µl of PI (50µg/ml) in HBS was added to samples to label non-viable cells. Flow cytometric analysis was performed on a FACSsort flow cytometer (Becton-Dickinson, San Jose, CA) equipped with an Ar-ion (λ_{excitation} 488nm) laser and a He-Ne (λ_{excitation} 635nm) laser. Fluorescence of YOYO (λ_{excitation} = 491nm and λ_{emission} = 509nm when bound to DNA) and FITC (λ_{excitation} = 494 nm and λ_{emission} = 519nm) were measured using a designated channel FL1 (λ_{emission} 530±15nm) to quantitate the number of cells that were fluorescent due to delivery facilitated by PFV. PI (λ_{excitation} = 535 nm and λ_{emission} = 617nm when bound to DNA) fluorescence was detected in FL2 (λ_{emission} 585±21nm). The data were acquired and analyzed using CellQuest software (Becton-Dickinson, San Jose, CA). Control samples, cells incubated with free YOYO and cells incubated with free FITC-labeled antisense oligonucleotides, were also assayed for background results. The flow cytometer was set to count 10,000 events representing cell numbers.

Prior to reading the cell samples, controls were performed with free YOYO (50nM) and PI (1µg/ml) added to separate flasks of cells permeabilized with 70% ethanol for 1 hour. The flow cytometer instrument settings were calibrated accordingly for each
cell line to optimize the FSC (forward scatter), SSC (side scatter), FL1-H (fluorescence channel 1) and FL2-H (fluorescence channel 2) parameters.

When analyzing the large amounts of data generated from flow cytometry of cell samples, there are numerous ways to express the data. Three possible ways of expressing data for analysis are dot plots, histograms or quadrant analyses (Figure 2.1). Dot plots are the simplest method of presenting bivariate data, where two measurements are made on each cell in the sample. The two measurements from each cell are displayed as a dot with X and Y coordinates proportional to the two measurements (Watson, 1991). These data can also be gated to exclude cell debris and particulate matter or to focus on a particular cell type, if the sample is heterogeneous (Figure 2.1A). Histograms, or two-dimensional frequency distributions, can provide the mean fluorescence statistical values from cell samples (Figure 2.1B). Therefore, a control sample, such as cells in the absence of a fluorescent marker, can be run through the flow cytometer and the data can be generated in the form of a histogram. Then a cell sample incubated with a fluorescent marker can be run through the flow cytometer and its data can generate a second histogram. The two histograms can then be compared statistically to determine how much of the fluorescent marker was associated with the second cell population compared to the control, in terms of mean fluorescence units. Alternatively, the data can be expressed in the form of quadrants (Figure 2.1C). For example, when using two fluorescent probes, the data generated from cells associated with the first probe can be plotted against the data generated from cells associated with the second probe. Subsequently, a plot can be generated that can be divided into four quadrants, or subgroups: in the first quadrant (lower right), cells that had taken up the first fluorescent
Three forms of expressing flow cytometric data are (A) dotplots, (B) histograms or (C) quadrant plots. Dot plots can plot one parameter against the next such as side scatter against forward scatter. As well, the data can be "gated" to focus on a single cell population or exclude cell debris. Histograms can compare fluorescence intensity of a negative control cell sample (left) to that of a positive cell sample (right), shown as a shift toward the right. And quadrant plots can be utilized to compare the detection of two fluorescent probes within the same cell sample. (From left to right) A cell sample negative for both probes, a cell sample only positive for probe 2, a cell sample positive for both probes and a cell sample only positive for probe 1. The cell population can be split up into more than one quadrant.
probe; in the second quadrant (upper left), cells that had taken up the second fluorescent probe; in the third quadrant (upper right), cells that had taken up both fluorescent probes; and in the final quadrant (lower left), cells that did not take up either fluorescent probe. By analyzing each quadrant, statistical values can be generated. For a majority of the studies mentioned in this thesis, the flow cytometric data will be expressed in terms of quadrant analyses.

2.10 ANALYTICAL METHODS

2.10.1 Phospholipid Quantitation

Phospholipid concentrations were determined by either assaying phosphorus content (Fiske and Subbarow, 1925) or by liquid scintillation counting of the vesicle samples using $^3$H-cholesteryl hexadecyl ether (CHE), diluted with distilled water and combined with liquid scintillation cocktail, on a Beckman LS-3801 liquid scintillation counter.

2.10.2 Quantitation of Mitoxantrone Concentration

To quantitate mitoxantrone, a spectrophotometric assay was employed as described previously (Chang et al., 1997). In brief, 200μl aliquots from each liposomal mitoxantrone preparation were diluted with 100μl of distilled water and 1.2 ml of 5% Triton X-100 (v/v). The mixture was heated to 60°C for 5 minutes to solubilize all vesicles and release encapsulated mitoxantrone. The samples were then allowed to cool to room temperature and their absorbances were read at 666.5 nm. Mitoxantrone concentrations were determined by interpolation using a standard curve (0-60 nmol mitoxantrone), which was read prior to assaying the samples.
2.10.3 Quantitation of YOYO-1 Iodide Concentration

YOYO-1 iodide encapsulated in liposomes was quantitated using a fixed DNA concentration of 2μg/ml. This was determined to be a sufficient DNA concentration for a maximum YOYO concentration of 200nM. The development of this assay is further discussed in Chapter 5. Vesicle samples (100μl) were combined with 300μl of Triton X-100 (10% in dH2O), 600μl of DNA (10μg/ml) and 2ml of HBS to make a final volume of 3ml. The samples were then vortexed well and transferred to a cuvette prior to measuring the fluorescence emission intensity. Fluorescence emission intensity was measured at 510 nm (slit width 2.5 nm) with excitation at 492 nm (slit width 2.5 nm) using a Perkin-Elmer LS-50 spectrofluorimeter. Each time YOYO-loaded vesicles were made, a standard curve was generated. Based on the standard curve, a linear regression analysis was performed using SigmaPlot for Windows software. Samples were measured in duplicate. From the linear regression equation generated, the YOYO encapsulated within vesicles was calculated based on fluorescence emission intensity values. As well, light-scattering controls were performed by adding “empty” vesicles to the mixture described above and measured for background intensity.

For the studies involving leakage of YOYO from PFVs, vesicles were incubated at 37°C in HBS and DMEM, supplemented with 10% FBS, and examined over a 24 hour timecourse. At 1, 2, 4, 8 and 24 hours, DNA (2μg/ml) was added to an aliquot of the incubation mixture. The mixture was vortexed and transferred to a quartz cuvette. YOYO fluorescence was then determined as described above. After the initial fluorescence reading, Triton X-100 (final concentration 1%) was added to the samples to disperse the vesicles and release all encapsulated YOYO. The samples were then read
again to obtain the maximal fluorescence value for each sample. YOYO release was calculated as the initial reading divided by the maximal reading multiplied by 100%. A correction was made for light scattering produced by intact vesicles.

2.10.4 Quantitation of Antisense Oligonucleotide Concentration

Concentrations of antisense oligonucleotide encapsulated in liposomal preparations were determined using a spectrophotometric method. 50μl aliquots of liposomal antisense samples were diluted with 200μl of distilled water, then 750μl of CHCl₃:MeOH (1:2.1 v/v) and 100μl MeOH were added to solubilize all vesicles and expose base pairs. In addition, a blank was made with the exact solvent composition, except that a 50μl aliquot of HBS was used in place of a sample. The mixtures were gently vortexed and read at an absorbance setting of 260 nm (A₂₆₀). Antisense concentration was calculated using the formula:

\[
[\text{antisense}] = A_\text{260} \times 29.27 \, (\mu g/ml) \times 1.1 \, \text{ml} / 0.05 \, \text{ml}
\]

This formula was developed on the basis of standard curves prepared with known concentrations of antisense oligonucleotides.

2.10.5 Vesicle Size Analyses: Quasi-Elastic Light Scattering (QELS)

Liposomal samples were analyzed to determine vesicle size distributions by quasi-elastic light scattering (QELS) using the Nicomp Submicron Particle Sizer Model 270 (Santa Barbara, CA, USA) as previously described (Kölchens et al., 1993).
2.11 DOWN-REGULATION OF bcl-2 mRNA USING FREE ANTISENSE OLIGONUCLEOTIDES, ANTISENSE-LIPOSOME COMPLEXES OR ANTISENSE OLIGONUCLEOTIDES ENCAPSULATED IN PFVS

Cells were cultured in 25cm² flasks at an initial density of 5 x 10⁵ cells/flask. Antisense oligonucleotides were delivered either as free oligonucleotides, by cationic liposome-complexes, or by PFVs. The control cells were treated with the same volume of HBS (PFV delivery) or distilled H₂O (liposome-complexes delivery). Using antisense/liposome complexes, cells were treated in a serum-free culture environment. Cells were incubated in serum-free media for four hours after which the media was replaced with serum-containing media. In the case of antisense delivery using PFVs, cells were maintained in serum-containing media. At 24 or 48 hours, media was removed and cells were directly lysed in the flask. Total RNA was extracted using TRIZOL® Reagent following the manufacturer’s instructions.

Quantitation of bcl-2 mRNA levels was carried out using reverse transcription-polymerase chain reaction (RT-PCR) following a standard procedure (Chelly and Kahn, 1994). Briefly, first strand cDNA was synthesized from 2.5μg total RNA using Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase and random hexamer in 50mM Tris-HCl (pH 8.3), 75mM KCl, 10mM DTT, and 2mM dNTPs. The reaction was allowed to proceed for 1 hour at 37°C and stopped by incubation of the reaction mixture at 95°C for 5 minutes. Reverse transcription reaction products subsequently underwent PCR amplification for the detection of specific genes. Aliquots (3μl) of RT reaction products were amplified separately for bcl-2 and β-actin (internal standard) by PCR in 20mM Tris-HCl (pH 8.4), 50mM KCl, 1.5mM MgCl₂, 0.8mM dNTPs, 0.25μM each of the gene specific primers and 1.5μ of Taq DNA polymerase. DNA amplification was
performed using a Perkin-Elmer DNA thermocycler under the following conditions: 30 seconds at 94°C followed by 1.5 minutes at 65°C for 20 to 35 cycles. Appropriate cycle numbers were determined experimentally. Amplified products (10μl) were electrophoresed in 1.5% (w/v) agarose gel stained by ethidium bromide. Levels of relative band intensity, corrected on the basis of β-actin levels, were quantitated using a digital camera (Eagle Eye™ II, Stratagene) coupling with ImageQuant software (Molecular Dynamic, CA). The final values of bcl-2 mRNA abundance were expressed as percentages of the untreated control cells.

### 2.12 STATISTICAL METHODS

For gene regulation studies, statistical analysis was evaluated with one way ANOVA using SigmaStat™ software. Differences among treatment groups were further evaluated with the Student-Newman-Keuls test. A statistically significant difference was reported if p < 0.05.
CHAPTER 3

CHARACTERIZATION OF DRUG BIOAVAILABILITY: COMPARISON OF CONVENTIONAL LIPOSOMES AND PROGRAMMABLE FUSOGENIC VESICLES

Experiments on the bioavailability of an anticancer agent, mitoxantrone, encapsulated in different carriers are described in this chapter. Bioavailability defined here refers to drug release from a liposomal carrier system; it is this drug pool that is considered to be available to cells (Mayer et al., 1994). A cytotoxicity or growth inhibition assay was used to assess a cell viability indicator following exposure to mitoxantrone added in free form as well as various liposomal forms. For the purpose of convenience, the term cytotoxicity will encompass inhibition of cell proliferation, cytostatic effects, cytotoxicity, cell death or a combination of two or more of the terms. Conventional liposomes, PFVs and free drug were compared in two different cell lines as a function of drug concentration and exposure time.

3.1 INTRODUCTION

Liposomal mitoxantrone has been well characterized. In previous studies, mitoxantrone has been complexed with anionic phosphatidic acid liposomes and encapsulated into DSPC:Chol and DMPC:Chol conventional liposomes, DSPC:Chol:PEG-PE sterically stabilized liposomes, and PFVs (Schwendener et al., 1991; Chang et al., 1997; Lim et al., 1997; Adlakha-Hutcheon et al., 1999). Mitoxantrone was first discovered by Zee-Cheng and Cheng in 1978 and was later found to be less cardiotoxic than doxorubicin, a commonly utilized anticancer agent (reviewed by Smith, 1983). Therefore, the rationale for its use as a therapeutic agent was to reduce
cardiotoxic side effects. As well, it is not capable of generating free radical damage in nonproliferating cells (Durr, 1984). Mitoxantrone can be entrapped within liposomes using transmembrane pH gradient-based techniques (Madden et al., 1990; Chang et al., 1997; Lim et al., 1997). As well, liposomal mitoxantrone exhibits improved antitumor efficacy and is less toxic than free mitoxantrone (Schwendener et al., 1991; Chang et al., 1997).

As for mitoxantrone, most drugs at sufficiently high concentrations can induce toxicity, which can be seen using cell lines in vitro. With cultured cell lines, drugs can prevent the growth or division of cells or may result in cell death. Assaying for changes in cell growth or cell number is a common and quantitative way to assess the effects a specific drug has on a particular cell line.

To expand on the information described in Section 1.3.2.1, cytotoxicity assays can determine the plasma membrane integrity, gross cell morphology, lysosomal activity, mitochondrial membrane potential and mitochondrial enzyme activity of cells. Plasma membrane integrity assays include: leakage of cytoplasmic enzymes such as glutamate pyruvate transaminase, creatine phosphokinase and lactate dehydrogenase (Mitchell et al., 1980); fluorescent carboxyfluorescein release (Kolber et al., 1988) or propidium iodide uptake (Nieminen et al., 1992); chromium-release (Brunner et al., 1968; Rosenberg et al., 1974); and trypan blue dye exclusion (Bhuyan et al., 1976). These assays all examine whether compounds can cross the plasma membrane as a result of drug cytotoxicity. Phase-contrast microscopy can also be used to visually examine the gross morphology of cells to detect lysis, membrane damage, apoptosis and the presence of vacuoles—often an indicator of drug cytotoxicity (Jiang and Acosta, 1993). Although
these assays are useful, changes in membrane permeability do not necessarily determine cell viability. As well, cell morphology also may not directly indicate the viability of a cell. For example, an inactive cell may appear structurally intact although it is actually non-viable.

In conjunction with these assays, other assays that measure cellular organelle enzymatic activity are a better assessment of cell viability. Such viability assays include neutral red uptake into lysosomes, tetrazolium compounds such as MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) measuring mitochondrial dehydrogenase activity, rhodamine 123 measuring mitochondrial membrane potential, and XTT (2,3-bis(2-methoxy-4-nitro-5sulphonyl)-5[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) which is similar to MTT. These assays all measure enzymatic activity in cellular organelles, which directly indicate whether the cells are active and therefore viable.

As shown in Figure 3.1, colorimetric assays such as the MTT-based assay involves the reduction of the tetrazolium salt and cleavage of the tetrazolium ring by active mitochondrial dehydrogenases within active cells to form blue formazan crystals within the cell (Slater et al., 1963; Mosmann, 1983; Carmichael et al., 1987; Heo et al., 1990). In this assay, MTT is added to the media and can permeate through cellular membranes to access the mitochondria. After the formation of water insoluble formazan crystals by active cells and accumulation within the cells, the crystals need to be solubilized. Following the solubilization step, the solution can be read spectrophotometrically. Unfortunately, the solubilization step may lead to inaccurate measurements because the formazan crystals may remain insoluble in organic solvents in
Figure 3.1: Structures of MTT and XTT Tetrazolium and Formazan.

Structures of two tetrazolium salt viability assay reagents and their respective formazan products which can be read spectrophotometrically (from Scudiero et al., 1988).
some cell lines (Alley et al., 1988). Usually solubilization is performed with dimethylsulfoxide (DMSO). However, the toxicity of DMSO makes its use undesirable. To overcome these limitations, an assay employing XTT has been utilized. This XTT-based assay allows for the formation of a water soluble formazan product that can permeate cell membranes and accumulate in the media (Scudiero et al., 1988). This eliminates the requirement for solubilization steps in the assay. As well, XTT is less toxic to cells and the media can be directly transferred to a cuvette and read on a spectrophotometer (Scudiero et al., 1988). In the studies reported in this chapter, this XTT assay was used to determine drug cytotoxicity.

To test the in vitro potential of PFVs and to distinguish them from drug encapsulated in conventional liposomes, the XTT assay was used to measure viability of two cell lines: a human cell line, 293 and a murine leukemia cell line, L1210. The 293 cell line is a permanent line of primary human embryonal epithelial kidney transformed by sheared human adenovirus type 5 (Ad 5) DNA (Graham et al., 1977). These cells are characterized as adherent, grown as a monolayer, and are tumorgenic when injected into mice. L1210 cells are murine lympholytic leukemia cells that are characterized as suspension, or non-adherent, and they are also tumorgenic (Law et al., 1949; Moore et al., 1966; Himmelfarb et al., 1967). These cell lines vary quite widely in their characteristics, which may allow extrapolation of results from these studies to a wide range of different cell lines. Furthermore, the assessment of cytotoxicity of liposomal drugs can be conveniently performed on these cell lines in culture, which may provide insight into the bioavailability of drugs from various liposomal carriers.
The bioavailability of a drug encapsulated within liposomes is an extremely important determinant of therapeutic activity. A previous study suggested that drug release is the dominating factor controlling the anticancer efficacy of liposomal mitoxantrone in tissues where carrier accumulation is rapid in vivo (Lim et al., 1997). Under conditions where drug accumulation within diseased tissue was slow, mitoxantrone-loaded vesicles, which exhibited increased drug release characteristics, produced greater delays in tumor growth even though other liposomal formulations engendered much higher levels of tumor-associated drug (Lim et al., 2000). Moreover, the antitumor efficacy of mitoxantrone was increased by the controlled destabilization of programmable fusogenic vesicles as compared to conventional liposomes in vivo (Adlakha-Hutcheon et al., 1999). These reports suggest that there should be an emphasis on designing liposomal formulations that optimize drug bioavailability after accumulation at disease sites. However, mitoxantrone bioavailability from liposomal carriers has not been examined at the cellular level in vitro.

The research presented in this chapter deals with the use of an XTT/formazan tetrazolium salt-based assay that can assess the cytotoxicity of liposomal drugs, such as mitoxantrone hydrochloride, in a cell culture assay. Ultimately, this provides us with an assessment of the bioavailability of liposomal drugs. Furthermore, this assay allows us to characterize and compare the bioavailability of mitoxantrone in different liposomal formulations. Two cell lines, 293 and L1210, were utilized to compare assay performance. These cell lines represent an adherent cell line and a suspension cell line, respectively.
3.2 RESULTS

3.2.1 Determination of the Growth Rate of HEK 293 Cells

To establish the concentration of cells to seed into 24-well plates over a timecourse of 96 hours for subsequent mitoxantrone cytotoxicity experiments, the growth rate of HEK 293 cells was determined. Growth rate of HEK 293 cells was determined by a simple procedure following cell passage described in Section 2.6. Cells were initially counted on a hemacytometer under a light microscope. To each new flask, approximately $1.6 \times 10^5$ cells were re-seeded. Subsequently, every 24 hours, cells from a single flask were counted.

As shown in Figure 3.2, after 24 hours, the cell number grew from approximately $1.6 \times 10^5$ cells to approximately $3.80 \times 10^5$ cells. After 48 hours, the cell number grew to approximately $1.36 \times 10^6$ cells. At 72 and 96 hours, the cell number reached approximately $3.9 \times 10^6$ cells and $4.5 \times 10^6$ cells, respectively. The growth curve was sigmoidal in shape, indicating an initial slow rate of growth followed by a rapid period of growth, then after reaching approximately $3.9 \times 10^6$ cells the growth rate began to slow again. From this curve, an estimate of the number of cells to seed into 24-well plates was determined.

3.2.2 Determination of Cell Growth in 24-Well Plates and Viability Assay Design and Protocol

To determine the growth of 293 cells in the wells of a 24-well plate and evaluate the performance or reliability of the XTT\textregistered formazan tetrazolium salt viability assay, the cells were initially seeded at a concentration from 0 to $3.0 \times 10^5$ cells per well, in triplicate. Each well had a total volume of 400μl, 200μl of media and 200μl of PBS.
Figure 3.2: Growth Rate of 293 Cell Line.

Typical growth rate of 293 human embryonic kidney epithelial cells. Cells were grown in 25cm² tissue culture flasks and counted every 24 hours for 4 days.
Cell media free of phenol red was used because this chemical interfered with the absorbance readings of the formazan product. The cells were allowed to recover from the trypsinizing/seeding process, which sometimes causes mechanical stresses to cells and shearing of cell membranes, and to reattach to the well surface. After a minimum of 8 hours, the XTT assay was employed as described in Section 2.7. From the absorbance readings and known cell concentrations seeded into each well, a standard curve was generated (Figure 3.3). The standard curve exhibited a linear regression between the cell number and absorbance value at 450nm. The $r^2$ value was 0.995 (SigmaPlot for Windows Version 4.00). A maximum absorbance value of 0.40 units was reached for $3.0 \times 10^5$ cells.

Based on the results of the standard curve and preliminary experiments, 293 cells were seeded at $2.0 \times 10^4$ cells per well for subsequent mitoxantrone cytotoxicity experiments. This cell concentration was used to allow for enough space to prevent any limitations on growth of the 293 cells such as lack of well space or lack of growth media over 96 hours.

Similarly, L1210 cells were seeded into wells of a 24-well plate to obtain a standard curve of absorbance at 450nm as a function of cell number (Figure 3.4). A maximum absorbance value of 0.8 units was reached with $3.0 \times 10^5$ cells. Based on the reported growth rate of these cells (Moore et al., 1966), about 10-12 hours doubling time, these cells were also initially seeded at $2.0 \times 10^4$ cells per well. However, preliminary studies indicated that L1210 cells were much more sensitive to mitoxantrone than 293 cells. Therefore, initial seeding of these cells was changed to $5.0 \times 10^4$ cells per well for mitoxantrone cytotoxicity experiments.
Figure 3.3: XTT/Formazan Standard Curve of 293 Cells.

Typical standard curve for the absorbances resulting from the bioreduction of XTT to formazan in cell culture media, read at 450nm, as a function of 293 cell number. Each point is the mean absorbance reading of three wells in a 24-well plate and the error bars represent the standard deviation.
Figure 3.4: XTT/Formazan Standard Curve of L1210 Cells.

Standard curve for the absorbances resulting from the bioreduction of XTT to formazan in cell culture media, read at 450nm, as a function of L1210 cell number. Each point is the mean absorbance reading of three wells in a 24-well plate and the error bars represent the standard deviation.
As shown in Figure 3.5A, the cells were passaged as described in Section 2.6, counted using a haemacytometer and diluted to a desired cell concentration based on calculations to achieve the initial cell concentrations mentioned earlier. Following the dilution of the cells, cells were seeded to the wells of 24-well plates at a volume of 200μl. The 24-well plates were then placed in an incubator a minimum of 8 hours at 37°C and 5% CO₂ to allow the cells to recover from the trypsinizing and seeding procedure. Wells with media free of cells and wells with PBS were also included into the 24-well plates to act as controls.

After a minimum of 8 hours, the cells were exposed to free or liposomal mitoxantrone (Figure 3.5B). Mitoxantrone test samples were prepared prior to the seeding of cell wells and diluted to various concentrations in HBS. Free or liposomal mitoxantrone was added to the respective, appropriately labeled wells in a volume of 200μl per well. Following cell seeding and test sample addition, the total well volume was 400μl. After test samples were applied to the wells at various concentrations, the 24-well plates were placed back into the incubator. Cells were continuously exposed to the mitoxantrone formulations for 24, 48, 72 and 96 hours. In addition, control wells contained either cells alone, cells with empty vesicles, or free drug without cells to account for increases or decreases in the absorbance due to the background readings.

At 24, 48, 72 and 96 hours, a set of 24-well plates was removed from the incubator and the XTT assay was employed as described in Section 2.7 to obtain absorbance values as indications of mitoxantrone cytotoxicity (Figure 3.5C).
A. Cells were harvested, counted, diluted and seeded to a fresh 24-well plate.

B. After a minimum 8 hours, cells were exposed to free mitoxantrone and mitoxantrone encapsulated into liposomes at various concentrations. Controls were added to subtract any increase in absorbance due to background readings.

C. XTT with 1% PMS (by weight) in fresh media (1mg/ml) was added directly to the cell media to assess the viability of the cells. The cell media was transferred to fresh test tubes containing 1% Triton X-100 in HBS following a 4 hour incubation period with XTT. The diluted solution was then transferred to a cuvette to be read on a spectrophotometer at 450nm.

Figure 3.5: Design and Protocol of XTT-Based Cytotoxicity Assay
3.2.3 Comparison of Mitoxantrone Cytotoxicity Against HEK 293 Cells

293 cells were continuously exposed for 24, 48, 72 and 96 hours to mitoxantrone in three forms in order to determine drug cytotoxicity as a percentage of the control: free drug, mitoxantrone encapsulated in conventional distearoylphosphatidylcholine (DSPC):Chol (55:45 mol%) large unilamellar vesicles (LUVs), and mitoxantrone encapsulated in PFVs (Figure 3.6). Two formulations of PFVs were utilized and they differed only by the acyl chain length of the polyethylene glycol (PEG)-lipid conjugate, PEG-DMPE and PEG-DSPE. See Introduction, Section 1.2.1, for the chemical structure of PEGs. No cytotoxicity was seen with empty PFVs and conventional liposomes.

With free mitoxantrone, the IC$_{50}$ concentrations ranged from 0.437μM at 24 hours to 0.017μM over 96 hours of continuous exposure (Table 3.1). These IC$_{50}$ data were obtained from time- and concentration-dependent cytotoxicity curves shown in Figure 3.6 (A-D). The most pronounced mitoxantrone cytotoxicity differential was seen between 24 and 48 hours of continuous exposure. The IC$_{50}$ concentrations differed by a factor of 10 over a period of 24 hours.

Table 3.1: IC$_{50}$ Concentrations of Mitoxantrone Inducing Cytotoxicity on 293 Cells

<table>
<thead>
<tr>
<th>Mitoxantrone Formulation</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
<th>96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Mitoxantrone</td>
<td>0.437μM$^a$</td>
<td>0.044μM</td>
<td>0.027μM</td>
<td>0.017μM</td>
</tr>
<tr>
<td>DOPE:Chol:DODAC:PEG-DSPE (30:45:15:10 mol%)</td>
<td>0.398μM</td>
<td>0.040μM</td>
<td>0.027μM</td>
<td>0.017μM</td>
</tr>
<tr>
<td>DOPE:Chol:DODAC:PEG-DMPE (30:45:15:10 mol%)</td>
<td>0.501μM</td>
<td>0.031μM</td>
<td>0.020μM</td>
<td>0.011μM</td>
</tr>
<tr>
<td>DSPC:Chol (55:45 mol%)</td>
<td>&gt; 10μM$^b$</td>
<td>&gt; 10μM</td>
<td>4.898μM</td>
<td>3.236μM</td>
</tr>
</tbody>
</table>

a. IC$_{50}$ concentrations (mitoxantrone concentration that cause 50% cytotoxicity compared to control) were calculated by interpolation from points on the cytotoxicity graphs using SigmaPlot linear regression analysis software.

b. Could not be measured even at a concentration of 10μM.
Figure 3.6: Comparison of Mitoxantrone Cytotoxicity Against 293 Cell Line.

Utilizing the XTT/formazan assay, cell survival was determined as a percentage of the control (100%) upon exposure to free mitoxantrone (●), mitoxantrone in PFVs containing PEG-DMPE (■) or PEG-DSPE (▲), or mitoxantrone in DSPC:Chol liposomes (♦) for 24 (A), 48 (B), 72 (C) and 96 (D) hours. No toxicity was seen with empty liposomes. Each point on the graph represented an average of triplicates.
Mitoxantrone encapsulated into PFVs shows similar IC$_{50}$ values as compared with free mitoxantrone (Table 3.1 and Figure 3.6: A-D). The most pronounced mitoxantrone cytotoxicity differential was also seen between 24 and 48 hours of continuous exposure for both of the PFV formulations tested. However, there was not a significant difference between the IC$_{50}$ of mitoxantrone encapsulated within the PEG-DMPE PFVs and the PEG-DSPE PFVs.

In contrast, when mitoxantrone is encapsulated in conventional liposomes, a significant reduction in cytotoxic activity is observed. As shown in Figure 3.6 (A-C), when 293 cells were exposed to mitoxantrone encapsulated in DSPC:Chol conventional liposomes at concentrations of 0.01, 0.1 and 1µM, mitoxantrone produced essentially no cytotoxicity over exposure times of 24, 48 and 72 hours. IC$_{50}$ values were almost 200-fold higher at 72 and 96 hours as compared with free mitoxantrone or mitoxantrone encapsulated in PFVs (Table 3.1). Hence, mitoxantrone cytotoxicity has been reduced when encapsulated into DSPC:Chol conventional liposomes. Only at the highest concentration of 10µM and also at 96 hours of continuous exposure to mitoxantrone at 1µM is significant cytotoxicity seen. This is consistent with previous studies involving conventional liposomes; whereby, limited drug release and little or no liposome-cell interactions occur, which inhibits the cytotoxic activity of an associated drug. Data demonstrating that drug encapsulated in PFVs is as active as free drug strongly suggest that this liposomal formulation enhances drug release.

3.2.4 Comparison of Mitoxantrone Cytotoxicity Against Murine L1210 Cells

Given the observation that the drug effects appear to be most significant within 48 hours of initial mitoxantrone exposure to 293 cells, seen in Figure 3.6 (A-D), this
timecourse was selected for the following experiment with L1210 cells.

To determine the extent of mitoxantrone cytotoxicity on L1210 cells, a similar protocol was performed as in section 3.2.3. However, as mentioned previously in this chapter, L1210 cells appeared to be much more sensitive to mitoxantrone. Therefore, mitoxantrone concentrations were reduced to a range from 0 to 50nM (Figure 3.7).

The IC$_{50}$ values for the various mitoxantrone formulations against the L1210 cell line are shown in Table 3.2. With free mitoxantrone, the IC$_{50}$ concentration was 2.159nM. Furthermore, in Figure 3.7, concentration-dependent cytotoxicity was apparent with free mitoxantrone. As with the 293 cell line, mitoxantrone encapsulated within PFVs produced cytotoxicity profiles comparable to that of free mitoxantrone. Furthermore, drug encapsulation within DSPC:Chol conventional liposomes again appears to greatly reduce mitoxantrone cytotoxicity with minor cytotoxicity seen even at 50nM. The significant difference in cytotoxicity between mitoxantrone encapsulated within DSPC:Chol conventional liposomes and within PFVs would suggest a significant difference in the bioavailabilities of the liposomal drug delivery formulations.

**Table 3.2: IC$_{50}$ Concentrations of Mitoxantrone Inducing Cytotoxicity on L1210 Cells**

<table>
<thead>
<tr>
<th>Mitoxantrone Formulation</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Mitoxantrone</td>
<td>2.159nM$^a$</td>
</tr>
<tr>
<td>DOPE:Chol:DODAC:PEG-DSPE (30:45:15:10 mol%)</td>
<td>4.863nM</td>
</tr>
<tr>
<td>DOPE:Chol:DODAC:PEG-DMPE (30:45:15:10 mol%)</td>
<td>5.129nM</td>
</tr>
<tr>
<td>DSPC:Chol (55:45 mol%)</td>
<td>$&gt;$ 50nM$^b$</td>
</tr>
</tbody>
</table>

a. IC$_{50}$ concentrations (mitoxantrone concentration that cause 50% cytotoxicity compared to control) were calculated by interpolation from points on the cytotoxicity graphs using SigmaPlot linear regression analysis software.

b. Could not be measured even at a concentration of 50nM.
Figure 3.7: Comparison of Mitoxantrone Cytotoxicity Against L1210 Cell Line.

Utilizing the XTT/formazan assay, cell survival was determined as a percentage of the control (100%) upon exposure to free mitoxantrone (●), mitoxantrone in PFVs containing PEG-DMPE (■) or PEG-DSPE (▲), or mitoxantrone in DSPC:Chol liposomes (◆) for 48 hours. Each point on the graph represented an average of triplicates.
3.3 DISCUSSION

Tetrazolium salt-based assays involving drugs and cultured cell lines have been commonly utilized in previous research; however, XTT has been utilized less extensively (Jones et al., 1997; Lappalainen et al., 1994; Horowitz et al., 1992; Gonzalez-Rothi et al., 1991). The data in this chapter suggest that XTT can be utilized for both adherent (293) and non-adherent, or suspension cell cultures (L1210). Therefore, this reagent provides a very simple, inexpensive, safe, effective and efficient means to detect cell viability and drug cytotoxicity for diverse cell lines. Furthermore, this assay was used to compare the bioavailability characteristics of an anticancer drug encapsulated in different liposomal drug delivery formulations. The IC₅₀ values of mitoxantrone measured for free drug, utilizing this assay, are comparable to other studies on the cytotoxicity of mitoxantrone against L1210 cells in cell culture (Traganos et al., 1980; Fry et al., 1985; Traganos et al., 1991).

This assay potentially appears suitable for cell lines of varying characteristics. As mentioned in the Introduction, the 293 cell line has very different characteristics than the L1210 cell line such as one cell line is of human origin and adherent, and the other cell line is murine and non-adherent, respectively. Although these cell lines differ, the XTT assay was able to characterize the cytotoxicity seen upon exposure to mitoxantrone encapsulated in different liposomal formulations with both cell lines. The major observed difference between the cytotoxicity effects was that the L1210 cell line was more sensitive than the 293 cell line to mitoxantrone (Tables 3.1 and 3.2; Figures 3.6B and 3.7). However, the cytotoxicity profiles of the mitoxantrone formulations were still similar when comparing the two studies. Therefore, this XTT cell culture assay may be
suitable for general utility to compare drug bioavailability of liposomal carriers and to compare different liposomal drug formulations on a variety of cell lines.

The subject of drug bioavailability from liposomal drug delivery systems has not been discussed extensively. The role of drug release from liposomes has recently become an important topic of discussion. Traditionally, liposomal carriers were designed to exhibit decreased plasma elimination rates and slow drug release rates to ensure preferential carrier and drug accumulation at disease sites such as tumors. However, it has been stated that there is no evidence suggesting that the encapsulated form of the drug is therapeutically active (Lim et al., 1997). Therefore, it is postulated that antitumor activity is mediated by free drug released from regionally localized liposomes (Mayer et al., 1994). Hence, when designing a liposomal drug delivery system, a balance between drug retention and drug release must be taken into consideration.

Studies that have examined the subject matter of drug bioavailability include experiments using liposomal mitoxantrone (Lim et al., 1997; Adlakha-Hutcheon et al., 1999, Lim et al., 2000). It has been demonstrated that the in vivo rate of mitoxantrone release from DMPC/Chol liposomes is at least 68-fold greater than that from DSPC/Chol liposomes; this resulted in improved antitumor efficacy against murine P388 cells or L1210 tumor cells inoculated intravenously in BDF1 mice, even though drug delivery was greater with DSPC/Chol liposomes (Lim et al., 1997). However, with the in vitro results included in this study, no substantial difference was seen between the rate of drug release rates from DMPC/Chol liposomes and DSPC/Chol liposomes. An in vitro assay, such as the XTT cytotoxicity assay, may be an improved alternative method of distinguishing drug release rates from liposomal carriers. More closely related to our
studies, it has been demonstrated *in vivo* that mitoxantrone encapsulated within PFVs improved the antitumor activity as compared with free mitoxantrone, mitoxantrone encapsulated in conventional liposomes, and mitoxantrone encapsulated in Stealth™ liposomes (Adlakha-Hutcheon *et al.*, 1999). As seen in Figures 3.6 and 3.7, *in vitro* characterization of mitoxantrone encapsulated in PFVs revealed a significant difference between the cytotoxicity of mitoxantrone encapsulated in DSPC:Chol conventional liposomes and in PFVs. These cell culture studies suggest that the cytotoxicity seen results from greater drug bioavailability from the PFVs, which is in agreement with the *in vivo* results mentioned in the previous study.

PFV-mediated increases in drug bioavailability likely occur as a consequence of increases in the drug pool available to the cultured cells. This is believed to be due to two possible mechanisms. First, direct fusion of the liposome with the cell membrane, or following endocytosis, may result in direct delivery of the drug to the cells. Second, destabilization of the PFVs due to loss of PEG, or due to culture-induced destabilization of the PFV, may cause an increase in drug concentration in the media surrounding the cells. Interestingly, no significant difference was seen between the PEG-DMPE and PEG-DSPE containing PFVs. Within 24 hours, PEG exchange from these vesicles may have resulted in vesicle destabilization, which may account for this observation.

As briefly discussed already, there needs to be a balance between drug retention properties and drug release characteristics when designing an effective liposomal drug delivery system. Specifically, the liposomal carriers should have sufficient time to accumulate at target sites and then release the maximum amount of encapsulated drug. In this chapter, we demonstrate that mitoxantrone encapsulated into PFVs allows for
increased drug bioavailability over conventional liposomes. If release from a liposomal carrier is indeed the dominating factor controlling the biological activity of a drug, further examination of the mechanism of action of PFVs would be important. These *in vitro* studies should also help to resolve whether the activity of PFVs is due to drug release in the media or direct fusion with cells.
CHAPTER 4
CHARACTERIZATION OF CONTROLLED FUSION AND INTRACELLULAR DELIVERY BY PROGRAMMABLE FUSOGENIC VESICLES WITH CULTURED CELLS UTILIZING PHASE CONTRAST AND FLUORESCENCE MICROSCOPY

In the previous chapter, the bioavailability of mitoxantrone encapsulated in both conventional liposomes and programmable fusogenic vesicles was investigated. It was concluded that mitoxantrone encapsulated in PFVs was more bioavailable than mitoxantrone encapsulated in DSPC:Chol conventional liposomes; however, the mechanisms mediating the enhanced activity of PFVs could not be determined clearly using an encapsulated cytotoxic drug. Therefore, having established the ability of PFVs to release mitoxantrone, subsequent studies focussed on characterizing controlled fusion and intracellular delivery by PFVs at the cellular level. In this chapter, a fluorescent lipid probe, Rh-PE, and an encapsulated fluorescent dye, YOYO-1 iodide, were utilized to visualize fusion and intracellular delivery under phase contrast and fluorescence microscopy. In subsequent chapters, other techniques such as flow cytometry will be employed to further characterize delivery by liposomal systems.

4.1 INTRODUCTION

As mentioned previously in Section 1.2.1.1, the lipid DOPE is often referred to as a “fusogenic” lipid because it triggers fusion with other membranes when it is a component of vesicle bilayers and when it undergoes a transition from the Lα, lamellar, liquid-crystalline phase to the HII, non-lamellar, inverted hexagonal phase. Mixtures of DOPE:Chol:DODAC together can form bilayers that are stable in distilled water;
however, in salt-containing solutions they rapidly destabilize (Holland et al., 2001). The inclusion of PEG-lipids promotes the stability of DOPE:Chol:DODAC vesicles in salt-containing solutions (Holland et al., 2001). It is expected that these vesicles should fuse with cellular membranes following PEG-lipid exchange. A common procedure for visualizing fusion events between liposomes and cellular membranes is to incorporate fluorescent probes into the liposomal bilayer and examine liposome interactions with cells under a fluorescent microscope (Struck and Pagano, 1980; Pagano et al., 1981a; Lelkes and Friedman, 1985; Wrobel and Collins, 1995). Fluorescent techniques should help to distinguish whether PFVs are fusing with the plasma membrane upon their exposure to cells and/or if fusion takes place following endocytosis of the vesicles into endosomes.

A frequently employed fluorescent probe that is often incorporated into the liposomal bilayer is 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-N-(Lissamine rhodamine B sulfonyl) (Rh-PE). From previous studies, it has been observed that the fluorescence of lipid probes such as Rh-PE can be self-quenched in the liposomal bilayer (Struck et al., 1981; Holland et al., 1996b; Holland et al., 2001). When the Rh-PE lipid probes are within close proximity of each other in the liposomal bilayer, their fluorescence is quenched by one another. Upon fusion with other vesicles or cellular membranes, dilution of the probe results in the increase of the physical distance between each probe and subsequent recovery of fluorescence (Holland et al., 2001). Using a similar assay, illustrated in Figure 4.1, Rh-PE can be incorporated into PFVs and used to visualize fusion events with cultured cells under the fluorescent microscope. In addition,
Figure 4.1: Concept of Rh-PE Fusion Assay

A. Prior to fusion, the close proximity of the Rh-PE probes incorporated into the PFV results in the self-quenching of fluorescence.

B. Upon exchange of the PEG-lipids out of the PFV, fusion occurs and lipid mixing between the vesicle outer membrane and the cell plasma membrane occurs. Ultimately, this results in the dilution of the probes, or dequenching, in the cell plasma membrane.

C. After fusion occurs, the physical distance between the Rh-PE probes increases which allows for the recovery of fluorescence. Fluorescent cell membranes should become visible under a fluorescence microscope.
the distribution of fluorescence can be examined to potentially elucidate the intracellular fate of PFVs.

In addition to fluorescent membrane probes incorporated into liposomal bilayers, encapsulated fluorescent markers have been used to show intracellular delivery. As mentioned before in Section 1.3.1.3, fluorescent labeled compounds and fluorescent probes have been encapsulated within liposomes to monitor intracellular delivery (Straubinger et al., 1985; Rao et al., 1997; Slepushkin et al., 1997). However, a potential disadvantage of using common fluorescent markers in earlier experiments originates from the fact that the probes were initially fluorescent or they became fluorescent upon probe dilution. Consequently, leakage of these fluorescent markers from the liposomal carriers, prior to fusion with cells, may falsely indicate intracellular delivery. YOYO®-1 iodide is a membrane-impermeant nucleic acid-binding dye that exhibits a 1000-fold increase in fluorescence when bound to single stranded DNA or RNA (Becker et al., 1994; Rye et al., 1993). Therefore, YOYO fluorescence is not detected unless intracellular delivery of the dye is achieved and it binds directly to nucleic acids (Figure 4.2). Moreover, not only would intracellular delivery be established, but cytoplasmic delivery would also be demonstrated upon the detection of YOYO. Furthermore, the majority of nucleic acids within the cell are found in the nucleus, which is also the target site of many therapeutic drugs. Hence, YOYO was encapsulated into PFVs to characterize their ability to deliver molecules directly to cultured 293 cells.

Controlled destabilization of PFVs containing PEG-PEs has been demonstrated in vivo resulting in enhanced antitumor activity of mitoxantrone (Adlakha-Hutcheon et al., 1999). As indicated in the previous chapter, it was further established that PFVs allowed
A. The PFV encapsulates YOYO, a membrane impermeant dye that fluoresces intensely once complexed with nucleic acids. Prior to fusion, the PFV, which also consists of the cationic lipid DODAC, is electrostatically attracted to the anionic cell plasma membrane.

B. Upon exchange of the PEG-lipids out of the PFV, fusion occurs and YOYO is delivered directly into the cell cytoplasm where it can diffuse to nucleic acids within the cell.

C. Upon complexing with nucleic acids, YOYO fluoresces within the cell, which can be seen under a fluorescence microscope.

Figure 4.2: Concept of YOYO Intracellular Delivery Assay
for increased mitoxantrone bioavailability compared to conventional liposomes. However, these studies do not establish the mechanisms of drug delivery that may occur between PFVs and cells. As well, controlled destabilization or controlled fusion has not been well characterized \textit{in vitro}. \textit{In vivo}, it is often difficult to examine the mechanisms of controlled delivery utilizing a liposomal drug delivery system. \textit{In vitro} methods, such as with fluorescent probes, would provide a much more direct and simpler approach to examine and provide evidence for controlled fusion with cells.

While studies have been performed with Rh-PE incorporated into liposomes to examine fusion (Struck \textit{et al.}, 1981; van Meer \textit{et al.}, 1985; Slepushkin \textit{et al.}, 1997), no experiments have been conducted on PFVs and cultured cells. Moreover, to the best of our knowledge, the use of encapsulated YOYO to evaluate liposome fusion and intracellular delivery with cells has not been described. Therefore, studies were conducted to examine controlled fusion and intracellular delivery by PFVs with cultured cells, employing Rh-PE and YOYO-1 iodide, respectively. Characterization of controlled fusion and intracellular delivery by PFVs, containing PEG-ceramides of varying acyl chain lengths, were completed using phase contrast and fluorescence microscopy. Furthermore, fluorescence distribution and intracellular localization of the probes was examined.
4.2 RESULTS

4.2.1 Characterization of Controlled PFV Fusion with 293 Cells Utilizing Rh-PE Under Phase Contrast and Fluorescence Microscopy

To examine controlled fusion, PFVs containing Rh-PE and PEG2000-ceramides of varying acyl chain length (C₈, C₁₄ and C₂₀) were incubated with cells at three phospholipid concentrations (25μM, 50μM and 100μM) for 4 hours. Cells were prepared for photography as described in Section 2.8.

At 25μM phospholipid concentration (Figure 4.3), substantially different patterns of fluorescence between the three pairs of phase contrast and fluorescent photographs could be seen. Cells incubated with PFVs containing PEG2000-C₈ ceramide (Figure 4.3A and B) revealed dense or concentrated clustered areas of fluorescence. Rapid fusion was expected with these vesicles because of the short acyl chain length of this PEG2000-ceramide. Likewise, the process of vesicle uptake or endocytosis is also very rapid (Wu et al., 1981; Daleke et al., 1990). Therefore, it was difficult to ascertain whether the fluorescence was at the surface of the cells or within the cells. The fluorescence may correspond to the membranes of intracellular bodies or clusters of fluorescent lipid on the surface of the cell. Cells incubated with PFVs containing PEG2000-C₁₄ ceramide (Figure 4.3C and D) also showed dense clustered areas of fluorescence; however, there were also areas of diffuse fluorescence. Furthermore, the intensity of fluorescence seemed slightly greater with these vesicles. Cells incubated with PFVs containing PEG2000-C₂₀ ceramide (Figure 4.3E and F) were significantly different in appearance: The fluorescence appeared to be much more diffuse and less intense.

At 50μM phospholipid concentration, toxic effects on the cells by the PFVs containing PEG2000-C₈ ceramide were seen (Figure 4.4A and B). The 293 cells, an
Figure 4.3: Controlled Fusion of Rh-PE PFVs Containing of PEG2000-Ceramides of Varying Acyl Chain Lengths at 25μM Phospholipid Concentration

Fluorescence (left column) and phase contrast (right column) micrographs of 293 cells incubated with PFVs incorporating Rh-PE and containing PEG2000-C₈ (A and B), C₁₄ (C and D) and C₂₀ (E and F) ceramides at a phospholipid concentration of 25μM at 37°C for 4 hours. The experiments were carried out in 25cm² flasks containing 3ml DMEM supplemented with 10% FBS, but photographed in D-PBS.
Figure 4.4: Controlled Fusion of Rh-PE PFVs Containing of PEG2000-Ceramides of Varying Acyl Chain Lengths at 50μM Phospholipid Concentration

Fluorescence (left column) and phase contrast (right column) micrographs of 293 cells incubated with PFVs incorporating Rh-PE and containing PEG2000-C₆ (A and B), C₁₄ (C and D) and C₂₀ (E and F) ceramides at a phospholipid concentration of 50μM at 37°C for 4 hours. The experiments were carried out in 25cm² flasks containing 3ml DMEM supplemented with 10% FBS, but photographed in D-PBS.
adherent cell line, became rounded and detached from the surface of the flask. As well, fluorescence microscopy showed intense fluorescence covering the entire surface of the cells. Cells incubated with PFVs containing PEG2000-C_{14} ceramide (Figure 4.4C and D) show punctate areas of fluorescence that looked like the lipid probe was internalized within the cell. Cells incubated with PFVs containing PEG2000-C_{20} (Figure 4.4E and F) ceramide once again show areas of diffuse fluorescence with less intensity.

At 100\mu M phospholipid concentration, the cells incubated with PFVs containing PEG2000-C_{8} ceramide (Figure 4.5A and B) showed dramatic signs of toxicity after 4 hours. The majority of the cells had become detached from the surface of the flask with cell remnants or debris floating within the media following a wash with HBS. The rounded cells floating in the media were intensely fluorescent, which covered the entire surface of the cell. Cell samples incubated with PFVs containing PEG2000-C_{14} ceramide (Figure 4.5C and D) again revealed some cells with punctate areas of fluorescence, as well as some cells with diffuse but intense fluorescence. In addition, some cells showed minor signs of rounding and detachment. Cells incubated with PFVs containing PEG2000-C_{20} (Figure 4.5E and F) resembled the photographs at 25\mu M and 50\mu M, exhibiting diffuse fluorescence; however, at 100\mu M, the intensity of the fluorescence was greater.

4.2.2 Characterization of Intracellular Delivery by PFVs to 293 Cells Utilizing YOYO-1 Iodide Under Phase Contrast and Fluorescence Microscopy

To examine and visualize intracellular delivery by PFVs, 293 cells were incubated with no dye, free YOYO (10nM), or YOYO encapsulated in PFVs containing PEG2000-C_{14} ceramide (10nM YOYO and approximately 25\mu M phospholipid) for 4 hours.
Figure 4.5: Controlled Fusion of Rh-PE PFVs Containing of PEG2000-Ceramides of Varying Acyl Chain Lengths at 100μM Phospholipid Concentration

Fluorescence (left column) and phase contrast (right column) micrographs of 293 cells incubated with PFVs incorporating Rh-PE and containing PEG2000-C₈ (A and B), C₁₄ (C and D) and C₂₀ (E and F) ceramides at a phospholipid concentration of 100μM at 37°C for 4 hours. The experiments were carried out in 25cm² flasks containing 3ml DMEM supplemented with 10% FBS, but photographed in D-PBS.
Control cells not incubated with either PFVs or free YOYO are shown in Figure 4.6 (A and B). Incubation of cells with free YOYO, shown in Figure 4.6 (C and D), revealed little or no fluorescence consistent with the fact that YOYO is non-membrane permeant. In comparison, incubation of the cells with YOYO encapsulated in PFVs, shown in Figure 4.6 (E and F), revealed fluorescence within cells with some localization at the nuclei. This demonstrated that YOYO required a carrier to facilitate its entry into 293 cells, whereupon it was able to bind to nucleic acids giving rise to the fluorescent species.

4.2.3 Characterization of Intracellular Delivery by PFVs Containing PEG2000-Ceramides of Varying Acyl Chain Length

To examine intracellular delivery, YOYO (50nM) encapsulated in PFVs containing PEG2000-ceramides of varying acyl chain length (C₈, C₁₄ and C₂₀) were incubated with 293 cells for 24 hours. Cells were also incubated with "empty" PFVs containing PEG2000-ceramides of varying acyl chain lengths as controls.

Cells incubated with PFVs containing PEG2000-C₈, shown in Figure 4.7 (A and B), exhibited intense fluorescence. Unfortunately, the cells were exposed to the respective phospholipid concentration of approximately 167μM. As seen with the Rh-PE PFVs containing PEG2000-C₈, phospholipid concentrations at 100μM have shown toxic effects (Figure 4.5 A and B) and the cells that exhibited fluorescence appeared very unhealthy or dying. Floating cells could be seen and their membranes appeared damaged. With PFVs containing PEG2000-C₁₄ (Figure 4.7 C and D), intense
Figure 4.6: Intracellular Delivery of YOYO Encapsulated in PFVs

Phase contrast (left column) and fluorescence (right column) micrographs of 293 cells incubated with either no dye (A and B), 10nM free YOYO (C and D) or 10nM YOYO encapsulated in PFVs (E and F) at 37°C for 4 hours. The experiments were carried out in 25cm² flasks containing 3ml DMEM supplemented with 10% FBS and photographed immediately following the incubation period.
Figure 4.7: Intracellular Delivery of YOYO Encapsulated in PFVs Containing PEG2000-Ceramides of Varying Acyl Chain Lengths

Phase contrast (left column) and fluorescence (right column) micrographs of 293 cells incubated with YOYO (50nM) encapsulated PFVs containing PEG2000-C₆ (A and B), C₁₄ (C and D) and C₂₀ (E and F) ceramides at 37°C for 24 hours. The experiments were carried out in 25cm² flasks containing 3ml DMEM supplemented with 10% FBS and photographed immediately following the incubation period.
fluorescence was also seen. However, a lower percentage of the cell population exhibited intense fluorescence as compared with the PFVs containing PEG2000-C₈. Interestingly, the cells that exhibited the highest levels of fluorescence intensity also became rounded and detached from the surface of the flask. Cells incubated with "empty" PFVs containing PEG2000-C₁₄ did not reveal significant toxicity after incubation for 24 hours. Therefore, the toxic effects may be at least partly attributed to the YOYO itself. In contrast to the C₈ and C₁₄ PEG-ceramides, the cells exposed to PFVs containing PEG2000-C₂₀ showed very faint signs of fluorescence. Only the outline of some of the cells could be seen under fluorescence microscopy.

4.3 DISCUSSION

The use of fluorescence microscopy and fluorescent compounds or probes is an established and powerful technique to investigate biochemical phenomena at the cellular level. Consequently, numerous studies have employed these techniques to examine liposome-cell interactions. For example, lipid membrane probes such as Rh-PE have been used in a number of studies involving fusion of liposomes with cultured cells (Struck et al., 1981; van Meer et al., 1985; Lee et al., 1992; Kirjavainen et al., 1996; Slepushkin et al., 1997; Cansell et al., 1999). These studies looked at fusion kinetics of various liposomes with cultured cells induced by the presence of divalent cations (e.g. Ca²⁺, Mg²⁺) or changes in pH and examined localization of fluorescence to indicate where liposomal lipids were distributed. Generally, most of the studies employed liposomal systems containing Rh-PE in combination with NBD-PE to measure resonance energy transfer (RET). RET is characterized by the transfer of light energy from one
fluorochrome (NBD-PE) to another molecule (Rh-PE) that is in close proximity (Uster and Pagano, 1986).

For the studies described in this chapter, Rh-PE allowed for the visual tracking of the PFVs to ascertain whether fusion with cellular membranes was occurring and, if so, where this fusion was occurring. Most importantly, the probe allowed for qualitative observations to distinguish differences between the extent of fusion and subsequent intracellular location of PFVs as a function of PEG-ceramide acyl chain length. The data reveal very different patterns of fluorescence resulting from the modification in the acyl chain length of the PEG-ceramide. Utilizing PFVs containing PEG2000-C₈ resulted in intense clusters of fluorescence (Figure 4.3 A and B). In contrast, PFVs containing PEG2000-C₂₀ gave rise to more diffuse or distributed fluorescence (Figures 4.3-4.5 E and F), while PFVs containing PEG2000-C₁₄ revealed an intermediate distribution of fluorescence (Figures 4.3-4.5 C and D). Comparing the cell photographs, the data shown at 4 hours are consistent with the exchange of PEG-lipids out of the PFVs according to acyl chain lengths (Silvius and Leventis, 1993; Holland et al., 1996b). The rapid exchange of the shorter acyl chain length PEG2000-C₈ ceramides from PFVs would be expected to result in rapid fusion with cellular membranes while the longer acyl chain length PEG2000-C₂₀ ceramides should result in slower fusion. The distribution of fluorescence seen appears to follow this pattern. Furthermore, the clustered or punctate fluorescence seen with PFVs containing PEG-ceramides of acyl chain lengths of C₈ and C₁₄ may indicate internalization of the liposomes, while the diffuse fluorescence seen with PFVs containing PEG2000-C₂₀ ceramides may reflect vesicles at the cell surface. As mentioned previously, endocytosis of lipid vesicles is the most commonly accepted
mechanism of liposome-cell interaction prior to fusion with intracellular membranes (Wrobel and Collins, 1995; Stirk and Baldeschwieler, 1987; Truneh et al., 1983). Therefore, endocytosis of PFVs may have resulted in the fluorescence of intracellular membranes subsequent to fusion with the endosomal membrane. Studies to determine endosomal uptake of vesicles were not conducted because the focus of the experiments in this chapter was to characterize fusion and intracellular delivery by PFVs. Nevertheless, the results suggest that Rh-PE transfer to the cell membranes had occurred to a great extent. Interestingly, exposing cells to PFVs containing PEG2000-C₈ ceramides, at 50μM and 100μM phospholipid concentration, appeared to induce toxicity. This was not unexpected because PFVs contain DODAC, a cationic lipid, that has detergent-like effects and cationic lipids are known to be toxic at high concentrations (Jungerman, 1970; Adams et al., 1977). Likewise, DOPE is a “fusogenic” lipid that may have the potential to disrupt the bilayer conformation of cellular membranes upon fusion.

Although differences in fluorescence distribution were evident between cells exposed to PFVs containing PEG-ceramides of varying acyl chain length, it was difficult to clearly differentiate between the intensity levels of the cell fluorescence seen. It would be expected that as the acyl chain length of the PEG-lipids increase (i.e. from C₈ to C₂₀), the fluorescence intensity should decrease because the extent of fusion decreases with increasing acyl chain length (Silvius and Leventis, 1993; Holland et al., 1996b; Holland et al., 2001). With the shorter acyl chain lengths of PEG-lipids, more fusion events should occur producing greater intensity levels of fluorescence than with longer acyl chain lengths of PEG-lipids. Still, the differences in fluorescence intensity between PEG-ceramides of varying acyl chain lengths were not as distinct as expected. Earlier studies
have demonstrated the spontaneous exchange of lipid monomers out of a liposomal membrane, which may occur when incorporating fluorescent lipid probes into the bilayers of liposomal carriers (Martin and Pagano, 1987; Pagano et al., 1983; Pagano et al., 1981a; Nichols and Pagano, 1981). This may lead to misinformation about lipid distribution, localization and concentrations. Rh-PE has been suggested to be a more stable probe in that it does not exchange as readily as other fluorescently labeled lipid probes (Silvius et al., 1987; Struck et al., 1981); however, this exchange process will likely depend on the lipid composition of the liposome used. The possibility of Rh-PE exchange from PFVs, therefore, cannot be ruled out.

As with fusion studies, intracellular delivery studies have often utilized fluorescent probes. As discussed in the introduction of this chapter, the fluorescent compounds or probes utilized are often initially fluorescent. The intracellular delivery studies conducted in this research utilizing YOYO are unique because dye binding to nucleic acids within cells is required to generate the fluorescent species and hence allow visualization. More importantly, YOYO must enter the cell cytoplasm to reach nucleic acids, thereby demonstrating cytoplasmic delivery. In this chapter, it is clearly shown that free YOYO is membrane-impermeant resulting in little or no fluorescence. Incubation of YOYO-encapsulated PFVs with cells, however, results in cytoplasmic delivery as shown by the appearance of YOYO fluorescence. Further, the fluorescence appeared to be localized at the nuclei of cells, which was expected because the majority of nucleic acids within a cell are found within the nucleus. Therefore, this technique is a new and effective way to determine both intracellular and cytoplasmic delivery.
The results suggest that cytoplasmic delivery could be convincingly demonstrated utilizing PFVs, prepared with PEG-ceramides of varying acyl chain lengths, which contained YOYO. The results in this chapter demonstrate that by varying the acyl chain composition of the PEG-ceramide, the extent of YOYO delivery to cells could be varied for a given timecourse. After 24 hours incubation, the most dramatic differences could be seen between the PFV formulations. The PFVs containing PEG2000-C₈ revealed intense fluorescence showing that a high concentration of YOYO was delivered to the cells. However, this PFV formulation was toxic to the cells. This is consistent with the observations made using similar PFV systems incorporating Rh-PE where the PFVs containing PEG2000-C₈ also caused cytotoxicity. Similarly, the PFVs containing PEG2000-C₁₄ also revealed intense cellular fluorescence with some cells, but other cells exhibited a lesser degree of fluorescence. Signs of toxicity, such as the rounding of cells and detachment from the surface of the flask, were observed when the cells were incubated with these PFVs; yet the degree of toxicity was lower than that observed for PFVs prepared with PEG2000-C₈. As mentioned in the Results section, "empty" PFVs containing PEG2000-C₁₄ at the same phospholipid concentration as YOYO-encapsulated PFVs did not demonstrate cytotoxicity. Therefore, YOYO intercalation with cellular nucleic acids may interrupt or halt cellular functions and may be responsible for the cytotoxicity observed. Finally, with PFVs containing PEG2000-C₂₀, only faint fluorescence was seen even after 24 hours. This is consistent with the much slower exchange of PEG2000-C₂₀ ceramide expected based on earlier studies (Silvius and Leventis, 1993; Holland et al., 1996b).
The results of these fluorescence studies show that fusion and cytoplasmic delivery could be demonstrated utilizing Rh-PE and YOYO incorporated into PFVs, respectively. Furthermore, it was established that the extent of fusion and cytoplasmic delivery was dependent on the acyl chain composition of the PEG2000-ceramides. The data from the Rh-PE assay studies were useful for visualization of fusion; however, the YOYO assay studies clearly provided a means to determine and distinguish cytoplasmic delivery. While the Rh-PE assay has been commonly utilized to demonstrate fusion, the YOYO assay seems to be more a reliable method to characterize and compare PFVs. As well, the YOYO assay described in this chapter could have general use as a cytoplasmic delivery assay for other liposomal formulations. For this reason, the following chapters provide a more quantitative analysis of YOYO delivery mediated by PFVs.
CHAPTER 5

CHARACTERIZATION AND DEVELOPMENT OF A QUANTITATIVE CYTOPLASMIC DELIVERY ASSAY UTILIZING YOYO-1 IODIDE ENCAPSULATED IN PFVS

The previous chapter dealt with the ability to visualize liposome-cell fusion and intracellular delivery between PFVs and HEK 293 cells utilizing fluorescence microscopy, which provided a qualitative characterization of liposome-cell interactions. This chapter describes the development of a quantitative assay to characterize cytoplasmic delivery using PFVs. The technique of flow cytometry was employed to measure the cell-associated fluorescence following incubation with YOYO-loaded PFV systems. This chapter will discuss preparation and analysis of YOYO-loaded PFVs, the stability of YOYO-loaded PFVs in saline and serum-containing solutions, and the quantitation of YOYO-associated cell fluorescence by flow cytometry.

5.1 INTRODUCTION

As mentioned in the previous chapter, YOYO is a membrane-impermeant nucleic acid dye that is essentially non-fluorescent in the absence of nucleic acids, but is strongly fluorescent upon binding to nucleic acids (Rye et al., 1992; Becker et al., 1994). The rationale for its use as an indicator of cytoplasmic delivery, therefore, is that it requires a carrier, such as a liposome, to facilitate its entry into cells and that fluorescence is detectable exclusively upon subsequent binding to nucleic acids within cells. Furthermore, other favorable characteristics of YOYO are that it forms very stable complexes with nucleic acids (binding constant estimated to $10^{10}$-$10^{12}$ M$^{-1}$) binding up to a 1:4 ratio of YOYO to nucleic acid, and picogram quantities of nucleic acids can be
detected using this dye employing a fluorometric assay (Larsson et al., 1995; Rye et al., 1993). Compared to other standard nucleic acid detection dyes, such as acridine orange, ethidium bromide and propidium iodide, YOYO has been shown to possess superior spectroscopic properties (Table 5.1). In addition, YOYO is more membrane-impermeant than standard nucleic acid dyes because it possesses four cationic charges (Figure 5.1), while other dyes generally have fewer cationic charges. This may also decrease its leakage rate from liposomes following encapsulation.

Flow cytometry has been utilized to follow intracellular delivery by liposomes in several previous studies (Truneh et al., 1983; Grosse et al., 1984; Lelkes and Friedman, 1985; Cudd and Nicolau, 1986; Truneh, et al., 1987; Bally et al., 1994; Longman et al., 1995; Tseng et al., 1997; Slepushkin et al., 1997). These studies have described the use of flow cytometry to characterize the intracellular delivery of doxorubicin, acridine orange complexed with DNA, ethidium monoazide, carboxyfluorescein and calcein into cells by liposomes. Although fluorescent compounds have been delivered to cell lines using liposomes, the key benefit to using YOYO is that it can clearly differentiate between cytoplasmic delivery by liposomes from uptake of liposomes into endosomes. As well, the importance of demonstrating cytoplasmic delivery is that many therapeutic compounds encapsulated in liposomes need to be released into the cytoplasm to produce biological effects. Combining the properties of YOYO and the technique of flow cytometry can provide an extremely powerful assay to measure cytoplasmic delivery.

This chapter describes studies conducted to develop a quantitative cytoplasmic delivery assay based upon the encapsulation of YOYO into PFVs and employing the technique of flow cytometry. Initial studies were required to characterize YOYO
Table 5.1: Comparison of YOYO-1 to Other Fluorescent Dyes When Bound to DNA

<table>
<thead>
<tr>
<th>Nucleic Acid Dye</th>
<th>Abs† (nm)</th>
<th>$\varepsilon_{\text{max}}^{+}$ (cm$^{-1}$M$^{-1}$)</th>
<th>Em† (nm)</th>
<th>Quantum§ yield ($\phi$)</th>
<th>Fluorescence enhancement (Ψ)</th>
<th>Binding affinity (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YOYO-1 Iodide*</td>
<td>491</td>
<td>99,000</td>
<td>509</td>
<td>0.52</td>
<td>460</td>
<td>6.0 x 10$^8$</td>
</tr>
<tr>
<td>Ethidium Bromide*</td>
<td>526</td>
<td>3,200</td>
<td>604</td>
<td>0.15</td>
<td>21</td>
<td>1.5 x 10$^5$</td>
</tr>
<tr>
<td>Acridine Orange*</td>
<td>502</td>
<td>53,000</td>
<td>538</td>
<td>0.43</td>
<td>1.5</td>
<td>3.1 x 10$^4$</td>
</tr>
<tr>
<td>Propidium Iodide+</td>
<td>535</td>
<td>5,400</td>
<td>617</td>
<td>0.2</td>
<td>20- to 30-fold</td>
<td>3.0 x 10$^5$</td>
</tr>
</tbody>
</table>

† Absorption and fluorescence emission maxima (respectively).
‡ Molar extinction coefficient (MEC).
§ Fluorescence quantum yield determined relative to fluorescein in 0.1 M NaOH (QY = 0.92).
* Information provided by Gurrieri et al., 1997.
+ Information provided by Haugland, 1999 (Molecular Probes).
Figure 5.1: Structure of Nucleic Acid Detection Dyes

The structure of the nucleic acid detection dyes (A) acridine orange, (B) ethidium bromide, (C) propidium iodide and (D) YOYO-1 iodide also known as oxazole yellow homodimer (from Haugland, 1999).
encapsulation into PFVs involving quantitation of YOYO concentration, YOYO encapsulation efficiency, and leakage of YOYO from these vesicles. Subsequently, experiments were performed to optimize and characterize the cytoplasmic delivery assay based on PFV-mediated delivery of YOYO.

5.2 RESULTS

5.2.1 Biophysical Characterization of YOYO-Loaded PFVs

Before beginning studies involving quantitative flow cytometry and cytoplasmic delivery to cells, experiments needed to be performed to characterize the biophysical properties of YOYO-loaded PFVs. These studies determined YOYO concentrations encapsulated in PFVs, the interaction between YOYO and PFVs, and the stability of YOYO encapsulated within PFVs.

5.2.1.1 Determination of an Optimal DNA Concentration for Maximum YOYO Fluorescence

A previous study has shown that the fluorescence intensity at a fixed YOYO concentration increases as the DNA concentration is increased (Rye et al., 1993). Thus, a fixed DNA concentration that was sufficient to maximize fluorescence for a range of YOYO concentrations needed to be determined in order to quantitate YOYO encapsulation in PFVs. In order to determine this optimal DNA concentration, studies were performed using various DNA concentrations from 0 to 2000ng/ml in 3ml HBS solutions containing the detergent Triton X-100 (1% final concentration by volume) and titrated against free YOYO at concentrations of 50nM, 100nM and 200nM. Triton X-100 was included in these solutions to disperse vesicles allowing for YOYO to be liberated
from the interior of the vesicles in subsequent studies. Figure 5.2 shows YOYO fluorescence intensity as a function of DNA concentration. As the concentration of DNA increased, for a fixed YOYO concentration, increases in fluorescence intensity were observed. For YOYO concentrations fixed at 50nM and 100nM, the fluorescence intensity appears to increase to a maximum fluorescence value. This provided an indication of the DNA concentration that was sufficient to allow for the majority of the dye to be bound at the YOYO concentrations used. At a fixed YOYO concentration of 200nM, the fluorescence intensity continued to increase as the DNA concentration was increased, but the intensity appeared to plateau as the DNA concentration was reaching 2000 ng/ml.

5.2.1.2 Development of a Standard Curve of YOYO Concentration

From the previous experiment, a fixed DNA concentration of 2000ng/ml was chosen for development of a YOYO standard curve measuring a dye range of 0-200nM. This standard curve was then used to determine the YOYO encapsulation efficiency in liposomes for subsequent studies. To generate a standard curve, fluorescence intensity was measured spectrofluorometrically as a function of increasing YOYO concentration from 0 to 200nM (Figure 5.3). The analytical method is detailed in Section 2.10.3. The standard curve exhibited a linear regression between the YOYO concentration and the measured fluorescence intensity. The r² value determined was 0.994.
Figure 5.2: Determination of an Optimal DNA Concentration for Maximum YOYO Fluorescence

Fluorometric measurements of YOYO fluorescence intensity as a function of increasing DNA concentrations from 0 to 2000ng/ml. Fixed concentrations of YOYO at 50nM (▲), 100nM (■) and 200nM (●) were utilized.
Figure 5.3: Standard Curve of Fluorescence Intensity as a Function of YOYO Concentration

Representative standard curve of fluorescence intensity as a function of increasing YOYO concentrations from 0 to 200nM utilizing a fixed DNA concentration of 2μg/ml. A linear regression analysis could generate an equation by which to interpolate concentrations of YOYO encapsulated within PFVs. Fluorescence measurements were taken as the mean and standard deviation of two experiments.
5.2.1.3 Determination of YOYO Encapsulation Efficiency and Vesicle Size for PFVs of Varying PEG-Ceramide Acyl Chain Length

Following the preparation of YOYO encapsulated PFVs with varying PEG-ceramide acyl chain lengths, encapsulation efficiencies were determined and vesicle sizes were analyzed using quasi-elastic light scattering (Table 5.2). The encapsulation efficiency was expressed as a ratio of YOYO concentration to phospholipid concentration.

Interestingly, YOYO encapsulation efficiency appeared to increase with increasing acyl chain length. In some other instances, the differences between the encapsulation efficiencies of PFVs containing PEG-ceramides of varying acyl chain length were not as dramatic. Nevertheless, the encapsulation of YOYO within PFVs was at concentrations that were sufficient for detection of fluorescence.

In terms of vesicle size, mean diameters did not seem to be dependent on acyl chain length of the PEG-ceramides. The “empty” PEG2000-C8 and C20 PFVs were of similar size, approximately 130nm, while the PEG2000-C14 PFVs were approximately 20nm smaller. When encapsulated with YOYO, the vesicle diameters of all the PFVs increased. While the standard deviations were large, the chi-squared values for the PFVs indicated that the vesicle populations were essentially homogenous.

5.2.1.4 Determination of YOYO Leakage from PFVs with PEG-Ceramide Varying Acyl Chain Lengths

To determine the stability of YOYO encapsulation within PFVs as a function of PEG-ceramide acyl chain length, PFV samples were incubated for a 24 hour timecourse at 37°C in either HBS or DMEM supplemented with 10% FBS, as described in Section 2.10.3. No dye leakage was seen from PFVs containing PEG-ceramides with C8, C14 or
Table 5.2: Example of YOYO Encapsulation Efficiency in PFVs with Varying Acyl Chain Lengths and Their Respective Size Analyses

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>YOYO Encapsulation Efficiency (nM YOYO/ mM Phospholipid)</th>
<th>Vesicle Mean Diameter* (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YOYO-Loaded DOPE:Chol:DODAC:PEG2000-C₈ Ceramide (35:45:15:5 mol%)</td>
<td>298 ± 92</td>
<td>137.8 (49.1), 1.2</td>
</tr>
<tr>
<td>YOYO-Loaded DOPE:Chol:DODAC:PEG2000-C₁₄ Ceramide (35:45:15:5 mol%)</td>
<td>473 ± 231</td>
<td>120.4 (42.5), 1.0</td>
</tr>
<tr>
<td>YOYO-Loaded DOPE:Chol:DODAC:PEG2000-C₂₀ Ceramide (35:45:15:5 mol%)</td>
<td>484 ± 244</td>
<td>183.1 (76.8), 0.2</td>
</tr>
<tr>
<td>“Empty” DOPE:Chol:DODAC:PEG2000-C₈ Ceramide (35:45:15:5 mol%)</td>
<td>NA</td>
<td>128.5 (44.6), 0.2</td>
</tr>
<tr>
<td>“Empty” DOPE:Chol:DODAC:PEG2000-C₁₄ Ceramide (35:45:15:5 mol%)</td>
<td>NA</td>
<td>111.2 (39.3), 0.3</td>
</tr>
<tr>
<td>“Empty” DOPE:Chol:DODAC:PEG2000-C₂₀ Ceramide (35:45:15:5 mol%)</td>
<td>NA</td>
<td>133.6 (48.8), 0.3</td>
</tr>
</tbody>
</table>

* The vesicle sizes are given with the standard deviation in brackets and the respective chi-squared values.

NA Not applicable.
C₂₀ N-acyl chain lengths in HBS (Figure 5.4A). This observed stability of PFVs in buffer is consistent with the lack of PEG-lipid exchange, and maintenance of vesicular structures because of the absence of hydrophobic binding sites to which the PEG-lipid can exchange (Holland et al., 1996b). In serum-containing media, PFVs containing PEG-ceramide C₈ showed an initial loss of approximately 50% encapsulated YOYO and a further slow rate of YOYO leakage over the remaining timecourse (Figure 5.4B). In contrast, PFVs containing PEG-ceramides C₁₄ or C₂₀ showed only slow release of YOYO over 24 hours even in the presence of serum.

5.2.2 Characterization of Cytoplasmic Delivery to 293 Cells Utilizing YOYO-Loaded PFVs

Once the initial studies characterizing YOYO encapsulated in PFVs and the stability of these systems in serum were completed, subsequent studies focussed on the development of a flow cytometry-based assay that could quantitate cytoplasmic delivery.

5.2.2.1 Influence of PFV Concentration on Cytoplasmic Delivery of YOYO

To determine the influence of PFV concentration on the cytoplasmic delivery of YOYO, 293 cells were incubated for 8 hours with a range of PFV concentrations (indicated as YOYO concentrations) and then analyzed by flow cytometry. In Figure 5.5, four vertical bars per sample are shown which represent the percentage of the cell population in the quadrants as discussed in Section 2.9 (Figure 2.1). In brief, YOYO indicates cytoplasmic delivery and propidium iodide (PI) indicates non-viable cells. The PI positive only quadrant (first bar) represents the percentage of non-viable cells in the total population, the PI and YOYO positive quadrant (second bar) represent the percentage of non-viable cells in the total population that have YOYO delivered to them,
Figure 5.4: Determination of YOYO Leakage from PFVs with Varying Acyl Chain Lengths

YOYO encapsulated in PFVs were incubated in (A) HBS or (B) DMEM supplemented with 10% FBS for 24 hours at 37°C. PFVs consisted of PEG-ceramide acyl chain lengths, (●) C8, (V) C14 and (■) C20. A baseline correction was made for fluorescence due to light-scattering by intact vesicles.
Figure 5.5: Influence of PFV Concentration on Cytoplasmic Delivery of YOYO

Quadrant analyses of increasing YOYO encapsulated in PFVs concentration incubated for 8 hours at 37°C. CO and FY represent control 293 cells not incubated with YOYO encapsulated in PFVs and 293 cells incubated with free YOYO (50nM), respectively. YOYO concentrations from 5nM to 25nM represent mean and standard deviation from two experiments. YOYO concentration at 50nM from single experiment.
the negative quadrant (third bar) represents the percentage of viable cells in the total population that do not show YOYO delivery, and the YOYO positive only quadrant (fourth bar) represents the percentage of viable cells in the total population that do show YOYO delivery. Control cells (CO) and cells incubated with 50nM free YOYO (FY) showed little or no fluorescence in either the YOYO or PI channels. This indicated that the cells were viable but there was no significant delivery of YOYO when added in free form. In comparison, cells incubated with YOYO encapsulated in PFVs (5nM to 50nM YOYO) showed significant cytoplasmic delivery of YOYO to viable cells. From 5nM to 25nM, the cytoplasmic delivery of YOYO increased from approximately 39% to 64% of the cell population above background cell fluorescence. Importantly, the percentage of PI-positive cells was very low for all concentrations of PFV-encapsulated YOYO.

5.2.2.2 Influence of “Sink” POPC:Chol Vesicles on the Cytoplasmic Delivery of YOYO Encapsulated in PFVs

To ensure the exchange of PEG-ceramides was not limited, a parallel experiment to that described in Section 5.2.2.1 was conducted with the addition of POPC:Chol (55:45 mol%) “sink” vesicles. These vesicles were added in an excess of 10-fold the phospholipid concentration of the PFVs to ensure that the PEG-ceramides could readily exchange from the PFVs.

Quadrant analyses of flow cytometric data, shown in Figure 5.6, again indicates that the control cells (CO) and cells incubated with 50nM free YOYO (FY) produce little or no fluorescence in the YOYO and PI channels. With YOYO encapsulated in PFVs, a progressive increase in the percentage of the cell population that were viable (PI negative) and positive for YOYO was seen as the concentration of YOYO increased.
Interestingly, at 50nM YOYO encapsulated in PFVs with the addition of “sink” vesicles, a small increase in the percentage of the cell population that were non-viable (PI positive) and YOYO positive was seen.

A direct comparison of the percentages of viable cells exhibiting cytoplasmic delivery (Figure 5.7) in the presence and absence of the “sink” vesicles reveals that the percentage of YOYO positive cells decreases in the presence of the “sink” vesicles, from 5nM to 15nM. This may be a consequence of “sink” vesicles binding to cells and inhibition of PFV-mediated YOYO delivery. Therefore, the addition of “sink” vesicles for subsequent studies was not considered necessary.

5.2.2.3 Comparison of Cytoplasmic Delivery by YOYO Encapsulated in PFVs and Free YOYO in the Presence of “Empty” PFVs

A study was performed to compare YOYO delivery to 293 cells when the dye was encapsulated in PFVs containing PEG2000-C14 ceramide or when free YOYO was incubated with cells in the presence of “empty” PFVs containing PEG2000-C14 ceramide (Figure 5.8). In this experiment, the equivalent PFV phospholipid concentration was used for YOYO-encapsulated PFVs and “empty” PFVs.

Exclusively examining viable cells exhibiting cytoplasmic delivery (PI negative and YOYO positive in quadrant analyses), both formulations displayed cytoplasmic delivery. Nevertheless, cytoplasmic delivery of YOYO was much more efficient when the dye was encapsulated, particularly at lower YOYO, and hence PFV, concentrations.
Figure 5.6: Influence of POPC:Chol “Sink” Vesicles on the Cytoplasmic Delivery of YOYO Encapsulated in PFVs

Quadrant analyses of increasing YOYO encapsulated in PFVs concentration incubated for 8 hours at 37°C with the addition of “sink” vesicles. All cell samples contained a phospholipid concentration of “sink” vesicles that was in excess of 10-fold the phospholipid concentration of the PFVs. Once again, CO and FY represent control 293 cells not incubated with YOYO encapsulated in PFVs and 293 cells incubated with free YOYO (50nM), respectively. YOYO concentrations from 5nM to 25nM represent mean and standard deviation from two experiments. YOYO concentration at 50nM from single experiment.
Figure 5.7: Comparison of the YOYO Positive Viable Cell Populations With and Without “Sink” Vesicles

Direct comparison of the percentage of YOYO positive viable (PI negative) cells incubated in the presence (O) and absence (●) of “sink” vesicles. The data was taken from the quadrant analyses shown in the two previous figures.
Figure 5.8: Comparison of Cytoplasmic Delivery by YOYO Encapsulated in PFVs Against Empty PFVs and Free YOYO

Representative quadrant analyses comparing YOYO encapsulated in PFVs, (●), and empty PFVs plus free YOYO, (○), incubated with 293 cells for 8 hours at 37°C. Only the percentage of viable (PI negative) cells positive for YOYO are shown. Increasing concentrations of YOYO from 0 nM to 25 nM were examined in this study. Similar results were obtained for a number of studies utilizing YOYO at a concentration of 10nM, encapsulated in PFVs against empty PFVs with free YOYO.
5.3 DISCUSSION

The development of a quantitative assay to characterize cytoplasmic delivery using PFVs was outlined in this chapter. It was based upon the technique of flow cytometry utilizing YOYO-1 iodide as an encapsulated fluorescent marker. The strengths of this assay are that it is simple to perform, cytoplasmic delivery to cells can be clearly identified due to the characteristics of YOYO, and flow cytometry can provide a wealth of information from small cell samples.

With respect to the characterization of free YOYO and YOYO encapsulated within PFVs, the sensitivity of YOYO to low concentrations of DNA was clearly demonstrated. In a previous study describing a fluorometric assay using YOYO, picogram quantities of DNA could be detected (Rye et al., 1993). In that study, a low concentration of YOYO at $2 \times 10^{-7}$ M (200nM) was utilized to produce a significant fluorescence yield once bound to DNA. Furthermore, in that study, a linear dependence of the fluorescence intensity of YOYO to the DNA concentration was shown. The sensitivity of YOYO was also demonstrated and reinforced by the results shown in this chapter. Here, it was shown that at even lower concentrations of YOYO, 50nM and 100nM, the sensitivity to low concentrations of DNA was still evident. When determining an appropriate DNA concentration that would allow for maximal fluorescence over a range of YOYO concentrations, it was established that low concentrations of YOYO could be utilized to produce detectable fluorescence yields. Consequently, a linear dependence of the fluorescence intensity to the YOYO concentration was demonstrated allowing for the production of a standard curve. The significance of these results was that even if low concentrations of YOYO were...
encapsulated in PFVs, this dye could be used for subsequent studies in order to quantitate YOYO encapsulated within PFVs because of its superior fluorometric properties. Other standard nucleic acid dyes such as acridine orange, ethidium bromide or propidium iodide do not produce such high fluorescence yields and need to be encapsulated in larger quantities within liposomes. Although there are studies describing the encapsulation of some of these dyes, in their free form, within liposomes (Haran et al., 1994; Silvander and Edwards, 1996; Clerc and Barenholz, 1998; Silvander et al., 1998; Bakker et al., 1998; Bisby et al., 2000), the majority of these studies do not involve intracellular delivery to cultured cells. Furthermore, YOYO clearly has superior properties, such as a much higher fluorescence enhancement and higher binding affinity, than standard nucleic acid detection dyes. In addition, YOYO was shown to be easily and stably encapsulated within PFVs. However, it was observed from the vesicle size analyses that the vesicles increased in size when encapsulated with YOYO. Alterations in the characteristics of the liposomes due to encapsulation usually would not be desired. Taking the standard deviation values into account, however, the vesicle sizes were not significantly different. More importantly, it was demonstrated that YOYO could be stably encapsulated within PFVs with relatively substantial encapsulation efficiencies. Other fluorescent probes often leak from vesicles incubated in salt solutions or cell media containing serum making detection of intracellular delivery unreliable (Bailey and Cullis, 1997). In HBS, YOYO leakage was not detected from PFVs containing PEG-ceramides with varying acyl chain lengths. In DMEM supplemented with heat-inactivated FBS, leakage of YOYO from the PFVs containing PEG2000-C₈ ceramide was observed. However, gradual leakage of YOYO was an expected characteristic of PFVs based upon the
exchange of PEG2000-ceramides out of the vesicles to serum proteins or cell membranes (Holland et al., 1996b; Adlakha-Hutcheon et al., 1999). As a consequence, the rate dependence of YOYO leakage was shown to be based upon the acyl chain composition of the PEG2000-ceramides utilized in PFVs and the relative stability of these vesicles in salt and serum containing solutions was demonstrated. Hence, the characteristics of YOYO make it an ideal fluorophore to encapsulate into PFVs and to characterize cytoplasmic delivery by PFVs.

Looking at the analysis of the quadrant data, the influence of PFV concentrations on the cytoplasmic delivery of YOYO to cells could be determined (Figure 5.5). Ultimately, this assay focused on the quantitation of viable cells positive for YOYO indicating cytoplasmic delivery based on the quadrant data analyses. As well, it appeared that very low concentrations of YOYO (5nM-25nM) encapsulated within PFVs could be utilized to detect fluorescence within cells demonstrating the sensitivity of flow cytometry. Consequently, the combination of a highly fluorescent nucleic acid dye and a highly sensitive fluorescence detection technique results in a powerful cytoplasmic delivery assay.

Finally, when the cytoplasmic delivery of YOYO encapsulated in PFVs was compared with free YOYO added with “empty” PFVs, it was clearly shown that the former resulted in improved cell labeling. It was previously mentioned that DOPE and DODAC could possibly permeabilize cell plasma membranes. With this in mind, it was possible that YOYO could gain entry into the cell by this mechanism over an 8-hour incubation period. However, it was obvious that cytoplasmic delivery was improved
when YOYO was encapsulated within PFVs. Therefore, this also shows that PFVs provide an excellent means to deliver YOYO to the interior of cells.

In this chapter, we have presented the quantitation of YOYO encapsulated within PFVs and the characteristics of these vesicles. A simple yet powerful flow cytometric based quantitative cytoplasmic delivery assay has been developed and described. This assay may possibly have the potential to be utilized for the comparison of cytoplasmic delivery by different liposomal formulations and the comparison of cytoplasmic delivery to different cell lines. Further studies are required to investigate the applications of this assay, as described in the following chapter.
CHAPTER 6

CHARACTERIZATION OF CYTOPLASMIC DELIVERY OF YOYO-1 IODIDE USING CONVENTIONAL LIPOSOMES, NON-FUSOGENIC LIPOSOMES AND PFVS TO TUMORIGENIC AND NON-TUMORIGENIC CELLS

The previous chapter discussed the development of a novel, flow cytometry-based, cytoplasmic delivery assay. It was concluded that the assay was convenient and allowed quantitation of the number of cells labeled with YOYO following cytoplasmic delivery. Building on this development, the present chapter describes studies that examined cytoplasmic delivery for a range of different liposomes and cultured cell lines. The objective of these experiments was to compare cytoplasmic delivery by PFVs with other liposomal drug delivery systems and characterize the role of individual PFV lipid components with respect to the efficiency of cytoplasmic delivery.

6.1 INTRODUCTION

As described in Section 1.1.4, there are many different types of liposomes. Because liposomes can be comprised of a wide range of lipids, they have the advantage of being versatile with respect to their applications for specific purposes. Unfortunately, this versatility results in numerous different formulations being used in different studies, making it exceedingly difficult to compare formulations. In addition, many different cell lines have been used for various studies involving cytoplasmic delivery by liposomes. Hence, it is difficult to keep track of specific liposomal formulations, their results on specific cell lines and it is difficult to make direct comparisons between these studies.
With respect to liposomal formulations, studies have characterized the delivery of various molecules using conventional liposomes. These studies have described attempts at delivery of various drugs, DNA and proteins (Kobayashi et al., 1977; Forssen and Tokes, 1979; Rahman et al., 1982; Cudd and Nicolau, 1985; Brown and Silvius, 1990; Schwendener et al., 1991; Gregoriadis et al., 1993). Studies with PFVs as drug carriers have demonstrated improved bioavailability of encapsulated mitoxantrone to cells in vitro and in vivo compared with conventional liposomes (Adlakha-Hutcheon et al., 1999). These studies, however, did not differentiate between mitoxantrone release (leakage) from PFVs and intracellular (cytoplasmic) delivery. The present studies, therefore, compared conventional DSPC:Chol liposomes and PFVs with respect to their cytoplasmic delivery capabilities. As well, specific lipid components of PFVs were examined to determine the importance of their role in efficient cytoplasmic delivery.

Many different types of cell lines have been utilized for delivery studies involving liposomes. Examples include some common cell lines such as J774, BHK, B16, L1210 and 293 cells (Snyderman et al., 1977; Floros et al., 1978; Talavera and Basilico, 1977; Kreider and Schmoyer, 1975; Fidler, 1975; Hu, 1965; Law et al., 1949; Graham et al., 1977). The J774 cell line are murine macrophage cells grown in suspension and characterized as being tumorigenic when injected into irradiated pristane primed BALB/c mice. BHK cells are an adherent hamster kidney cell line that is an efficient host for transfection studies and is not tumorigenic. The B16 cell line are adherent murine melanoma cells which are tumorigenic in syngeneic mice. L1210 and 293 cells have been described in Chapter 3. These cell lines were studied to determine how cell characteristics might influence cytoplasmic delivery.
6.2 RESULTS

6.2.1 Comparison of YOYO Delivery to 293 Cells with PFVs, Non-Fusogenic Cationic Vesicles and Conventional Liposomes

To compare the cytoplasmic delivery of PFVs with non-fusogenic cationic vesicles and conventional liposomes, YOYO cytoplasmic delivery to 293 cells was examined over a 4 hour timecourse. The non-fusogenic cationic vesicles were of similar composition to that of PFV systems with the exception that DOPE was replaced by DOPC. The conventional liposomes consisted of DSPC:cholesterol (45:55 mol%). As clearly shown in Figure 6.1, PFV systems deliver YOYO (10nM) to a much larger percentage of the cell population (~57%) compared to non-fusogenic cationic vesicles (~14%) at 4 hours. In the case of conventional liposomes encapsulating YOYO, no significant delivery was seen.

6.2.2 Influence of DODAC Concentration on Cytoplasmic Delivery of YOYO by PFVs

DODAC is a cationic lipid that was utilized in the PFV formulation. To examine the effect that the concentration of DODAC has on intracellular (cytoplasmic) delivery, YOYO (10nM) encapsulated in PFVs containing PEG2000-C_{14} ceramide with DODAC concentrations from 0 to 15 mol% of total lipid were incubated for 4 hours with 293 cells.

From Figure 6.2, no cytoplasmic delivery of YOYO was seen when cells were incubated with PFVs containing 0 mol% and 5 mol% DODAC. This agrees with the idea that a cationic lipid component may be required to allow for electrostatic interaction with cells. At 10 mol% and 15 mol% DODAC, YOYO fluorescence was seen in 23% and 67% of the total cell sample population, respectively. For PFVs containing DODAC
Figure 6.1: Comparison of YOYO Delivery to 293 Cells with PFVs, Non-Fusogenic Cationic Vesicles and Conventional Liposomes

YOYO (final concentration 10nM) encapsulated vesicles were incubated with 293 cells for 4 hours at 37°C. (A) Flow cytometric quadrant analysis was used to examine live cells positive for delivery of YOYO and (B) mean fluorescence intensity values from respective cell populations. Samples include 293 cells without YOYO, (○), free YOYO, (▼), YOYO encapsulated in PEG2000-C$_{14}$ ceramide PFVs, (▲), YOYO encapsulated in non-fusogenic DOPC:cholesterol:DODAC:PEG2000-C$_{14}$ ceramide vesicles (▲), YOYO encapsulated in conventional DSPC:cholesterol vesicles (■).
Figure 6.2: Influence of DODAC Concentration on Intracellular Delivery of YOYO by PFVs

YOYO (final concentration 10nM) encapsulated in PEG2000-C_{14} ceramide PFVs, containing varying DODAC concentrations from 0 to 15 mol\%, were incubated with 293 cells for 4 hours at 37°C. (A) Flow cytometric quadrant analysis was used to examine live cells positive for delivery of YOYO and (B) mean fluorescence intensity values from respective cell populations. In this experiment, single samples were incubated with duplicate flasks of cells and the results are expressed as a mean with the error bars representing the standard deviation.
concentrations at 10 mol% and below, minimal propidium iodide uptake was seen, indicating no cytotoxicity according to analysis of quadrant data. Interestingly, at 15 mol% DODAC, 10% of the total cell sample population was both positive for PI and YOYO. This may again indicate toxicity by DODAC or YOYO as previously mentioned in Chapter 4.

6.2.3 Influence of PEG-Ceramide Acyl Composition on YOYO Delivery to 293 Cells

The rate of PEG-ceramide exchange, and hence rate of PFV destabilization, is determined by the ceramide N-acyl chain length (Holland et al., 1996b; Silvius and Leventis, 1993). Accordingly, the rate of YOYO cytoplasmic delivery should also be dependent on PEG-ceramide acyl chain length. This dependency was confirmed by the results shown in Figure 6.3. PFV systems containing PEG2000-C8 ceramide provide rapid YOYO delivery to 293 cells consistent with the expected rapid exchange of PEG-ceramides with short N-acyl chains. PFVs containing PEG2000-C14 ceramide exhibit a lag period of approximately 1 to 2 hours, after which a rapid increase in YOYO delivery to cells was observed. In contrast, PFVs containing PEG2000-C20 ceramide show little YOYO delivery up to 24 hours. This observation is consistent with the expected slow exchange of PEG2000-C20 ceramides (Holland et al., 1996). As in previous studies, intracellular uptake of free YOYO was very slow and even at 24 hours only a small percentage of the cell population (~15%) was positive.
Figure 6.3: Influence of PEG-Ceramide Acyl Composition on YOYO Delivery to 293 Cells

(A) Flow cytometric quadrant analysis was used to examine live cells positive for delivery of YOYO and (B) mean fluorescence intensity values from respective cell populations. YOYO (final concentration 10nM) encapsulated in PFVs containing PEG2000-C₈, (■), C₁₄, (◊) and C₂₀, (▲) were incubated with 293 cells over a 24 hour timecourse at 37°C. Control samples include 293 cells without YOYO, (●), and free YOYO, (▽).
6.2.4 Comparison of YOYO Delivery to Tumorigenic and Non-Tumorigenic Cells Using PFVs

To examine the cytoplasmic delivery of YOYO to HEK 293, B16, BHK, L1210 and J774 cell lines, PFVs containing PEG2000-C14 ceramide were employed. YOYO concentrations of 0nM, 10nM, 25nM and 50nM encapsulated in PFVs were incubated with the various cell lines for 4 hours. Prior to the reading the cell samples, controls were performed with free YOYO (50nM) and PI (1μg/ml) added to separate flasks of cells permeabilized with 70% ethanol for 1 hour. The flow cytometer instrument settings were calibrated accordingly for each cell line to optimize the FSC (forward scatter), SSC (side scatter), FL1-H (fluorescence channel 1) and FL2-H (fluorescence channel 2) parameters.

As shown in Figure 6.4, efficient cytoplasmic delivery of YOYO using PFVs was seen with BHK, J774 and 293 cell lines. The BHK cell line showed the highest percentage of YOYO-labeled cells, approximately 77% positive at 50nM YOYO. The 293 and J774 cell lines showed approximately 40-50% of cells positive for YOYO at 25nm. Interestingly, little or no cytoplasmic delivery was seen with the L1210 (~9%) or B16 (~2%) cell lines.

6.3 DISCUSSION

The general application of the flow cytometry-based cytoplasmic delivery assay described in Chapter 5 was illustrated in this chapter. This assay was shown to be useful for three purposes: (1) comparing cytoplasmic delivery by different liposomal formulations; (2) establishing the importance of individual lipid components in the PFV formulation; (3) comparing cytoplasmic delivery to various cell lines.
YOYO (final concentration 10nM) encapsulated in PFVs containing PEG2000-C14 ceramide were incubated with cells over a 4 hour timecourse at 37°C. (A) Flow cytometric quadrant analysis was used to examine live cells positive for delivery of YOYO and (B) mean fluorescence intensity values from respective cell populations. The five cell lines utilized were 293 (●), B16 (▽), BHK (▲), L1210 (○) and J774 (■).
Firstly, a clear difference was seen in the cytoplasmic delivery capabilities of different liposomal formulations. As expected, conventional DSPC:Chol liposomes provided little or no delivery of YOYO to 293 cells. This is consistent with previous studies which have shown the ability of conventional liposomes to retain their encapsulated contents and may compromise their ability to make encapsulated contents readily bioavailable. The results of this experiment demonstrate that cytoplasmic delivery would be difficult or significantly reduced using conventional liposomes. Previous studies have proposed that these liposomes are taken up by endocytosis (Pagano and Weinstein, 1978; Lee et al., 1993; Miller et al., 1998). Unfortunately, conventional liposomes are not believed to be able to destabilize endosomal membranes and, consequently, encapsulated contents may be subjected to degradative enzymes found within lysosomes instead of being released into the cell cytoplasm. With the non-fusogenic cationic liposomes containing DOPC in place of DOPE, cytoplasmic delivery was seen to a low percentage of the cell population. Vesicles composed of DOPC readily form bilayers and therefore are usually inherently stable. However, the inclusion of a cationic lipid, DODAC, in this formulation may have allowed for the permeabilization of cell plasma membranes or endosomal membranes to some degree, allowing for the release of YOYO into the cell cytoplasm. In comparison, cytoplasmic delivery by PFVs was superior to conventional liposomes and non-fusogenic cationic liposomes. This observation is consistent with PFVs being able to introduce their contents to the cell cytoplasm. This result agrees with earlier studies, which have also demonstrated an improvement in delivery by DOPE-containing liposomal drug delivery systems over
DOPC-containing liposomal systems (Chu et al., 1990; Farhood et al., 1995; Lutwyche et al., 1998).

Other lipid components in the PFV formulation were also shown to be essential to the cytoplasmic delivery capabilities of these vesicles. Formulations involving cationic liposome-DNA complexes have utilized high concentrations of cationic lipids, up to a 1:1 ratio of neutral lipid (DOPE) to cationic lipid. This has often resulted in aggregation of these complexes, in saline and serum-containing solutions, in addition to being highly toxic to cells (Lasic, 1997). Employing this assay, it was shown that PFVs, which utilize lower concentrations of cationic lipid than complexes, could demonstrate cytoplasmic delivery. As well, studies performed have shown that cationic lipids, when incorporated in PFVs systems, are far less toxic to cells than in cationic lipid complexes (unpublished studies by Dr. Qiang Hu). Although cationic lipids can be toxic, the use of cationic lipids in the PFV formulation was shown to be essential for cytoplasmic delivery. In addition, PEG2000-ceramides were also shown to be important to the delivery process. Specific to the PFV formulation, different extents of cytoplasmic delivery could be demonstrated by simple modifications in the acyl chain composition of the PEG2000-ceramides. This was also demonstrated qualitatively by fluorescence microscopy in Chapter 4. However, here the use of flow cytometry allows for quantitative comparisons. By combining the qualitative data from fluorescence microscopy and quantitative data using the flow cytometry-based cytoplasmic delivery assay, a convincing display of cytoplasmic delivery by PFVs was established.

In regard to the application of this assay to examine cytoplasmic delivery by PFVs to various cell lines, substantial differences between cell lines were observed. The
BHK cell line has demonstrated in previous studies its efficiency for transfection (Holland et al., 1976; Chen and Okayama, 1987; Bichko et al., 1994; Teifel and Friedl, 1995). The studies presented here also suggested that cytoplasmic delivery to the BHK cell line could be readily performed and this cell line showed the highest percentage of YOYO-positive cells of the cell lines studied. Hence, there may be some correlation between BHK cells' ability to exhibit cytoplasmic delivery in addition to being efficient hosts for transfection studies. Similarly, J774 cells and 293 cells also demonstrated efficient cytoplasmic delivery by PFVs.

Examining the B16 and L1210 cells, little or no cytoplasmic delivery was observed. The B16 cell line are melanoma cells which produce melanin, a pigment that darkens skin or hair. Melanin was clearly visible in the cell media and the cell membranes appeared to have darkened during cell passages. As a consequence, this may have resulted in the quenching and lack of detection of YOYO fluorescence. Also with L1210 cells, no cytoplasmic delivery of YOYO was detected. A reasonable explanation for these results still has not been elucidated.

The general application of this flow cytometry-based cytoplasmic delivery is quite extensive. The four studies presented in this chapter have illustrated the usefulness of this assay to examine various liposomal formulations, lipid components of the PFV formulation and various cell lines. Although more studies need to be performed to thoroughly characterize all the possible applications of this assay, it can be concluded that this assay does exhibit strong potential to characterize cytoplasmic delivery by liposomal drug delivery systems to various cell lines. Furthermore, in detailing the applications of this assay, the characterization of PFVs as cytoplasmic delivery carriers was also
accomplished. These vesicles may have the capacity to efficiently deliver a wide variety of molecules to cells *in vivo* with the function of being general cytoplasmic delivery vehicles. Up to this point, PFVs have been shown to deliver the anticancer agent mitoxantrone and the fluorophore YOYO which are both relatively small molecules. It would be of interest to determine if PFVs are effective at delivering larger macromolecules.
CHAPTER 7
CHARACTERIZATION OF ANTISENSE OLIGONUCLEOTIDE DELIVERY
UTILIZING PROGRAMMABLE FUSOGENIC VESICLES

In previous chapters, the delivery of various small molecules to cultured cell lines utilizing conventional liposomes and PFVs was examined. From these studies, results established that PFVs allowed for increased drug bioavailability of mitoxantrone and improved intracellular delivery of YOYO compared with conventional liposomes. Delivery of large polar molecules encapsulated within liposomes is a more difficult task. In this chapter, intracellular delivery of antisense oligonucleotides to cultured cells utilizing PFVs will be characterized. Furthermore, the biological effects of a specific antisense construct encapsulated in PFVs will be assessed. The objective of these experiments was to determine if PFVs would be suitable delivery vehicles for large polar molecules.

7.1 INTRODUCTION

As mentioned previously in Section 1.4.3, the site of action of antisense oligonucleotides is cytosolic mRNA. Due to the fact that oligonucleotides are polyanionic, they do not readily diffuse through cell membranes. In vitro, cationic liposomes complexed with antisense oligonucleotides have been commonly utilized to deliver these molecules to various cell lines. Unfortunately, these complexes are not suitable for systemic delivery because they are highly unstable, often forming large aggregates that are cleared rapidly from the circulation (Litzinger 1997; Litzinger et al., 1996; Zelphati et al., 1998). Furthermore, these complexes can cause significant organ toxicity, particularly in the liver, as well as toxicities associated with complement
activation (Plank, 1996; Filion and Phillips, 1997; Zelphati et al., 1998). Therefore, new formulations involving the encapsulation of antisense oligonucleotides within liposomes are currently under investigation.

In terms of specific proto-oncogenes, a commonly studied antisense oligonucleotide target is the \textit{bcl}-2 gene. It has been identified as a regulator of apoptosis and it appears to be a suppressor of cell death (Konopleva \textit{et al.}, 2000). In human melanomas, over-expression of Bcl-2 protein has been detected in up to 90% of all cases (Cerroni \textit{et al.}, 1995; Morales-Ducret \textit{et al.}, 1995; Plettenberg \textit{et al.}, 1995). The 518A2 is one representative example of human melanoma derived cell line that over-expresses Bcl-2. Consequently, the effect of an antisense construct to the \textit{bcl}-2 proto-oncogene encapsulated in PFVs on 518A2 cells was studied in experiments described in this chapter.

This chapter characterizes the delivery of antisense oligonucleotides encapsulated in PFVs. The delivery of fluorescein isothiocyanate (FITC)-labeled antisense oligonucleotides encapsulated in various PFV formulations was characterized and compared to free antisense oligonucleotides utilizing the methods of flow cytometry and fluorescence microscopy. The biological effects of antisense constructs against the proto-oncogene, \textit{bcl}-2, were also examined. Reverse polarity oligonucleotide constructs, which have the same base sequence as the primary antisense construct except that the 3'-end and the 5'-end are switched, were examined for non-specific down-regulation effects.
7.2 RESULTS

7.2.1 Comparison in Delivery of Antisense Oligonucleotides Encapsulated in PFVs Against Free Antisense Oligonucleotides to Cells In Vitro

The delivery of FITC-labeled antisense oligonucleotides to the gene stop codon of epithelial growth factor receptor (EGFR) by PFVs to 293 cells was examined by flow cytometry. This antisense sequence was selected as a representative sequence and delivery of this FITC-labeled sequence will provide results comparable to those that would be obtained for other sequences, including the bcl-2 targeted antisense. Free FITC-EGFR, free FITC-EGFR added with empty PFVs, and FITC-EGFR encapsulated in PFVs were incubated with cells over a 4-hour timecourse and intracellular delivery was compared. As shown in Figure 7.1, cells exposed to free antisense showed only low levels of fluorescence as compared with control cells. In contrast, cells incubated with PFVs encapsulating antisense showed approximately 20-fold higher mean fluorescence compared to those cells treated with free antisense. Interestingly, cells incubated with free antisense plus empty PFVs also demonstrated an increase in fluorescence, albeit to lower levels than were achieved for FITC-EGFR encapsulated in PFVs.

7.2.2 Influence of PFVs Containing PEG2000-Ceramides of Varying Acyl Composition on Antisense Oligonucleotide Delivery to 293 Cells

To characterize the influence of the PEG2000-ceramide acyl chain composition on the delivery of antisense molecules to cells in culture, FITC-EGFR encapsulated in PFVs containing PEG2000-ceramides with acyl chain lengths of C8, C14 and C20 were incubated with 293 cells over a 4-hour timecourse.

As shown in Figure 7.2, all cells incubated with PFV formulations exhibited greater mean fluorescence intensity values as compared with free antisense. Comparing
Figure 7.1: Comparison in Delivery of Antisense Oligonucleotides Encapsulated in PFVs Against Free Antisense Oligonucleotides to Cells In Vitro

FITC-labeled antisense encapsulated in PFVs were incubated with 293 cells for 4 hours at 37°C. Mean fluorescence values were determined by flow cytometry upon exposure to free antisense (▽), free antisense plus empty PFVs (■) and antisense encapsulated in PFVs (○). Untreated cells were presented as the control (•).
Figure 7.2: Influence of PFVs Containing PEG2000-Ceramides of Varying Acyl Composition on Antisense Oligonucleotide Delivery to 293 Cells

FITC-labeled antisense encapsulated in PFVs were incubated with 293 cells for 4 hours at 37°C. Antisense was delivered as free antisense (\(\nabla\)), by PFVs containing PEG-C_{6} (\(\bullet\)), PEG-C_{14} (\(\blacktriangledown\)) and PEG-C_{20} (\(\blacksquare\)) ceramides. Untreated cells were presented as control (O).
the three formulations of PFVs, differences in the cellular mean fluorescence could be seen over the duration of 4 hours. When PEG-C\textsubscript{14} PFVs delivered antisense to cells, mean cellular fluorescence increased progressively over 4 hours. In comparison, mean cellular fluorescence was initially high and then a gradual decrease was seen with PFVs containing PEG-C\textsubscript{8} and PEG-C\textsubscript{20} ceramides. Following the 4-hour incubation period, cells exposed to FITC-EGFR encapsulated in PFVs containing PEG-C\textsubscript{14} exhibited mean cellular fluorescence exceeding 2-fold greater intensity than cells exposed to FITC-EGFR encapsulated in PFVs containing PEG-C\textsubscript{8} or C\textsubscript{20}.

### 7.2.3 Examination of Antisense Intracellular Distribution Following Delivery by PFVs with PEG2000-Ceramides of Varying Acyl Composition to 293 Cells

To examine the localization and intracellular distribution of antisense molecules upon delivery by PFVs to cells, a study was conducted similar to that mentioned in Section 7.2.2. Once again, PFVs containing PEG2000-ceramides of acyl chain lengths C\textsubscript{8}, C\textsubscript{14} and C\textsubscript{20} were utilized; however this time, 293 cells were examined under phase contrast and fluorescence microscopy following a 4-hour incubation period.

In this study, differences in antisense distribution were readily apparent between the three PFV formulations (Figure 7.3). Following the antisense delivery by PFVs containing PEG2000-C\textsubscript{8} and PEG2000-C\textsubscript{20}, FITC fluorescence appeared to be primarily localized on the cell surface. The difference between these two formulations was that fluorescence with PEG2000-C\textsubscript{8} PFVs appeared to be more punctate, while the fluorescence with PEG2000-C\textsubscript{20} PFVs appeared more evenly distributed over the cell surface. A significant difference was apparent when cells incubated with PEG2000-C\textsubscript{14} PFVs were examined. The level of fluorescence appeared to be much higher, consistent
FITC-labeled antisense encapsulated in PFVs were incubated with 293 cells for 4 hours at 37°C. Antisense was delivered by PFVs containing PEG2000-C₈ (A and B), PEG2000-C₁₄ (C and D) and PEG2000-C₂₀ (E and F) ceramides.

Figure 7.3: Examination of Antisense Intracellular Distribution Following Delivery by PFVs with PEG2000-Ceramides of Varying Acyl Composition to 293 Cells
with flow cytometry data, as well the fluorescence appeared to be diffuse, most likely indicating the internalization of the antisense oligonucleotides and cytoplasmic delivery. Comparing these results with the data from flow cytometric analysis, the initially high fluorescence seen at 0.5 hours with the PEG2000-C_{8} and PEG2000-C_{20} PFVs, in the previous study, may be due to adsorption of PFVs on the cell surface rather than intracellular delivery of antisense oligonucleotides.

7.2.4 Antisense Effects on \textit{bcl-2} mRNA expression

Based on the results of the flow cytometric and fluorescence microscopy studies, the PEG-C_{14} PFVs could clearly demonstrate effective intracellular delivery of antisense oligonucleotides. Following these studies, the next objective was to examine the biological effects of antisense oligonucleotides on target genes upon delivery by PEG2000-C_{14} PFVs. Consequently, studies were performed to examine the effects of an 18-mer antisense construct targeting the proto-oncogene \textit{bcl-2}. In previous studies, this antisense construct has been shown to exhibit biological effects (Jansen \textit{et al.}, 1998). When delivered as cationic liposome-antisense oligonucleotide complexes, this antisense has successfully inhibited gene expression at both the mRNA and protein levels as well as preventing cell growth by inducing apoptosis in human melanoma cell lines (Jansen \textit{et al.}, 1998). One of the human melanoma cell lines described in the study by Jansen \textit{et al.} was the 518A2 cell line. Therefore, antisense effects on this cell line were examined.

To confirm that the antisense construct was effective, an initial study was performed exposing 518A2 cells to cationic liposome-antisense complexes at a final oligonucleotide concentration of 0.5\textmu M for 24 hours. As shown in Figure 7.4, \textit{bcl-2} mRNA levels were reduced by 60% utilizing the cationic liposome-antisense complexes.
Figure 7.4: Antisense Effects on bcl-2 mRNA Expression in 518A2 Cells Utilizing Cationic Liposome-Antisense Oligonucleotide Complexes

Adapted from experiments performed by Dr. Qiang Hu (Hu et al., 2001). 518A2 cells were incubated with bcl-2 antisense oligonucleotide (AS), cationic liposomes, reverse polarity oligonucleotide-liposome complexes (RP-C) or antisense-liposome complexes (AS-C) for 24 hours at a final AS concentration of 0.5μM. Control cells were treated with equal volume of distilled water. The abundance of bcl-2 mRNA was quantified by RT-PCR. Values presented as means ± SEM (n=4). * denotes significant difference between AS-C and the rest of treatment groups (p < 0.05).
as compared to the control and free antisense. In addition, the reverse polarity control oligonucleotide (RP) produced 30% reduction in mRNA levels. A subsequent study was then performed to examine the effectiveness of the bcl-2 antisense construct encapsulated in PFVs. Cells were exposed to free antisense, empty PFVs, control RP encapsulated in PFVs, and bcl-2 antisense oligonucleotides encapsulated in PFVs. Cells were incubated for 24 and 48 hours at two antisense concentrations, 0.5μM and 1.0μM (Figure 7.5). After 48 hours treatment with bcl-2 antisense oligonucleotides encapsulated in PFVs, an antisense specific down-regulation effect was clearly detected at both concentrations. At an antisense oligonucleotide concentration of 0.5μM, the mRNA level was decreased by approximately 20% compared to the control and free antisense-treated cells. Interestingly, with the control RP encapsulated in PFV treated cells, an up-regulation of bcl-2 mRNA was seen. When the oligonucleotide concentration was increased to 1.0μM, the mRNA level was reduced by approximately 25% compared to the control and free antisense treated cells. Conversely, the control RP encapsulated in PFV treated cells again demonstrated an up-regulation of mRNA levels. At 24 hours, it was evident that no down-regulation effects were observed. It was also evident from this study, based on the data at 24 hours, that a lag period existed between the initial treatment of cells with bcl-2 antisense encapsulated in PFVs and the actual detection of down-regulation. With antisense delivery using cationic liposome complexes, this lag phase was not evident.
Figure 7.5: Antisense Effects on bcl-2 mRNA Expression in 518A2 Cells Utilizing Antisense Encapsulated in PFVs containing PEG2000-C_14 Ceramide

Adapted from experiments performed by Dr. Qiang Hu (Hu et al., 2001). 518A2 cells were exposed to HBS (control, open bar), free antisense (shaded), empty PFVs (diagonal hatched), RP oligonucleotide encapsulated in PFVs (horizontal hatched) or antisense encapsulated in PFVs (cross hatched) at a final antisense concentration of 0.5μM (A) or 1.0μM (B). Values presented as means ± SEM (n=3-5). * denotes significant difference between antisense oligonucleotide PFVs and RP oligonucleotide encapsulated PFVs (p < 0.05).
7.3 DISCUSSION

The results reported in this chapter demonstrate that PFVs could effectively deliver antisense oligonucleotides to cells in culture. In particular, PFVs containing PEG2000-C_{14} ceramide led to optimal delivery over the timecourse examined. As well, cytoplasmic delivery of antisense oligonucleotides was exhibited with this PFV formulation. With the PEG2000-C_{8} ceramide, the short acyl chain resulted in rapid exchange from the PFVs leading to rapid vesicle destabilization. It is likely that this also led to the aggregation of these vesicles and potentially inhibited vesicle uptake and subsequent antisense delivery. With the PEG2000-C_{20} ceramide, the longer acyl chain would not be expected to exchange out of the PFVs over the timecourse examined (Silvius and Leventis, 1993; Holland et al., 1996b). Accordingly, vesicle destabilization would not occur, and hence liposome-cell interactions would be inhibited and antisense oligonucleotide delivery would not be expected. The experimental findings in this chapter support this concept. However, for in vivo studies, PFVs containing PEG-ceramides with longer acyl chain lengths exhibiting slower vesicle destabilization rates may be required to allow for longer circulation lifetimes and increased accumulation at tumor sites.

In terms of the biological effects of antisense oligonucleotides, the down-regulation of bcl-2 mRNA levels utilizing bcl-2 antisense encapsulated in PEG-C_{14} PFVs was demonstrated. The method of down-regulation detection demonstrates cytoplasmic delivery because the antisense must access the cytoplasm to decrease mRNA levels. This is important because delivery to the cytoplasm is essential for the antisense oligonucleotides to bind to mRNA and elicit their biological effects, which leads to their
use as therapeutic agents. Specific antisense effects could be seen as compared with control reverse polarity (RP) oligonucleotides encapsulated in PFVs. Compared to cationic liposome complexes, PFV systems required a longer time period before decreases in mRNA levels were observed. This lag period may be related to the time required for PFV destabilization. Furthermore, increasing the concentration of \textit{bcl-2} antisense oligonucleotides in PFVs from 0.5\mu M to 1.0\mu M only led to a minor decrease in mRNA levels. This may reflect the saturation of \textit{in vitro} binding sites for PEG-ceramide resulting in decreased vesicle destabilization. Additional studies are required to further investigate this matter.

Antisense oligonucleotides complexed with cationic liposomes have been shown to elicit biological effects \textit{in vitro} resulting in down-regulation of cellular mRNA levels (Lebedeva \textit{et al.}, 2000). The data in this chapter confirm the idea that cationic liposome complexes are effective for \textit{in vitro} down-regulation studies. Nevertheless, cationic liposome complexes are not suitable for systemic delivery of antisense molecules because serum components bind to the complexes inhibiting their interactions with cells (Zelphati \textit{et al.}, 1998). Furthermore, other disadvantages of cationic liposome complexes mentioned earlier are their toxicity and rapid clearance from the circulation. In this chapter, PFVs have been shown to deliver antisense oligonucleotides and elicit biological effects in \textit{in vitro} cell culture models. As well, PFVs containing PEG ceramides with longer acyl chains, C_{14} and C_{20}, have been previously shown to be stable in serum containing media, they are relatively less toxic to cells, and they contain PEG-ceramides which would allow for longer circulation lifetimes. Therefore, antisense oligonucleotide
encapsulation in PFVs may be better adapted for \textit{in vivo} utility. Likewise, PFVs may be potentially suitable for general intracellular delivery of large polar molecules.
CHAPTER 8
SUMMARY

The studies presented in this thesis focused on the characterization of liposomal drug delivery systems utilizing cell culture models. In the first chapter, I hypothesized that the characterization of liposomal systems could be improved by developing and utilizing assays specifically designed to examine drug bioavailability and intracellular delivery (fusion and cytoplasmic delivery). These are characteristics that can, if enhanced, lead to the design of improved liposomal formulations as candidates for the \textit{in vivo} delivery of drugs. Programmable fusogenic vesicles (PFVs) were examined as a representative drug delivery system with potential intracellular delivery capabilities. The results presented in this thesis support my hypothesis.

Assays were utilized to characterize four areas of liposome delivery. The first assay was designed to characterize drug bioavailability from liposomal systems. The second assay examined the ability of programmable fusogenic vesicles to fuse with cultured cells. The third assay was developed to characterize cytoplasmic delivery to cells by liposomes. The final study examined the delivery of large polar molecules such as antisense oligonucleotides to cells \textit{in vitro}.

Drug bioavailability from liposomes is believed to be the most important factor in determining the biological activity of the drug (Lim \textit{et al.}, 1999). A cytotoxicity assay was employed to characterize this property of liposomal carriers, in particular PFVs and conventional DSPC:Chol vesicles. Mitoxantrone, an anticancer drug, was encapsulated in these systems as a representative drug. PFV systems improved mitoxantrone bioavailability compared to a conventional liposome drug delivery formulation. From
these studies, I established that drug bioavailability from liposomal systems could be compared by utilizing the XTT cytotoxicity assay described in Chapter 3.

In Chapter 4, I wanted to examine the PFVs' potential to fuse with cells and demonstrate cytoplasmic delivery of encapsulated molecules under fluorescence microscopy. Using the fluorescent lipid probe, Rh-PE, we were able to demonstrate that PFVs could fuse with cultured cells. More importantly, the use of the nucleic acid dye, YOYO-1 iodide, to examine cytoplasmic delivery proved to be a better assay because cytoplasmic delivery by PFVs was clearly established.

To measure the cytoplasmic delivery of YOYO to cells in vitro, a quantitative flow cytometry assay was employed. In the development of this assay, I first demonstrated the stability of YOYO encapsulation within PFVs exposed to saline and serum-containing solutions. In Chapter 6, I used this flow cytometry assay for cytoplasmic delivery to compare different liposomal formulations. I found that cytoplasmic delivery of YOYO was greatly enhanced when encapsulated in PFVs, as compared with encapsulation in conventional DSPC:Chol liposomes or non-fusogenic (DOPC containing) cationic vesicles. The importance of individual lipid components in the PFV formulation to the cytoplasmic delivery of YOYO was also demonstrated.

The final set of studies examined the delivery and biological activity of antisense oligonucleotides. Delivery of fluorescent-labeled antisense oligonucleotides could be visualized and quantitated using fluorescence microscopy and flow cytometry, respectively. The PFV formulation with PEG2000-C_{14} ceramide exhibited the greatest levels of delivery compared to PFV formulations containing PEG2000-C_{8} and PEG2000-C_{20} ceramides. An assay that measures mRNA down-regulation was then employed to
examine the biological effects of antisense oligonucleotides to the proto-oncogene *bcl*-2 delivered to cells by PFVs. Antisense oligonucleotides encapsulated in PFVs resulted in down-regulation of mRNA levels by 20% compared to the control and free antisense. This observation also reconfirms the cytoplasmic delivery of molecules, such as antisense oligonucleotides and YOYO-1 iodide, by PFVs.

The results summarized above illustrate the importance of developing specific assays for the characterization of liposomal drug delivery systems. By developing specific assays to examine different aspects of liposome-cell interactions, I was able to characterize and compare liposomal drug delivery systems. In turn, this brings us closer to designing optimal liposomal formulations. Taking the PFVs as an example of a novel formulation, we demonstrate that this formulation improves drug bioavailability, fuses with cultured cells, allows for cytoplasmic delivery of encapsulated contents as well as delivers large polar molecules. The significance of these results is that all these characteristics are conducive to the design of a desirable drug delivery carrier with potential for *in vivo* applications.

It is important to note that a single assay does not fully characterize a liposomal formulation, but rather, utilizing a variety of analytical techniques provides a more complete picture of the liposomal formulation and its potential as an *in vivo* drug carrier. Therefore, utilizing delivery assays in combination to fully characterize a liposomal formulation should be encouraged.

The research presented in this thesis outlines the development and application of cell culture techniques to characterize liposomal drug delivery systems. In particular, programmable fusogenic vesicles were investigated. The positive results reported in this
thesis will hopefully lead to further characterization of effective liposomal drug delivery systems similar to PFVs. Ultimately, it is hoped that delivery systems characterized by the aforementioned cell culture assays can lead to liposomal drug formulations with applications for clinical use.
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