

The Development and Characterisation of an Anionic Liposome Gene Transfer Vector

A thesis submitted to the University of Manchester for the degree of Doctor of
Philosophy in the Faculty of Science and Engineering

by

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June 2000

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Contents

Figures	9
Tables	15
Equations	16
Abstract	17
Declaration	19
Copyright	20
Acknowledgements	21
The Author	22
Abbreviations	23

CHAPTER 1 *Introduction*

1.1. Gene Therapy	27
1.2 Nucleic acids for gene transfer	29
1.2.1 Cytotoxicity of lipopolysaccharide contamination of plasmid DNA	29
1.2.1.2 Suppression of lipopolysaccharide cytotoxicity <i>in vivo</i>	30
1.2.2 Immunogenicity of plasmid DNA isolated from a bacterial source	32
1.2.3 Production of low immunogenicity vector DNA	33
1.2.4 Effect of DNA supercoiling on vector properties	33
1.2.5 Antisense oligonucleotide optimisation	34
1.3 Targeted gene delivery	35

1.3.1 Initial DNA-targeting protocols	36
1.3.2 Poly-L-lysine conjugates as mediators of gene transfer: targeting of asialoglycoprotein receptor	37
1.3.2.1 Optimisation of asialoglycoprotein receptor targeting	38
1.3.3 Targeting of the transferrin receptor	40
1.3.4 Miscellaneous ligands targeted using poly-L-lysine conjugates	40
1.3.5 Preparation of poly-L-lysine/DNA polyplexes	41
1.3.5.1 Morphology of polyplexes	42
1.3.6 An inspection of poly-L-lysine as a vector component	43
1.3.6.1 Heterogeneity of poly-L-lysine preparations	43
1.3.6.2 Toxicity of poly-L-lysine	43
1.3.6.3 Poly-L-lysine has little membrane fusion activity	44
1.3.7 Artificial peptides as mediators of gene transfer	45
1.3.7.1 DNA-condensing peptides for gene transfer	49
1.3.7.2 Peptides with membrane fusion activity	49
1.3.7.3 Peptides with nuclear localisation activity	51
1.3.7.4 Future problems of artificial peptide-based vectors	52
1.4 Structures of anionic liposome/DNA complexes	53
1.4.1 Advances in anionic liposome-DNA association	53
1.4.1.1 Encapsulation model of DNA association (Lee and Huang, 1996)	54
1.4.1.2 Surface-association model of DNA association (Hagstrom <i>et al.</i> , 1996)	55
1.4.3 Additional evidence for the structure of anionic liposome complexes	56
1.5 Project aims	59

CHAPTER 2 Materials and Methods

2.1 Materials	61
2.2 Methods	64
2.2.1 Bacterial cell strains	64

2.2.2 Plasmid DNA	64
2.2.3 Preparation of liposomes	65
2.2.3.1 Vesicles by the Extrusion Technique (VETs)	65
2.2.3.2 Immunoliposome preparation using a French Press	66
2.2.4 Polyplex and lipopolyplex preparation	67
2.2.5 Electron microscopy	68
2.2.5.1 Cryo transmission electron microscopy	68
2.2.5.2 Standard transmission electron microscopy	69
2.2.6 Particle size determination by Photon Correlation Spectroscopy	69
2.2.7 Zeta potential measurement	70
2.2.8 Agarose gel electrophoresis	71
2.2.9 Liposome permeability measurements	71
2.2.10 Sensitivity of DNA complexes to pronase enzyme	72
2.2.11 Maintenance of mammalian cell lines	73
2.2.12 Jurkat cell transfection	73

CHAPTER 3 *Characterisation of DNA-Poly-L-lysine complexes*

3.1 Overview	76
3.1.1 Aspects of Nomenclature	76
3.2 Results	78
3.2.1 Physical characterisation of DNA-Poly-L-lysine complexes (polyplexes)	78
3.2.1.1 Electron microscopy of polyplexes	78
3.2.1.2 Size and zeta potential analysis of polyplexes	80
3.2.1.2.1 PLK ₅ -DNA polyplexes	80
3.2.1.2.2 PLK ₄₅ -DNA polyplexes	80
3.2.1.2.3 PLK ₉₉ -DNA polyplexes	81
3.2.1.2.4 PLK ₅₃₁ -DNA polyplexes	81

3.2.1.3 Gel electrophoresis analysis of polyplexes	86
3.2.1.3.1 PLK ₅ -DNA polyplexes	86
3.2.1.3.2 PLK ₄₅ -DNA polyplexes	86
3.2.1.3.3 PLK ₉₉ -DNA polyplexes	87
3.2.1.3.4 PLK ₅₃₁ -DNA polyplexes	87
3.3 Discussion	89
3.3.1 Electron microscope analysis of polyplexes	89
3.3.1.2 Order and control of DNA condensation	90
<i>Summary</i>	91
3.3.2 Size and Zeta potential analysis of polyplexes	92
3.3.2.1 Primary stages of DNA condensation	92
3.3.2.2 Polyplexes carrying a low zeta potential	93
3.3.2.3 Saturation of plasmid DNA with bound poly-L-lysine	93
3.3.2.4 Benefits of zeta potential and PCS analysis of polyplexes	94
<i>Summary</i>	94
3.3.3 Agarose gel electrophoresis analysis of polyplexes	96
3.3.3.1 Why are highly condensed polyplexes completely retarded by the gel matrix?	96
<i>Summary</i>	97
3.3.4 Condensation of plasmid DNA using PLK ₅	98
<i>Summary</i>	99

CHAPTER 4 *Characterisation of Lipopolyplexes*

4.1 Overview	101
4.2 Results	103
4.2.1 Liposome and lipopolyplex permeability to glucose	103
4.2.3 Sensitivity of lipopolyplexes to pronase enzyme digestion	110
4.2.3 Cryo TEM of lipopolyplexes	111
4.3 Discussion	114
4.3.1 Lipopolyplex formation and structure	114
4.3.2 Physical properties of lipopolyplexes	117

CHAPTER 5 *Transfection of Jurkat cells using Lipopolyplexes*

5.1 Transfection rationale _____ 120

5.2 Results _____ 123

5.2.1 Primary screen of gene transfer activity of individual liposome formulations using PLK₉₉ as a DNA condensing agent _____ 123

5.2.1.1 DOPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer 124

5.2.1.2 DOPE/DOPC/oleic acid anti-CD3 liposome mediated gene transfer ____ 125

5.2.1.3 DMPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer 126

5.2.1.4 DLPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer 126

5.2.1.5 DLPE/DLPC/oleic acid anti-CD3 liposome mediated gene transfer _____ 127

5.2.2 Secondary screen of liposome formulations for gene transfer activity _____ 142

5.2.2.1 PLK₅ DNA condensing agent _____ 144

5.2.2.1.1 DOPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer
_____ 144

5.2.2.1.2 DOPE/DOPC/oleic acid anti-CD3 liposome and DMPE/cholesterol/oleic
acid anti-CD3 liposome mediated gene transfer _____ 144

5.2.2.2 PLK₃₆ DNA condensing agent _____ 151

5.2.2.2.1 DOPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer
_____ 151

5.2.2.2.2 DOPE/DOPC/oleic acid anti-CD3 liposome and DMPE/cholesterol/oleic
acid anti-CD3 liposome mediated gene transfer _____ 152

5.2.2.3 PLK₅₃₁ DNA condensing agent _____ 157

5.2.2.3.1 DOPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer
_____ 157

5.2.2.3.2 DOPE/DOPC/oleic acid anti-CD3 liposome mediated gene transfer ____ 157

5.2.2.3.3 DMPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer
_____ 158

5.2.2.4 PLR₁₇ DNA condensing agent _____ 165

5.2.2.4.1 DOPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer
_____ 165

5.2.2.4.2 DOPE/DOPC/oleic acid anti-CD3 liposome mediated gene transfer ____ 165

5.2.2.4.3 DMPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer	165
5.2.3 Targeted transfection of Jurkat cells	173
5.2.3.1 Targeted transfection of Jurkat cells in serum-free media	175
5.2.3.2 Targeted transfection of Jurkat cells in the presence of serum	175
5.2.3.2.1 DOPE/cholesterol/oleic acid liposome mediated gene transfer	175
5.2.3.2.2 DOPE/DOPC/oleic acid liposome and DMPE/cholesterol/oleic acid liposome mediated gene transfer	176
5.2.3.3 DOPE/DOPC/oleic acid liposome targeted transfection of Jurkat cells	179
5.3 Discussion	182
5.3.1 Analysis of transfection data	182
5.3.2 Primary transfection screen	185
5.3.2.1 Transfection of Jurkat cells using PLK ₉₉ polyplexes	185
<i>Summary</i>	186
5.3.2.2 Gene transfer properties of DOPE/cholesterol/oleic acid liposomes	187
<i>Summary</i>	189
5.3.2.3 Gene transfer properties of DOPE/DOPC/oleic acid liposomes	190
<i>Summary</i>	192
5.3.2.4 Gene transfer properties of DMPE/cholesterol/oleic acid liposomes	192
<i>Summary</i>	193
5.3.2.5 Gene transfer properties of DLPE/cholesterol/oleic acid liposomes and DLPE/DLPC/oleic acid liposomes	193
<i>Summary</i>	194
5.3.3 Secondary transfection screen	195
5.3.3.1 Transfection of Jurkat cells using lipopolyplexes containing PLK ₅ - and PLK ₃₆ -condensed DNA	195
<i>Summary</i>	197
5.3.3.2 Transfection of Jurkat cells using lipopolyplexes containing PLK ₅₃₁ -condensed DNA	198
<i>Summary</i>	200
5.3.3.4 Transfection of Jurkat cells using lipopolyplexes containing PLR ₁₇ -condensed DNA	201
<i>Summary</i>	202
5.3.4 Targeted transfection of Jurkat cells	204
<i>Summary</i>	205
5.3.5 Targeted transfection of Jurkat cells in the presence of serum	206

<i>Summary</i>	207
5.3.6 Targeted transfection using DOPE/DOPC/oleic acid/DSPE-PEG- anti CD3 liposomes	209
<i>Summary</i>	209
 CHAPTER 6 <i>Discussion and Conclusion</i>	
6.1 Discussion and Conclusion	211
6.1.1 Characterisation studies	211
6.1.2 Gene transfer studies	213
6.2 Future work	219
 References	 224
 Appendix 1. Bacteriological Media	 238
Appendix 2. Calculating the number of antibody molecules per liposome	239

Figures

Figure 1.1 Structure of lipopolysaccharide and ceramide	31
Figure 1.2 Structure of poly-L-lysine (PLK _n) backbone	37
Figure 1.3 Helical grid representation of KALA the cationic amphipathic peptide	51
Figure 1.4 Possible mechanism for the formation of LPD complexes	57
Figure 1.5 Neutral 'helper' lipids	58
Figure 3.1 Electron micrographs of PLK ₉₉ polyplexes	79
Figure 3.2 Analysis of (a) particle size and (b) zeta potential of PLK ₅ polyplexes	82
Figure 3.3 Analysis of (a) particle size and (b) zeta potential of PLK ₄₅ polyplexes	83
Figure 3.4 Analysis of (a) particle size and (b) zeta potential of PLK ₉₉ polyplexes	84
Figure 3.5 Analysis of (a) particle size and (b) zeta potential of PLK ₅₃₁ polyplexes	85
Figure 3.6 Agarose gel electrophoresis of PLK/DNA polyplexes	88
Figure 4.1 Permeability plots for DOPE/cholesterol/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C	105
Figure 4.2 Permeability plots for DOPE/DOPC/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C	106
Figure 4.3 Permeability plots for DMPE/cholesterol/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C	107
Figure 4.4 Permeability plots for DMPE/DMPC/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C	107
Figure 4.5 Permeability plots for DLPE/cholesterol/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C	108

Figure 4.6	Permeability plot for DLPE/DLPC/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C	108
Figure 4.7	Agarose gel electrophoresis of polyplexes and lipopolyplexes treated with pronase. (a) DOPE/cholesterol/oleic acid lipopolyplexes (b) DOPE/DOPC/oleic acid lipopolyplexes	112
Figure 4.8	Cryo electron micrographs of lipopolyplexes	113
Figure 4.9	Possible models for the interaction between polyplexes and anionic liposomes	115
Figure 5.1	The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₉₉ -condensed pEGlacZ, on gene transfer activity in Jurkat cells	128
Figure 5.2	Extractable cellular protein content of Jurkat cell populations transfected using DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₉₉ -condensed pEGlacZ	129
Figure 5.3	Particle diameters of DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes used for the transfection of Jurkat cells	130
Figure 5.4	The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₉₉ -condensed pEGlacZ, on gene transfer activity in Jurkat cells	131
Figure 5.5	Extractable cellular protein content of Jurkat cell populations transfected using DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₉₉ -condensed pEGlacZ	132
Figure 5.6	Particle diameters of DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes used for the transfection of Jurkat cells	133
Figure 5.7	The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₉₉ -condensed pEGlacZ, on gene transfer activity in Jurkat cells	134

Figure 5.8 Extractable cellular protein content of Jurkat cell populations transfected using DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₉₉ -condensed pEGlacZ _____	135
Figure 5.9 Particle diameters of DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes used for the transfection of Jurkat cells _____	136
Figure 5.10 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DLPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₉₉ -condensed pEGlacZ, on gene transfer activity in Jurkat cells _____	137
Figure 5.11 Extractable cellular protein content of Jurkat cell populations transfected using DLPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₉₉ -condensed pEGlacZ _____	138
Figure 5.12 Particle diameters of DLPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes used for the transfection of Jurkat cells _____	139
Figure 5.13 Extractable cellular protein content of Jurkat cell populations transfected using DLPE/DLPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₉₉ -condensed pEGlacZ _____	140
Figure 5.14 Particle diameters of DLPE/DLPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes used for the transfection of Jurkat cells _____	140
Figure 5.15 Particle diameters of PLK ₉₉ polyplexes used in the preparation of lipopolyplexes for transfection of Jurkat cells _____	141
Figure 5.16 The effect of PLK ₉₉ -polyplex charge ratio on the extractable cellular protein content of polyplex-transfected Jurkat cell populations _____	141
Figure 5.17 The effect of polylysine/DNA charge ratio on the diameter of PLK ₅ -polyplexes prepared in 10mM HEPES pH8.0/5% glucose _____	146
Figure 5.18 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₅ -condensed pEGlacZ, on gene transfer activity in Jurkat cells _____	147

Figure 5.19 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₅ -condensed pEGlacZ _____	148
Figure 5.20 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₅ -condensed pEGlacZ _____	149
Figure 5.21 Extractable cellular protein content of Jurkat cell populations transfected using DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₅ -condensed pEGlacZ _____	150
Figure 5.22 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₃₆ -condensed pEGlacZ, on gene transfer activity in Jurkat cells _____	153
Figure 5.23 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₃₆ -condensed pEGlacZ _____	154
Figure 5.24 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₃₆ -condensed pEGlacZ _____	155
Figure 5.25 Extractable cellular protein content of Jurkat cell populations transfected using DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₃₆ -condensed pEGlacZ _____	156
Figure 5.26 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₅₃₁ -condensed pEGlacZ, on gene transfer activity in Jurkat cells _____	159
Figure 5.27 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₅₃₁ -condensed pEGlacZ _____	160
Figure 5.28 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK ₅₃₁ -condensed pEGlacZ, on gene transfer activity in Jurkat cells _____	161

- Figure 5.29** Extractable cellular protein content of Jurkat cell populations transfected using DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₅₃₁-condensed pEGlacZ _____ 162
- Figure 5.30** The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₅₃₁-condensed pEGlacZ, on gene transfer activity in Jurkat cells _____ 163
- Figure 5.31** Extractable cellular protein content of Jurkat cell populations transfected using DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₅₃₁-condensed pEGlacZ _____ 164
- Figure 5.32** The effect of PLR/DNA charge ratio and lipid/DNA weight ratio of DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLR₁₇-condensed pEGlacZ, on gene transfer activity in Jurkat cells _____ 166
- Figure 5.33** Extractable cellular protein content of Jurkat cell populations transfected using DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLR₁₇-condensed pEGlacZ _____ 167
- Figure 5.34** The effect of PLR/DNA charge ratio and lipid/DNA weight ratio of DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLR₁₇-condensed pEGlacZ, on gene transfer activity in Jurkat cells _____ 168
- Figure 5.35** Extractable cellular protein content of Jurkat cell populations transfected using DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLR₁₇-condensed pEGlacZ _____ 169
- Figure 5.36** Extractable cellular protein content of Jurkat cell populations transfected using DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLR₁₇-condensed pEGlacZ _____ 170
- Figure 5.37** The effect of PLK₅-polyplex charge ratio on the extractable cellular protein content of polyplex-transfected Jurkat cell populations _____ 171
- Figure 5.38** The effect of PLK₃₆-polyplex charge ratio on the extractable cellular protein content of polyplex-transfected Jurkat cell populations _____ 171
- Figure 5.39** The effect of PLK₅₃₁-polyplex charge ratio on the extractable cellular protein content of polyplex-transfected Jurkat cell populations _____ 172

Figure 5.40	The effect of PLR ₁₇ -polyplex charge ratio on the extractable cellular protein content of polyplex-transfected Jurkat cell populations _____	172
Figure 5.41	Targeted transfection of Jurkat cells using DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes in serum-free media (a) β -galactosidase activity (b) extractable cellular protein content ____	177
Figure 5.42	Targeted transfection of Jurkat cells using DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes in the presence of 10% FCS. (a) β -galactosidase activity (b) extractable cellular protein content _	178
Figure 5.43	Figure 5.1 Targeted transfection of Jurkat cells using DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes in serum-free media. (a) β -galactosidase activity (b) extractable cellular protein content _____	180
Figure 5.44	Particle diameters of DOPE/DOPC/oleic acid/DSPE-PEG 40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes used for the targeted transfection of Jurkat cells _____	181
Figure 5.45	The effect of cell viability on the interpretation of experimental transfection data _____	184
Figure 6.1	Possible influences of vector components on cellular endocytic pathway for the delivery of gene transfer complexes _____	216

Tables

Table 1.1	Comparative properties of synthetic DNA-condensing agents	45
Table 1.2	Developments in peptide receptor-mediated gene transfer complexes	46
Table 4.1	Properties of liposomes used in characterisation studies	102
Table 4.2	Permeability coefficients of liposomes and lipopolyplexes of different liposome compositions	109
Table 5.1	Properties of anti-CD3 liposomes used in the primary transfection screen	123
Table 5.2	Properties of anti-CD3 liposomes used in the secondary transfection screen	142
Table 5.3	Properties of polyplexes used in the secondary transfection screen	143
Table 5.4	Properties of liposomes used for the targeted transfection of Jurkat cells	173
Table 5.5	Properties of lipopolyplexes used for targeted transfection of Jurkat cells	174
Table 5.6	Properties of DOPE/DOPC/oleic acid liposomes used for the targeted transfection of Jurkat cells	179
Table 6.1	Optimum lipopolyplex formulations for transfection of Jurkat cells	214
Table 6.2	Phase transition temperatures of phosphatidylethanolamine (PE) helper lipids and their relationship to observed transfection activities	220

Equations

Equation 2.1	Stokes-Einstein equation _____	69
Equation 2.2	Henry Equation _____	70
Equation 2.3	Smoluchowsky approximation of the Henry Equation _____	71
Equation 2.4	Permeability of liposomes to an entrapped solute _____	72
Equation 2.5	Permeability coefficient calculation _____	72
Equation 2.6	Expansion of permeability coefficient _____	72
Equation 2.7	Internal radius of a liposome _____	72
Equation 4.1	Lipopolyplex permeability _____	104
Equation 4.2	Modified permeability equation _____	104

Abstract

An artificial gene transfer vector has been developed that utilises an anionic liposome as the DNA carrier. Gene transfer vectors based on pH-sensitive anionic liposomes should show few interactions with the negatively charged surface of non-targeted cells. Any interaction and hence gene transfer to a cell could be controlled by the presence of targeting ligands on the liposome surface. Individual components of the vector were characterised, their interactions with each other, and the effect of vector composition on its physical properties and gene transfer activity.

Plasmid DNA was condensed using the cationic peptide poly-L-lysine (PLK_n). A 7.6kb plasmid was condensed using PLK₉₉ into rod- and spherical-shaped particles (polyplexes). Plasmid DNA was shown to have a strongly negative zeta potential in deionised water. The surface charge can be readily neutralised by the addition of poly-L-lysine. Stable particles can be formed of small size (~100nm) when they carry a high zeta potential. Condensed-DNA particles having a low zeta potential tended to form large visible aggregates. Plasmid DNA complexes appear to become saturated with poly-L-lysine at high charge ratios (PLK/DNA), possibly leading to the presence of free poly-L-lysine in solution. The average molecular weight of the poly-L-lysine condensing agent appears to have little effect on the size and zeta potential of the polyplexes formed. However, poly-L-lysine of a very low degree of polymerisation only condenses DNA effectively in low ionic strength media. Agarose gel electrophoresis was shown to be an inefficient measure of the level of DNA condensation. Condensed-DNA particles with a positive zeta potential were used for the interaction with anionic liposomes.

Several anionic liposome formulations were produced and their interactions with polyplexes (forming lipopolyplexes) studied. The liposomes differed in the structure of the phosphatidylethanolamine (PE) lipid and the presence of either cholesterol or a phosphatidylcholine (PC) lipid. The structure of the lipopolyplexes appears to be independent of lipid composition of the liposomes. Liposomes retain their structural integrity throughout their interaction with polyplexes. The interaction of liposomes and polyplexes appears to be a more gentle interaction than that of cationic liposomes

with DNA. Retention of structural integrity suggests that the polyplex particle is located on the outer surface of the liposome and not internalised within its aqueous core. Polyplexes associated with liposomes are still susceptible to degradative enzymes. A lipid permeability barrier is maintained throughout the interaction with the polyplex, but becomes more permeable.

CD3⁺ T lymphocytes (Jurkat cells) were successfully transfected using a lipopolyplex gene transfer vector. Polyplexes alone show no gene transfer activity. Polyplexes containing high molecular weight poly-L-lysine are cytotoxic to Jurkat cells. Lipid composition of the lipopolyplexes has a greater influence than the DNA condensing agent on its total gene transfer activity. DOPE (dioleolphosphatidylethanolamine)/cholesterol/oleic acid (40:40:20 mol%) liposomes deliver the highest levels of transfection in Jurkat cells. Optimum transfection was noted using lipopolyplexes containing low molecular weight poly-L-lysine DNA-condensing agents. Increased transfection may be due to the low cytotoxicity of the peptides. Additional cytotoxicity may have been conferred by the transferred DNA itself. Non-colloidal aggregates of lipopolyplexes confer only low levels of gene transfer. Liposomes lacking DOPE, or containing DOPE-bilayer stabilising lipids such as DOPC (dioleoylphosphatidylcholine) confer little gene transfer activity. Liposomes of this type probably lack a pH-sensitive function and are unable to successfully deliver their DNA load to the cell.

DOPE/cholesterol/oleic acid liposomes associated with PLK₃₆-condensed DNA were shown to transfect Jurkat cells in a ligand-dependent manner in the presence of 10% serum. Cytotoxicity associated with the vector was reduced in the presence of serum. It was calculated that in a transfection incubation there were approximately 5×10^5 DNA particles per targeted cell.

Further suggestions are made for the optimisation of the liposome vector. A correlation is suggested between transfection activity and the H_{II} phase transition temperature of the PE helper lipid component of the vector.

Declaration

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Acknowledgements

I thank my supervisor at the University of Manchester Dr Malcolm N. Jones for his constant help and encouragement throughout the past three years. I also thank Dr Neil Weir, my supervisor at Celltech Chiroscience, for his helpful advice, and hospitality during my visit.

I would also like to thank Dr Cath Catterall, Dr Terry Baker, Dr Ken Crook and Alison Turner at Celltech for their help, advice and discussion.

I thank Dr Alan Jacob for his useful contributions throughout my studies and for acting as my advisor.

I wish to thank Dr Mike Kaszuba for his (all too!) frequent assistance with the Zetasizer.

Dr Roland Ennos provide assistance with statistical analysis of the data.

I thank Chris Gilpin for performing the cryo-TEM preparation and examination.

The work carried out during the past three years would not have been possible without generous funding from the BBSRC and Celltech Chiroscience.

Tesca, Matt, Christelle, Hee Jeong, Arthur, Khalid, Patrick and other more temporary residents of Lab 2.530 are thanked for providing a good working atmosphere.

Lorcan, Joanne and Peter, "The Chorlton Set?" helped in other, slightly less academic ways during my tenure at number 73.

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For the next three years he carried out research for PhD under the supervision of Dr Malcolm N. Jones in the Division of Biochemistry at the University of Manchester.

Abbreviations

A-T	adenine-thymine base-pair
ACM	adherent cell monolayer
ANOVA	analysis of variance
ASOR	asialoorosomucoid
ATCC	American type culture collection
BCA	bicinchoninic acid
bp	base pair
CCV	clathrin coated vesicle
CD3	Cluster of Differentiation number 3 cell surface marker
CHO	Chinese Hamster Ovary cells
D	diffusion coefficient
Da	Dalton
DC-Chol	3, β [N-(N',N' dimethylaminoethane)-carbamoyl] cholesterol
DLPC	dilauroylphosphatidylcholine
DLPE	dilauroylphosphatidylethanolamine
DMEM	Dulbecco's Modified Eagle's Medium
DMPC	dimyristoylphosphatidylcholine
DMPE	dimyristoylphosphatidylethanolamine
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOPC	dioleoylphosphatidylcholine
DOPE	dioleoylphosphatidylethanolamine
DPPC	dipalmitoylphosphatidylcholine
DSPE	distearoylphosphatidylethanolamine
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay
EU	endotoxin units
FCS	Foetal Calf Serum
f(κ a)	Henry Function
GFP	green fluorescent protein

H _{II}	lipid inverted hexagonal non-bilayer phase
HCMV	human cytomegalovirus
HEPES	N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid]
HIV	human immunodeficiency virus
HPO ₄ ²⁻	hydrogen phosphate
HRP	Horse Raddish peroxidase
IgG	immunoglobulin G
ISS	immunostimulatory sequence
KDO	3-deoxy-D- <i>manno</i> -octulosonic acid
L _L	permeability of liposomes
L _α	lipid lamellar bilayer phase
LB	Luria Bertani
LBP	lipopolysaccharide binding protein
LE	late endosome
LPD	liposome-entrapped polycation-condensed DNA complexes
LPS	lipopolysaccharide
Mb	megabase pairs
MLVs	Multilamellar vesicles
MWCO	molecular weight cut off
mV	millivolts
\overline{M}_w	average molecular weight
NBS	new-born bovine serum
NIH	National Institute of Health
NLS	nuclear localisation signal
OD	optical density
P _L	permeability coefficient
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCS	Photon Correlation Spectroscopy
PE	phosphatidylethanolamine
PEG	poly(ethylene glycol)
pEGlacZ	plasmid pEGlacZ
PLK _n	poly-L-lysine of chain length n

PLR _n	poly-L-arginine of chain length n
PMA	phorbol 12-myristate 13-acetate
RNA	ribonucleic acid
rpm	revolutions per minute
SATA	N-succinimidyl S-acetylthiocyanate
SDS	sodium- <i>n</i> -dodecyl sulphate
<i>T_h</i>	lipid lamellar to hexagonal phase transition temperature
TBE	Tris Borate EDTA buffer
TCR	T cell receptor
TE	Tris EDTA buffer
TEM	Transmission Electron Microscopy
TNF	tumour necrosis factor
U _E	Electrophoretic mobility
ULVs	unilamellar vesicles
VETs	vesicles by the extrusion technique
V _o	void volume

Chapter 1

Introduction

1.1. Gene Therapy

“If one considers the purpose of the drug to be to restore the normal function of some particular process in the body, then DNA would be the ultimate drug”

Aposhian, 1970

Gene therapy is a set of approaches to the treatment of human disease based on the transfer of genetic material. The notion of utilising genes to treat disease is one that goes back several decades to when early genetic principles were discovered. Gene therapy represents a new branch of medicine that holds the possibility of revolutionising the way we treat human disease. Most illnesses arise, at least in part, due to the dysfunction of one or more genes, including such conditions such as cancer. The replacement of defective genes with corrective copies, or the expression of therapeutic gene products in cancerous cells, could lead to the cure, or at least remission from diseases that are at present invariably fatal.

Many of the genetic-based diseases remain untreatable by the latest generation of conventional drugs. Many conventional drugs often act as a rather blunt instrument, in contrast to the specific nature of gene therapy theory. Treatment by gene therapy is dependent on a knowledge of the genetic basis of the disease. In addition to the identification of the therapeutic gene, it must be delivered to the target site efficiently and accurately. Inefficient delivery will not only lead to less genetic material being delivered to the target cells, but may lead to the expression of toxic products in bystander cells (Miller and Vile, 1995). Much current work in the gene therapy field involves the optimisation of gene delivery systems (vectors) to maximise their therapeutic gene delivery *in vivo*.

Liposome-based gene transfer systems offer significant advantages over many of the existing viral protocols. Liposomes hold the promise of being able to carry DNA of almost unlimited size. Unlike many virus-based protocols, liposomes do not have the capacity to generate infectious particles and invoke little immune response (Crystal, 1995). Liposome-DNA particles are technically easier to construct than viral vectors. Scale-up of non-infectious plasmid DNA is less hazardous than mass production of viral material (Felgner, 1995).

The following section describes the developments that have been made toward the production of a target-specific gene transfer vector, based on plasmid DNA, peptide and anionic liposomes. The philosophy behind the work for this thesis was made succinctly by the NIH panel investigating research on gene therapy (Orkin and Motulsky, 1995):

“In order to confront the major outstanding obstacles to successful somatic gene therapy, greater focus on basic aspects of gene transfer, and gene expression within the context of gene transfer approaches, is required. Such efforts need to be applied to improving vectors for gene delivery, enhancing and maintaining high level expression of genes transferred to somatic cells, achieving tissue-specific and regulated expression of transferred genes, and directing gene transfer to specific cell types”.

1.2 Nucleic acids for gene transfer

The majority of artificial gene delivery vectors utilise plasmid DNA, either in a 'naked' form for nucleic acid vaccination, or complexed with lipids or cationic peptides. The DNA used in gene delivery protocols needs to be highly purified and free of potentially harmful contaminants. Plasmid DNA is usually produced in the host bacterium *Escherichia coli*. The very nature of plasmid isolation protocols exposes the final product to many deleterious agents and cellular contaminants. Many of these contaminants can co-purify with plasmid DNA using the variety of conventional isolation techniques. Major contaminants of plasmid DNA include host genomic DNA, host protein, host RNA and lipopolysaccharide (LPS) (Prazeres *et al.*, 1999).

1.2.1 Cytotoxicity of lipopolysaccharide contamination of plasmid DNA

Bacterial endotoxin or lipopolysaccharide (LPS) is potentially the most serious contaminant of plasmid DNA. Lipopolysaccharide can generate a toxicity to mammalian cells if present during transfection (Cotten *et al.*, 1994). Lipopolysaccharides are outer membrane components of Gram-negative bacteria. Lipopolysaccharides are unique molecules in a biological sense, and structurally can be defined by three distinct regions (Figure 1.1). The cytotoxic activity of LPS appears to be conferred by the lipid A component of the molecule (Cotten and Saltik, 1997). Lipid A is unique among bacterial lipids in containing hydroxy fatty acids (Hammond *et al.*, 1984). The molecular structure of lipid A appears to confer the cytotoxic response. It is thought that lipid A may mimic intracellular ceramide (Figure 1.1) and activate the downstream components of an apoptosis pathway (Obeid *et al.*, 1993). Ceramide is released from the cellular membrane by sphingomyelinase activity following cellular exposure to a number of signals including tumour necrosis factor (TNF) and ionising radiation (Obeid *et al.*, 1993).

Lipopolysaccharide is generally thought to mediate its cytotoxicity to mammalian cells through its interaction with LPS binding protein (LBP) and cell surface CD14. However, cells lacking CD14 show toxicity to LPS during transfection (Cotten *et al.*,

1994). This cytotoxicity requires intracellular delivery of LPS. Transfection is bypassing the surface receptor and directly activating a cytoplasmic response. Simple exposure of CD14⁺ cells to LPS does not induce a cytotoxic response.

The polyanionic nature of LPS facilitates its co-purification with plasmid DNA (Wils *et al.*, 1997). Methods have been described to eliminate LPS toxicity from DNA preparations (Cotten *et al.*, 1994). Ideally, a batch of plasmid DNA should contain <0.1EU (endotoxin units) per µg of plasmid DNA (Horn *et al.*, 1995).

1.2.1.2 Suppression of lipopolysaccharide cytotoxicity *in vivo*

Despite it being possible to produce endotoxin-free plasmid DNA, it is not always possible to remove endotoxin from the gene delivery environment. In order to correct the genetic defect in cystic fibrosis, efforts have concentrated on delivering genes to the lung. However, the pulmonary environment in cystic fibrosis patients can contain large quantities of LPS derived from chronic *Pseudomonas* infection (Cotten and Saltik, 1997).

Addition of a number of compounds that block LPS toxicity such as lactoferrin (Apelmek *et al.*, 1994), pentamidine (David *et al.*, 1994), apolipoprotein A1 (Flegel *et al.*, 1993), and the cyclic antibiotics polymyxin B and polymyxin E (Morrison and Jacobs, 1976) can improve transfection activity of DNA preparations contaminated with LPS (Cotten and Saltik, 1997). Polymyxin B itself shows no cytotoxicity at high concentrations in primary cell culture (Cotten and Saltik, 1997).

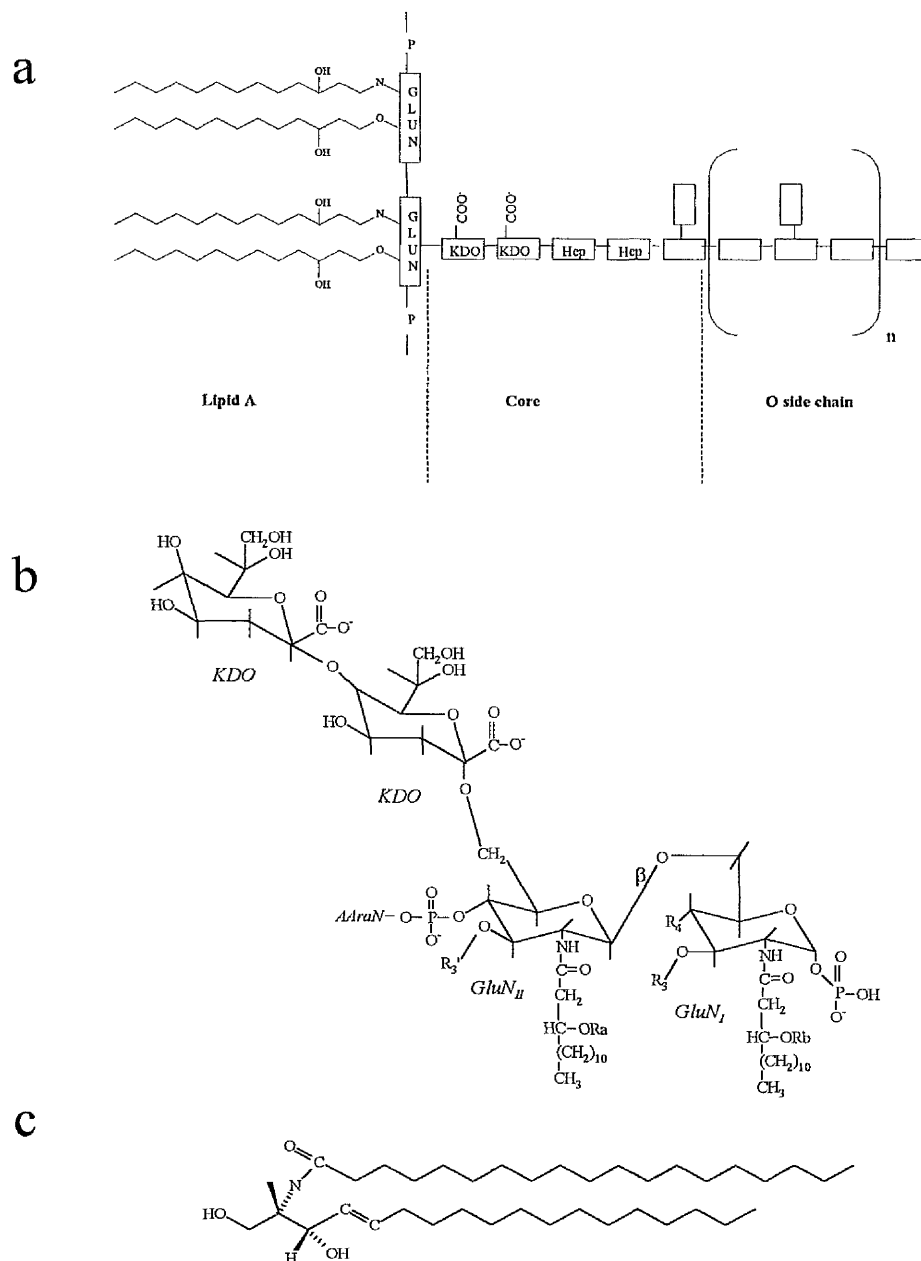


Figure 1.1 Structure of bacterial lipopolysaccharides and ceramide. (a) generalised structure of bacterial lipopolysaccharides. The structure of the lipid A region is highly conserved amongst a wide range of Gram-negative bacteria. Similarly little variation is found in the inner core (KDO-heptose) region. Considerable variation is present in the monosaccharides that constitute the outer core and O-side chain regions. (b) structure of the lipid A of *Salmonella* species. The two glucosamine sugars are amide linked at the 2-position to 3-OH fatty acids, which may themselves bear ester-linked fatty acids (Ra and Rb). Additional acylation by ester-linked 3-OH fatty acids may occur at the 3 and 3' positions. The sugar 4-amino-arabinose may be substituted at the 4' of the glucosamine II and ethanolamine at the 1 position of glucosamine I. The KDO disaccharide (not strictly part of lipid A) is attached to glucosamine II 6. The 4' position of glucosamine I is probably unsubstituted (Hammond *et al.*, 1984). (c) typical ceramide structure.

1.2.2 Immunogenicity of plasmid DNA isolated from a bacterial source

Plasmid DNA isolated from a bacterial source can induce a number of deleterious responses when introduced into mammalian cells. Injection of pure plasmid DNA backbone (LPS free) into muscle cells causes a widespread inflammation with a mononuclear infiltrate (McMahon *et al.*, 1998). Despite the apparent homogeneous chemical nature of nucleic acids between species, chemical modification of bases and the frequency of particular base sequences differ in mammalian and bacterial DNA.

DNA from bacteria has potent immunostimulatory activities that include polyclonal B cell activation, induction of cytokines, and stimulation of a specific antibody response (Messina *et al.*, 1991; Tokunaga *et al.*, 1992; Krieg *et al.*, 1995; Halpern *et al.*, 1996; Klinmann *et al.*, 1996).

Structural motifs common in bacterial DNA, but almost absent from mammalian genomes cause these immune responses. The general structure of the motifs are two 5' purines, an unmethylated CpG dinucleotide, and two 3' pyrimidines (Yamamoto *et al.*, 1988, 1992; Krieg *et al.*, 1995). These immunostimulatory sequences (ISSs) are more common in bacterial than mammalian DNA because of differences in cytosine methylation as well as CpG suppression in mammalian DNA (Bird, 1997). In certain sequence contexts such signals are probably recognised as a pathogen-associated molecular pattern or PAMP (Medzhitov and Janeway, 1997). Other ISSs may contain extended runs of dG sequences, and may act as B cell mitogens (Pisetsky and Reich, 1993). The inflammatory response to plasmid DNA can, in some cases, be more serious than a similar response to LPS (McMahon *et al.*, 1998).

Plasmid DNA isolated from a bacterial source can be modified to lessen its immunogenic properties. One or more of the following techniques could be applied: (1) methylation of CpG motifs; (2) the use of methylation insensitive promoters, and (3) the use of antibiotic resistance genes and promoter elements containing fewer immunostimulatory sequences.

Methylation of DNA can be problematic. The methylation of DNA rendered it less soluble (McMahon *et al.*, 1998) and may down regulate certain promoter sequences (Muiznieks and Doerfler, 1994). Antibiotic selection markers such as the ampicillin resistance gene contain more ISSs than the tetracycline resistance gene and thus elicits

a stronger immune response when present in an otherwise identical vector (Sato *et al.*, 1996).

1.2.3 Production of low immunogenicity vector DNA

Ideally, vectors should be produced without immunostimulatory sequences and in a host that provides the correct covalent modification of the DNA. Darquet *et al.* (1997) have developed a technique for producing safer genetic material for gene transfer. They developed a vector, a 'supercoiled minicircle', that in its final form lacks both a bacterial origin of replication and antibiotic resistance gene selection markers. The vector itself replicates in its bacterial host by a recombination event. The deleted sequences contain a number of immunostimulatory sequences and are deleterious to transfection. In addition, antibiotic resistance markers could be disseminated to endogenous Enterobacteriaceae following clinical use of vectors containing such genes (Darquet *et al.*, 1997).

1.2.4 Effect of DNA supercoiling on vector properties

The tertiary structure of plasmid DNA may affect its activity. DNA supercoiling can impact on promoter activity and overall gene expression from the plasmid. Transfection activity was shown to be 50-100 fold greater for supercoiled plasmid DNA over that of plasmid in its linear form (Buttrick *et al.*, 1992). In addition, the level of supercoiling can allow transcriptional control of certain eukaryotic promoters (Dunnaway and Ostrander, 1993). Negative supercoiling favours DNA-histone interactions (Patterton and von Holt, 1993), this may assist the association of vector DNA with condensing or targeting peptides. DNA supercoiling is difficult to quantify and control; over-vigorous plasmid preparation steps may result in shearing and loss of supercoiling of plasmid DNA.

There is anecdotal evidence of the need for DNA supercoiling in plasmid DNA vectors for efficient gene transfer, but it is unlikely that DNA is delivered to the nucleus in a supercoiled state. Plasmid DNA exposed to cytoplasmic nucleases is likely to be nicked and revert to a relaxed form (Prazeres *et al.*, 1999). Analysis of peptide or lipid complexed with plasmid DNA often show heterogeneous populations

of complexes, this may be related to the variable level of supercoiling present in the plasmid preparation. Standardisation of plasmid preparation to produce more uniform DNA has not yet been achieved (Smith *et al.*, 1997).

1.2.5 Antisense oligonucleotide optimisation

A second type of nucleic acid which may be used for gene transfer applications are antisense oligonucleotides. Antisense oligonucleotides are short synthetic single-stranded sequences of DNA that can bind to complementary sequences of DNA or RNA, thereby preventing transcription and translation respectively (Helene and Toulme, 1990). Oligonucleotides can be chemically modified, in contrast to plasmid DNA, to become more resistant to degradation by nucleases (Maurer *et al.*, 1999). Replacement of a non-esterified oxygen atom of the phosphodiester backbone of the oligonucleotide with sulphur increases its intracellular half-life by more than 50 fold (Hoke *et al.*, 1991; Fisher *et al.*, 1993; Crooke *et al.*, 1998; Hope *et al.*, 1998). Serum half-life *in vitro* is also greatly extended (Campbell *et al.*, 1990; Akhtar *et al.*, 1991; Gilar *et al.*, 1997). These phosphorothioate oligodeoxynucleotides are the most frequently used class of oligonucleotides (Maurer *et al.*, 1999).

1.3 Targeted gene delivery

The ideal gene therapy vector must possess specific properties and fulfill a number of stringent criteria (Hodgson, 1995):

- It should be able to transfer DNA up to the megabase pair (Mb) range. Delivery of minichromosomes and other large constructs may be necessary.
- It should be able to transfect quiescent cells.
- It should be targetable to specific cell/tissue types.
- It should be applicable for *in vivo* gene delivery.
- It should lead to sustained gene expression in the targeted cell type.
- It must not be oncogenic.
- The possibility of integration into the host genome should be retained if required.

In addition, a number of specific cellular barriers must be overcome to deliver nucleic acid-based drugs. DNA is a large anionic molecule and does not readily interact with the negatively charged surface of eukaryotic cells. Cell surfaces contain a number of anionic species such as carboxylic acid groups of sialic acid attached to glycoproteins, sulphate groups of proteoglycans and phosphate groups of glycerophosphates (Singh *et al.*, 1992). The negative charge of DNA may however be advantageous, as few non-specific interactions with the cell surface will occur without specific targeting entities. It would however appear that most of the negative charge of the DNA needs to be masked if effective gene transfer is to occur (Zauner *et al.*, 1998). To fulfill these requirements, DNA needs to be condensed to a size that allows it to be taken up by the cell. The condensed DNA particle must also be able to bind to a specific receptor on the cell surface. Until the development of cell-specific promoters and gene expression, this is the most promising method of delivering targeted gene expression. Importantly, following receptor binding, the cell surface molecule must be internalised by the cell. Some targeted molecules such as CD4 on the surface of T lymphocytes are not internalised upon binding of anti-CD4 antibodies (Puls and Minchin, 1999). The CD4/T cell receptor complex is internalised at a very low rate as its aggregation and internalisation into coated-pits is inhibited by cytoplasmic factors (Veillette *et al.*, 1988).

Following internalisation, DNA complexes must escape from the vesicular system, enter the cytoplasm, and be effectively targeted to the nucleus. In vivo, particles must be able to permeate the tissue of interest in addition to withstanding the biologically harsh environment of the bloodstream. Importantly, the vector should not cause inflammation or other immunological responses (Zauner *et al.*, 1999).

Receptor-mediated gene transfer can fulfill a number of these requirements. The vectors are relatively simple structures, where targeting molecules are associated with DNA (oligonucleotides, plasmid DNA) via cationic DNA-condensing peptides that interact electrostatically with the nucleotide backbone. This peptide-DNA complex is commonly known as a polyplex (Felgner *et al.*, 1997). The interaction of cationic peptides with DNA causes it to condense into small structures with lower intrinsic charge than the original DNA molecule. The vectors themselves, in theory, could be customised to target individual cell-surface receptors. As the DNA is not encapsulated into e.g. a viral capsid, the size of the DNA carried by the vector is limited only to the size of a condensed-DNA particle that can be endocytosed by the target cell. Due to their lack of replicative function there is no danger of the vector generating infectious agents.

1.3.1 Initial DNA-targeting protocols

The prospect of targeting DNA molecules to specific cell-surface receptors was first mooted by Cheng *et al.* in 1983. They covalently conjugated α_2 -macroglobulin to DNA. It was speculated that targeted DNA molecules would bind to a specific cell surface receptor and subsequently be internalised. The structure of the DNA was not affected by the chemical modification procedure as specific 'modification sequences' were inserted into a plasmid vector for the coupling of the targeting protein. Preliminary results showed that α_2 -macroglobulin-DNA conjugates were taken up, at least partially, by cells expressing the α_2 -macroglobulin receptor. Similar experiments had demonstrated that a Horse Radish peroxidase (HRP)- α_2 -macroglobulin conjugate, bound to, and was internalised by fibroblast cells (Dickson *et al.*, 1981).

The DNA- α_2 -macroglobulin conjugates developed by Cheng *et al.* (1983) were probably limited in that the DNA was not in a condensed form, and therefore unable to escape degradation in the lysosomal pathway. The large, polymorphous nature of

the uncondensed DNA particles probably limited the extent to which they could be internalised by the cells.

1.3.2 Poly-L-lysine conjugates as mediators of gene transfer: targeting of asialoglycoprotein receptor

The groundbreaking observations describing ligand targeting of DNA by Cheng *et al.* led to the development of improved conjugate systems. Wu and Wu (1987) hypothesised that DNA condensed using poly-L-lysine would form small, soluble complexes as it binds to DNA in an electrostatic manner (Li *et al.*, 1973). This soluble system was in contrast to the calcium phosphate precipitation technique for the transformation of mammalian cells prevalent at the time (Graham and Van der Eb, 1973). Insoluble complexes would have little application for *in vivo* treatments and show no target specificity.

Poly-L-lysine itself could be used as a multifunctional molecule. The ϵ -amino group of poly-L-lysine is protonated at physiological pH (Figure 1.2). The amino groups of poly-L-lysine could readily interact with polyanions such as DNA, and in addition provides a readily available chemical group to couple target ligands. Additionally, poly-L-lysine is readily degraded by the cell and had, at the time, found application as a small-drug delivery agent (Arnold, 1985).

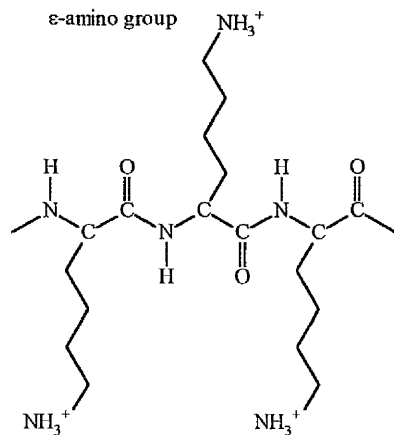


Figure 1.2 Structure of poly-L-lysine (PLK_n) backbone.

The transfection complexes formed by Wu and Wu (1987) were <200nm in diameter and were retarded in the loading wells of an agarose gel during electrophoresis.

The targeting of the soluble DNA carrier system was achieved by the coupling of asialoorosomucoid (ASOR) to the poly-L-lysine. The ASOR was desialyated to enable recognition by asialoglycoprotein receptor, differentially expressed on the surface of parenchymal liver cells. Asialoglycoprotein receptor is a paradigm for receptor-mediated gene delivery for a number of reasons:

1. The limited tissue distribution of expression enables specific hepatocyte targeting.
2. The receptor has a high affinity for targeting molecules.
3. Rapid internalisation rate from the cell surface (Perales *et al.*, 1994b).

Differentiated hepatocytes that are involved in the clearance of 'aged' glycoproteins (desialated) from the bloodstream would take up the DNA complexes containing ASOR as a targeting ligand. Despite the fact that binding to asialoglycoprotein receptor directs bound ligands to degradative pathways, their saturation with DNA complexes should allow sufficient DNA to escape and lead to gene expression.

The experiments of Wu and Wu (1987) demonstrated the potential of a receptor-mediated gene transfer system:

- Asialoglycoprotein receptor expressing cells were transformed, whereas non-expressing cells were not.
- Transfection could be inhibited by free ASOR.
- Soluble complexes were formed, which probably have a low net charge.

The specificity of the transfection was probably conferred due to the complexes being slightly anionic, therefore preventing non-specific interactions with the cell surface.

Wu and Wu (1988) went on to demonstrate that similar poly-L-lysine/ASOR complexes could transfect hepatocytes *in vivo*.

1.3.2.1 Optimisation of asialoglycoprotein receptor targeting

Further characterisation of asialoglycoprotein receptor-targeting complexes was performed by Perales *et al.* (1994a, 1997). They simplified the poly-L-lysine conjugate by using galactose-substituted PLK₁₆₀ as the targeting peptide. The galactose-terminated poly-L-lysine/DNA complexes were taken up by parenchymal liver cells *in vivo* via the asialoglycoprotein receptor. It was also noted that no

complexes could be detected in the spleen or lungs, areas containing large numbers of macrophages, which might take up galactose-terminated glycoproteins. They postulated that the small size (10-12nm diameter spheres), negative charge, and homogeneity of the transfecting complexes were necessary properties for receptor-mediated gene transfer. In addition, Perales *et al.* (1997) determined that each condensate probably contains a single plasmid molecule. It was thought that only particles in the range 10-22nm in diameter could be taken up by endocytosis of the asialoglycoprotein receptor, and as such large aggregates of complexes are observed not to transfect.

Many of the structure-function relationships suggested by Perales *et al.* (1994a, 1997) are apparently contradicted by Hashida *et al.* (1998). They similarly substituted PLK₆₃ with galactose residues and targeted DNA complexes to hepatocyte cells expressing asialoglycoprotein receptor. Optimal complexes for targeted transfection were assembled at a ratio of poly-L-lysine/DNA of 0.6 (wt/wt). These complexes had a zeta potential of -20mV and a size of 180nm. This suggests that previous estimates for the maximum size of particle to be endocytosed via asialoglycoprotein receptor were incorrect. It is possible that the 180nm particles were not involved in transfection, and that smaller complexes in a heterogeneous population are the transfection competent particles.

Chiou *et al.* (1994) demonstrated that most of the *in vivo* transfection activity of receptor-mediated gene transfer vectors was probably related to the ability of the cationic peptide (in this case PLK₁₀₀ and PLK₁₉₇-ASOR conjugates) to condense DNA into a nuclease resistant form. It was determined that plasmid DNA does not dissociate from the polyplex in the presence of serum, and that the half-life of peptide-condensed DNA was greatly extended over that of naked DNA in the presence of serum. The peptide condensing agent was however less able to protect the DNA from endonucleolytic activity. Protection from nuclease activity is dependent on the chain length of the poly-L-lysine used to condense the DNA (Kwoh *et al.*, 1999). Small poly-L-lysines (PLK₁₉, PLK₄₈) provide no protection for plasmid DNA even at high charge ratios. Discrete complexes are formed using small poly-L-lysines, but of larger size than those prepared using poly-L-lysine of a longer chain length (PLK₁₂₅); this appears to expose sensitive areas of DNA to nuclease activity.

It is thought that the size, shape and surface chemistry of condensed DNA particles influences their interaction with serum components and cells (Kwoh *et al.*, 1999). For

in vivo transfection using receptor-mediated gene delivery, all polyplexes have possessed a slight negative charge (Wu and Wu, 1988; Perales *et al.*, 1994b). Positively charged complexes probably readily interact with serum opsonins and are readily removed from the circulation. Complexes may be prevented from transfecting their target cells by either being directed to the reticuloendothelial system (RES) following opsonisation, or exposure to nucleases rendering their DNA load inactive. Kwoh *et al.* (1999) have conjugated poly(ethylene glycol) (PEG) to their polyplexes to afford steric stabilisation to neutral polyplexes. The PEG component of the polyplexes has a similar effect to that grafted onto the surface of liposomes (Čeh *et al.*, 1997); the steric stability of conjugates in saline was greatly enhanced. The addition of PEG to the DNA polyplexes does however lessen the interaction with the target cell surface.

1.3.3 Targeting of the transferrin receptor

Transferrin has also been applied as a ligand to mediate receptor-specific gene delivery. Transferrin modified poly-L-lysine (PLK₂₇₀)-DNA complexes have been used for the transfection of erythroblast cells (Wagner *et al.*, 1990). Optimum transfection was observed with neutral complexes, and little cell death was noted. However, transfection was greatly enhanced by the addition of chloroquine to aid the escape of the DNA complexes from acidified vesicles.

The 'transferrinfection' system was further characterised (Wagner *et al.*, 1991). Full DNA condensation was required for optimum transfection. Receptor-mediated gene delivery required 10-20 transferrin molecules per condensed DNA particle, which were of size 80-100nm torroids. It was also established that non-transferrin conjugated poly-L-lysine could not mediate transfection when used alone to condense DNA, although its presence in transferrin-conjugated complexes could, in fact enhance transfection.

1.3.4 Miscellaneous ligands targeted using poly-L-lysine conjugates

Poly-L-lysine is an extremely versatile molecule, and has been coupled to a large variety of ligands. Targeting of individual cell-surface markers often reveals new requirements and inadequacies of the receptor-mediated gene delivery system.

Erbacher *et al.* (1997) used partially glyconylated poly-L-lysine (PLK₁₉₀) to generate polyplexes for the transfection of hepatocytes. Polyplexes were thought to be internalised by the cells by receptor-independent endocytosis, as the gluconyl group has no specific receptor on a hepatocyte cell surface. It is also suggested that small particles (25-50nm) with a negative surface potential are optimal for transfection.

Poly-L-lysine conjugates have also been used for the specific transfection of T lymphocytes (Buschle *et al.*, 1995, Puls and Minchin, 1999). It is interesting to contrast the targeting of either CD3 or CD4 utilised by the two groups. Targeting of CD3 and CD4 was achieved using specific monoclonal antibodies conjugated to poly-L-lysine, which in turn was used to generate condensed-DNA particles. Anti-CD3-targeted complexes could readily target and transfect CD3-expressing T lymphocytes (Buschle *et al.*, 1995). In contrast, CD4-expressing T lymphocytes were only transfected to a low level with anti-CD4 complexes (Puls and Minchin, 1999). It was noted that CD3 is rapidly recycled from the cell surface, enabling receptor-mediated endocytosis of anti-CD3 targeted complexes. The CD4 molecule is inhibited from being internalised by internal cellular mechanisms (Veillette *et al.*, 1988). Stimulation of CD4 internalisation using phorbol ester treatment resulted in higher transfection levels being observed in treated cells (Puls and Minchin, 1999). This demonstrates the importance of targeting the correct cell surface ligand is of as great an importance in vector design as vector composition.

1.3.5 Preparation of poly-L-lysine/DNA polyplexes

Poly-L-lysine-DNA complexes (polyplexes) have been formed by a variety of methods. Each method produces polyplexes with different physical properties.

1. Flash mixing in 150mM NaCl (Wagner *et al.*, 1990, 1991).

- Dilute DNA solution (<20µg/ml)
- PLK₂₅₀ added at a charge ratio (poly-L-lysine NH₃⁺/DNA PO₄⁻)

'Doughnut' structures (torroids?) are often formed; the sample often contains a heterogeneous population of complexes. Particle aggregation is often noted, possibly

because of the neutral charge ratio of the polyplexes. Higher DNA concentrations can be used when the poly-L-lysine is modified with transferrin (300µg/ml); an increase in particle size is noted at higher DNA concentrations. Particle size is independent of DNA size.

2. One-step dialysis (Wu and Wu, 1987).

- Dialysis of poly-L-lysine/DNA solution from 2M→150mM NaCl
- DNA concentration 500µg/ml

Particles produced by this method are not well characterised, although they are not excluded by a 200nm pore filter.

3. Excess salt addition (Perales *et al.*, 1994a).

- Poly-L-lysine added to DNA in a 0.5M NaCl solution with mixing
- Aggregates are then disaggregated by the addition of excess NaCl
- DNA concentration up to 1mg/ml

Particles formed using this method are very small (15-30nm) and are the most uniform particles generated by any technique. It has been determined that each particle probably contains a single plasmid molecule (Perales *et al.*, 1997).

4. Flash mixing in deionised water/ low ionic strength media (Lee and Huang, 1996; Wolfert and Seymour, 1996; Xu *et al.*, 1998).

- Low DNA concentration required

Rod or spherical particles are formed. Particles can appear very heterogeneous. This method is now commonly used for polyplex preparation, possibly because of its simplicity and the greater electrostatic interactions in low ionic strength media.

1.3.5.1 Morphology of polyplexes

The morphology of condensed DNA particles can be most easily observed by negative stain electron microscopy. The morphologies observed do not appear to be related to either particle preparation methods or reaction conditions. Torroids are the most frequently observed form, although rod-shaped species (and hybrids between the two) are often observed. The size of the DNA, presence of alcohol in the solvent,

vigorousness of the preparation method and the observation method affect the morphology of the DNA condensates observed (Bloomfield, 1996).

1.3.6 An inspection of poly-L-lysine as a vector component

Poly-L-lysine conjugates for the generation of targeted polyplexes have received enormous attention in recent years. Polyplexes have been developed that can give rise to targeted gene expression *in vivo* (Wu and Wu, 1988). However, poly-L-lysine-based gene-transfer systems suffer from a number of potentially serious flaws.

1.3.6.1 Heterogeneity of poly-L-lysine preparations

The very chemical nature of the poly-L-lysine used in the generation of the majority of targeted polyplexes may be the cause of both variability of sample preparations and the heterogeneity of individual polyplex preparations. Commercially available poly-L-lysine is synthesised by the polymerisation of the N-carboxy-anhydride of lysine (Smith *et al.*, 1998). Individual preparations of poly-L-lysine, with a stated degree of polymerisation have been shown to contain 30 oligopeptides (Dolnik and Navotny, 1993). As poly-L-lysines of different chain lengths have different DNA condensing properties (Wolfert and Seymour, 1996) and afford different degrees of nuclease protection (Ziady *et al.*, 1999), polyplexes generated from such preparations will have heterogeneous properties that will be batch dependent.

1.3.6.2 Toxicity of poly-L-lysine

Poly-L-lysine has been shown to be toxic to a variety of mammalian cell types. Poly-L-lysines of $\overline{M}_w > 25,000$ (PLK₁₂₀) can be highly cytotoxic in nM concentrations (Morgan *et al.*, 1988). Indeed, poly-L-lysine was shown to have antitumour activity *in vivo* (Arnold *et al.*, 1979). As large amounts of poly-L-lysine are required to condense plasmid DNA, toxic concentrations of the agent may occur. Plank *et al.* (1996) also determined that poly-L-lysine activated the complement system *in vitro*. Complement activation could however be reduced using oligolysines of short chain lengths. Poly-L-lysine/DNA polyplexes also bind large amounts of serum albumin in the presence

of serum (Dash *et al.*, 1999). The binding of serum proteins to polyplexes severely reduces their transfection activity.

1.3.6.3 Poly-L-lysine has little membrane fusion activity

Potentially the greatest drawback of poly-L-lysine conjugates used for gene transfer is the relative lack of gene expression observed in targeted cells in comparison to the amount of DNA delivered. Studies have shown that the majority of the DNA delivered to the cells is trapped in acidified vesicles. In vesicles such as endosomes the DNA is still topologically outside of the cell and must escape the vesicle to enter the cytoplasm and be passaged to the nucleus. This bottleneck to gene transfer occurs because most of the trapped material is delivered to degradative lysosomes. Poly-L-lysine/ligand mediated gene transfer can be improved 100-fold by the addition of endosmolytic agents (Wagner, 1998). These agents include peptides, glycerol, bacterial proteins and adenovirus particles. The use of adenovirus particles to enhance transfection is limited by immune responses to such particles (Curiel *et al.*, 1991). In addition to immunological problems this type of enhanced vector may be overcomplicated and difficult to both characterise and optimise. Most studies *in vitro* of receptor-mediated gene transfer utilise chloroquine in the transfection medium.

1.3.7 Artificial peptides as mediators of gene transfer

The combination of factors against poly-L-lysine-based vectors has led to the design of a number of artificial synthetic peptides, to carry out both the function of poly-L-lysine and provide additional functions such as membrane lysis and nuclear localisation. In natural proteins, it can be seen that enzyme active sites, receptor-binding sites and antibody binding regions usually contain 10-20 functional amino acids (Stryer, 1988). Therefore, the primary function of poly-L-lysine, DNA condensation, should be able to be carried out by a small, chemically defined peptide. Artificial peptides have a number of promising qualities that should prove superior to heterogeneous poly-L-lysine preparations (Table 1.1). Indeed recent research developments suggest that defined-synthetic peptides are gaining favour over poly-L-lysine for gene delivery (Table 1.2).

Table 1.1 Comparative properties of synthetic DNA-condensing agents

Poly-L-lysine	Artificial peptides
Heterogeneity of polymer	Defined chemical synthesis/rigorous characterisation
Ease of modification	Inclusion of reactive amino acids for modification
DNA condensing conferring nuclease resistance	DNA condensing with small polymers
Heterogeneity of polyplexes	Heterogeneity of polyplexes (?)
Toxic in low concentrations	Immunologically and toxicologically inert
Little (in any) membrane fusion activity	Membrane fusion peptides, natural or synthetic
Little NLS activity	NLS peptides e.g. SV40 large-T antigen
Monofunctional DNA-condensing properties	Multifunctional peptides
Specific ligand targeting	Difficulty of ligand targeting ?

Table 1.2 Developments in peptide receptor-mediated gene transfer complexes

Receptor	Ligand/peptide	Model system/cell type	Significance	Complex properties	Reference
α_2 -macroglobulin	α_2 -macroglobulin covalent conjugate to plasmid DNA	Swiss 3T3-4 fibroblasts	Limited receptor-specific uptake of complexes	Plasmid DNA still active following conjugation	Cheng <i>et al.</i> (1983)
Asialoglycoprotein	Asialoorosomucoid (ASOR)/PLK ₃₃₀	HepG2	Receptor-specific targeting and gene expression	Complexes retarded by agarose gel electrophoresis	Wu and Wu (1987)
Asialoglycoprotein	Asialoorosomucoid (ASOR)/PLK ₃₃₀	Rat liver hepatocytes/ <i>in vivo</i>	<i>In vivo</i> targeting of complexes to rat liver	-	Wu and Wu (1988)
Transferrin	Human transferrin/PLK ₂₇₀ and PLK ₉₀	Avian erythroblasts K5562	Transfection enhanced by chloroquine	-	Wagner <i>et al.</i> (1990)
Transferrin	Transferrin/PLK ₃₅ , PLK ₉₀ , PLK ₂₀₀ , PLK ₄₅₀	Avian erythroblasts K5562	Non-conjugated cation enhances transfection	DNA condensed into 'doughnut' complexes	Wagner <i>et al.</i> (1991)
Asialoglycoprotein	Galactose covalently linked to PLK ₁₀₀	Liver hepatocytes/ <i>in vivo</i>	Liver targeting. No non-specific expression	10-12nm spheres/ anionic complexes	Perales <i>et al.</i> (1994a)
CD3	Anti-CD3 antibody/ influenza peptide INF5	Jurkat cells/ primary lymphocytes	CD3-specific gene expression	-	Buschle <i>et al.</i> (1995)

Receptor	Ligand/peptide	Model system/cell type	Significance	Complex properties	Reference
None	YKAK ₈ WK + endosome release peptide	HepG2	Condensing/lytic activity in separate peptides	-	Gottschalk <i>et al.</i> (1996)
None	CWK _n	HepG2/Cos-7	Tryptophan residue gives smaller particles. Cysteine for ligand binding	53-231nm (spheres?)	Wadwha <i>et al.</i> (1997)
Receptor-independent endocytosis	PLK ₁₉₀ partially gluconylated	HepG2 etc.	Partial charge reduction by gluconylation increases transfection	Anionic complexes 25-50nm	Erbacher <i>et al.</i> (1997)
None	KALA peptide binds DNA/permeabilises membranes	C2C12	10:1 charge ratio (peptide/DNA) required for optimum transfection	-	Wyman <i>et al.</i> (1997)
Epidermal growth factor (EGF)	EGF/PLK ₁₉ , PLK ₁₁₄ , PLK ₂₄₄ , PLK ₁₁₁₆	H1299	High M _w PLK better for transfection (+ chloroquine)	50-110nm spheres	Xu <i>et al.</i> (1998)
Asialoglycoprotein	Galactose modified PLK ₆₃	HepG2	Targeting requires particles with negative zeta potential	Complexes 180nm/-20mV	Hashida <i>et al.</i> (1998)
Serpin enzyme complex receptor (SEC-R)	a.a. 346-374 of α_1 antitrypsin/PLK ₃₆ , PLK ₂₅₆	HuH7 cells	High M _w PLK confers greater DNase protection/transfection	17nm (PLK ₂₅₆) 24nm (PLK ₃₆) spheres	Ziady <i>et al.</i> (1999)

Receptor	Ligand/peptide	Model system/cell type	Significance	Complex properties	Reference
Transferrin	Transferrin/PLK	Primary human fibroblasts	Nuclear targeting role for PLK	-	Zauner <i>et al.</i> (1999)
Asialoglycoprotein	Asialoorosomucoid/PLK/poly(ethylene glycol)	Hepatocytes	Stabilisation of complexes in serum for neutral complexes	Spheres/rods	Kwoh <i>et al.</i> (1999)
Asialoglycoprotein	D-KS copolymer/galactose/PEG ₅₀₀₀	Mouse liver cells/ <i>in vivo</i>	GFP expression in liver	-	Hisayasu <i>et al.</i> (1999)
Transferrin	Transferrin/arg-lys polymer	K562	Little complement activation	Torroids/spheres 50-80nm	Plank <i>et al.</i> (1999)
None	Peptide NLS of SV40 large-T antigen/HIV-1 gp41 fusion protein	HS-68/NIH 3T3 fibroblasts	No chloroquine/adenovirus required	-	Morris <i>et al.</i> (1999)
CD4	Anti-CD4 antibody/PLK ₂₆₈	Jurkat cells	CD4-specific gene expression <i>in vitro</i> / Targeting only of CD4 <i>in vivo</i>	Anionic?	Puls and Minchin (1999)

1.3.7.1 DNA-condensing peptides for gene transfer

A number of artificial peptides have been designed and synthesised that can condense DNA into particles and are applicable for gene transfer. These peptides often contain lysine, arginine or ornithine repeats that have a high affinity for DNA molecules.

Wadhwa *et al.* (1997) show that the peptide **Cys-Trp-Lys_n** (CWK_n) can condense DNA into spherical particles 53-231nm in diameter. They discovered that a minimum of n=13 lysines was required to adequately condense the DNA. It is suggested that alkylated CWK₁₈ is better than PLK₁₉ for transfection and shows low toxicity. The tryptophan residue enhances transfection, possibly by increasing the binding affinity of the peptide to the DNA. The polyplexes do lack an endosome escape mechanism and required chloroquine for full functionality.

Branched lysine, ornithine and arginine peptides of the general structure **(X_n)₂XGGC**, where X is either of the three basic amino acids, have also been shown to mediate transfection (Plank *et al.*, 1999). The branched arrangement of the peptide was designed to enable attachment of targeting ligands at a sufficient distance from the DNA-binding region. These branched peptides were shown to be weak activators of complement in contrast to poly-L-lysine. Again, the lack of an endosmolytic function to the vector necessitated the addition of chloroquine to the transfection medium.

A novel galactose-modified **D-Lys-D-Ser** copolymer with a PEG conjugate has also been shown to mediate transfection (Hisayasu *et al.*, 1999). The conjugate could target DNA to hepatocytes *in vivo* and generate gene expression. It is unknown as to the component of the vector that mediates endosomal escape.

1.3.7.2 Peptides with membrane fusion activity

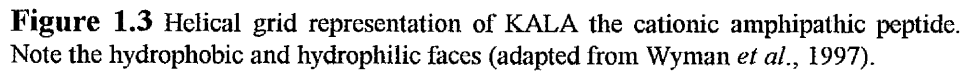
Artificial peptides have also been designed to permeate cellular membranes and aid polyplex escape into the cytoplasm. This type of vector must also contain peptides to condense DNA, or contain a multifunctional protein that can condense DNA and permeabilise membranes.

Gottschalk *et al.* (1996) designed a two-peptide vector system. The peptide **YKAK₈WK** ('K8') interacts electrostatically with plasmid DNA and condenses it

into small particles (<150nm), of positive surface potential. A second, lytic-peptide **GLFEALLELLESLWELLLEA** ('JTS-1') with a net negative charge, was added to the cation-condensed particles. The peptide JTS-1 is amphipathic and based on the structural features of influenza virus fusion systems (Duguid *et al.*, 1999). It was noted that K8/DNA polyplexes lacked transfection activity, but that K8/DNA/JTS-1 polyplexes had greatly enhanced transfection activity. The K8 peptide showed little cytotoxicity in comparison with PLK₁₀₀. Vectors having a positive, neutral or negative zeta potential show equal transfection activity (Duguid *et al.*, 1999). In addition, transfection activity was shown to be related to a combination of DNA dose, particle zeta potential, lytic peptide content and number of lysine residues in the condensing peptide.

The two-component vector developed by Gottschalk and colleagues appears very promising. The ability of the particles of opposite surface potentials to transfect cells does lead one to question, how non-specific transfection will be reduced to enable target-specific gene transfer.

DNA-binding and membrane permeabilising functions can be combined in a single peptide (Wyman *et al.*, 1997). The cationic amphipathic peptide 'KALA' with the sequence **WEAKLAKALAKALAKHLAKALAKALKACEA** (Figure 1.3) is an efficient mediator of transfection. The peptide undergoes an α -helix to random-coil transition with a fall in pH from 7.5 to 5. Transfection complexes do however require a great excess of peptide (and hence positive charge) to mediate transfection. Specific targeting of this type of complex may prove problematic.



DNA that has been transported to the cell surface, internalised, and then released into the cytoplasm must, in most circumstances, be transported to the nucleus for gene expression to occur. The cytosol is not a friendly environment for plasmid DNA (Lechardeur *et al.*, 1999) and provides a further barrier to gene transfer (Zabner *et al.*, 1995). Passage to the nucleus may be assisted by the presence of Nuclear Localisation Signals (NLSs) in the DNA-condensing peptide.

Phe-Lys-Lys-Arg-Lys-Val

Lys-Lys-Lys-Tyr-Lys-Leu-Lys

have been shown to target viral genomes to the nucleus of mammalian cells. The presence of lysine residues in such sequences has led to the suggestion that poly-L-lysine may confer a NLS function (Zauner *et al.*, 1999). However, differentiating between NLS function and the nuclease protection role of poly-L-lysine (extending cytosol residence time) has not been proven.

The potential advantages of NLS sequences in vector peptides are self-explanatory, and Morris *et al.* (1999) have developed a trifunctional peptide to mediate gene delivery. The peptide 'MPG' has intrinsic DNA-binding functions as well as the membrane fusion peptide sequence from HIV-glycoprotein (hydrophobic) and the NLS from SV-40 large T-antigen (hydrophilic) in a 27 amino acid sequence. This non-toxic peptide confers nuclease resistance to plasmid DNA upon condensation into particles 200-300nm in diameter. The vector has been able to deliver plasmid DNA to several cell lines. As with all the artificial peptide vectors developed so far, no specific targeting activity was shown by the vector.

1.3.7.4 Future problems of artificial peptide-based vectors

Artificial peptide vectors have many potential advantages over those produced from poly-L-lysine. However, a number of issues remain unresolved (that previously applied to poly-L-lysine vectors).

Despite the well-defined nature of the peptides, the polyplexes derived from them are often as, if not more, heterogeneous as those produced using poly-L-lysine. Small vectors, such as those produced by Perales *et al.* (1994a) using poly-L-lysine, have yet to be produced using artificial peptides. As the development of artificial peptide vectors is in its early stages, protocols for producing uniform polyplexes have yet to be developed. It may also suggest that the common factor in the two types of peptide vector, plasmid DNA, may cause the majority of the heterogeneity observed.

It is of concern that all the artificial peptide vectors so far developed show high levels of non-specific interaction/membrane lysis with the 'targeted' cells. Perhaps vectors that only interact with cells in the presence of targeting ligand (Wu and Wu, 1987, Perales *et al.*, 1994a; Puls and Minchin, 1999) are more desirable.

1.4 Structures of anionic liposome/DNA complexes

A fundamental disadvantage to using anionic or neutral liposomes to carry large polynucleotides such as plasmid DNA is that the internal diameter of the liposome is often less than the longest dimension of a typical plasmid DNA molecule (Felgner, 1995).

Recent work has shown that neutral and anionic vesicles can incorporate 44-55% and 45-63% of a 6kb plasmid (depending on DNA concentration) using a dehydration-rehydration technique (Gregoriadis *et al.*, 1996). The majority of plasmid DNA is protected from DNase degradation. The encapsulation efficiencies are consistent with those shown by a number of pioneering studies (summarised in Mok *et al.*, 1999).

Anionic or neutral vesicles would not be expected to interact with plasmid DNA in an electrostatic manner. It is likely therefore, that plasmid DNA associated with anionic or neutral liposomes after separation procedures such as centrifugation is encapsulated into the liposome's aqueous core. However, a minority of plasmid DNA does appear to be associated, in part, with the outer leaflet of the vesicles. This DNA appears to be a fraction of 'associated' DNA that is susceptible to DNase degradation (Gregoriadis *et al.*, 1996). It is unknown if the fraction of DNase susceptible DNA is entirely bound to the surface of the vesicles or partially encapsulated, exposing an area of the plasmid to the surrounding aqueous environment. Surface association of plasmid DNA, although unlikely, may be aided by the presence of divalent cations (Mn^{2+} , Ca^{2+} , Mg^{2+}) in the interaction buffer. Divalent cations can bridge phosphate groups on DNA and liposomes (Bichenikov *et al.*, 1988). It is possible that vesicles with surface associated DNA could transfect mammalian cells in the absence of serum nucleases. However, DNA surrounded by a complete lipid bilayer would be protected from serum nucleases. This type of vector would be ideal for systemic gene delivery (Chonn and Cullis, 1998).

1.4.1 Advances in anionic liposome-DNA association

New methods have been developed to allow greater association between DNA and anionic liposomes. A cationic peptide (e.g. poly-L-lysine) is used as a bridging molecule between anionic liposome and anionic DNA. Two preliminary structural

models have been suggested: complete engulfment of a peptide-condensed DNA molecule by an anionic liposome (Lee and Huang, 1996) and association of the peptide-condensed DNA particle with the liposome surface (Hagstrom *et al.*, 1996).

1.4.1.1 Encapsulation model of DNA association (Lee and Huang, 1996)

Lee and Huang (1996) generate liposome-entrapped polylysine-condensed DNA (LPD) particles in a two stage process. Poly-L-lysine is added to plasmid DNA prior to the addition of anionic liposomes, generating rod-like complexes of condensed DNA as observed by negative stain electron microscopy. The condensed DNA, carrying an excess of positive charges, is then added to DOPE/cholesteryl hemisuccinate/folate-poly(ethylene glycol)-phosphatidylethanolamine (6:4:0.01 mole ratio) anionic liposomes generating a ternary complex.

The size, overall surface potential, and transfection efficiency of the ternary complexes is dependent on the lipid/DNA ratio in the final complex. Complexes having low net charge tended to aggregate and form large particles with low transfection activity. Complexes having a net positive or negative charge formed small, stable particles, which were efficient at transfection.

Electron micrographs of the ternary complexes show small particles (<100nm) with an electron dense core surrounded by a low density coating. Similar structures were observed by Gao and Huang (1996), where anionic DNA-poly-L-lysine complexes were associated with cationic liposomes (DC-Chol/DOPE, 40/60 mol%). Lee and Huang deduce that the particles observed by electron microscopy are condensed DNA particles encapsulated in a lipid bilayer. The ternary particles are very heterogeneous, and appear to show populations of DNA particles engulfed to different extents by lipid membranes. It cannot be determined if the electron dense core observed in the ternary complexes is indeed condensed DNA. The poly-L-lysine condensed DNA prior to addition to the anionic liposomes forms rod-like structures, all of which appear to form spherical particles upon engulfment. Indeed, many of the particles shown by Gao and Huang appear to have no lipid associated with them at all. It appears that particles of heterogeneous lipid content and hence charge are present in the population.

It is unclear if only the lipid-associated particles confer gene transfer activity. It is undoubtedly the case that the lipid is associated with the DNA particles, the dehydrated nature of the microscopic preparation may create an 'engulfed' appearance to the complex where the liposome has dried on to the surface of the condensed DNA.

The complexes themselves are sensitive to the presence of serum (Lee and Huang, 1997). This would suggest that the DNA particle is in fact located on the external surface of the liposome, exposed to deleterious factors, and not engulfed within the aqueous core. However, DNA condensed using agents such as poly-L-lysine is resistant to nuclease degradation (Chiou *et al.*, 1994). In addition, transfection in the presence of serum was restored by addition of an amphipathic lytic peptide to the poly-L-lysine/DNA complex during vector assembly. This suggests that serum was disrupting the pH-sensitive liposome component of the vector involved in endosome escape, and does not infer a surface location for the DNA complex.

1.4.1.2 Surface-association model of DNA association (Hagstrom *et al.*, 1996)

Hagstrom *et al.* (1996) have generated ternary vectors using plasmid DNA condensed with a novel DNA-binding protein associated with anionic liposomes. Fundamentally, they suggest that the condensed-DNA particle is associated with the outer surface of the liposome and is not encapsulated. Evidence for the surface model is that the DNA in the complex is still DNase sensitive, is still able to intercalate ethidium bromide, and is visible on the outer surface of the liposome by electron microscopy.

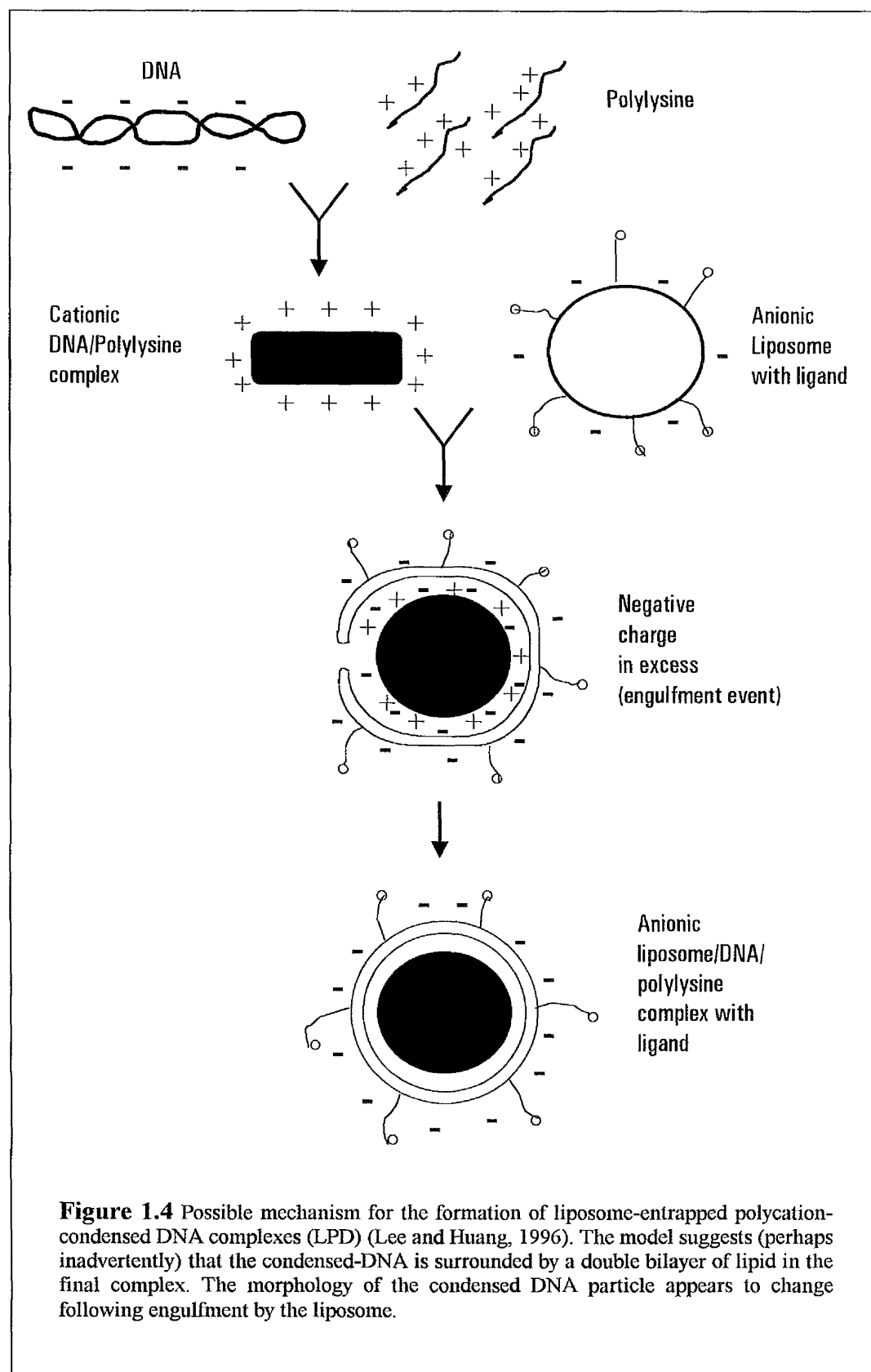
The surface interaction can possibly be explained by examining the protocol by which the complexes were prepared. Lee and Huang (1996) used a two-step process to prepare their ternary complexes. Initially, DNA/poly-L-lysine complexes are prepared by direct addition of both components. The electron-dense particles visible by electron microscopy are similar to those generated by e.g. Wagner *et al.* (1991), and a majority of other studies. Poly-L-lysine/DNA particles are then mixed with anionic liposomes to generate ternary complexes. Hagstrom *et al.* (1996) first mix the DNA condensing agent with the anionic liposomes before addition of plasmid DNA. Using this method the electron-dense condensed DNA particles do not appear to form. If the DNA-condensing peptide first associates with the surface of the liposome the DNA

may associate in a thin layer with the surface of the liposome. As some of the intended DNA-condensing charge associated with the peptide will be bound to the liposome, insufficient charge may be available to ensure total DNA condensation (as is evident by its DNase sensitivity). A different mode of interaction may be formed if the vector components were mixed in an order similar to that used by Lee and Huang (1996). This method may also generate a more practical vector, as it appears to provide no protection for its associated DNA, which is important for *in vivo* application.

1.4.3 Additional evidence for the structure of anionic liposome complexes

Poly-L-lysine condensed plasmid DNA has been associated with pH-sensitive anionic liposomes of the formulation oleic acid/DOPE/cholesterol (2:2:1 mole ratio) (Tsai *et al.*, 1999) (see Figure 1.5 for lipid structures). It is suggested that the encapsulation of prepared DNA condensates by anionic liposomes is unlikely as both are of similar size (measured by dynamic light scattering). The encapsulation model, such as that suggested by Lee and Huang (1996) (Figure 1.4), describes a situation where condensed DNA particles undergo a change in morphology during their interaction with anionic liposomes. This mechanism may allow apparently incongruous condensed DNA plasmid particles to be encapsulated within a vesicle of similar size.

Plasmid DNA condensed using the cyclic peptide gramicidin S associates with anionic lipid dispersions in a different manner (Legendre *et al.*, 1998). An amorphous dispersion of DOPE forms neat spherical structures (100-400nm in diameter) when mixed with plasmid DNA condensed using gramicidin S. Plasmid DNA condensed using gramicidin S only forms into thick cylindrical structures.



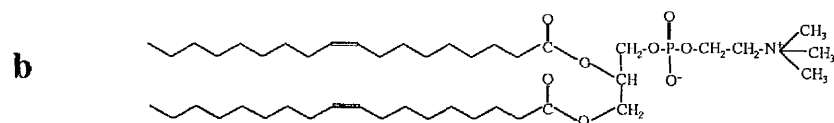
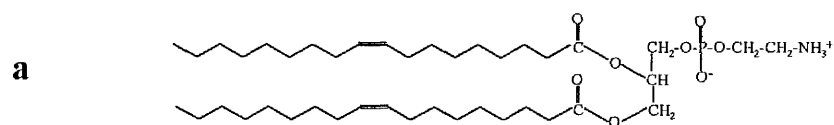


Figure 1.5 Neutral 'helper' lipids and cholesterol. (a) DOPE, dioleoylphosphatidylethanolamine (b) DOPC, dioleoylphosphatidylcholine (c) cholesterol.

1.5 Project aims

The aims of the project were two-fold:

1. To characterise poly-L-lysine (PLK)-DNA particles (polyplexes), and their interactions with anionic liposomes (forming lipopolyplexes). Determine the effect of PLK polymer length on the physical properties of polyplexes such as particle size and morphology, and zeta potential. Determine the physical structure and properties of lipopolyplexes, and the effect of lipid composition on such structures.
2. To determine the properties of lipopolyplexes necessary for efficient gene transfer in Jurkat cells. Factors to be studied include DNA condensing agent, liposome composition and lipopolyplex formulation. Show specificity of gene transfer to a known cell surface epitope and the effect of transfection medium composition on gene transfer activity and specificity.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Materials

The materials that were used in this work were obtained from the following companies:

Sigma-Aldrich Company Ltd., Poole, England.

Bacteriological agar, boric acid, cholesterol, dilaurylphosphatidylcholine (DLPC C12:0), dimyristoylphosphatidylcholine (DMPC C14:0), dioleoylphosphatidylcholine (DOPC C18:1), dilauroylphosphatidylethanolamine (DLPE C12:0), dimyristoylphosphatidylethanolamine (DMPE C14:0), dioleoylphosphatidylethanolamine (DOPE C18:1), ethylenediaminetetraacetic acid (CuNa₂EDTA cupric disodium salt), glucose solution (45%), kanamycin sulphate, β -mercaptoethanol, oleic acid, poly-L-lysine (hydrobromide salt) average molecular weight 1,000, 7,500, 9,500, 20,700, 111,000, pronase (Protease Type XIV from *Streptomyces griseus*), Sephadex G50, Triton X-100 (t-octylphenoxypolyethoxyethanol).

BDH Laboratory Supplies, Poole, England.

Bromophenol Blue, chloroform, ethylenediaminetetraacetic acid (EDTA disodium salt), ethanol, ethidium bromide, Folin & Ciocalteu's phenol reagent, glucose, HEPES (N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid]), isopropanol, methanol, sodium hydroxide, sodium carbonate, sodium chloride, sodium-*n*-dodecyl sulphate, sucrose, Tris(hydroxymethyl)methylamine.

Agar Scientific, Stanstead, England.

Formvar.

Amersham-Pharmacia Biotech, Little Chalfont, England.

[³H]-cholesterol, [³H]-dipalmitoylphosphatidylcholine (DPPC), [¹⁴C]-D-glucose, PD10 columns, Sepharose CL4B/4B.

Anachem, Luton, England.

Midi Horizontal Gel Tank (H3-SET).

Becton Dickinson Labware, Franklin Lakes, NJ, USA.

Falcon Tubes 50ml.

Boehringer Mannheim GmbH, Mannheim, Germany.

Agarose MP, β -galactosidase ELISA kit (catalogue number 1 539 426).

Life Technologies Ltd., Frederick, MD, USA. (Gibco).

Glutamine, Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's PBS, penicillin, streptomycin.

Helena Biosciences, Newcastle upon Tyne, England.

Foetal calf serum, newborn calf serum.

Lipex Biomembranes Inc., Vancouver, BC, Canada.

Extruder for VET preparation.

Malvern Instruments, Malvern, England.

Malvern ZetaSizer 3000, dts5050 latex standard for zeta potential measurement.

Millipore Product Division, Bedford, MA, USA.

Millex filter 0.2 μ m, 0.45 μ m. Millipore Elix3 deionised water preparation.

Oxoid Ltd., Basingstoke, England.

Tryptone, Yeast extract.

Pierce & Warriner, Chester, England.

BCA protein assay, BSA protein standards (23309), Hydroxylamine, SATA (N-succinimidyl S-acetylthiocyanate).

Poretics Corporation, Livermore, USA.

Chapter 2 Materials and Methods

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Polycarbonate membrane filters (25mm diameter, 100nm pores).

Qiagen, Crawley, England.

Qiagen Plasmid Mega Kit (12162), EndoFree™ Plasmid Giga Kit (12391), SuperFect™ Transfection Reagent (301 305).

Shearwater Polymers, Huntsville, AL, USA.

Distearoylphosphatidylethanolamine (DSPE)-PEG-maleimide. (DSPE-2000).

Spectrum Medical Industries Inc., Houston, TX, USA.

Spectra/Por® MWCO 3,500 CE (cellulose ester) dialysis membrane (131 162).

University of Southampton, Hampshire, England.

R17 polypeptide (poly-L-arginine).

Whatman International Ltd., Maidstone, England.

Anatop Plus filters 0.02μm.

2.2 Methods

2.2.1 Bacterial cell strains

Escherichia coli XL-1 Blue containing the plasmid pEGlacZ was grown on LB agar supplemented with kanamycin at a concentration of 10µg/ml (Appendix 1). Agar plates were incubated at 37°C for 18h. Plates were then stored at 4°C for up to one week.

Cultures for large-scale plasmid preparation were prepared by inoculating 15ml of LB broth in a 50ml Falcon Tube with a colony from a freshly grown agar plate. The inoculum was incubated at 37°C for 6h with vigorous shaking (200rpm). Kanamycin was then added to the culture at a final concentration of 10µg/ml and further incubated for 2h at 37°C. This late log-phase culture (~8h) was used to inoculate 500ml of fresh LB broth containing 10µg/ml kanamycin. This culture was incubated at 37°C for 15-17h with vigorous shaking before harvesting.

2.2.2 Plasmid DNA

The plasmid pEGlacZ was used for the preparation of polyplexes and in the transfection studies. The plasmid pEGlacZ is a 7676bp construct of the plasmids pSVβgal (Promega) and pGFPN1 (Clontech) and was supplied by Celltech Chiroscience (Slough, England). The plasmid pEGlacZ contains the *E. coli* β-galactosidase gene under the control of a truncated HCMV promoter (found in pGFPN1). In addition, the plasmid contains a kanamycin resistance gene for selection in a bacterial system (*E. coli*).

Plasmid DNA was purified using Qiagen ion-exchange chromatography columns. This technique is modified from the alkaline lysis method developed by Birnboim and Doily (1979). Plasmid DNA used for transfection studies was prepared using the Qiagen Giga EndoFree™ kit.

Purified plasmid DNA eluted from the ion-exchange columns was precipitated by the addition of isopropanol at a final concentration of 40% (vol/vol), and precipitated DNA pelleted by centrifugation (20,000g, Beckman JA-20 rotor) for 30min at 4°C. The DNA pellet was washed with 2.5ml of 70% (vol/vol) ethanol in TE buffer

(10mM Tris, 1mM EDTA pH8.0) and repelleted. The pellet was then air-dried at 25°C to remove any residual alcohol. Plasmid DNA was resuspended in a total of 1.5ml TE buffer.

The plasmid DNA concentration was determined by a spectrophotometric assay at 260nm, where 50µg/ml DNA = 1 OD unit. Plasmid DNA was subject to electrophoresis through a 0.8% agarose gel to determine plasmid integrity and confirm the absence of contaminating host genomic DNA and RNA species. Plasmid DNA was diluted if necessary, to 1.5mg/ml in TE buffer pH8.0 and filtered through a 0.45µm Millex disc filter into a sterile universal. The concentration of the DNA was then reassayed, losses due to filtration were between 10-20%. Plasmid DNA was then stored at 4°C.

2.2.3 Preparation of liposomes

Liposomes without conjugated antibodies were prepared using the vesicle extrusion technique (Olsen *et al.* 1979; Hope *et al.* 1985; Mayer *et al.* 1986). Immunoliposomes were prepared using a French Press (Barenholz *et al.* 1979; Pagano and Weinstein, 1978). All liposomes contained a phosphatidylethanolamine (PE) lipid with either cholesterol or a phosphatidylcholine (PC) lipid. Anionic liposomes were made by the addition of oleic acid to the lipid mixture.

2.2.3.1 Vesicles by the Extrusion Technique (VETs)

The lipid composition of all VETs used in the characterisation and transfection studies are shown in Table 4.1 (section 4.1, page 102).

A thin lipid film (total lipid mass 30-60mg + 5µCi [³H]-DPPC) was formed in a 250ml round bottom flask by rotary evaporation (60°C) from 20ml organic solvent (chloroform-methanol, volume ratio 4:1). Lipid films that were not evenly distributed over the surface of the flask were redissolved in solvent and reformed. The lipid film was rehydrated in deionised water containing 0.01M NaOH at a final lipid concentration of 10mg/ml. Multilamellar vesicles (MLVs) were formed by gentle swirling of the hydrating solvent over the lipid film for 5-10min at 60°C. The vesicles were extruded at 60°C through two stacked polycarbonate filters (0.1µm pore size,

Poretics) under a nitrogen pressure (100-300psi) using a Lipex Biomembranes Inc. Extruder. The resultant VETs were passed through the filters a further four times. The lipid concentration was determined by scintillation counting of $2 \times 10 \mu\text{l}$ liposome samples, where $1 \text{ mg lipid} = 0.167 \mu\text{Ci } [^3\text{H}]\text{-DPPC label}$ (for a 30mg total lipid VET preparation).

2.2.3.2 Immunoliposome preparation using a French Press

The required lipids (180 μmol s) were dissolved in dry chloroform with $1 \mu\text{Ci } [^3\text{H}]\text{-cholesterol}$. Each liposomal lipid formulation (Table 4.1, page 102) was prepared with the substitution of 0.1mol% cholesterol or phosphatidylcholine lipid with DSPE-PEG₂₀₀₀-maleimide for antibody coupling. Lipid films were formed in a 100ml round bottom flask by rotary evaporation at 60°C. The lipid film was redissolved in exactly 5ml organic solvent (chloroform-methanol, volume ratio 4:1), and $2 \times 20 \mu\text{l}$ samples were removed for scintillation counting of $[^3\text{H}]\text{-cholesterol}$ to determine the initial lipid concentration. A thin lipid film was then reformed by rotary evaporation at 60°C.

Lipid films were rehydrated with 4ml deionised water and 30 μl 1M NaOH at 60°C and vigorous agitation using 1.5-2mm glass beads to produce MLVs. MLVs were downsized by French Press (SLM Aminco) (1000psi) to produce unilamellar vesicles (ULVs). Twenty microlitres of ULVs were removed for scintillation counting to determine the final lipid concentration. To minimise hydrolysis of reactive maleimide groups, antibody coupling was carried out promptly.

SATA (N-succinimidyl S-acetylthioacetate) derivatised antibodies (450 μl , 10mg/ml; OKT3 anti-CD3, 2A non-CD3 specific) were reacted with 50 μl of hydroxylamine solution (0.5M hydroxylamine, 10mM EDTA, 0.5M NaOH pH 7.5) to expose reactive sulfhydryl groups with 2h incubation at 25°C. Hydrolysed acetate groups were separated from activated antibodies using PD10 gel filtration columns (Sephadex G25M) equilibrated with 50mM HPO_4^{2-} , 1mM EDTA pH8.0. Antibody concentration was determined spectrophotometrically at 280nm, $\text{OD}/1.43 = 1 \text{ mg/ml antibody}$. Antibodies with reactive sulfhydryl groups were coupled to maleimide containing liposomes at a ratio of 25 $\mu\text{g protein}/\mu\text{mol lipid}$ and incubated at room temperature overnight with end over end mixing.

Unreacted liposome maleimide groups were quenched by addition of β -mercaptoethanol to a final concentration of 6mM with incubation at room temperature for 30min. Uncoupled antibodies and β -mercaptoethanol were removed using a Sepharose 4B gel filtration column equilibrated with 10mM HEPES pH8.0. Liposome fractions from the void volume (V_0) were pooled and sterile filtered using a 0.22 μ m Millex-GV filter. Liposome concentration was determined by the scintillation counting of the [3 H]-cholesterol label.

Immunoliposome-coupled antibody concentration was determined by the separation of 100 μ l of liposomes and unreacted liposomes on a Sepharose CL-4B gel filtration column equilibrated with deionised water adjusted to pH8.0 with NaOH. One hundred microlitres of the separated liposomes were assayed for [3 H]-cholesterol by scintillation counting to determine the lipid concentration. A second 100 μ l of liposomes was assayed using the colorimetric protein assay developed by Wang and Smith (1975). A calibration curve was set up using antibody standards in deionised water containing 1.97, 4.93 and 9.86 μ g of antibody in 100 μ l. Five hundred microlitres of Wang and Smith's reagent (250mg CuEDTA, 2% (w/v) sodium carbonate, 0.1M NaOH in 1L deionised water) was added to each sample and incubated at room temperature for 10min. Following addition of 500 μ l of 10% (w/v) SDS and 50 μ l Folin-Ciocalteu's reagent (50% vol/vol) samples were mixed vigorously and incubated for a further 30min at room temperature. After incubation the absorbance of the sample was measured at 700nm and the protein concentrations determined using the calibration curve. Antibody coupling ratios were determined in terms of μ g antibody/ μ mol lipid. The weight-average number of IgG antibodies per vesicle was determined using the BBC basic program "D-LIPPRO" based on an IgG having a molecular weight of 150,000Da (Appendix 2).

2.2.4 Polyplex and lipopolyplex preparation

Stock solutions of poly-L-lysine, poly-L-arginine salts were prepared in deionised water at a concentration of 1mg/ml. These solutions were filtered using a 0.02 μ m Anotop plus filter. Peptide solutions were stored at 4°C. Peptides for transfection studies were prepared in 10mM HEPES pH8.0/5% glucose and filtered in a similar manner.

To calculate the net charge of the polyplex (positive charge equivalents/negative charge equivalents), the charge ratio, the number of negative charge equivalents carried by the DNA was calculated using an average molecular weight per nucleotide monomer of 331, each of which bears one negative charge. Therefore, pEGlacZ, a 7676bp plasmid, carries 15,352 negative charges per molecule and there are 1.8196×10^{15} negative charges per microgramme of plasmid DNA. The hydrobromide salts of poly-L-lysine and poly-L-arginine have a formula weight per NH_3^+ of 208.9 and 262.5 respectively. For example the number of charges carried by a 20.7kDa poly-L-lysine polymer is 99.1.

Stock plasmid DNA solutions were diluted to a working concentration of 120 $\mu\text{g/ml}$ in deionised water. The necessary amount of condensing peptide for the required calculated charge ratio was made up in an equivalent volume in deionised water. Polyplexes were then formed by drop-wise addition of the plasmid solution to the peptide solution with vortexing (final DNA concentration 60 $\mu\text{g/ml}$).

Liposomes were added to the polyplexes to form lipopolyplexes at various lipid/DNA ratios (wt/wt). Liposome stocks were diluted in 10mM HEPES pH8.0 to the required concentration in a volume half that of the polyplex solution. The polyplex solution was added drop wise to the liposome solution with vortexing.

Polyplex size and lipopolyplex size and zeta potential were measured immediately after complex formation.

Polyplexes for transfection studies were prepared at a final DNA concentration of 30 $\mu\text{g/ml}$. All peptides, plasmid DNA and liposomes were diluted using 10mM HEPES pH8.0/5% glucose.

2.2.5 Electron microscopy

2.2.5.1 Cryo transmission electron microscopy

Carbon-coated copper grids were rendered hydrophilic using a glow discharge chamber. Samples were applied to the grid and snap-frozen in liquid propane cooled with liquid nitrogen. Samples were viewed on a cold stage using a Philips 420 transmission electron microscope and images taken using a 1024 \times 1024 CCD camera.

2.2.5.2 Standard transmission electron microscopy

Samples were blotted onto formvar-coated copper grids and stained with a 1% solution of uranyl acetate. The samples were then examined using a Philips 400 transmission electron microscope.

2.2.6 Particle size determination by Photon Correlation Spectroscopy

The sizes of all particles were measured by Photon Correlation Spectroscopy (PCS) using either a Malvern Zetasizer 3000 or a Coulter N4 Plus Submicron Particle Sizer analyser. Size measurements were performed in 10mM HEPES pH8.0 or deionised water. Polyplex samples were sized without further dilution. All sizing was carried out at 25°C. Each particle was subject to 3 size measurements with a mean and standard deviation calculated from these values.

Particles in suspension undergo Brownian motion at a rate determined by the diffusion coefficient D of the suspended particles. Smaller particles that have less inertia and are affected more by collisions with the solvent molecules, move around more readily, and hence have a large diffusion coefficient. PCS effectively calculates D by analysing the variation of laser light scattering intensity from the sample at time t and $t+\delta t$. For a smaller particle the scattered light intensity variation will be more rapid as the particle is diffusing more rapidly. By analysis of the scattered light for various values of δt using a correlator the diffusion coefficient of the suspended particle can be calculated. The radii of the suspended particles is related to the diffusion coefficient by the Stokes-Einstein equation (2.1):

$$D = \frac{kT}{6\pi\eta R_h} \quad \text{Equation 2.1}$$

Where R_h is the hydrodynamic radius of the scattering molecule, η is the viscosity of the medium, k is Boltzmann's constant and T is the absolute temperature. The parameters of viscosity and refractive index used were those of water at a temperature at 25°C. The viscosity was $0.8904\text{Nm}^{-2}\text{s}^{-1}$ and the refractive index was 1.33.

As particle samples are often polydisperse, a range of diffusion coefficients are calculated. The correlation technique used by PCS produces a single correlation function giving a Z-average particle diameter. The particle size distribution is fitted to a normal distribution by cummulants analysis giving a mean particle diameter and standard deviation.

2.2.7 Zeta potential measurement

Zeta potential was measured by Laser-Doppler velocimetry using a Malvern Zetasizer 3000. Samples (2-3ml) were prepared by dilution in their constitutive buffer (usually 10mM HEPES pH8.0 or deionised water). The Zetasizer was calibrated before each series of measurements using the standard latex dts5050 which gives a zeta potential of $-50 \pm 5\text{mV}$. The measuring cell was cleaned by frequent flushing with absolute alcohol. Samples were measured ten times at an attenuator frequency of 1000Hz or using the Fast Field Reversal technique.

The Zetasizer measures the electrophoretic mobility (U_E) of the particles and calculates the zeta potential of the particles using the Henry equation (2.2):

$$U_E = \frac{2\varepsilon_0\varepsilon_r\zeta}{3\eta} \cdot f(\kappa a) \quad \text{Equation 2.2}$$

Where ε_0 is the permittivity of the vacuum, ε_r is the relative permittivity of water, η is the viscosity of water, and ζ is the zeta potential. $f(\kappa a)$ is the Henry function where a is the particle radius and $1/\kappa$ the Debye length.

At very low ionic strengths the Debye length (a measure of the thickness of the electrical double layer) is large, $a/(1/\kappa)$ is small and $f(\kappa a) \rightarrow 1$. This is the Hückel approximation. This approximation is used for samples suspended in deionised water to calculate the zeta potential.

At higher ionic strength the electrical double layer is condensed, $1/\kappa$ is reduced $a/(1/\kappa)$ is large, and $f(\kappa a) \rightarrow 1.5$. This is the Smoluchowski approximation and the Henry equation is simplified to (2.3):

$$U_E = \frac{\epsilon_0 \epsilon_r \zeta}{\eta} \quad \text{Equation 2.3}$$

This equation was modified to calculate the zeta potential of particles suspended in 10mM HEPES pH8.0 (I=7.38mM as HEPES is a zwitterion (pK_a=7.55)) and f(κa)=1.3.

2.2.8 Agarose gel electrophoresis

Analysis of DNA samples was carried out on 0.8% agarose gels. Samples were mixed with 0.1 volumes of 6× gel loading buffer (40% sucrose, 0.25% (w/v) bromophenol blue in deionised water) before being applied to the gel. Electrophoresis was carried out at 4.5Vcm⁻¹ for 1-3h in 0.5×TBE buffer (90mM Tris base, 90mM Boric acid, 2.5mM EDTA) containing 0.4µg/ml ethidium bromide. DNA bands were visualised by UV illumination. Gel images were captured using an Ultra Violet Products (Cambridge, England) digital camera and analysed using Grab-IT Annotating Grabber v2.04.6 software.

2.2.9 Liposome permeability measurements

The permeability of liposomes and lipopolyplexes to encapsulated [¹⁴C]-glucose was measured using a similar dialysis technique to that of Johnson and Bangham (1969). Lipid films (60µg) were resuspended in 6ml deionised water containing 0.01M NaOH, 4.4µM glucose and 10µCi [¹⁴C]-glucose with VETs being prepared in the standard manner (2.2.3.1).

Unencapsulated [¹⁴C]-glucose was separated on a Sephadex G50 gel filtration column equilibrated with 10mM HEPES pH8.0. Lipid concentration in the liposome fractions and encapsulation of [¹⁴C]-glucose was determined by scintillation counting.

Liposomes samples (liposomes only, lipopolyplexes) in 3ml 10mM HEPES pH8.0 was placed in dialysis bags cut from Spectra/Por[®] tubing (MWCO 3,500) and sealed with plastic clips. Samples were incubated in 30ml 10mM HEPES pH8.0 in a 50ml Falcon Tube. The time interval between collection of the VET samples and dialysis

was typically 1-2h. Glucose diffusion from the dialysis bag was assayed from 0min to 6h from 0.3ml samples of dialysate assayed for [¹⁴C]-glucose.

The experiments were interpreted using the equation (2.4):

$$\ln\left(1 - \frac{dpm_o v_o}{dpm_i v_2}\right) = -Lt \quad \text{Equation 2.4}$$

where dpm_o and dpm_i is the total count rate outside the bag and inside the bag respectively, v_o is the total volume of the system and v₂ the volume outside the dialysis bag. The parameter L relates to the permeability of the liposomes. The permeability of the liposomes calculated can be used to determine the permeability coefficient P (cm h⁻¹) from (2.5):

$$P = \frac{Lv_c}{S_c} \quad \text{Equation 2.5}$$

where S_c is the liposome surface area and v_c the volume of the liposomes.

If the liposomes have an internal radius R (2.6):

$$P = \frac{4\pi R^3 L}{3} \bigg/ 4\pi R^2 = \frac{LR}{3} \quad \text{Equation 2.6}$$

For a liposome of bilayer thickness h_b the internal radius is given by (2.7):

$$R = \frac{\bar{d}_w}{2} - h_b \quad \text{Equation 2.7}$$

Where \bar{d}_w is the weight average diameter and h_b=7.5nm.

2.2.10 Sensitivity of DNA complexes to pronase enzyme

DNA polyplexes and lipopolyplexes were exposed to pronase enzyme digestion. Samples containing 1.44μg of DNA were incubated with pronase (Protease XIV, Sigma) at a concentration of 100μg/ml (0.56 Units/ml) in a final sample volume of 50μl for 30min at 37°C. To solubilise lipid in lipopolyplex samples Triton X-100 was

added at a final concentration of 0.5% (vol/vol) mixed vigorously by vortex and samples incubated at 37°C for 30min prior to pronase addition. Samples were then subject to electrophoresis on a 0.8% agarose gel for 3h (2.2.8).

The sensitivity of samples to pronase degradation was estimated from the generation, visualised on the gel, of migrating plasmid DNA species, as plasmid DNA condensed using poly-L-lysine at a charge ratio of 2.1 is completely retarded in the gel loading well.

2.2.11 Maintenance of mammalian cell lines

Jurkat cells (E6 from ATCC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum (FCS). All medium was supplemented with penicillin (10µg/ml), streptomycin (10µg/ml) and L-glutamine (1mM). Small-scale cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. Large-scale cultures for transfection studies were incubated in sealed 1L roller flasks (Corning 430195) with the medium saturated with 5% CO₂ in air.

2.2.12 Jurkat cell transfection

Two hundred microlitres of liposome complexes (each containing 4µg of DNA) prepared in 10mM HEPES pH8.0/5% glucose were plated in 24 well plates (in triplicate). Jurkat cells were harvested and resuspended in fresh DMEM at a concentration of 2×10^6 cells/ml. To each well was added 1×10^6 cells (0.5ml) and the plates incubated at 37°C for 3h. After 3h a further 1ml of DMEM containing 17% FCS was added to the cells (final FCS concentration 10%) and they were incubated for a further 15h at 37°C. For transfection in the presence of 10% FCS, 1ml of DMEM containing 17% FCS was added to the complexes at the same time as the cells and incubated for 18h at 37°C.

Cells were harvested by centrifugation and washed three times in Dulbecco's PBS (1ml at 4°C). Cell pellets were lysed in 400µl of 1× Boehringer Mannheim lysis buffer (diluted in PBS) with vigorous shaking for 20min. Cell debris was pelleted and 400µl cell lysates were removed and stored at 70°C.

Cell lysates were then assayed for β -galactosidase expression and protein concentration.

The level of β -galactosidase expression was assayed using a β -galactosidase ELISA kit (Boehringer Mannheim) with the following modifications. The wells of the assay plate were blocked using 200 μ l newborn bovine serum (NBS) diluted 1:5 in Dulbecco's PBS with 2h incubation. A calibration curve of β -galactosidase standards was prepared using the supplied enzyme at concentrations of 1030, 515, 257.7, 129, 64.5, 32.3, 16.1 and 0pg/ml (in triplicate) in 1 \times lysis buffer. One hundred microlitres of unknown sample were added to each well and incubated with shaking (300rpm) for 1h. After coupling of the peroxidase enzyme-coupled antibody, 200 μ l of substrate (H_2O_2 :TMB, 1:1 (vol/vol)) was added and incubated with shaking for 10min. The absorbance from each well was measured using a plate reader set at 645/492nm and the β -galactosidase concentration calculated using the calibration curve.

Protein concentration was assayed using the BCA protein plate assay. A standard curve was set up using BSA with concentrations of 1200, 1000, 800, 600, 400, 200, 100 and μ g/ml in 1 \times lysis buffer (in triplicate). Fifty microlitres of each sample was plated on a 96 well Nunc plate (Nalge Nunc International) and 200 μ l BCA reagent (29.4ml Solution A, 0.6ml Solution B) added. Following incubation for 30min at 37°C the absorbance from each well was measured using a plate reader (Multiskan Ascent Labsystems) at 540/450nm and the protein concentration determined using the calibration curve.

Superfect™ Transfection Reagent (Qiagen) was used as a transfection positive control for Jurkat cells. Twenty microlitres of Superfect were added to 5 μ g of DNA in 60 μ l of DMEM and incubated at room temperature for 10min. A further 350 μ l of medium was added to the reagent and then added to the Jurkat cells. After 3h incubation at 37°C a further 1ml of DMEM containing 17% FCS was added to the cells.

Chapter 3

Characterisation of DNA-Poly-L-lysine complexes

3.1 Overview

The gene transfer vector employed in this study will incorporate a pH-sensitive liposome. These liposomes have been shown to mediate gene transfer, but suffer from poor encapsulation efficiency due to the large size and negative charge of plasmid DNA (Wang and Huang, 1987). Standard methods that improve DNA encapsulation such as freeze-thaw and sonication may cause severe DNA damage (Zhou *et al.*, 1992). A recent development associated anionic liposomes with poly-L-lysine condensed DNA via charge interactions (Lee and Huang, 1996) and delivered efficient gene transfer. This method of DNA “encapsulation” will be used in the present study.

A primary aim will be to characterise these poly-L-lysine-condensed DNA particles, and their interactions with a variety of anionic liposome formulations.

The effect of poly-L-lysine (PLK_n) chain length on the physical properties of the resultant PLK-DNA complexes was studied. Particle size and morphology was measured using photon correlation spectroscopy (PCS) and electron microscopy. The charge of the particles was studied by zeta potential measurement with more qualitative observations using agarose gel electrophoresis.

3.1.1 Aspects of Nomenclature

Following Felgner *et al.* (1997) a strict nomenclature for the description of artificial gene transfer vectors will be followed by the present study.

- A “**polyplex**” is a cationic polymer-nucleic acid complex.
- A “**lipoplex**” is a cationic lipid-nucleic acid complex.

An anomaly arises in the description of an anionic lipid-cationic polymer-nucleic acid complex, the subject of the present studies. Felgner *et al.* do not offer a term to describe this type of complex. They do however describe the following:

- A “**lipopolyplex**” is a cationic lipid-cationic polymer-nucleic acid complex.

As no cationic lipid complexes are discussed in the experimental section, the term “lipopolyplex” will be used to refer to such a complex containing anionic lipid.

The composition of PLK-DNA complexes will be described in terms of the net calculated charge of the system, the “charge ratio”, where:

$$\text{Charge ratio} = \frac{\text{Positive charge equivalents of the cationic component}}{\text{Negative charge equivalents of the nucleic acid component}}$$

Compositions containing an excess of the cationic polymer have a charge ratio of greater than one, formulations containing more negative than positive charges have a charge ratio of less than one (Felgner *et al.*, 1997).

The composition of polyplex-lipid complexes will be described in terms of the lipid/DNA weight ratio.

3.2 Results

3.2.1 Physical characterisation of DNA-Poly-L-lysine complexes (polyplexes)

3.2.1.1 Electron microscopy of polyplexes

The structure of DNA-poly-L-lysine polyplexes was examined by electron microscopy. Polyplexes prepared using PLK₉₉ and having a charge ratio of 1.6 were observed in a fully hydrated state by cryo transmission electron microscopy (Figure 3.1a-c). The polyplexes appeared as electron-dense polymorphic rod structures. Further, the polyplexes were very heterogeneous, and varied in length from 53-160nm but had a more uniform width of 23-31nm. Many of the elongated rods show lateral banding along their entire length. A smaller number of polyplexes appear as small spheres of diameter 18-30nm.

Preparations of polyplexes were also observed following uranyl acetate staining. These samples were viewed in a dehydrated state by standard transmission electron microscopy. Figure 3.1d-e show PLK₉₉ polyplexes having a charge ratio of 2.1. Rod-shaped species were most common, but a small number of apparently spherical complexes are also present. The rod-shaped complexes are highly heterogeneous, varying in length from less than 50nm in length of more than 120nm, but of uniform width of approximately 20nm. The particles positively stained with the uranyl acetate and few structural details are visible.

Similar particles were not visible in samples of non-condensed DNA.

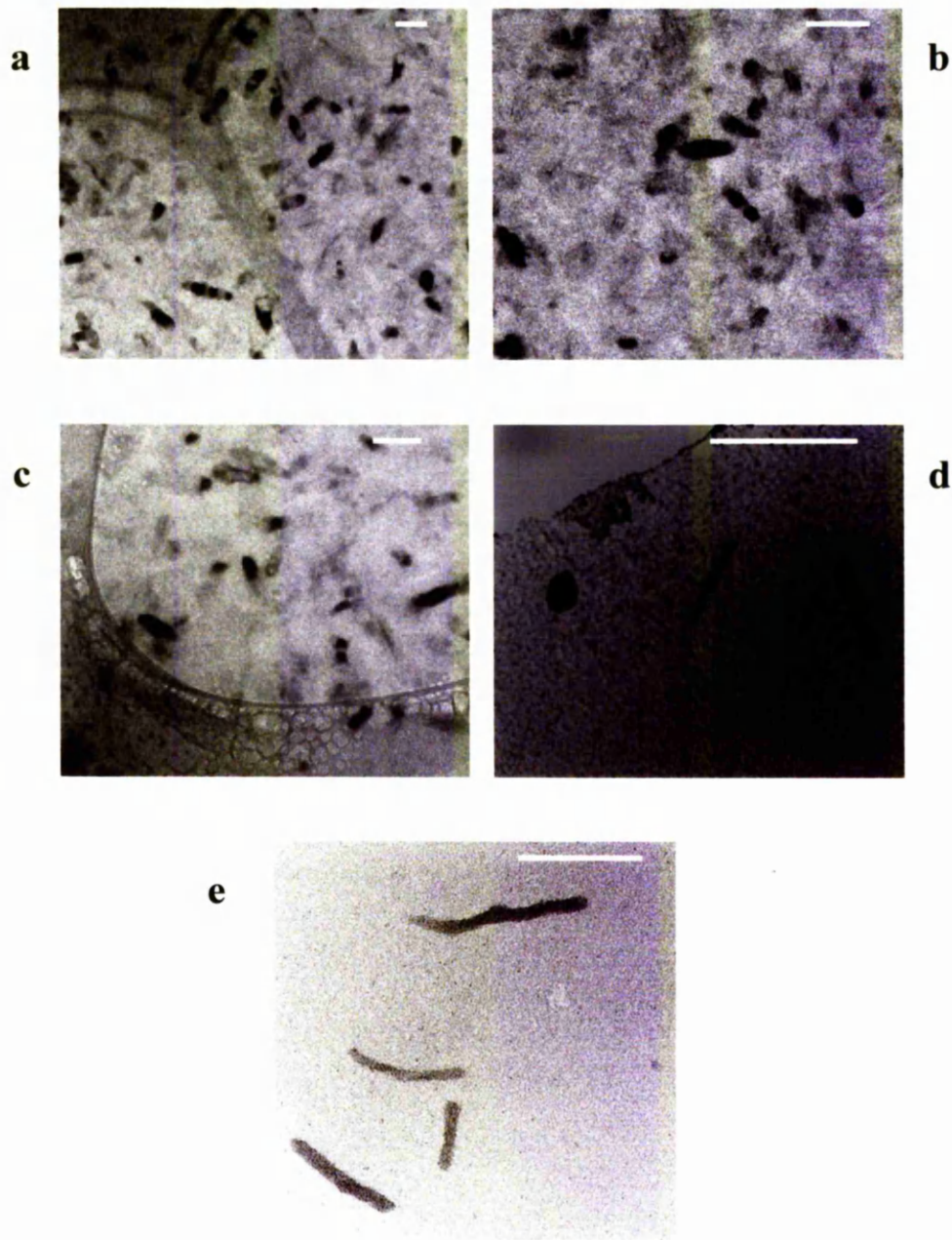


Figure 3.1 Electron micrographs of PLK₉₉ polyplexes. (a-c) Cryo TEM of PLK₉₉ polyplexes having a PLK/DNA charge ratio of 1.6. (d-e) PLK₉₉ polyplexes having a charge ratio of 2.1 stained with 1% uranyl acetate. Polyplexes were prepared at a final DNA concentration of 60µg/ml in deionised water. Scale bar indicates 100nm.

3.2.1.2 Size and zeta potential analysis of polyplexes

The interaction of DNA and PLK in deionised water was monitored at different charge ratios ($\text{NH}_3^+/\text{PO}_4^-$). The particles generated at each of these charge ratios were analysed for both particle size and zeta potential.

Polyplexes were prepared at a final DNA concentration of 60 $\mu\text{g/ml}$. This DNA concentration generated sufficient light scattering species to allow accurate determination of size and zeta potential using the Malvern Zetasizer 3000.

3.2.1.2.1 PLK₅-DNA polyplexes

Polyplexes prepared using PLK₅ having a charge ratio of less than 1.7 form large aggregates that are unsuitable for size measurement by PCS. Stable particles of 882 \pm 33nm are formed at a charge ratio of 1.7. An increase in the charge ratio from 1.7 to 2.38 results in a decrease in particle size of 760nm. Further increases in charge ratio from 2.8 to 4 resulted in only a small decrease in the particle diameter (Figure 3.2a).

All the stable polyplexes formed had a positive zeta potential. As the charge ratio is increased from 1.7 to 4 the zeta potential of the resultant particles increases from +14mV to +40mV. No zeta potential measurements were made of the aggregating particles prepared at charge ratios of less than 1.7 (Figure 3.2b).

3.2.1.2.2 PLK₄₅-DNA polyplexes

In the charge ratio range of 0.5 to 1, polyplex particle size increased from 139 to 199nm. A peak in particle size occurs at a charge ratio of 1.2. These particles formed larger insoluble aggregates following analysis of the particle size. Small particles were again observed between a charge ratio range of 1.3 to 2 (Figure 3.3a).

Zeta potential analysis showed that polyplexes having a charge ratio of between 0.5 and 1 have a negative surface charge (-57mV to -42mV). When the charge ratio is increased from 1 to 1.2 the polyplexes go from having a negative to a positive zeta potential. Polyplexes having a charge ratio of 1.2 that formed large aggregates have a very low zeta potential (+8mV). Increasing the charge ratio from 1.2 to 2 causes the particles to carry a slightly more positive zeta potential (Figure 3.3b)

3.2.1.2.3 PLK₉₉-DNA polyplexes

When PLK₉₉ polyplexes have a charge ratio of between 0.16 to 0.8 measured particle size varies between 221 and 296nm, with a slight peak in particle size noted at a charge ratio of 0.48. Larger particles of size 411nm were formed at a charge ratio of 0.95. Particle size then decreased between the range of charge ratios 1.1 to 1.4. A further increase in charge ratio did not result in smaller particles being formed. The size of plasmid DNA only (charge ratio 0) could not be obtained by PCS measurement (Figure 3.4a).

Zeta potential analysis showed that when the charge ratio was increased from 0 to 0.95 the particles had a less negative zeta potential. At a charge ratio of approximately 1 (calculated charge neutrality) the sign of the particle charge changed from negative to positive. The large particles formed at a charge ratio of 0.95 were shown to have a lower zeta potential than each of the other particles formed (Figure 3.4b). All particles formed at charge ratios greater than 0.95 had a similar positive zeta potential. The zeta potential of naked DNA was shown to be -108mV (Figure 3.4b).

3.2.1.2.4 PLK₅₃₁-DNA polyplexes

Particle size remains relatively unchanged in the charge ratio range of 0.5 to 1. A distinct peak in particle size is noted with polyplexes having a charge ratio of 1.2. Smaller particles were again noted between the charge ratios of 1.3 to 2 (Figure 3.5a). Surface charge analysis showed that polyplexes having charge ratios between 0.4 and 2 had a decreasingly negative zeta potential. The sign of the particles zeta potential changes from negative to positive between a charge ratio of 1.2 and 1.3. Polyplexes having a charge ratio of 1.2, that form large aggregating particles, have a very low zeta potential. Increasing the charge ratio from 1.3 to 2 gives the particles an increasingly positive zeta potential (Figure 3.5b).

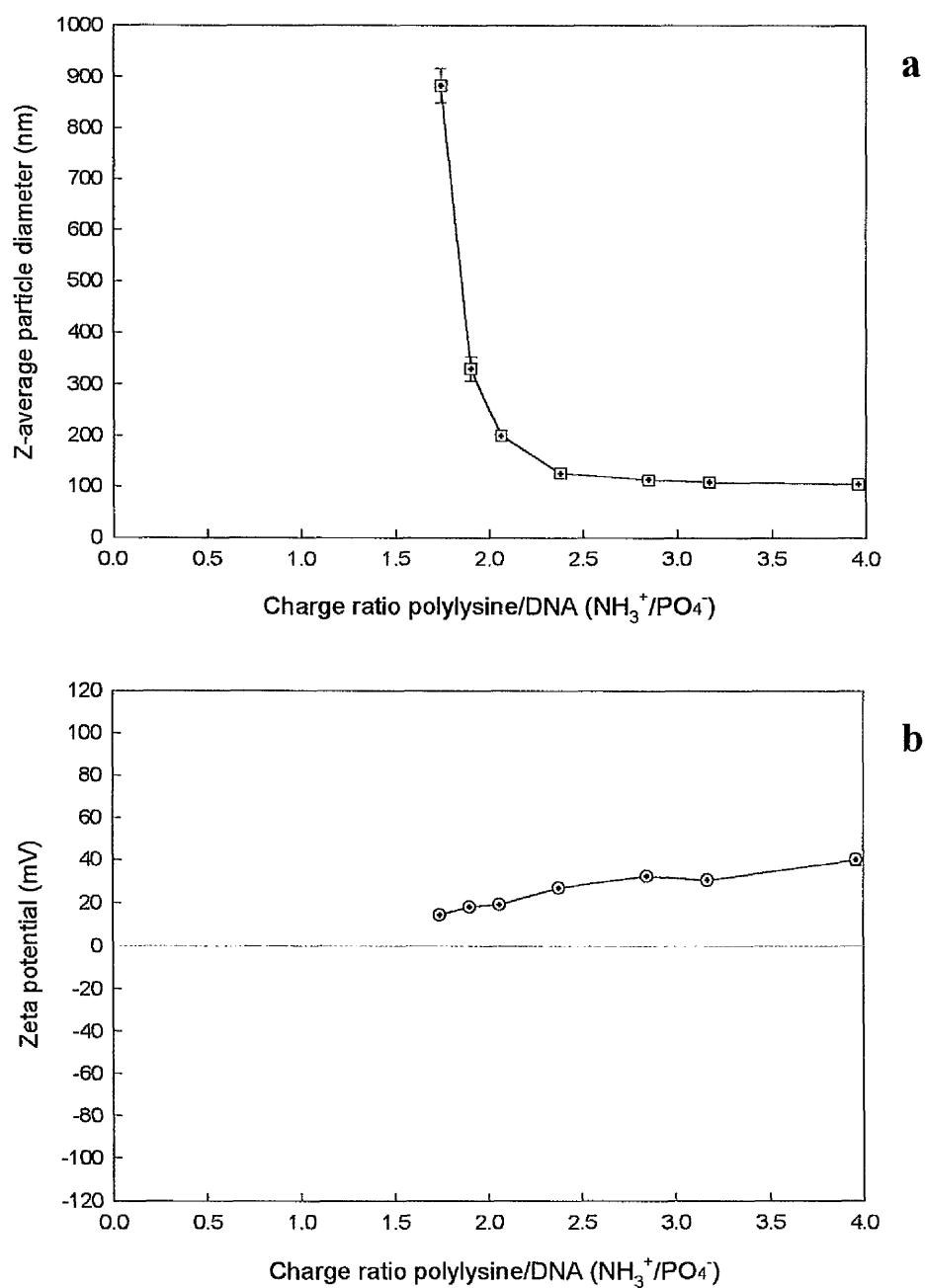


Figure 3.2 Analysis of (a) particle size and (b) zeta potential of PLK₅ polyplexes. Polyplexes were prepared at a final DNA concentration of 60 $\mu\text{g}/\text{ml}$ in deionised water pH7.5. Zeta potential calculated using the Hückel approximation $f(\kappa a)=1$. Results show mean of 3 measurements \pm s.d.

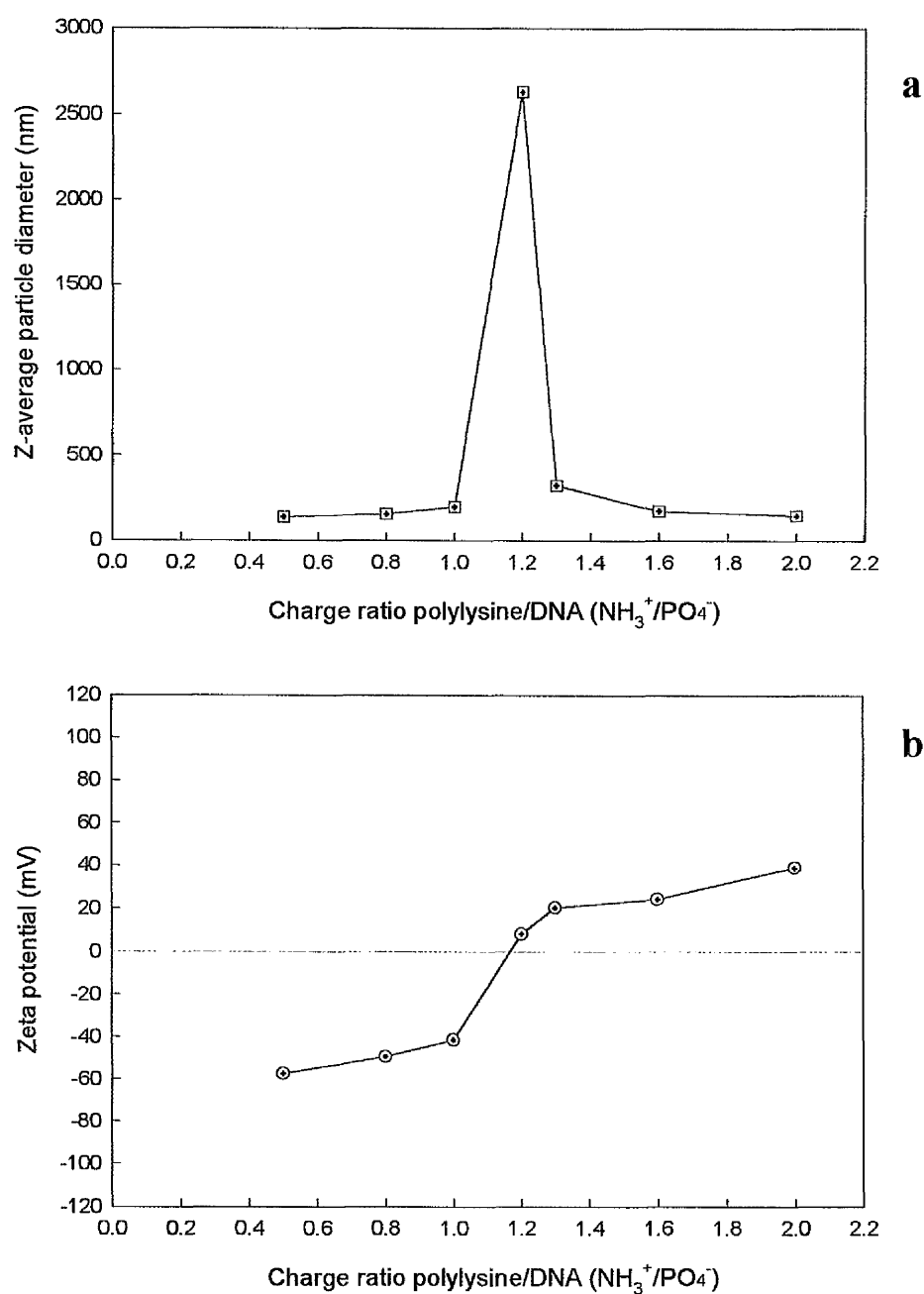


Figure 3.3 Analysis of (a) particle size and (b) zeta potential of PLK₄₅ polyplexes. Polyplexes were prepared at a final DNA concentration of 60 $\mu\text{g}/\text{ml}$ in deionised water pH7.5. Zeta potential calculated using the Hückel approximation $f(\kappa a)=1$. Results show mean of 2 measurements \pm s.d.

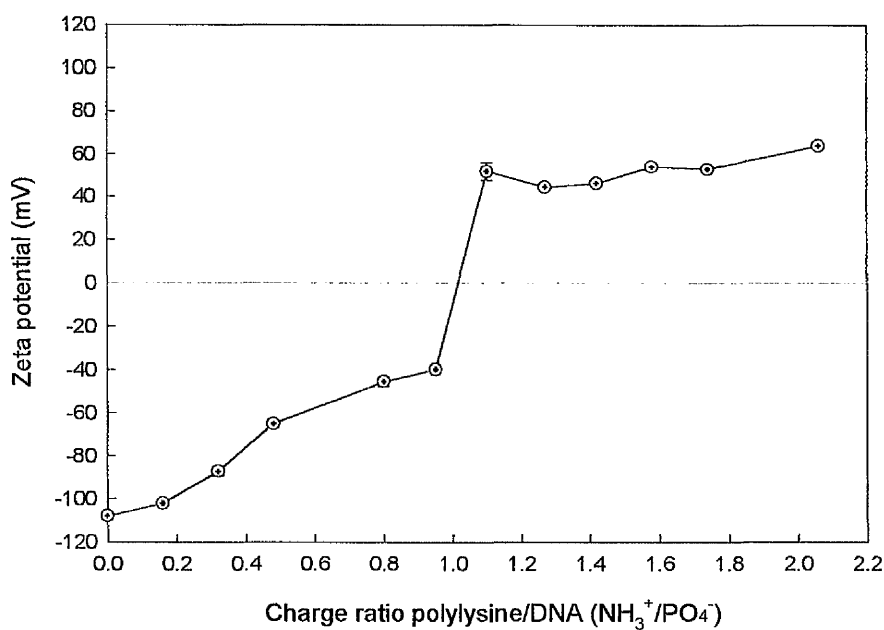
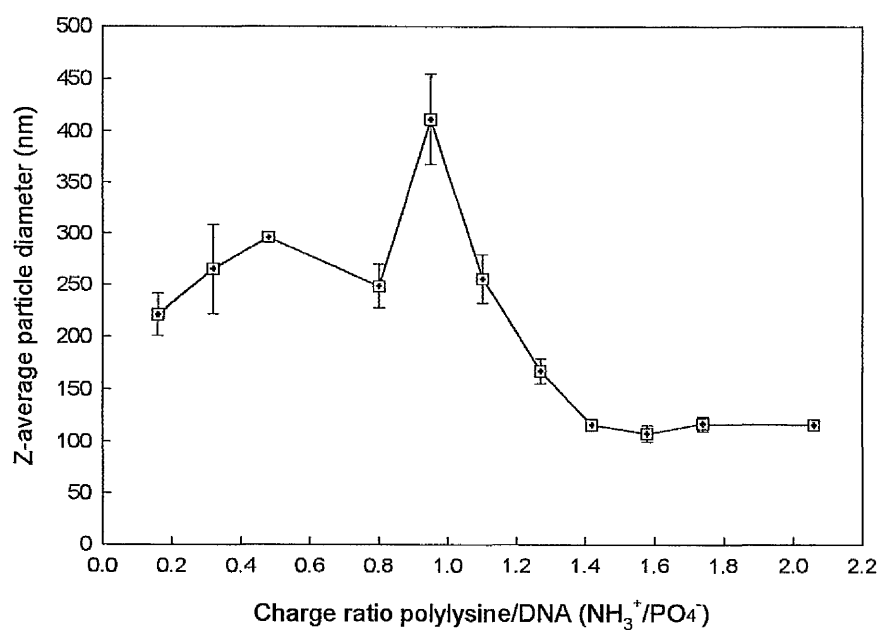


Figure 3.4 Analysis of (a) particle size and (b) zeta potential of PLK₉₉ polyplexes. Polyplexes were prepared at a final DNA concentration of 60 $\mu\text{g}/\text{ml}$ in deionised water pH7.5. Zeta potential calculated using the Hückel approximation $f(\kappa a)=1$. Results show mean of 3 measurements \pm s.d.

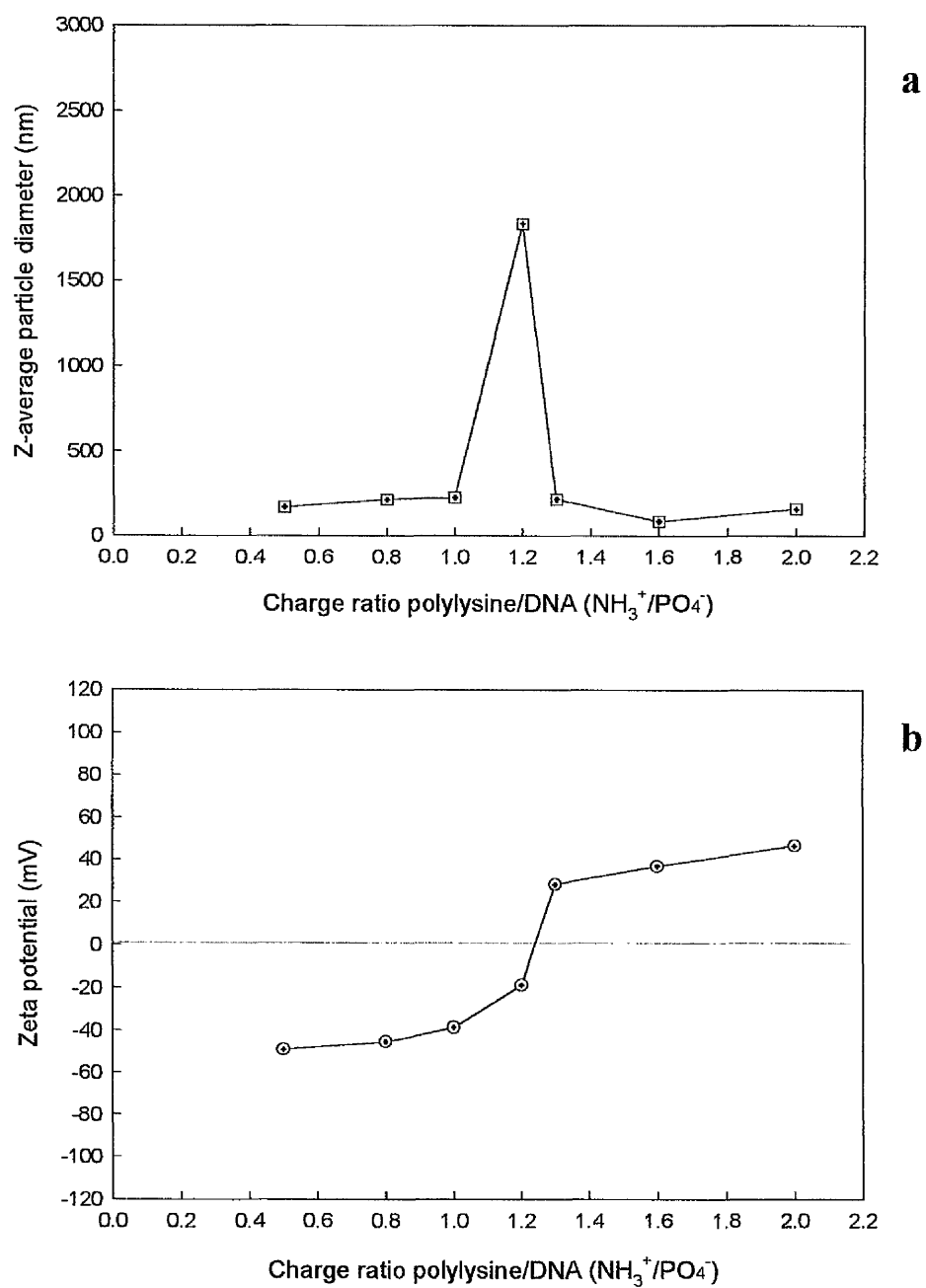


Figure 3.5 Analysis of (a) particle size and (b) zeta potential of PLK₅₃₁ polyplexes. Polyplexes were prepared at a final DNA concentration of 60 $\mu\text{g}/\text{ml}$ in deionised water pH7.5. Zeta potential calculated using the Hückel approximation $f(\kappa a)=1$. Results show mean of 3 measurements \pm s.d.

3.2.1.3 Gel electrophoresis analysis of polyplexes

3.2.1.3.1 PLK₅-DNA polyplexes

Figure 3.6a shows PLK₅ polyplexes analysed by agarose gel electrophoresis and stained with ethidium bromide. Lane 1 shows plasmid DNA (pEGlacZ 7676bp) with no added poly-L-lysine. Most of the DNA migrates as a single band of supercoiled plasmid with a lesser amount of nicked-circular plasmid.

Addition of increasing amounts of PLK₅ causes the plasmid DNA to become more retarded by the gel matrix. A number of additional DNA bands are visible in the polyplex samples prepared at charge ratios of 0.48, 0.8 and 1.1, running behind the leading band. Polyplexes having a charge ratio of 1.74 have been shown to have a positive zeta potential of +14mV, albeit in low ionic strength media (Figure 3.2b), yet the visible DNA band is migrating as if it were an anionic species (toward the cathode). Polyplexes having charge ratios in the range 0.16 to 1.4 have been shown to form large aggregates that would not be expected to penetrate the gel matrix (Figure 3.2a).

3.2.1.3.2 PLK₄₅-DNA polyplexes

Addition of PLK₄₅ to plasmid DNA at a charge ratio of 0.16 causes a small retardation of the supercoiled DNA band but no retardation of the nicked-circular band. At charge ratios of 0.48 and 0.8 a smeared band is visible behind a less distinct leading band. Small amounts of ethidium bromide fluorescence are visible in the loading well from apparently electroneutral complexes. Non-migrating species only are observed at charge ratios of 0.8, 1.1, 1.4 and 1.74. Lessening amounts of ethidium bromide fluorescence are visible in each of the loading wells. Polyplexes having a charge ratio of 1.4 and 1.74 have been shown to have a positive zeta potential in low ionic strength media (Figure 3.3b), but no species were observed migrating toward the anode (Figure 3.6b).

3.2.1.3.3 PLK₉₉-DNA polyplexes

Addition of PLK₉₉ to plasmid DNA at a charge ratio of 0.16 causes no retardation of the supercoiled DNA band. Further addition of PLK₉₉ at a charge ratio of 0.48 causes little increase in retardation of the leading DNA band. However, the fluorescent intensity of the bands is reduced compared to uncondensed plasmid DNA, and some ethidium bromide fluorescence is visible in the loading well. Polyplexes having a charge ratio of 0.8 appear retarded in the loading well, with a feint smear of migrating species. Polyplexes having a charge ratio in the range 1.1 to 1.74 are apparently retarded in the loading well despite having small size and strongly positive zeta potentials (Figure 3.4). None of these species were observed migrating toward the anode (Figure 3.6c).

3.2.1.3.4 PLK₅₃₁-DNA polyplexes

The addition of PLK₅₃₁ to plasmid DNA at a charge ratio of 0.16 caused no retardation of the supercoiled DNA band and no apparent reduction in fluorescence intensity (Figure 3.6d). Further addition of PLK₅₃₁ at a charge ratio of 0.48 causes slight (increase in) retardation of the leading DNA band. The fluorescent intensity of the band is greatly reduced and a small amount of fluorescence is visible in the loading well. Only a feint smeared band is visible migrating into the gel at a charge ratio of 0.8, a majority of the ethidium bromide fluorescence appears to be present in the loading well. At a charge ratio of 1.1 all DNA species appear to be completely retarded in the loading well. However, at greater charge ratios no ethidium bromide fluorescence is visible from migrating DNA bands or retarded in the loading well. These species have been shown to have strongly positive zeta potentials (Figure 3.5b).

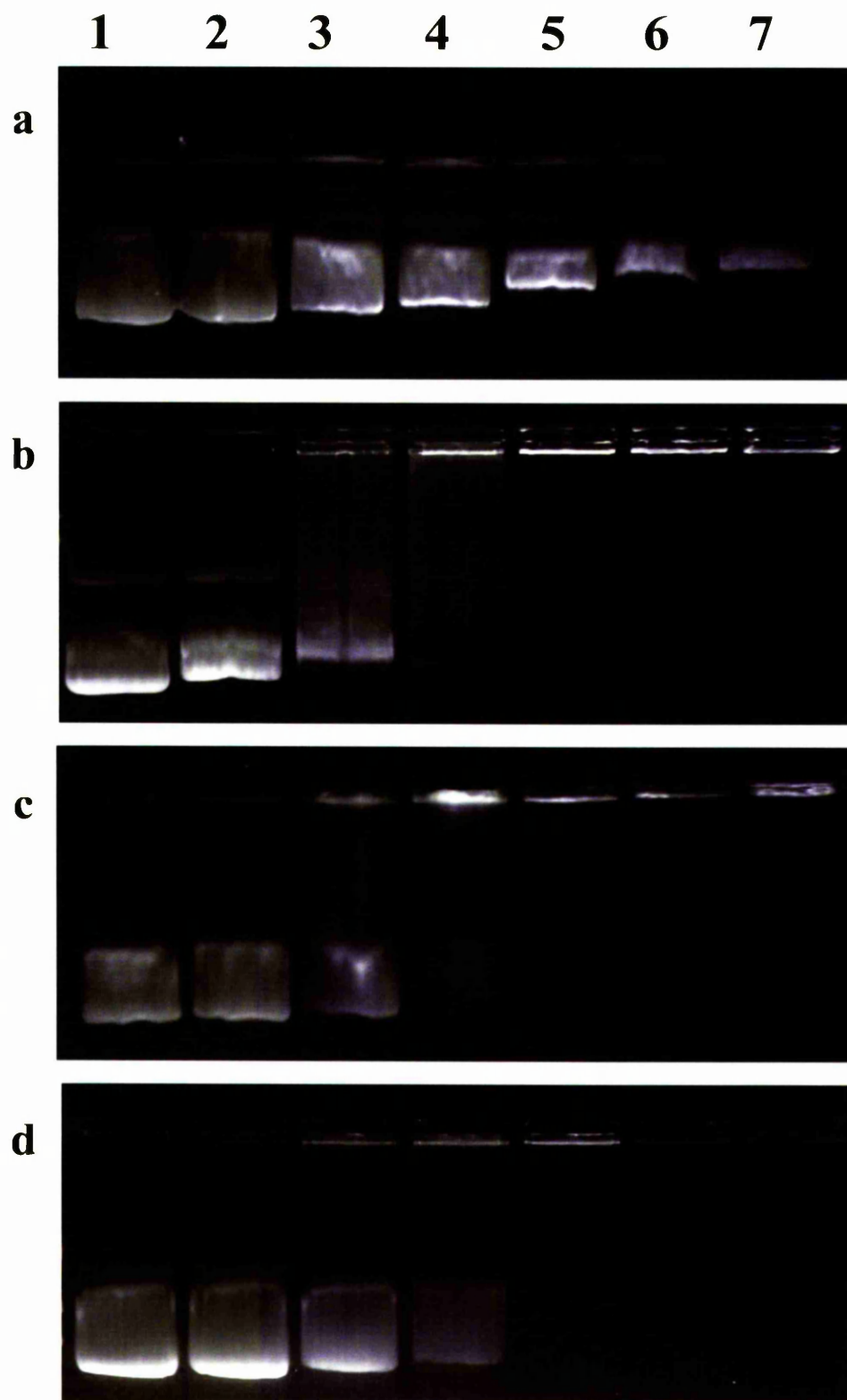


Figure 3.6 Agarose gel electrophoresis of PLK/DNA polyplexes. Gels a, b, c and d are for polyplexes formed with PLK₅, PLK₄₅, PLK₉₉ and PLK₅₃₁ respectively. Lanes 1-7 represents PLK and DNA mixed at an $\text{NH}_3^+/\text{PO}_4^-$ charge ratio of 0 (control), 0.16, 0.48, 0.8, 1.1, 1.4 and 1.74 respectively.

3.3 Discussion

Plasmid DNA has been condensed using poly-L-lysine to generate cationic DNA-poly-L-lysine complexes (polyplexes) that will more readily interact with anionic liposomes than DNA in its native form. Ideally, the condensed DNA particles will be of small size, so not to make the ternary vector of liposome and poly-L-lysine-condensed DNA of such size that it is unable to be endocytosed by the target cell (Perales, 1994b).

3.3.1 Electron microscope analysis of polyplexes

Polyplexes prepared using the plasmid pEGlacZ (7.6kb) and PLK₉₉ at a charge ratio of 1.6 were visualised by cryo-TEM. By using this technique the staining and drying artifacts found in conventional TEM are avoided and samples are viewed in a near native state (Gustafsson *et al.*, 1995).

The polyplexes observed formed two overlapping populations. The more common species were electron dense rods that varied in length from 53-160nm. A smaller population of near-spherical complexes of 18-30nm in diameter was also observed. The areas of light and dark banding visible on some of the polyplexes possibly indicate different levels of DNA condensation (see Figure 3.1a). Poly-L-lysine has been shown to bind preferentially to A-T rich sequences in DNA (Leng and Felsenfield, 1966).

No aggregates of small polyplex species were observed in the preparations, these have been noted by other groups (Tang and Szoka, 1997) indicating instability in the polyplex suspension.

A number of polyplex morphologies have been noted using electron microscopy. The polyplexes prepared here most closely resemble those in Lee and Huang (1996). They condensed plasmid of unstated size with PLK₁₂₃ at a charge ratio of 1.1 and generated rod-like particles of $109\text{nm} \pm 36\text{nm}$ in length and 15nm in diameter. The particles were prepared in deionised water at a final DNA concentration of 20µg/ml. Kwoh *et al.* (1999) also prepared polyplexes in deionised water, the polyplexes formed were of two morphological populations, torroids (60-65%) and rods. A torroidal morphology is that most commonly observed in polyplex preparations (Tang and Szoka, 1997;

Plank *et al.*, 1999; Duguid *et al.*, 1998; Perales *et al.*, 1997; Wagner *et al.*, 1991; Ziady *et al.*, 1999). The torroids vary in diameter from 20 to over 100nm. Most of the torroidal morphologies were observed in samples prepared in physiological saline or high salt concentrations, although Kwoh *et al.* (1999) observed torroids in samples prepared in deionised water. It is possible that the small complexes visible in Figure 3.1a-c are torroidal, but fine details of their morphology are not discernable.

Whether DNA condenses into torroids or rods is dependent on a number of as yet poorly defined parameters. Short DNA strands (<700bp) form rod structures, but if the \overline{M}_w of the DNA species is increased the proportion of torroids in the preparation increases greatly (Perales, 1994b). A high proportion of rod-structures were noted when DNA was condensed by $\text{Co}(\text{NH}_3)_6^{3+}$ in the presence of alcohol (methanol, ethanol and isopropanol) (Arscott *et al.*, 1990). It is thought the synergy between the condensing ligand and the alcohol may act locally to destabilise the DNA double helix, permitting fold backs and allowing rod-like condensed structures (Bloomfield, 1996). It is interesting to note that both ethanol and isopropanol are used in the procedure for isolating plasmid DNA (2.2).

As can be seen in Figure 3.1d-e, polyplexes visualised by standard electron microscopy techniques have rod structures that are narrower and less “rounded” than those visualised by cryo-TEM. It would be interesting to see if the torroidal structures in the literature are subject to artifacts due to the staining and dehydration procedures employed.

3.3.1.2 Order and control of DNA condensation

Individual polyplex particles are visible using electron microscopy, but do these particles contain an individual plasmid molecule? Bloomfield (1996) notes that although condensation of single molecules has been observed in very dilute DNA solutions, it is more common with plasmid sized DNA for several molecules to be incorporated into a single torroidal structure. Kwoh *et al.* (1999) noted that polyplex size was dependent on the DNA concentration in the preparation. They suggested that the smaller particles formed at low DNA concentrations contained a smaller number of DNA molecules. The heterogeneous nature of the polyplexes observed by electron microscopy (Figure 3.1) indicates that each may contain a different number of plasmid molecules.

Perales *et al.* (1997) calculated that the 20.7nm torroids formed from a 7.4kb plasmid condensed with poly-L-lysine contain a single plasmid molecule. This suggests that the small complexes visible in Figure 3.1a-c of 18-30nm in diameter possibly contain a single plasmid molecule. If the DNA has a similar condensed volume as that calculated by Perales *et al.* (1997), the rod structures of 160nm could contain several plasmid molecules.

It is important to distinguish the term "DNA condensation" as it is used here from its strict definition. DNA condensation is the dramatic reduction in the volume occupied by a DNA molecule (Bloomfield, 1996). The condensed DNA particles generated in this study probably occupy a greater volume than an individual supercoiled plasmid because many contain more than one plasmid molecule (e.g. see micrographs in Gustafsson *et al.*, 1995). The process that is occurring here is more correctly DNA aggregation and condensation where the aggregate is a finite size.

The successful delivery of a small, unimolecular complex or a large multimolecular complex to a transfected cell could confer different levels of observed transfection. The preparation of the condensed polyplexes must therefore be reproducible in order to reduce the variability between transfection experiments.

Summary

- Plasmid DNA is condensed into heterogeneous structures by poly-L-lysine that have rod-shaped or, less frequently, spherical morphologies.
- Condensed DNA particles probably contain a variable number of plasmid molecules dependent on their size, this may affect reproducibility of transfection experiments.
- Staining and drying of polyplex samples affects their visible morphology as viewed by electron microscopy.

3.3.2 Size and Zeta potential analysis of polyplexes

By using zeta potential analysis, a measure of the surface potential of the polyplexes formed at a specific charge ratio can be determined. If the zeta potential is used in conjunction with a measurement of particle diameter, small cationic polyplexes can be generated for interaction with anionic liposomes.

A number of trends are noted when poly-L-lysine is added to plasmid DNA:

- Small particles are formed at charge ratios of 0.1 to ~1. These polyplexes have a negative zeta potential.
- There is a step-wise fall in the zeta potential of polyplexes from a charge ratio of 0 to ~1.
- At a charge ratio of approximately 1, large aggregates of polyplexes are formed. These polyplexes have a very low zeta potential.
- Above a charge ratio of ~1 small particles are formed. These polyplexes have a positive zeta potential.
- The positive zeta potential of these polyplexes does not increase greatly at higher poly-L-lysine contents
- Poly-L-lysine of a chain length of 5 (PLK₅) does not form small polyplexes below a charge ratio of 1.7. Small polyplexes with positive zeta potentials are formed above a charge ratio of 2.

3.3.2.1 Primary stages of DNA condensation

Plasmid DNA dissolved in deionised water has a strongly negative zeta potential (-108mV). A molecule carrying such a high charge density would carry with it a number of counter ions to nullify charge, even in a deionised solvent. The presence of these counter ions could help to explain the small change in zeta potential with the initial addition of poly-L-lysine to naked DNA (Figure 3.4b). Further addition of poly-L-lysine causes a stepwise fall in zeta potential, suggesting the plasmid has not been saturated by the previous addition. Each of the poly-L-lysine charges is probably involved in charge neutralisation on the DNA backbone, with the excess negative charges on the plasmid giving the particle a negative zeta potential. Polyplexes that

have a calculated charge ratio with negative species in excess each have negative zeta potentials.

3.3.2.2 Polyplexes carrying a low zeta potential

At a calculated charge ratio of 1 to 1.25, depending on the poly-L-lysine species, large particles are produced. Similar observations have been noted using poly-L-lysines of various chain lengths and plasmids of different sizes (e.g. Pouton *et al.*, 1998; Lee and Huang, 1996; Xu *et al.*, 1998). These particles could be the result of either the swelling of individual particles, or the aggregation of small individual complexes.

At a calculated charge ratio of 1, the particles formed would be expected to have a neutral overall charge. It can be seen in Figures 3.2 to 3.5 that the large particles also have a low zeta potential, much lower than that of the particles forming smaller species.

It is known that stable colloidal suspensions have zeta potentials of more than +30mV or more negative than -30mV. The large particles described here possess a zeta potential that makes them prone to aggregation. It would appear that at calculated charge ratios approaching 1 small polyplexes having a very low zeta potential are formed. These polyplexes form unstable suspensions and therefore aggregate into large particles. It can be seen that particles having the lowest zeta potential form the largest aggregates (Figures 3.3-3.5).

3.3.2.3 Saturation of plasmid DNA with bound poly-L-lysine

Small particles are produced when the particles (polyplexes) have a strongly positive zeta potential. The transition from negative to positively charged species occurs at a calculated charge ratio of approximately 1 to 1.25 depending on the poly-L-lysine condensing agent. For a polyplex to carry a positive zeta potential, the poly-L-lysine charges must neutralise the DNA charges and confer the positive surface potential. This probably involves a number of poly-L-lysine molecules being partly bound to the DNA backbone, and being partly free in the surrounding milieu.

Little increase in zeta potential is noted as charge ratio is raised from 1 to 2 (see also Xu *et al.*, 1998 and Tang and Szoka, 1997). This suggests the DNA is largely

saturated with poly-L-lysine at the lowest charge ratio that confers a positive zeta potential. This can be compared with the addition of poly-L-lysine to initially naked DNA when the zeta potential is lowered incrementally by 68mV indicating many available poly-L-lysine binding sites (e.g. Figure 3.4b). Despite the apparent near saturation at high charge ratios, a small increase in the zeta potential is noted compared to complexes prepared at lower charge ratios but still with a positive zeta potential. Trubetskoy *et al.* (1999a) have suggested a model of polyplex formation where a polyplex having a calculated charge ratio of 1 has a measurable positive zeta potential. They suggest an inner core of DNA charges not all of which are directly neutralised by poly-L-lysine charges, surrounded by an outer layer of excess poly-L-lysine charges conferring the positive zeta potential. At higher charge ratios, perhaps more poly-L-lysine molecules could be associated with this outer layer conferring a slightly higher zeta potential.

3.3.2.4 Benefits of zeta potential and PCS analysis of polyplexes

The measurement of zeta potential allows the rapid determination of the polyplex surface potential. This determines which polyplexes have a positive surface potential and will readily interact with anionic liposomes. The calculated $\text{NH}_3^+/\text{PO}_4^-$ charge ratio gives an indication as to the particle's charge at high and low ratios. However, at calculated charge ratios of approximately 1 the calculated charge ratio does not always describe the correct surface potential of the particle. This may be especially important in high ionic strength solutes where the electrostatic interaction between poly-L-lysine and the DNA may be reduced.

PCS measurement of polyplex size suggests average particle sizes similar to those observed by electron microscopy (cf. Figure 3.1a-c). PCS allows the measurement of many more particles than by EM, giving a more representative sample. As the majority of the particles have a rod-shaped morphology, the average particle diameter given by PCS will be of a sphere of equivalent diameter (e.g. $100\text{nm} \times 20\text{nm}$ rod = 39.1nm equivalent sphere diameter).

Summary

- Poly-L-lysine (PLK) can condense plasmid DNA into small particles at high and low charge ratios, generating particles with positive and negative zeta potentials respectively.
- Condensed plasmid DNA particles having low zeta potentials form unstable suspensions and are prone to immediate aggregation.
- Small, positively charged polyplexes are produced by PLK₄₅, PLK₉₉ and PLK₅₃₁ at calculated charge ratios greater than one, and are suitable for association with anionic liposomes.
- Plasmid DNA becomes saturated with poly-L-lysine at high charge ratios.
- Zeta potential and PCS analysis allow rapid (quantitative) determination of polyplex characteristics.

3.3.3 Agarose gel electrophoresis analysis of polyplexes

A further technique to monitor DNA condensation by peptides is agarose gel electrophoresis (e.g. Wu and Wu, 1987). Polyplexes prepared using poly-L-lysines of different chain lengths were analysed in this manner (Figure 3.6).

It is thought that DNA complexes will be retarded in the loading well of the gel when the charge on the DNA is neutralised by the condensing peptide.

The analysis by agarose gel electrophoresis has a number of flaws in determining the surface potential (neutralisation) of the polyplex DNA. Complexes that appear retarded in the loading well (ethidium bromide fluorescence in the well) do not necessarily have a neutral surface potential when measured in their preparatory buffer. For example, when PLK₉₉ was mixed with DNA at a charge ratio of 1.1 (Figure 3.6c Lane 5) gel analysis suggests a neutral polyplex is generated. However, zeta potential analysis shows the particles have a surface potential of +51mV (Figure 3.4b). It appears that polyplexes containing more than a defined amount of poly-L-lysine cannot penetrate the gel matrix. Indeed, polyplexes carrying a positive zeta potential would be expected to be seen migrating toward the cathode, the opposite polarity to naked plasmid DNA.

Secondly, the ethidium bromide used to visualise the polyplexes in the agarose gel is readily excluded from DNA as its level of condensation increases (Pouton *et al.*, 1998). This is shown most notably by condensation of DNA using PLK₅₃₁ in Figure 3.6d. The total amount of ethidium bromide fluorescence visible in each lane is reduced as the amount of poly-L-lysine added to the DNA is increased. Indeed, at a charge ratio of 1.74 no ethidium bromide fluorescence is visible from the polyplexes. It is possible therefore that highly condensed polyplexes having positive zeta potentials are migrating toward the cathode but cannot be visualised using ethidium bromide staining. However, when Wu and Wu (1987) radiolabelled the poly-L-lysine condensing agent they noted all radioactivity remained associated with non-migrating DNA species in the loading well, and no radiolabelled DNA species migrated into the gel.

3.3.3.1 Why are highly condensed polyplexes completely retarded by the gel matrix?

Surprisingly, the reason why condensed DNA does not readily migrate into an agarose gel matrix has not been widely discussed. The explanation of charge neutrality is an overly simplistic observation (e.g. Wu and Wu, 1987) as many of the non-migrating species carry positive and negative zeta potentials. It seems most likely that condensed plasmid DNA forms rigid structures that are unable to penetrate the gel matrix as efficiently as the more flexible naked plasmid DNA. Above an as yet undefined level of condensation polyplex species superficially appear charge neutral. As both complexes with negative and positive zeta potentials are retarded it is unlikely there is any electrostatic interaction with the gel matrix (pure agarose is charge neutral). Very low concentration agarose gels (e.g. 0.1-0.2%) still do not allow penetration by the highly condensed DNA particles (data not shown). Complexes having a zeta potential of -50mV can enter the matrix readily (low condensation) whereas complexes having a positive zeta potential of $+50\text{mV}$ (high condensation) cannot. The less condensed polyplexes having negative zeta potentials intercalate greater amounts of ethidium bromide (see Figure 3.6c and d). It is also likely that polyplexes with a zeta potential of -30 to $+30\text{mV}$ (measured in deionised water) would aggregate in the presence of high ionic strength TBE buffer that bathes the gel. The buffer salts would cause shrinkage of the electrical double layer surrounding the polyplexes, lowering the zeta potential and making them prone to aggregation. Large aggregates are unlikely to enter the gel matrix.

Polyplexes that show migrating species and non-migrating species probably contain an heterogeneous population of complexes having slightly different levels of DNA condensation or perhaps DNA supercoiling.

Summary

- Agarose gel electrophoresis analysis cannot be used to accurately determine the surface charge of a polyplex.
- Zeta potential analysis shows that polyplexes that are apparently charge neutral by gel electrophoresis have a high surface charge.
- Condensed DNA particles do not readily intercalate ethidium bromide.
- Structural characteristics of condensed DNA may prevent it penetrating an agarose gel matrix.

3.3.4 Condensation of plasmid DNA using PLK₅

Each of the trends in DNA condensation using poly-L-lysine, observed by PCS, zeta potential and less strictly agarose gel electrophoresis applies to the polymers PLK₄₅, PLK₉₉ and PLK₅₃₁. These polymers appear to condense DNA in a similar manner, producing polyplexes of similar size and zeta potential at equivalent charge ratios. However, the polymer PLK₅ behaves somewhat differently. Using PLK₅, small stable polyplexes having negative zeta potentials could not be generated (Figure 3.2b). In addition, small particles having positive zeta potentials were only generated with charge ratios of greater than 2.4. These polyplexes have low zeta potentials.

Small poly-L-lysines would appear not to bind as avidly to DNA as poly-L-lysine having longer chain lengths. Large polymers will have cooperative binding between each of the positive charge-bearing groups, increasing the strength of the interaction. Small polymers on the other hand have fewer charges per molecule and will bind in a weaker manner, being readily displaced. At low charge ratios, small polymers may not be present in sufficient amounts to condense DNA into small particles instead causing aggregation of a number of less condensed species.

Plank *et al.* (1999) show that complexes formed using small DNA binding ligands are readily dissociated in the presence of an electric field. This can be confirmed by studying the gel electrophoresis analysis of PLK₅ polyplexes (Figure 3.6d). At low charge ratios large aggregates were seen to be formed, the gel however shows small particles readily entering the gel matrix. When an electric field is applied to these particles the poly-L-lysine will readily dissociate, leaving small complexes containing little poly-L-lysine that can readily enter the gel.

In addition, a polyplex having a charge ratio of 1.74 has been shown to have a zeta potential of +14mV (Figure 3.2b), yet it migrates as a negatively charged species. This suggests the majority of the poly-L-lysine has dissociated from the polyplex due to the electric field applied across the gel. It must be added however, that the zeta potential of the polyplexes in TBE electrophoresis buffer may be somewhat lower than that measured in deionised water, due to its high ionic strength.

Interestingly, the polyplexes are exposed to a greater potential difference during the zeta potential measurement and yet do not appear to dissociate using this technique. This may be simply due to the short time course of zeta potential measurement. Alternatively, the greater resistance to migration the gel has on plasmid DNA

compared to a small poly-L-lysine molecule may aid their dissociation when the polyplex becomes trapped in the matrix. The poly-L-lysine having low affinity to the DNA would be stripped off the polyplex and be attracted to the cathode, leaving essentially naked-plasmid DNA free to migrate into the gel matrix toward the anode.

Summary

- PLK₅ has a low affinity for plasmid DNA and can only form small stable polyplexes at high charge ratios with positive zeta potentials.
- Polyplexes prepared using small DNA binding ligands readily dissociate during agarose gel electrophoresis.

Chapter 4

Characterisation of Lipopolyplexes

4.1 Overview

The interaction between cationic liposomes (or lipids) and DNA has been widely studied both biophysically (e.g. Eastman *et al.*, 1997) and by a variety of microscopy techniques (e.g. Rädler *et al.*, 1997; Gustafsson *et al.*, 1995). However, little information has been gathered regarding the structure-function relationships between DNA-cationic liposome complexes and their gene transfer activities. Electron microscopy studies of cationic liposome/DNA complexes (lipoplexes) show a heterogeneous population of structures, and it is not known if the gene transfer activities of these populations differ. Separation of these physicochemically similar populations to study their individual properties is impractical.

Recent work has begun to concentrate on the reproducible production of artificial gene transfer vectors and removing heterogeneity in the preparation (e.g. Wheeler *et al.*, 1999). In addition, vectors are being characterised in terms of their size, zeta potential and several other physical properties in relation to their gene transfer activity (Duguid *et al.*, 1998).

In this study a complex between an anionic liposome and poly-L-lysine-condensed DNA will be used for transfection. The structure of this type of complex has not been specifically determined. A number of studies using analogous gene transfer vectors have suggested possible models for the interaction of condensed DNA (polyplexes) with anionic liposomes. Lee and Huang (1996) suggest that the poly-L-lysine-condensed DNA particle is internalised by the liposome, shielding it from the external environment. This model is based entirely on electron micrographs of dehydrated lipopolyplex samples, and it is not obvious that the polyplex is contained entirely within a lipid bilayer. Alternatively, Hagstrom *et al.* (1996) suggest that the DNA particle is exposed on the liposome surface where it is susceptible to DNase degradation. These particles were not prepared by addition of preformed condensed DNA particles to liposomes and may form a different type of interaction to that described by Lee and Huang.

This study will aim to characterise the interaction between poly-L-lysine-condensed DNA particles (polyplexes) and anionic liposomes of various compositions. Any differences in structures due to a change in lipid composition will be related to the transfection activity obtained using the complexes (lipopolyplexes).

The liposomes of composition DOPE/cholesterol/oleic acid (40:40:20 mol%) have been shown to have high levels of gene transfer activity (Wang and Huang, 1987). The effect of lipid acyl chain length, and substitution of the cholesterol component for a PC lipid in the liposome formulation, on the interaction with condensed DNA and ultimately with mammalian cells (for transfection) will be studied. Table 4.1 shows the six different liposome formulations tested and their properties.

Table 4.1 Properties of liposomes used in characterisation studies

Liposome Formulation (40:40:20 mol%)	Z-average diameter (nm) ^a	Average lipid M _w	Lipid molecules/liposome ^b
DOPE/cholesterol/oleic acid	192.5 ± 71.2 200.4 ± 51.5	508.78	429,860 468,307
DOPE/DOPC/oleic acid	154.2 ± 42.9	668.54	271,146
DMPE/cholesterol/oleic acid	131 ± 42.9	465.54	192,372
DMPE/DMPC/oleic acid	113.2 ± 52.5	582.02	141,104
DLPE/cholesterol/oleic acid	174.8 ± 30.8	443.10	352,430
DLPE/DLPC/oleic acid	107.9 ± 33.1	537.14	127,378

^a determined by PCS. ± s.d. of unimodal distribution.

^b determined using 0.5nm²/lipid molecule for lipid headgroup area and lipid bilayer thickness of 7.5nm.

4.2 Results

4.2.1 Liposome and lipopolyplex permeability to glucose

If the interaction of liposome and condensed-DNA polyplexes involves a surface binding of the two components it would be expected that the integrity of the lipid bilayer would be retained throughout. However, if the condensed-DNA is internalised by the liposome, the dynamics of the interaction suggest the membrane would have to rupture and then perhaps reform. By entrapping a labelled solute in the aqueous core of the liposome, the extent of solute release (or retention) during liposome/condensed-DNA interaction can be monitored.

Two permeabilities per liposome formulation were determined.

- Permeability of liposomes in the absence of condensed-DNA.
- Permeability of condensed-DNA/liposome complexes (lipopolyplexes).

Condensed-DNA polyplexes were prepared by addition of PLK₉₉ to 120µg of plasmid DNA at a charge ratio of 2.1:1.

The ratio of liposomes per condensed DNA particle was calculated by determining the following:

Each lipopolyplex preparation contains 120µg of 7676bp plasmid DNA. Assuming each plasmid forms a single polyplex upon condensation, the maximum number of polyplexes to react with the liposomes is 1.422×10^{13} .

The number of lipid molecules per liposome can be calculated by determining the inner and outer surface area of the liposome assuming a bilayer thickness of 7.5nm, and a single lipid molecule occupies 0.5nm^2 . The number of liposomes added to the polyplexes is further determined by calculating the total number of lipid molecules added to the condensed-DNA using the average lipid M_w (Table 4.1).

The permeability experiments were interpreted using Equation 2.4.

The slope of the left hand side of Equation 2.4 versus time relates to the permeability of the liposomes/lipopolyplexes.

The permeability of liposomes only (L_L) and liposomes + condensed-DNA (L_{LD}) were determined separately using each liposome formulation (Figures 4.1-4.6, Table 4.2). The permeability was used to calculate the permeability coefficient (P) of each

system using Equations 2.5-2.7 where R was calculated from the values in Table 4.1 and h_b is taken as 7.5nm.

The observed permeability coefficient of liposomes + condensed-DNA (\bar{P}_{obs}) determined experimentally is the number average of the permeability coefficients of the liposomes (P_L) and lipopolyplexes ($P_{L/D}$):

$$\bar{P}_{obs} = P_L \frac{n_L}{n_L + n_{L/D}} + P_{L/D} \frac{n_{L/D}}{n_L + n_{L/D}} \quad \text{Equation 4.1}$$

Where n_L and $n_{L/D}$ are the numbers of liposomes and lipopolyplexes respectively.

Then:

$$\bar{P}_{obs} = P_L \frac{x}{1+x} + P_{L/D} \frac{1}{1+x} \quad \text{Equation 4.2}$$

If x is the ratio of the number of liposomes (n_L) to lipopolyplexes ($n_{L/D}$) i.e. $x = n_L/n_{L/D}$.

Using Equation 4.2 permeability coefficients of lipopolyplexes ($P_{L/D}$) can be determined (Table 4.2).

There is no large initial release of glucose following condensed-DNA addition to liposomes of any composition. Lipopolyplexes of all lipid compositions are each more permeable to glucose than their component liposomes (55-1028% more permeable). Lipopolyplexes retain a permeability barrier following addition of condensed-DNA. An average value of L_D and $L_{L/D}$ was determined for DOPE/cholesterol/oleic acid (40:40:20 mol%) liposomes using the slopes generated from the liposomes described in Table 4.1. The permeability coefficient of DLPE/DLPC/oleic acid liposomes was not determined.

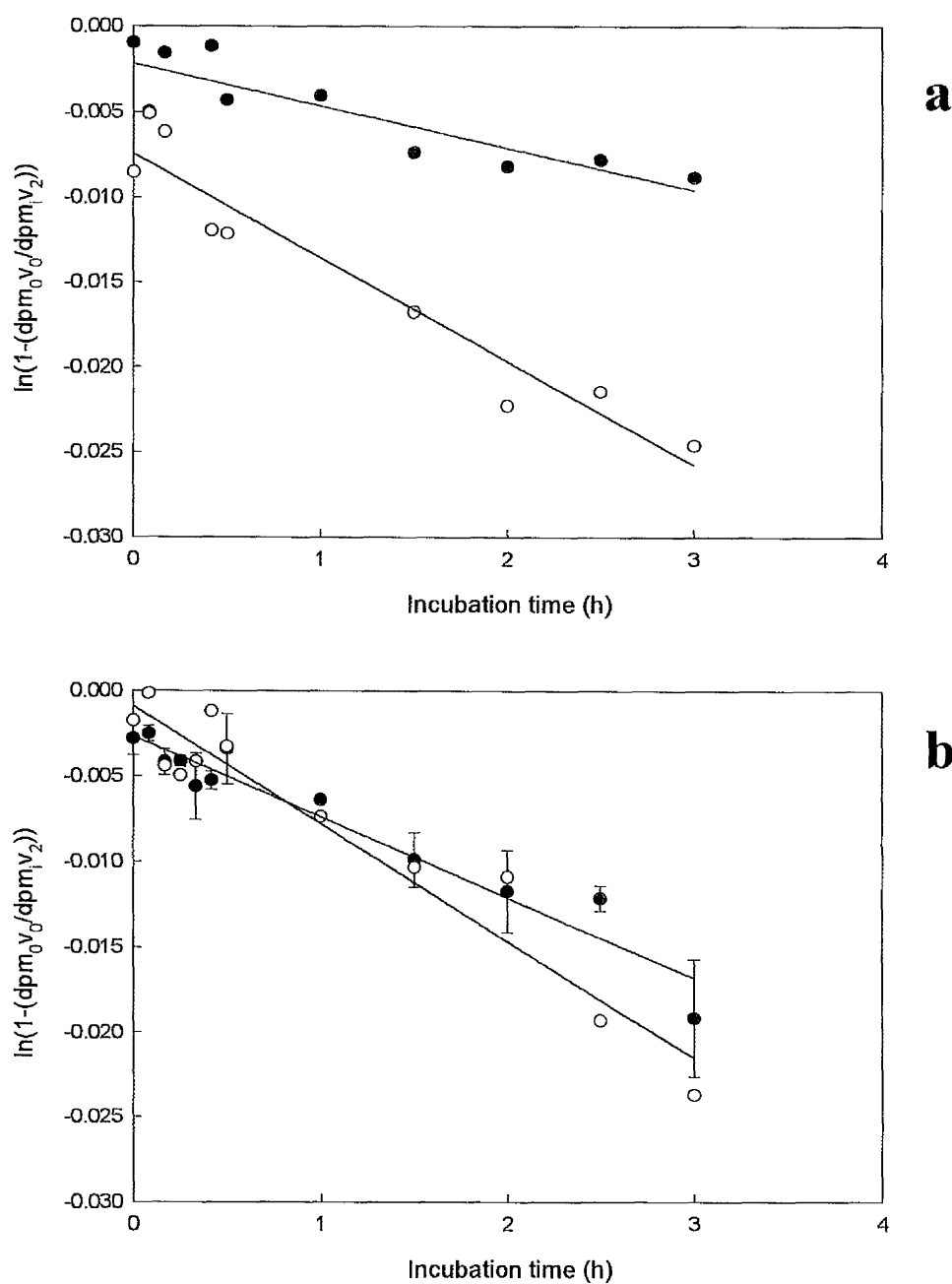


Figure 4.1 Permeability plots for DOPE/cholesterol/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C. Solid points are permeabilities of free liposomes (L_L). Open points are permeabilities of liposomes + polyplexes (charge ratio 2.1, PLK₉₉) (L_{LD}). (a) liposome diameter 192.3nm. (b) liposome diameter 200.4nm open points, 187.1nm diameter solid points. The points were calculated by application of Equation 2.4. Ratio of liposomes/polyplexes, (a) 0.8 (b) 0.76. Data show mean \pm s.d. ($n=2$) for (b) free liposomes.

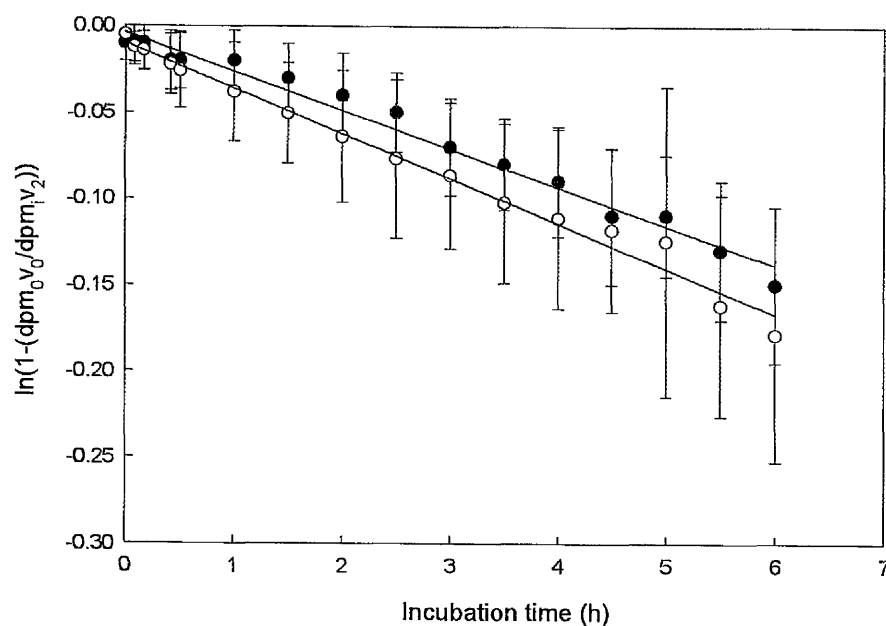


Figure 4.2 Permeability plots for DOPE/DOPC/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C. Solid points are permeabilities of free liposomes (L_L). Open points are permeabilities of liposomes + polyplexes (charge ratio 2.1, PLK₉₉) ($L_{L/D}$). The points were calculated by application of Equation 2.4. Ratio of liposomes/polyplexes, 2.37. Data show mean \pm s.d. (n=3).

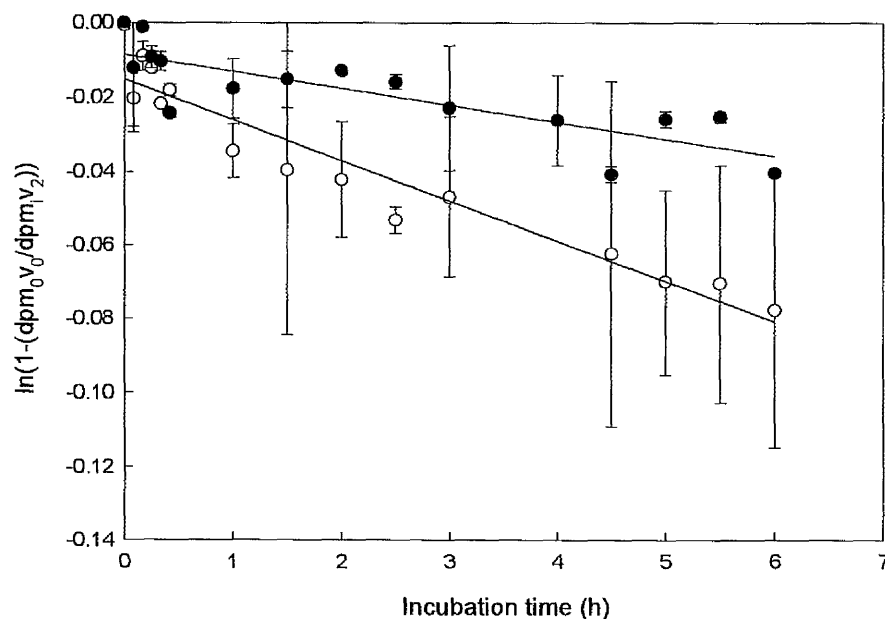


Figure 4.3 Permeability plots for DMPE/cholesterol/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C. Solid points are permeabilities of free liposomes (L_t). Open points are permeabilities of liposomes + polyplexes (charge ratio 2.1, PLK₉₉) (L_{tD}). The points were calculated by application of Equation 2.4. Ratio of liposomes/polyplexes, 1.9. Data show mean \pm s.d. ($n=2$).

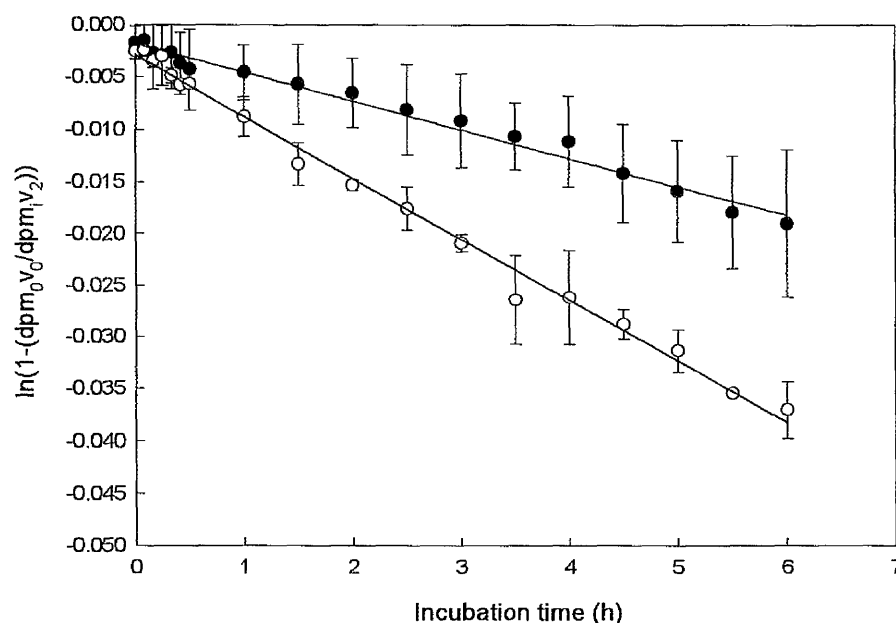


Figure 4.4 Permeability plots for DMPE/DMPC/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C. Solid points are permeabilities of free liposomes (L_t). Open points are permeabilities of liposomes + polyplexes (charge ratio 2.1, PLK₉₉) (L_{tD}). The points were calculated by application of Equation 2.4. Ratio of liposomes/polyplexes, 7.9. Data show mean \pm s.d. ($n=3$).

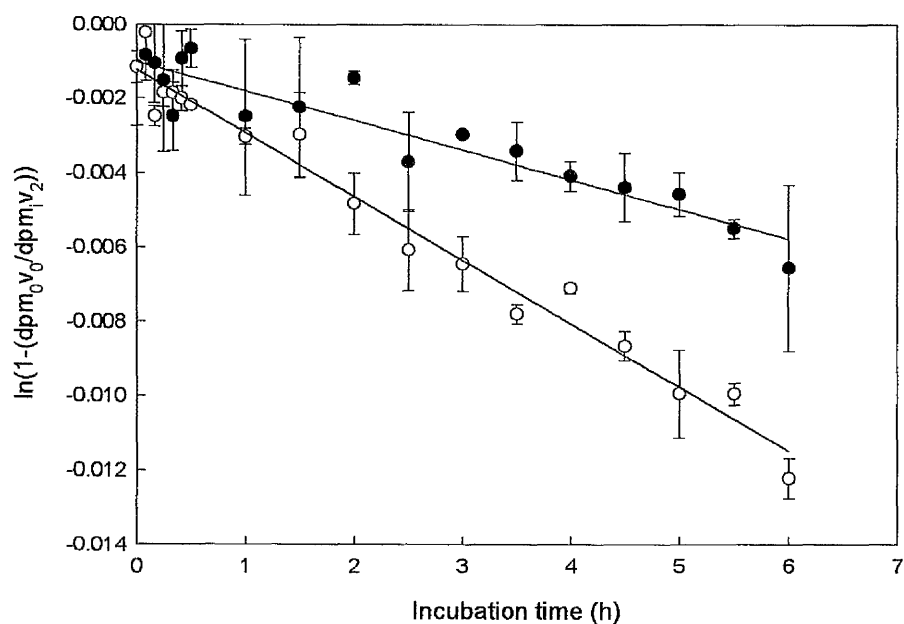


Figure 4.5 Permeability plots for DLPE/cholesterol/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C. Solid points are permeabilities of free liposomes (L_L). Open points are permeabilities of liposomes + polyplexes (charge ratio 2.1, PLK₉₉) (L_{LD}). The points were calculated by application of Equation 2.4. Ratio of liposomes/polyplexes, 3.35. Data show mean \pm s.d. (n=3).

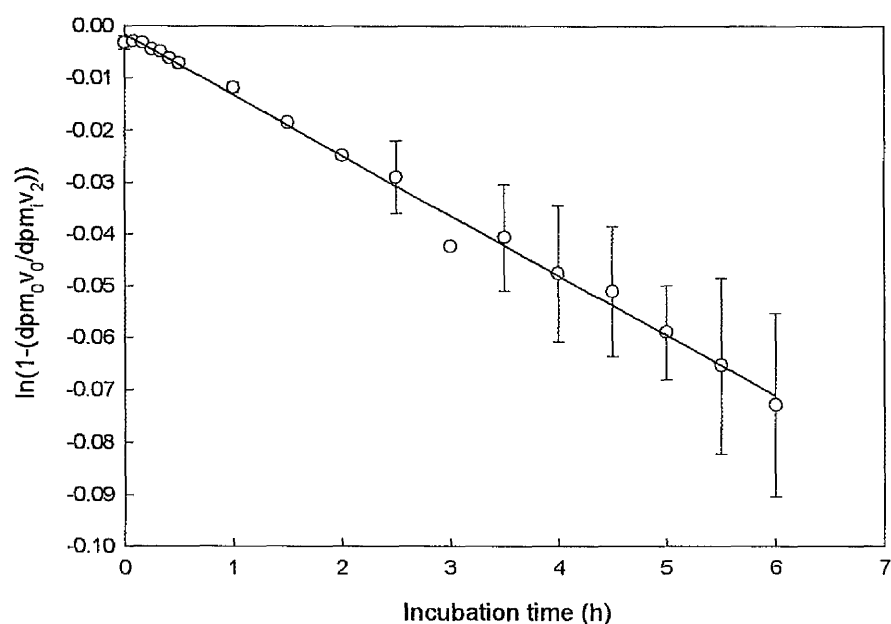


Figure 4.6 Permeability plot for DLPE/DLPC/oleic acid liposomes (40:40:20 mol%) for glucose leakage at 25°C. Open points are permeabilities of liposomes + polyplexes (charge ratio 2.1, PLK₉₉) (L_{LD}). The points were calculated by application of Equation 2.4. Ratio of liposomes/polyplexes, 8.28. Data show mean \pm s.d. (n=2).

Table 4.2 Permeability coefficients of liposomes and lipopolyplexes of different lipid compositions

Liposome Formulation (40:40:20 mol%)	L_L (h^{-1})	L_{LD} (h^{-1})	$10^8 P_{obs}$ ($cm\ h^{-1}$)	$10^8 P_L$ ($cm\ h^{-1}$)	$10^8 P_{LD}$ ($cm\ h^{-1}$)	P_{LD}/P_L	Liposomes/lipopolyplexes (x)	% change in P after polyplex addition
DOPE/cholesterol/oleic acid	0.0036 ± 0.00045	0.0066 ± 0.001	1.99 ± 0.3	1.04 ± 0.13	2.750 ± 0.42	2.64	0.8	164
DOPE/DOPC/oleic acid	0.0225 ± 0.0008	0.0262 ± 0.0008	6.07 ± 0.185	5.22 ± 0.186	8.085 ± 0.24	1.54	2.37	55
DMPE/cholesterol/oleic acid	0.0046 ± 0.0008	0.011 ± 0.0009	2.1 ± 0.17	0.89 ± 0.154	4.399 ± 0.35	4.94	1.9	394
DMPE/DMPC/oleic acid	0.0027 ± 0.0001	0.0059 ± 0.0001	0.97 ± 0.016	0.45 ± 0.017	5.078 ± 0.086	11.28	7.9	1028
DLPE/cholesterol/oleic acid	0.008 ± 0.0001	0.0017 ± 0.0001	0.45 ± 0.027	0.21 ± 0.0027	1.254 ± 0.074	5.97	3.35	497
DLPE/DLPC/oleic acid	N.D.	0.0116 ± 0.0002	1.79 ± 0.031	N.D.	N.D.	N.D.	8.28	N.D.

-N.D. -not determined

-all values show mean ± s.d (n=2 or 3).

4.2.3 Sensitivity of lipopolyplexes to pronase enzyme digestion

Figure 4.7 shows DOPE/cholesterol/oleic acid and DOPE/DOPC/oleic acid lipopolyplexes and their component species treated with the proteolytic enzyme pronase and further analysed by agarose gel electrophoresis. Lipopolyplexes were prepared using PLK₉₉-condensed DNA having a charge ratio of 2.1 and had a lipid/DNA ratio of 36. Polyplexes were prepared at an identical charge ratio.

Plasmid DNA (7676bp pEGlacZ) with no added poly-L-lysine migrates as a single band of supercoiled plasmid DNA with a lesser amount of nicked-circular plasmid (e.g. Figure 4.7b, Lane 1). Addition of poly-L-lysine (PLK₉₉) to plasmid DNA at a charge ratio of 2.1 (forming polyplexes) causes apparent retardation of the DNA species in the loading well, probably due to DNA charge neutralisation (see 3.3.3.1). Incubation of the polyplexes with pronase enzyme regenerates apparently uncondensed plasmid DNA species that can migrate into the gel (Lane 3). A larger proportion of the plasmid DNA is now in the nicked-circular form compared to untreated plasmid DNA. The leading band of migrating DNA species, apparently supercoiled plasmid is more retarded by the gel matrix than the analogous band in an untreated plasmid DNA preparation (Lane 1).

Lipopolyplexes formed from polyplexes having a charge ratio of 2.1 show no migrating DNA bands when analysed by agarose gel electrophoresis (Lane 4). The amount of ethidium bromide fluorescence observed in the loading well is either greater than (Figure 4.7a) or less than (Figure 4.7b) that shown by polyplexes only (Lane 2). Treatment of lipopolyplexes with 0.5% Triton X-100 causes a change in the intensity of ethidium bromide fluorescence observed in the loading well, but no DNA species are observed migrating into the gel matrix (Lane 5). Incubation of lipopolyplexes with pronase enzyme releases migrating species of plasmid DNA from the lipopolyplex, a majority of which appears to be supercoiled plasmid DNA. The amount of ethidium bromide fluorescence observed from the migrating species is slightly less than that seen in a sample containing an identical amount of untreated plasmid DNA (Lane 1). A small amount of ethidium bromide fluorescence is still apparent in the loading well of the gel (Lane 6). Solubilisation of the lipid component of the lipopolyplex using Triton X-100 prior to pronase digestion appears to render all DNA species able to migrate into the gel matrix. The majority of the migrating DNA

species are observed as an apparent supercoiled plasmid band with a smaller amount of nicked circular plasmid DNA (Lane 7).

Lipopolyplexes prepared from other liposome compositions described in Table 4.1 show identical trends when treated with pronase enzyme (data not shown).

4.2.3 Cryo TEM of lipopolyplexes

The structure of lipopolyplexes was examined by cryo TEM. Lipid vesicles examined using this technique display a distinct lipid membrane surrounding an aqueous core (Figure 4.8). Preliminary studies of lipopolyplexes could not determine the presence of distinct condensed-DNA particles such as those found when observing polyplexes (Figure 3.1). It was not possible to determine if condensed DNA was associated with the lipid membrane in an ordered manner that rendered both indistinguishable. Lipopolyplexes tended to form aggregates of vesicles not visible in liposome only samples.

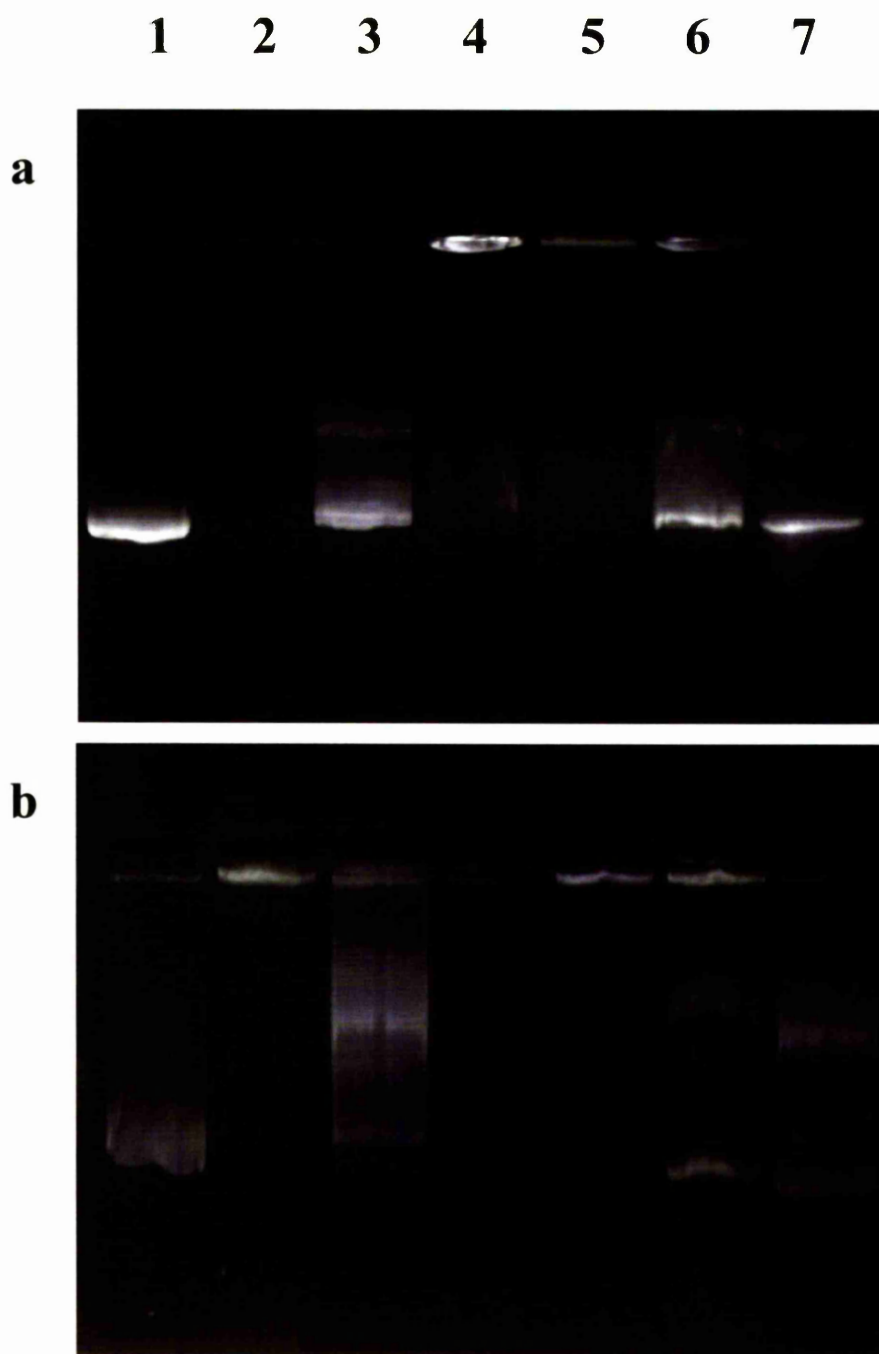


Figure 4.7 Agarose gel electrophoresis of polyplexes and lipopolyplexes treated with pronase. (a) DOPE/cholesterol/oleic acid lipopolyplexes (b) DOPE/DOPC/oleic acid lipopolyplexes. **Lane 1:** pEGlacZ only. **Lane 2:** PLK₉₉ polyplexes having a charge ratio of 2.1. **Lane 3:** PLK₉₉ polyplexes + pronase. **Lane 4:** Lipopolyplexes having a charge ratio of 2.1 and a lipid/DNA ratio of 36. **Lane 5:** Lipopolyplexes + 0.5% Triton X-100. **Lane 6:** Lipopolyplexes + pronase. **Lane 7:** Lipopolyplexes + 0.5% Triton X-100 + pronase.

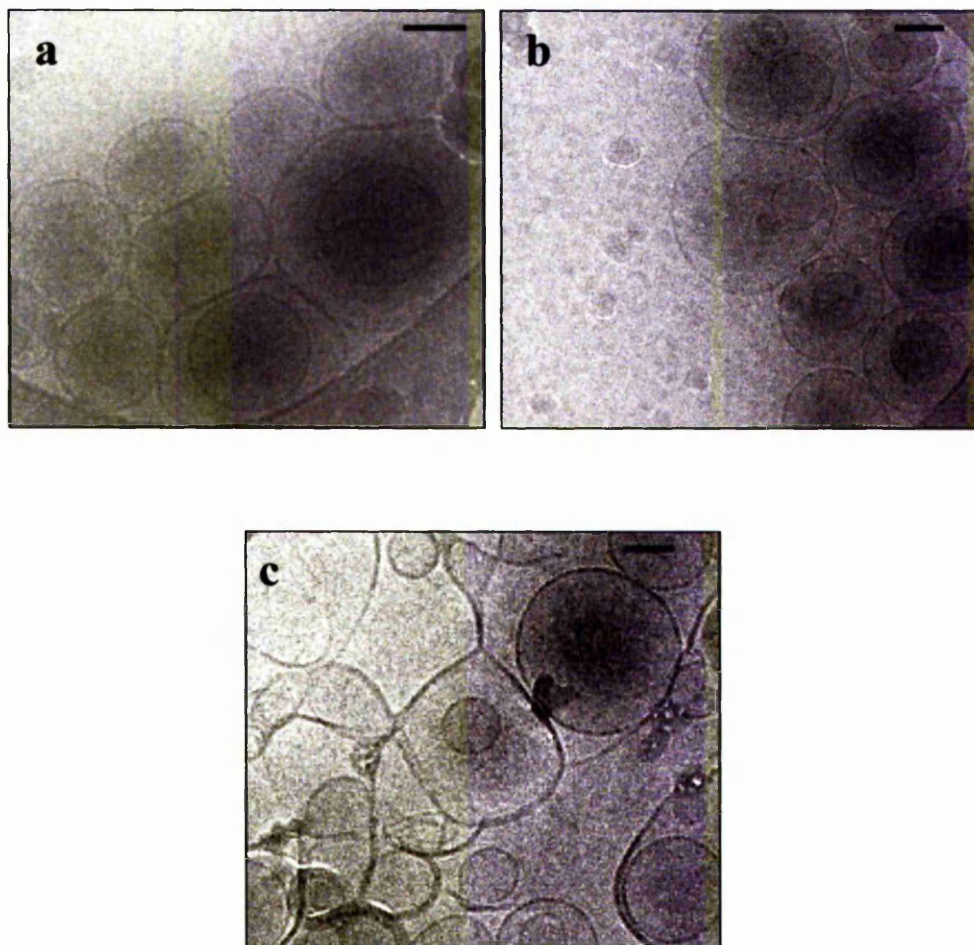


Figure 4.8 Cryo electron micrographs of (a+b) DMPE/DMPC/oleic acid (40:40:20 mol%) liposomes. (c) Lipopolyplexes of DMPE/cholesterol/oleic acid liposomes (40:40:20 mol%) and PLK₉₉-condensed pEGlacZ (charge ratio PLK/DNA 1.6, lipid/DNA ratio 36). Scale bar indicates 100nm.

4.3 Discussion

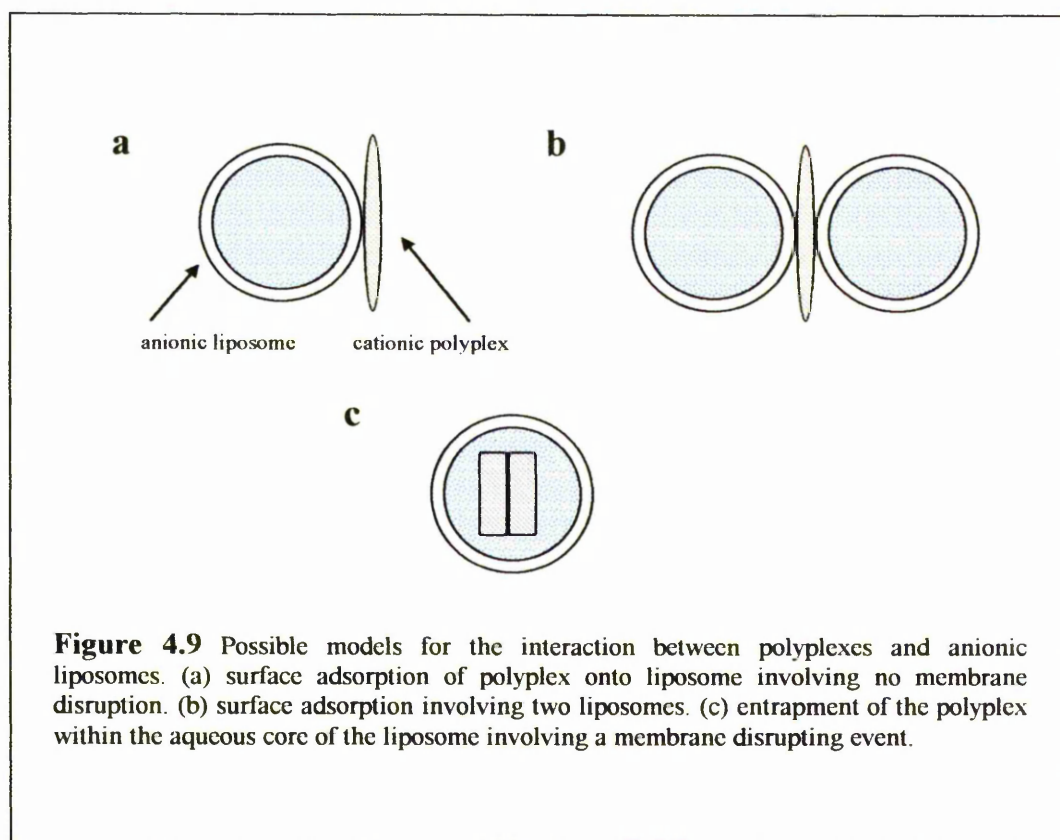
4.3.1 Lipopolyplex formation and structure

To determine the nature of the interaction between cationic polyplexes and anionic liposomes, a labeled solute was encapsulated in the aqueous core of the liposomes. Lee and Huang (1996) suggest that the polyplex particle is engulfed by the liposome and encapsulated within its aqueous core. It is implicit in such an engulfment event that a pinching (breaking) of the lipid membrane would need to occur for successful encapsulation to take place. If the membrane were to rupture the contents of the liposome's aqueous core would be released. If the polyplex were simply adsorbed onto the surface of the liposome (e.g. Hagstrom *et al.*, 1996) the membrane would be expected to maintain its integrity throughout the interaction.

By examining the permeability plots of the various lipopolyplexes (Figures 4.1-4.6) it can be seen that there is no large efflux of ^{14}C -glucose from the liposomes following polyplex addition. A large-scale release of solute would be noted by an initial steep gradient to the permeability plot. Liposomes of all formulations tested retain their integrity during the interaction with polyplexes. All the liposome formulations contain the same amount of the charged-species oleic acid, which probably contributes the majority of the interaction with the polyplex.

It was noted that cationic liposomes interacting with DNA undergo radical topological transitions, and fuse into condensed multilamellar structures (Rädler *et al.*, 1998). Peptide-condensed DNA appears to interact with anionic liposomes in a manner that retains their integrity of the original liposomes. Micrographs of such complexes often show individual liposomes interacting with an indeterminate number of DNA particles (Hagstrom *et al.*, 1996; Lee and Huang, 1996). Cationic liposomes appear to interact with peptide-condensed DNA in a similar manner to anionic liposomes, their integrity apparently being maintained (Gao and Huang, 1996). Additionally, Sorgi *et al.* (1997) observe that particle size of protamine-condensed DNA/cationic liposome complexes are six times smaller than equivalent DNA/cationic liposome complexes. It is possible that a large number of liposomes with a relatively low charge density are required to neutralise the high charge carried by an individual plasmid. The localisation of several liposomes around a single DNA molecule may lead to their

coalescence and fusion into larger structures. The condensation of DNA with peptides may enable a more gentle interaction with the liposomes by lowering the charge density carried by the DNA. Possible models for the interaction of anionic liposomes and condensed-DNA are shown in Figure 4.9.



It appears that liposome membrane integrity is maintained throughout lipopolyplex formation. The exact location of the polyplex in relation to the lipid component cannot be determined using the solute leakage model. Figure 4.7 shows that polyplexes prepared from PLK₉₉ having a charge ratio of 2.1 are susceptible to pronase enzyme digestion. This releases the DNA in a form that migrates into an agarose gel (Lane 3). The slight retardation of this band in comparison to untreated supercoiled DNA may be due to the presence of small lysine peptides or lysine monomers generated by the pronase enzyme, still associating with the plasmid DNA. Similarly, a majority of the condensed-DNA associated with lipopolyplexes is released following their incubation with pronase enzyme. A smaller amount of DNA that is still retarded within the gel loading well appears to be inaccessible to the pronase enzyme. Solubilisation of the lipid using Triton X-100 appears to render this

condensed DNA susceptible to pronase degradation. It would appear that the lipopolyplexes are a heterogeneous population of structures. The majority appear to have a polyplex exposed on the liposome surface that is readily degraded by the pronase enzyme (Figure 4.9b). A smaller number appear to have a polyplex that is shielded from pronase degradation by the lipid (liposome) component. This structure may have the polyplex sandwiched between a number of liposomes, or be encapsulated within the aqueous core of an individual liposome. Such engulfment events could be missed by the solute release assay as they would be greatly outnumbered by the species that undergo a surface interaction. Similar heterogeneous populations of lipopolyplexes were observed by Hagstrom *et al.* (1996). They noted that the condensed-DNA particles were not encapsulated by the liposomes, were still susceptible to DNase degradation and often interacted with more than one liposome.

Agarose gel analysis also shows the relative level of DNA condensation in polyplexes is different from that observed in lipopolyplexes. Semi-quantitative observations show that DNA in DOPE/cholesterol/oleic acid lipopolyplexes is less condensed than in polyplexes (as determined by ethidium bromide intercalation, Figure 4.7a, Lanes 2 and 4). Addition of Triton X-100 to the lipopolyplexes appears to allow the DNA to undergo further condensation, but not to the extent observed in polyplexes. Conversely, DOPE/DOPC/oleic acid lipopolyplexes appear to contain DNA in a more condensed form than in its component polyplexes. The level of condensation is apparently reduced when the lipopolyplexes are treated with Triton X-100. Both lipopolyplexes appear equally susceptible to pronase degradation and the reduction in ethidium bromide fluorescence observed in DOPE/DOPC/oleic acid lipopolyplexes (Lane 4, Figure 4.7b) does not appear to be related to a greater encapsulation of the polyplex DNA.

Cryo-electron microscopy studies of lipopolyplexes were inconclusive in determining their structure. It is perhaps significant that highly condensed DNA structures observed in polyplex preparations (Figure 3.1) were not observed in lipopolyplex preparations (Figure 4.8). No liposomes with a high electron density core were noted, this may possibly be observed following DNA encapsulation. It can be speculated that the condensed-DNA is associated in a thin layer with the lipid membrane rendering them both indistinguishable using this technique (e.g. Gustafsson *et al.*, 1995).

4.3.2 Physical properties of lipopolyplexes

Lipopolyplexes of each liposome composition tested appear to have similar structures. Lipopolyplexes retain the permeability to ^{14}C -glucose found in their component liposomes. The permeability barrier is however more leaky than that of untreated liposomes of identical composition. The extent of the increase in permeability is dependent on the exact liposome composition (Table 4.2). Liposome compositions containing cholesterol (40 mol%) that have a relatively low permeability coefficient, such as DLPE/cholesterol/oleic acid ($0.21 \pm 0.0027 \times 10^{-8}$), show the greatest increase in permeability upon addition of polyplexes (497%). More permeable cholesterol containing liposomes such as DOPE/cholesterol/oleic acid become more permeable, but to a lesser extent (164% increase). In absolute values however, DOPE/cholesterol/oleic acid lipopolyplexes are still twice as permeable as those prepared from DLPE/cholesterol/oleic acid liposomes. Liposomes of the formulation DMPE/DMPC/oleic acid undergo a dramatic increase in permeability following polyplex addition. In general it appears that the permeability of less permeable liposomes is more greatly affected by polyplexes.

The mechanism by which polyplexes render liposomes more permeable, in an apparently stable manner is not known. It has been determined that polycationic amino acids such as poly-L-lysine can cause leakage and fusion of anionic liposomes due to binding to their surface, possibly altering the lipid phase transitions (Epanand and Lim, 1995). Polyplexes containing poly-L-lysine could perhaps have similar structural effects on a lipid membrane rendering them more permeable.

The retention of a permeability barrier by the lipopolyplex is perhaps significant. A number of molecules could be entrapped in the aqueous core of the liposome and be co-delivered with the DNA during transfection (Wasan *et al.*, 1998). Any entrapped molecules would be retained during lipopolyplex formation. Such molecules could include nuclease inhibitors that would be delivered in a site-specific manner in high concentrations.

Summary

- Polyplexes probably adsorb onto the surface of anionic liposomes.

- Polyplexes are still susceptible to pronase degradation when associated with anionic liposomes.
- Lipopolyplexes are more permeable than their constituent liposomes.
- Lipopolyplexes maintain a permeability barrier to entrapped solutes.

Chapter 5

Transfection of Jurkat cells using Lipopolyplexes

5.1 Transfection rationale

A series of transfection experiments were designed to determine the properties of liposome-poly-L-lysine-DNA complexes (lipopolyplexes) necessary for high gene transfer activity in Jurkat cells. The effect the following variables/characteristics have on gene transfer activity were investigated:

- DNA condensing agent: degree of polymerisation of poly-L-lysine (PLK), comparison with poly-L-arginine (PLR) of defined chain length, condensing agent/DNA charge ratio, polyplex (poly-L-lysine (or poly-L-arginine)/DNA complex) size and charge.
- Liposome composition: acyl chain length and saturation of the phosphatidylethanolamine (PE) lipid, presence of cholesterol, presence of a phosphatidylcholine (PC) lipid.
- Lipopolyplex formulation: DNA/lipid weight ratio, size and surface charge.
- Targeting ligand: Specificity of gene transfer activity to the presence of a known cell surface receptor.

Liposomes of compositions described previously (4.1) were prepared with surface conjugated anti-CD3 antibodies (2.2.3.2). The liposomes were associated with polyplexes of various compositions to form lipopolyplexes, which were then used to transfect a CD3-expressing Jurkat cell line. Jurkat cells were used as a model for the transfection of human T cells.

The CD3 molecule makes an excellent target for receptor-targeted gene transfer for the following reasons: (i) CD3 molecules are present on the surface of >95% of circulating human peripheral T cells, 10-40,000 copies of the CD3 molecule are present on the surface of a mature T cell. (ii) Soluble antibodies binding to CD3 are readily endocytosed by the T cell. (iii) Turnover of the CD3/T cell receptor (TCR) complex from the cell surface (i.e. internalisation) involves up to 420,000 molecules/cell per 24h (Buschle *et al.*, 1995).

Each transfection experiment used a single DNA condensing agent. The condensing agent was used to form polyplexes having different PLK(R)/DNA charge ratios, which were subsequently mixed with liposomes at various DNA/lipid weight ratios to form lipopolyplexes. A reaction matrix of four charge ratios and four lipid ratios was chosen to generate sixteen lipopolyplex preparations per liposome formulation. The

four polyplex PLK(R)/DNA charge ratios chosen included that of a single anionic polyplex (DNA PO_4^- charges in excess) thought not to interact electrostatically with anionic liposomes, and three cationic polyplexes (poly-L-lysine NH_3^+ charges in excess) which would interact more favorably with the anionic liposomes. The range of lipid/DNA weight ratios chosen generated lipopolyplexes with either an overall positive surface charge, or a negative surface charge.

All experimental variables were compared in a single series of transfections to remove unknown variables such as lipopolyplex batch preparation and Jurkat cell batch. DNA concentration ($4\mu\text{g}/\text{transfection}$) and initial Jurkat cell number (1×10^6) remained constant throughout (see protocol 2.2.12).

The efficiency of gene transfer was assessed by assaying β -galactosidase enzyme production in the transfected cells. Any recombinant β -galactosidase gene expression would be derived from the transfected plasmid. The β -galactosidase expression was assayed from cell lysates and described in terms of picogrammes β -galactosidase/well. This term describes the total mass of β -galactosidase enzyme produced by the transfected cell population at the time of cell harvesting, 18h post-transfection. This figure is calculated from the raw data of pg/ml β -galactosidase enzyme in the cell lysate, generated by the enzyme assay procedure (2.2.12).

Extractable cellular protein content can be used to give an indication as to the number of cells present at the end of the transfection procedure. With comparison to a non-challenged control population of cells, any growth inhibitory effects of the lipopolyplexes (and to some extent cytotoxic effects) were ascertained. The effect of the transfection process on cell number of the transfected Jurkat cell population was assessed by monitoring the extractable cellular protein content of the cell lysates (2.2.12) (Yang and Huang, 1997).

A transfection positive control, a commercially available dendrimer preparation (SuperFect, Qiagen), was used throughout to determine that the target cells could express the transgene. The positive control had transfection activity of $>100\text{pg}/\text{well}$ β -galactosidase enzyme in each transfection experiment, using the transfection protocol described (2.2.12). Jurkat cells display no background recombinant β -galactosidase expression ($<6.4\text{pg}/\text{well}$) at the minimum detection limit for the β -galactosidase ELISA procedure.

The following series of experiments determined the optimal lipopolyplex formulation, ultimately for targeted transfection of Jurkat cells in the presence of 10% serum.

1. **Primary screen of liposome formulations**, gene transfer efficiency in serum free media. Determine which liposome formulations confer gene transfer activity. Lipopolyplexes were formed using poly-L-lysine of 99 amino acid chain length (PLK₉₉) as the DNA condensing agent. Polyplexes formed using poly-L-lysine of this chain length have been shown to have gene transfer activity *in vitro* by a number of groups (e.g. Wagner *et al.*, 1990, Perales *et al.*, 1994b; Erbacher *et al.*, 1997). Liposome formulations having no gene transfer activity were removed from subsequent screens.
2. **Secondary screen**. Using a reduced number of liposome formulations, repeat the transfection protocol using PLK₅, PLK₃₆, PLK₅₃₁ and poly-L-arginine (PLR₁₇) as DNA condensing agents.
3. **Using the previous screens, determine the optimum lipopolyplex formulations for Jurkat cell transfection in the absence of serum using the following parameters.** (i) Liposome composition. (ii) Condensing agent type/size. (iii) Ratios of charge (PLK(R)/DNA) and lipid/DNA. Show the specificity of the transfection to the CD3 epitope in the presence and absence of 10% serum in the transfection media.

5.2 Results

5.2.1 Primary screen of gene transfer activity of individual liposome formulations using PLK₉₉ as a DNA condensing agent

Five liposome formulations were prepared with conjugated anti-CD3 antibodies (Table 5.1). The liposome formulation DMPE/DMPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) described in Chapter 4, was not successfully coupled with the anti-CD3 antibody. Addition of reactive antibody to this liposome formulation caused aggregation of the liposomes into large precipitates. No transfection data was obtained using this preparation.

Table 5.1 Properties of anti-CD3 liposomes used in the primary transfection screen

Liposome formulation (40:39.9:20:0.1 mol%)	Particle diameter (nm) ^a	Zeta potential (mV) ^b	Antibody coupling ratio ($\mu\text{g}/\mu\text{mol}$)	Weight average Antibody molecules/liposome ^c	Liposome batch age (days) ^d
DOPE/cholesterol/oleic acid/DSPE-PEG/anti-CD3	124.1 ± 45	-46.8 ± 3.0	26	22	49
DOPE/DOPC/oleic acid/DSPE-PEG/anti-CD3	108.7 ± 39.9	-44.3 ± 3.6	21.7	13	49
DMPE/cholesterol/oleic acid/DSPE-PEG/anti-CD3	147.3 ± 36.3	-49.9 ± 0.5	16.98	28	42
DLPE/cholesterol/oleic acid/DSPE-PEG/anti-CD3	109 ± 33.2	**	21.106	10	47
DLPE/DLPC/oleic acid/DSPE-PEG/anti-CD3	108.7 ± 38.3	**	9.61	4.3	47

^a determined by PCS. \pm s.d. of unimodal distribution.

^b in 10mM HEPES pH8.0. Zeta potential calculated using $f(\kappa a)=1.3$. s.d. is for $n=5$.

^c calculated based on M_w of IgG=150,000 using D-LIPPRO computer program.

^d days stored at 4°C since preparation.

** not determined.

Polyplexes were formed using PLK₉₉. The PLK/DNA charge ratio at which neutral polyplexes are formed, and then aggregate, was determined ($\sim 1.4 \text{ NH}_3^+/\text{PO}_4^-$, data not shown). A single charge ratio below that of the aggregation point (0.8; PO_4^- in excess), and three above it (1.6, 3.3, and 4.9; NH_3^+ in excess), were chosen for polyplex formation. Lipopolyplexes were formed by the addition of liposomes at lipid/DNA weight ratios of 4, 8, 16 and 32 to each polyplex preparation (see 5.1).

5.2.1.1 DOPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer

Liposomes of the formulation DOPE/cholesterol/oleic acid have greater gene transfer activity than each of the other liposome formulations assayed using PLK₉₉ as the DNA condensing agent. Jurkat cells transfected using PLK₉₉ polyplexes (no lipid) display no β -galactosidase expression at each PLK/DNA charge ratio, showing the lipid component of the lipopolyplex is essential for successful transfection (data not shown). The lipopolyplex PLK/DNA charge ratio has a greater influence on the success of transfection than the total lipid content (Figure 5.1). Minimal β -galactosidase expression is noted in Jurkat cells transfected using lipopolyplexes with a PLK/DNA charge ratio of 0.8, at each lipid ratio. The highest β -galactosidase expression levels were observed in Jurkat cells transfected using lipopolyplexes having a PLK/DNA charge ratio 1.6, and lipid/DNA ratio of 8 (871pg/well). Jurkat cells transfected using lipopolyplexes having a lipid/DNA ratio of 16 and 32 and PLK/DNA charge ratio of 1.6 also display high levels of β -galactosidase expression. Gene transfer activity is lower using lipopolyplexes having a PLK/DNA charge ratio of greater than 1.6. Lipopolyplexes that aggregate before addition to the targeted cells, or during subsequent storage, have low gene transfer activity.

The extractable cellular protein content of a Jurkat cell population is reduced, compared to that of the control population, when transfected using lipopolyplexes having a PLK/DNA charge ratio of 1.6, 3.3, and 4.9 (Figure 5.2). Jurkat cell populations similarly show a lower extractable cellular protein content when transfected using PLK₉₉ polyplexes having a high PLK/DNA charge ratio (Figure 5.16), but display no detectable β -galactosidase expression. Increasing the lipid content of lipopolyplexes having a fixed PLK/DNA charge ratio, does not cause a

further decrease in the extractable cellular protein content of the transfected cell populations.

Lipopolyplex size was measured by PCS (2.2.6). Particle diameter was measured using 200µl of lipopolyplex not used in the transfection procedure. The particles had been stored at 25°C for 48h prior to measurement. Observations of particle aggregation during lipopolyplex formation, and at the time of the particle size measurement, were noted.

The particle diameters of the polyplexes that were used in the preparation of the lipopolyplexes are shown in Figure 5.15. Polyplex diameter decreases with an increase in PLK₉₉ content. The lipopolyplexes are each of greater diameter than the component liposomes ($124.1 \pm 45\text{nm}$). The measured diameter of the lipopolyplex is not simply the sum of the diameters of the liposome and the polyplex. At a PLK/DNA charge ratio of 0.8, lipopolyplex diameter decreases with an increase in lipid/DNA ratio. Lipopolyplexes aggregate when prepared at a single lipid/DNA ratio-PLK/DNA charge ratio combination. This can be observed at the PLK/DNA charge ratios of 1.6, 3.3, and 4.9. This aggregation process indicates the formation of electroneutral lipopolyplexes. The aggregation point is at a high lipid/DNA ratio when the PLK/DNA charge ratio of the lipopolyplex is increased. Particle diameters of non-aggregating lipopolyplexes are greater if their lipid/DNA ratio-PLK/DNA charge ratio combination is similar to that of aggregating lipopolyplexes (Figure 5.3).

5.2.1.2 DOPE/DOPC/oleic acid anti-CD3 liposome mediated gene transfer

Minimal gene transfer activity is shown by lipopolyplexes prepared using DOPE/DOPC/oleic acid liposomes at each of the assayed charge and lipid ratios (Figure 5.4). Lipopolyplexes having a PLK/DNA charge ratio of 0.8 (anionic polyplexes) show similar gene transfer activity to those with higher charge ratios (cationic polyplexes). In contrast, gene transfer activity of DOPE/cholesterol/oleic acid liposomes is far greater using lipopolyplexes having a PLK/DNA charge ratio >0.8 (Figure 5.1). Lipopolyplex aggregation does not have an inhibitory effect on gene transfer activity when using DOPE/DOPC/oleic acid liposomes.

Extractable cellular protein content of Jurkat cell populations is greatly reduced when transfected using lipopolyplexes having a high PLK/DNA charge ratio. The

extractable cellular protein content of Jurkat cells transfected using lipopolyplexes having PLK/DNA charge ratio of 0.8 and 1.6 are similar to those of the control population of cells. In Jurkat cell populations transfected using lipopolyplexes having a PLK/DNA charge ratio of 3.3 or 4.9, lower extractable cellular protein contents are observed (Figure 5.5).

Particle diameter measurements show lipopolyplex size to be greater than that of the component liposomes ($108 \pm 39.9\text{nm}$) (Figure 5.6). No lipopolyplex aggregation is noted using the PLK/DNA charge ratio of 0.8, particle diameter is smaller at high lipid/DNA ratios. Particle diameter of lipopolyplexes having a PLK/DNA ratio of 4.9 increases markedly at high lipid/DNA ratios, but no particle aggregation is noted. Particle diameters of lipopolyplexes of equivalent lipid/DNA and PLK/DNA charge ratio are similar to those prepared using DOPE/cholesterol/oleic acid liposomes.

5.2.1.3 DMPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer

DMPE/cholesterol/oleic acid lipopolyplexes have gene transfer activity when prepared at PLK/DNA charge ratios of 3.3 or 4.9. However, the level of β -galactosidase expression observed in the transfected Jurkat cells is 80-fold less than that obtained using the optimum lipopolyplex formulation prepared from DOPE/cholesterol/oleic acid liposomes using the PLK₉₉ DNA condensing agent (Figure 5.7 and Figure 5.1). Lipopolyplex preparations containing aggregates confer similar levels of β -galactosidase expression to transfected Jurkat cells as colloidal preparations

Jurkat cell populations transfected using lipopolyplexes having a high PLK/DNA charge ratio generally display lower extractable cellular protein contents than those transfected using low charge ratio lipopolyplexes (Figure 5.8).

Lipopolyplexes are of similar size to the component liposomes ($147.3 \pm 36.3\text{nm}$). Larger particle diameters are observed at, and close to, the PLK/DNA charge ratio-lipid/DNA ratio combination that causes lipopolyplex aggregation (Figure 5.9).

5.2.1.4 DLPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer

Jurkat cells transfected using DLPE/cholesterol/oleic acid lipopolyplexes display no β -galactosidase expression above the minimum detectable limit of expression. This is true of each PLK/DNA and lipid/DNA ratio tested (Figure 5.10). Lipopolyplexes of formulations 0.8 charge ratio/32 lipid ratio and 3.3 charge ratio/32 lipid ratio were not tested.

The extractable cellular protein contents of Jurkat cell populations are lower when lipopolyplexes having a high PLK/DNA charge ratio are used for transfection (Figure 5.11).

Lipopolyplex size is greater than that of the component liposomes ($109 \pm 33.2\text{nm}$) (Figure 5.12).

5.2.1.5 DLPE/DLPC/oleic acid anti-CD3 liposome mediated gene transfer

A smaller number of lipopolyplex compositions were tested using the DLPE/DLPC/oleic acid liposome formulation. Four PLK/DNA charge ratios were used, but at only in combination with one lipid/DNA ratio each. No β -galactosidase expression was detected in Jurkat cells transfected using each lipopolyplex composition tested (data not shown).

The extractable cellular protein content of Jurkat cell populations is lower when transfected with lipopolyplexes having a high PLK/DNA charge ratio (Figure 5.13).

DLPE/DLPC/oleic acid lipopolyplex sizes are shown in Figure 5.14.

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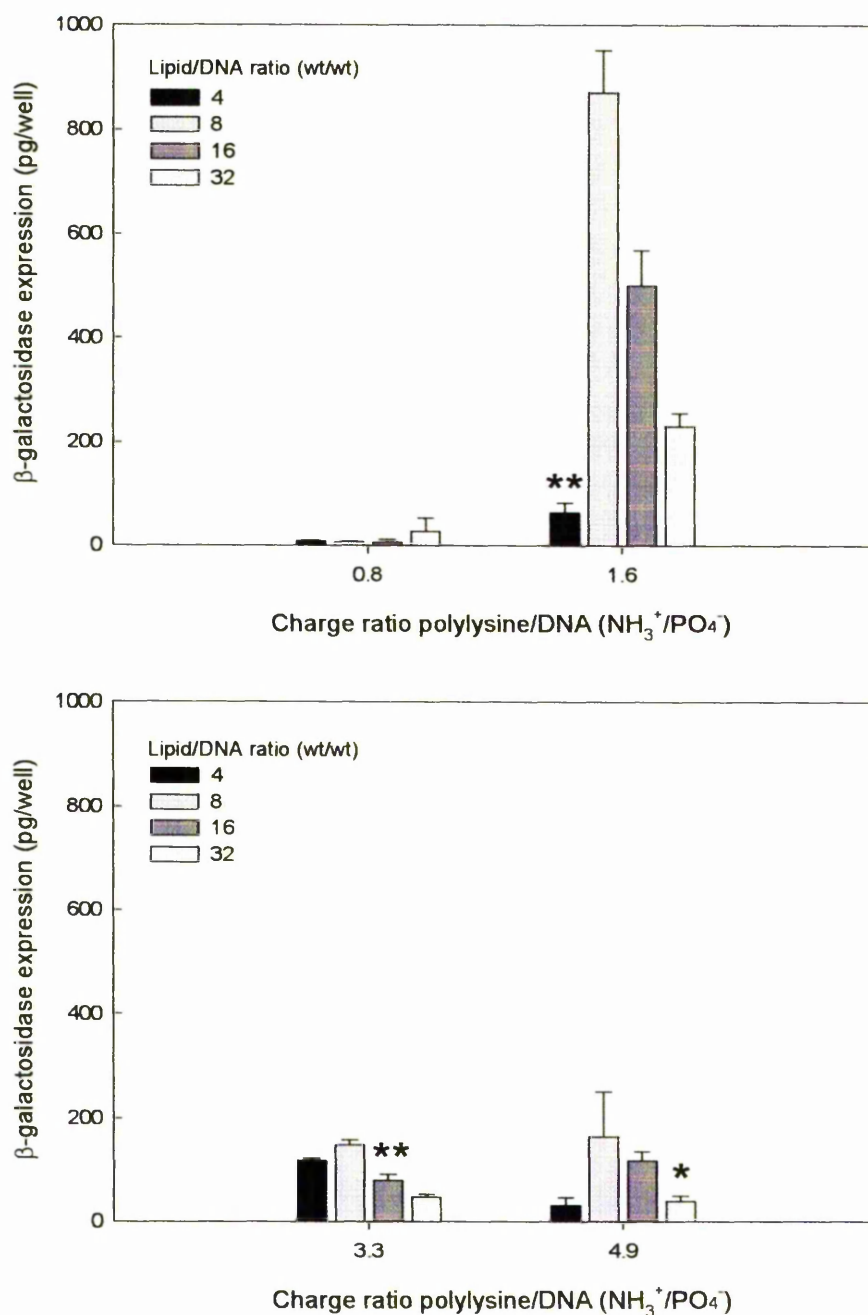


Figure 5.1 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of **DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** prepared using **PLK₉₉-condensed pEGlacZ**, on gene transfer activity in Jurkat cells (antibody coupling ratio 26 $\mu\text{g}/\mu\text{mol}$ lipid). β -galactosidase concentration in cell lysates was assayed 18h post-transfection.*- lipopolyplexes formed visible aggregates before addition to cells.**- lipopolyplexes formed visible aggregates after 48h storage at 25°C. Data show mean \pm s.d. (n=3).

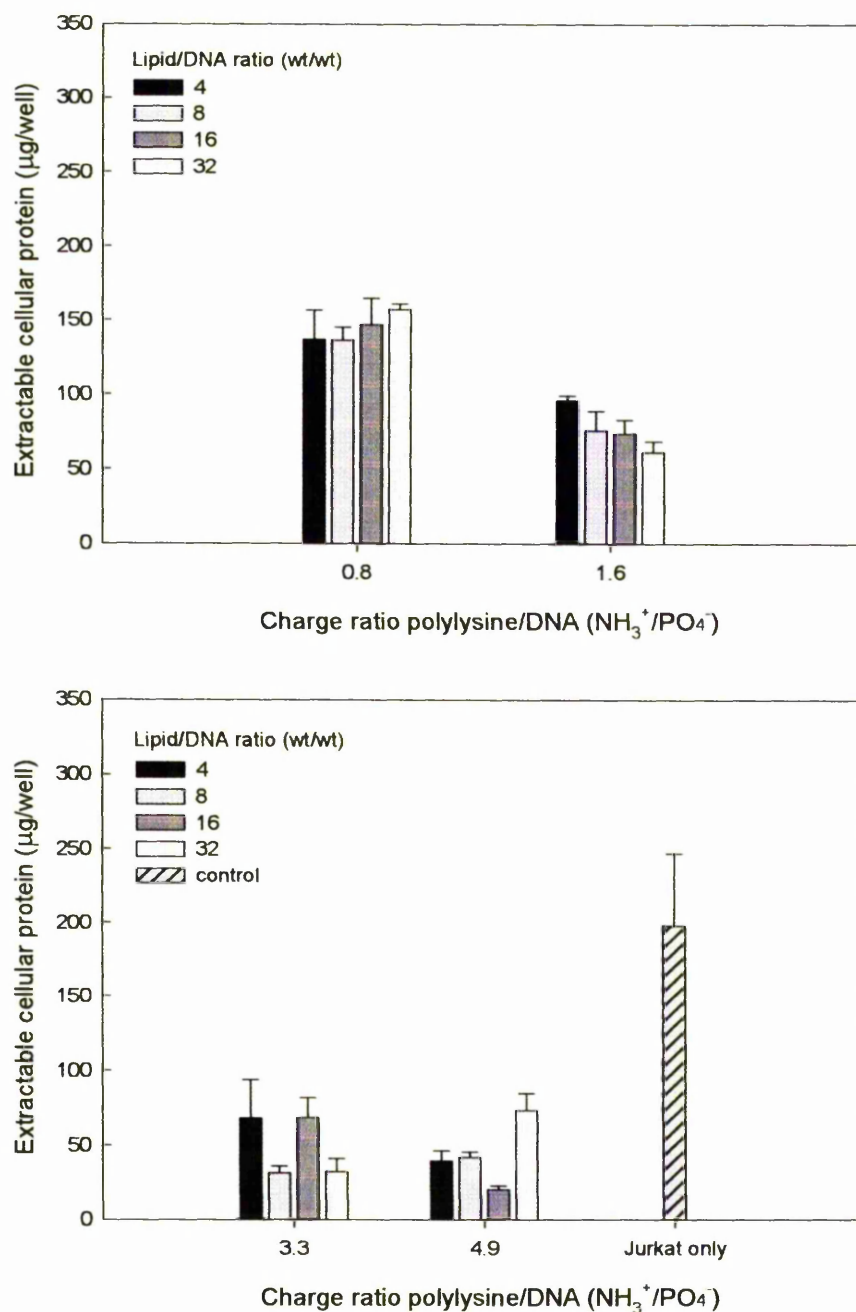


Figure 5.2 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₉₉-condensed pEGlacZ (see Figure 5.1). Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

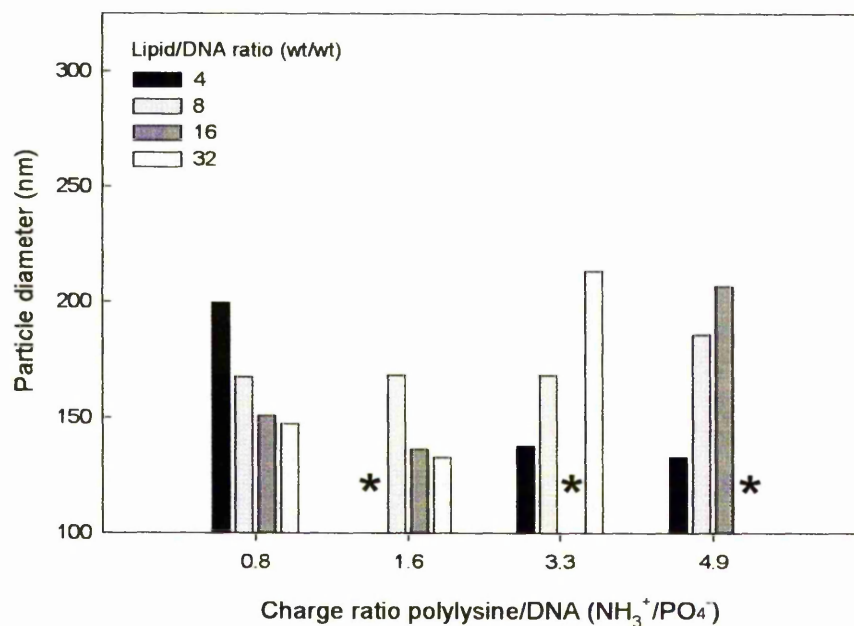


Figure 5.3 Particle diameters of **DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** used for the transfection of Jurkat cells (see Figure 5.1). Size determined by PCS 48h post-transfection. *-visible aggregates present, not suitable for PCS measurement ($>2\mu\text{m}$). Diameter of component liposomes-124.1nm.

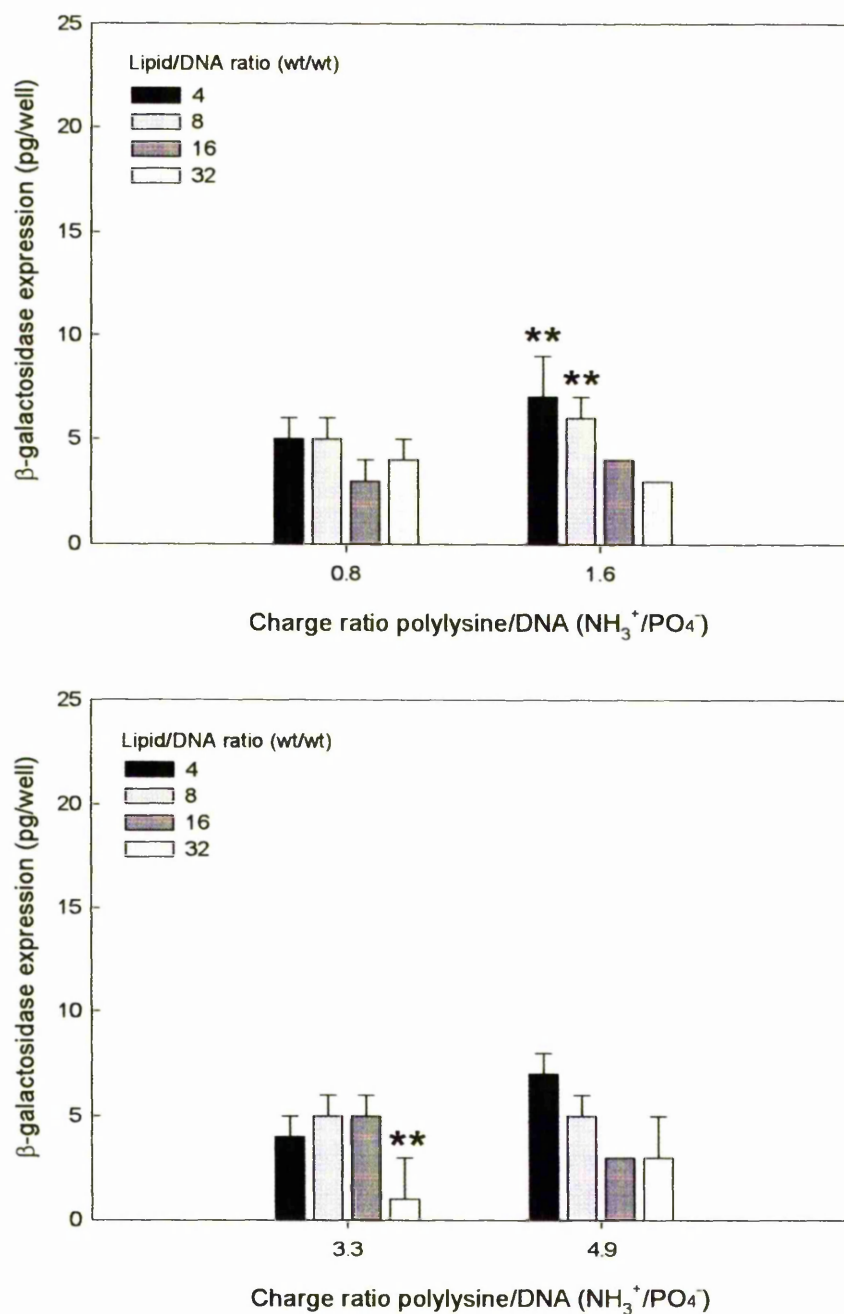


Figure 5.4 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₉₉-condensed pEGlacZ, on gene transfer activity in Jurkat cells (antibody coupling ratio 21.7 $\mu\text{g}/\mu\text{mol}$ lipid). β -galactosidase concentration in cell lysates was assayed 18h post-transfection. *- lipopolyplexes formed visible aggregates before addition to cells. **- lipopolyplexes formed visible aggregates after 48h storage at 25°C. Data show mean \pm s.d. (n=3). Note change of scale of y-axis compared to Figure 5.1.

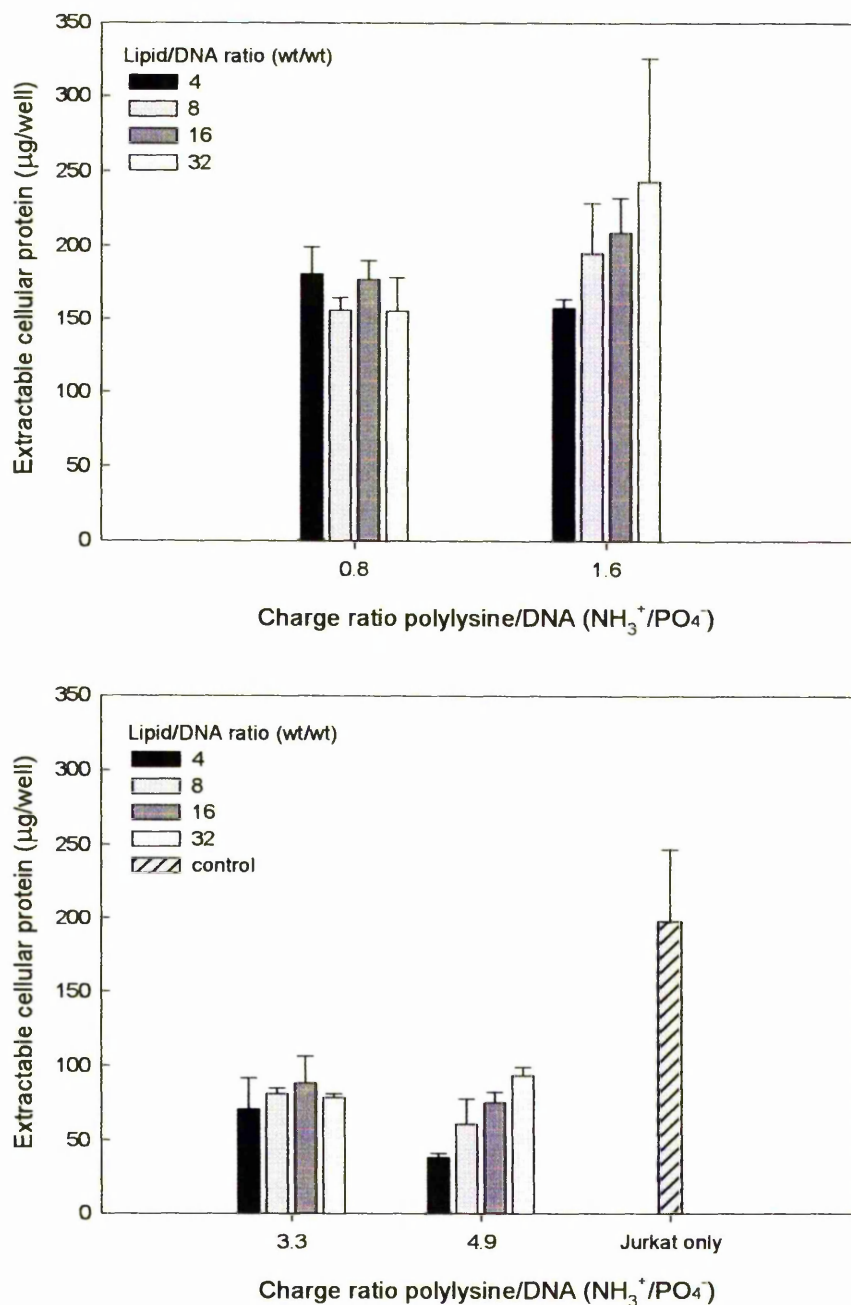


Figure 5.5 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₉₉-condensed pEGlacZ (see Figure 5.4). Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

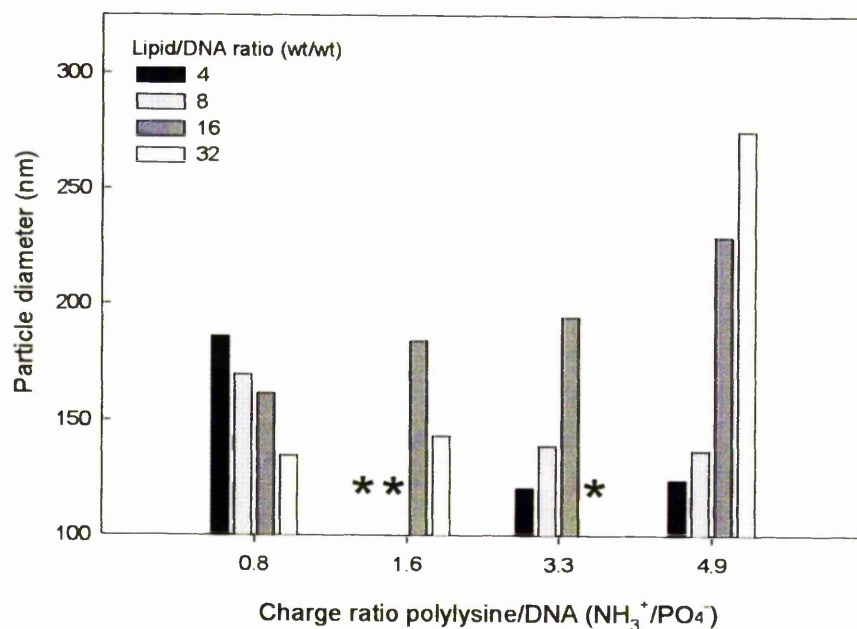


Figure 5.6 Particle diameters of **DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** used for the transfection of Jurkat cells (see Figure 5.4). Size determined by PCS 48h post-transfection. *-visible aggregates present, not suitable for PCS measurement ($>2\mu\text{m}$). Diameter of component liposomes-108nm.

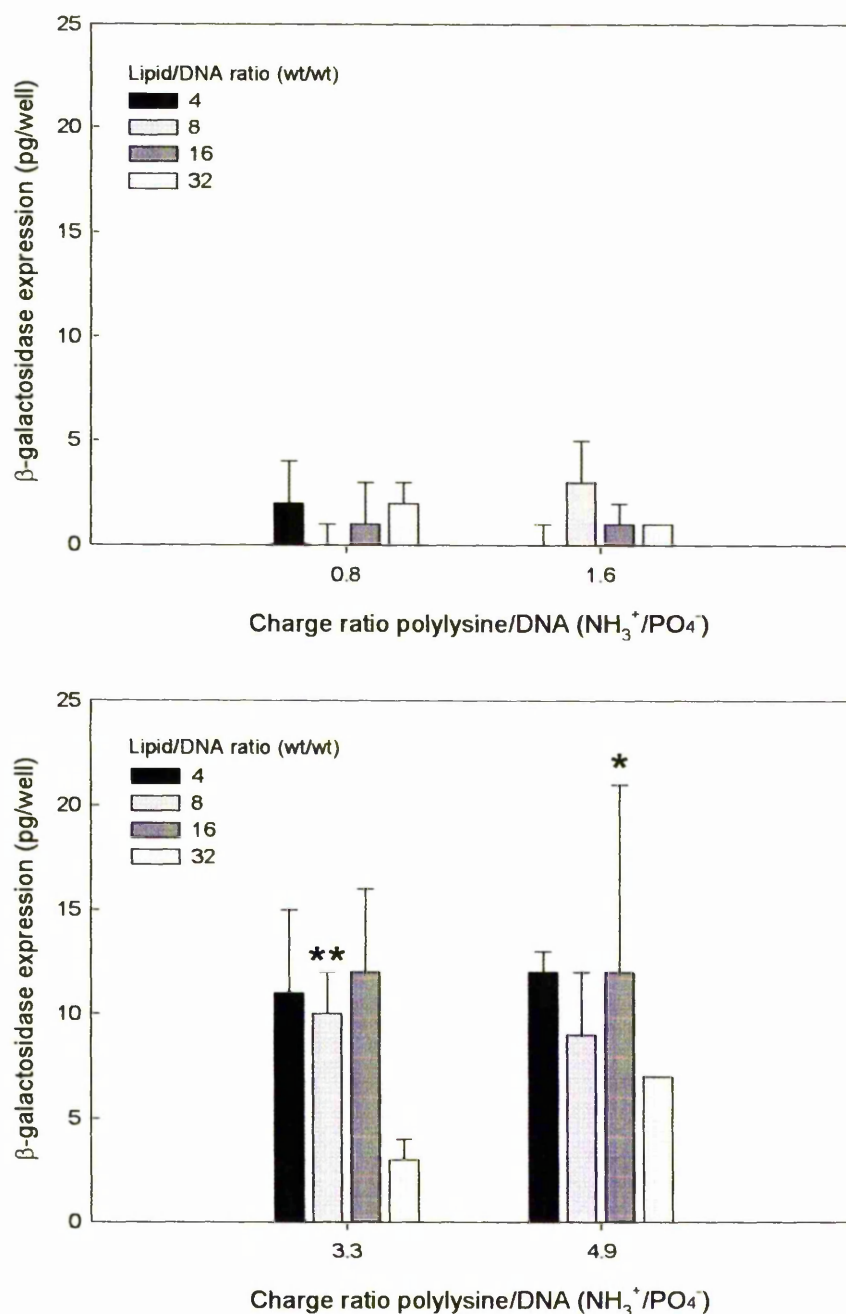


Figure 5.7 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₉₉-condensed pEGlacZ, on gene transfer activity in Jurkat cells (antibody coupling ratio 16.98 $\mu\text{g}/\mu\text{mol}$ lipid). β -galactosidase concentration in cell lysates was assayed 18h post-transfection.*- lipopolyplexes formed visible aggregates before addition to cells.**- lipopolyplexes formed visible aggregates after 48h storage at 25°C. Data show mean \pm s.d. (n=3). Note change of scale of y-axis compared to Figure 5.1.

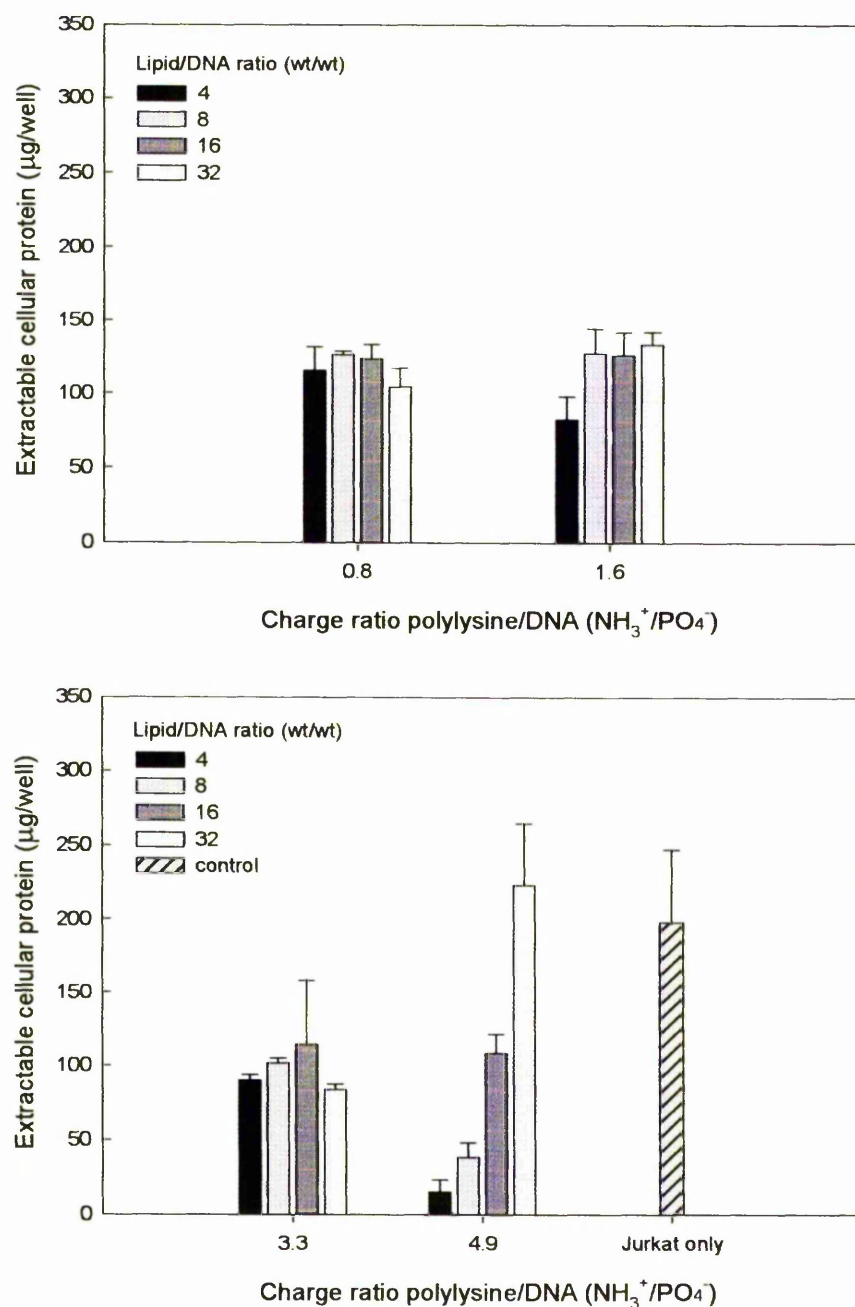


Figure 5.8 Extractable cellular protein content of Jurkat cell populations transfected using DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₉₉-condensed pEGlucZ (see Figure 5.7). Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

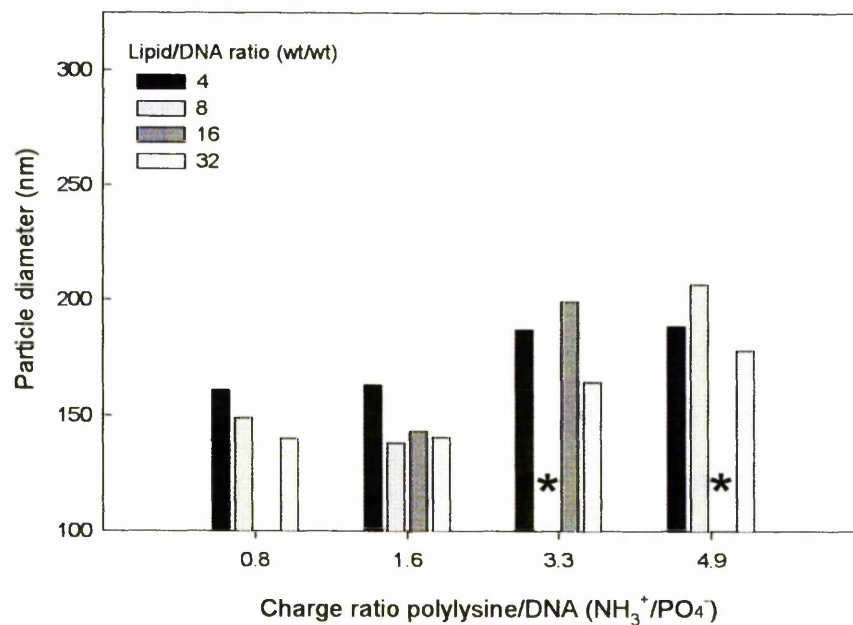


Figure 5.9 Particle diameters of **DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** used for the transfection of Jurkat cells (see Figure 5.7). Size determined by PCS 48h post-transfection. *-visible aggregates present, not suitable for PCS measurement (>2 μ m). Diameter of component liposomes-147.3nm.

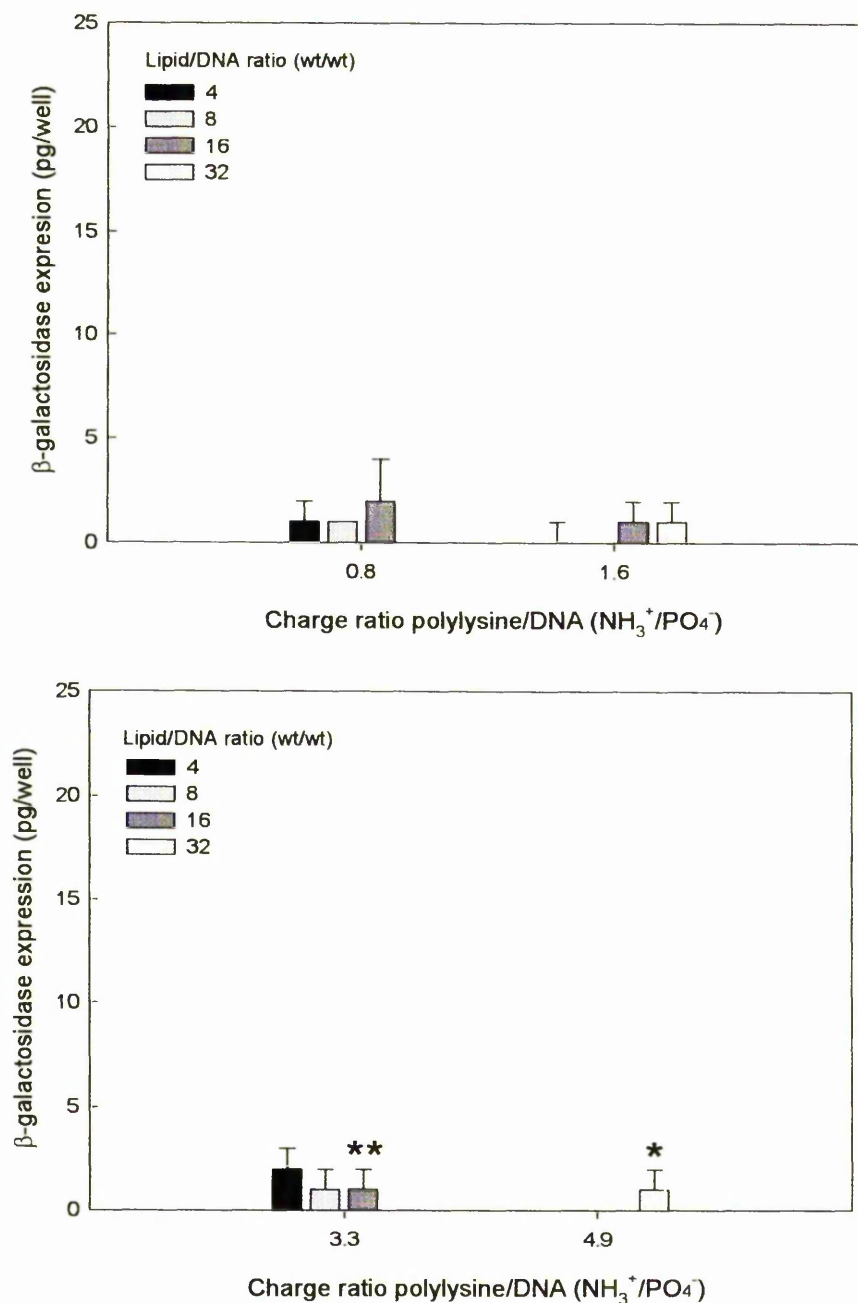


Figure 5.10 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DLPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₉₉-condensed pEGlacZ, on gene transfer activity in Jurkat cells (antibody coupling ratio 21.106 $\mu\text{g}/\mu\text{mol}$ lipid). β -galactosidase concentration in cell lysates was assayed 18h post-transfection.*- lipopolyplexes formed visible aggregates before addition to cells.**- lipopolyplexes formed visible aggregates after 48h storage at 25°C. Data show mean \pm s.d. (n=3). Note change of scale of y-axis compared to Figure 5.1.

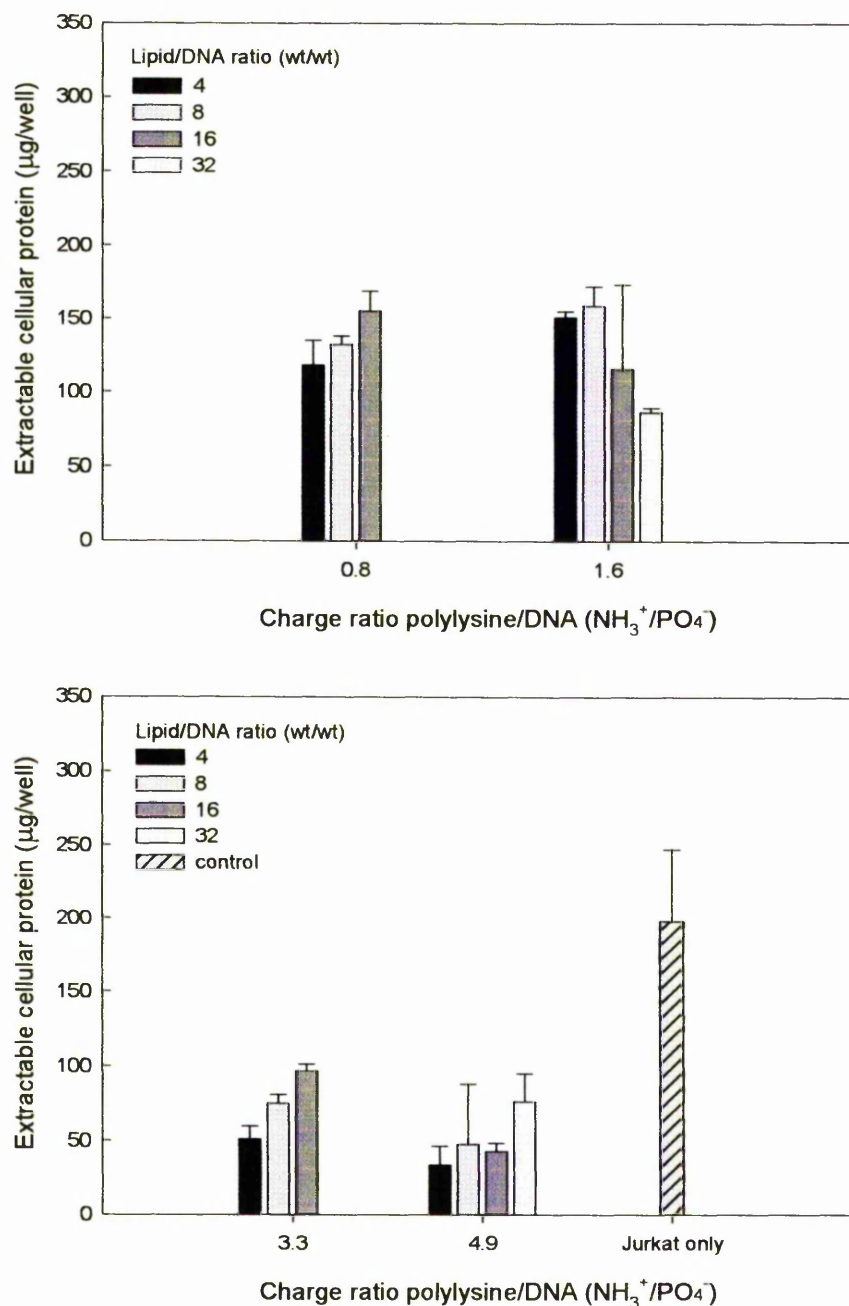


Figure 5.11 Extractable cellular protein content of Jurkat cell populations transfected using DLPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₉₉-condensed pEGlacZ (see Figure 5.10). Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

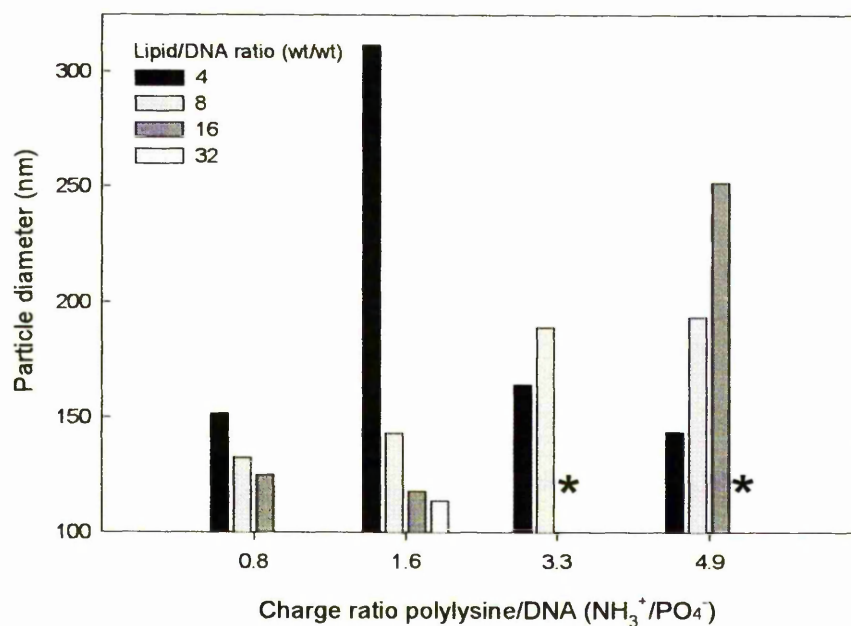


Figure 5.12 Particle diameters of **DLPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** used for the transfection of Jurkat cells (see Figure 5.10). Size determined by PCS 48h post-transfection. *-visible aggregates present, not suitable for PCS measurement ($>2\mu\text{m}$). Diameter of component liposomes-109nm.

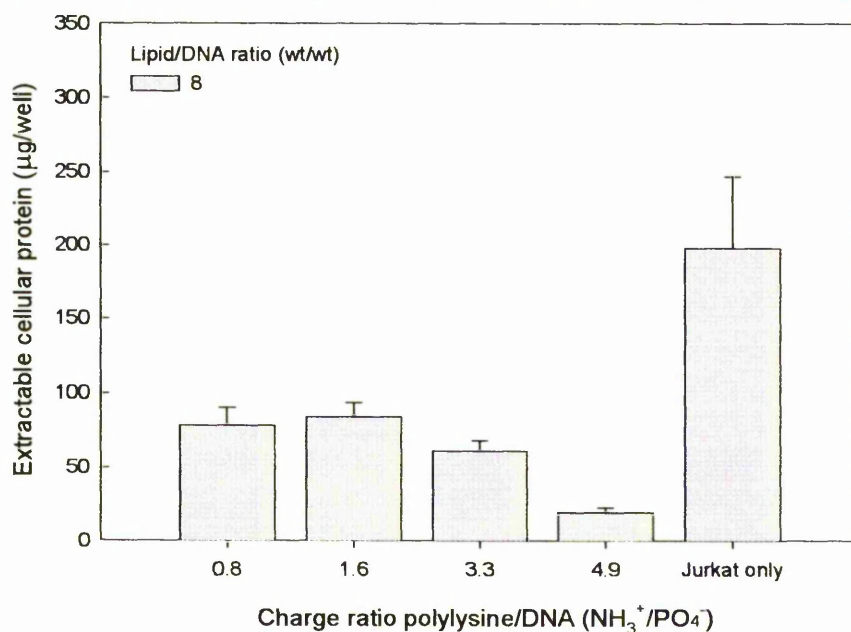


Figure 5.13 Extractable cellular protein content of Jurkat cell populations transfected using **DLPE/DLPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** prepared using **PLK₉₉-condensed pEGlacZ**. Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

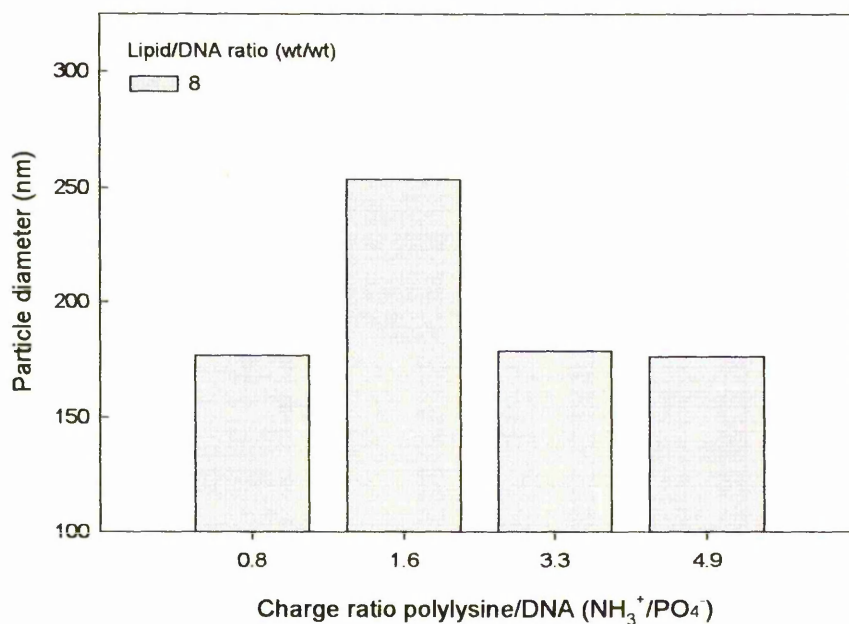


Figure 5.14 Particle diameters of **DLPE/DLPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** used for the transfection of Jurkat cells. Size determined by PCS 48h post-transfection. Diameter of component liposomes-108.7nm.

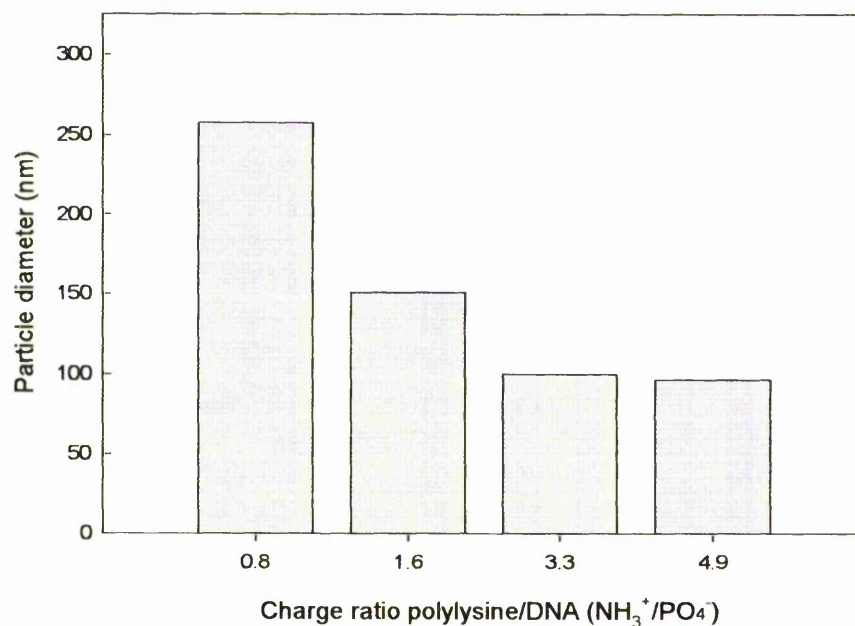


Figure 5.15 Particle diameters of PLK_{99} -polyplexes used in the preparation of lipopolyplexes for transfection of Jurkat cells. Size determined by PCS 48h post-transfection.

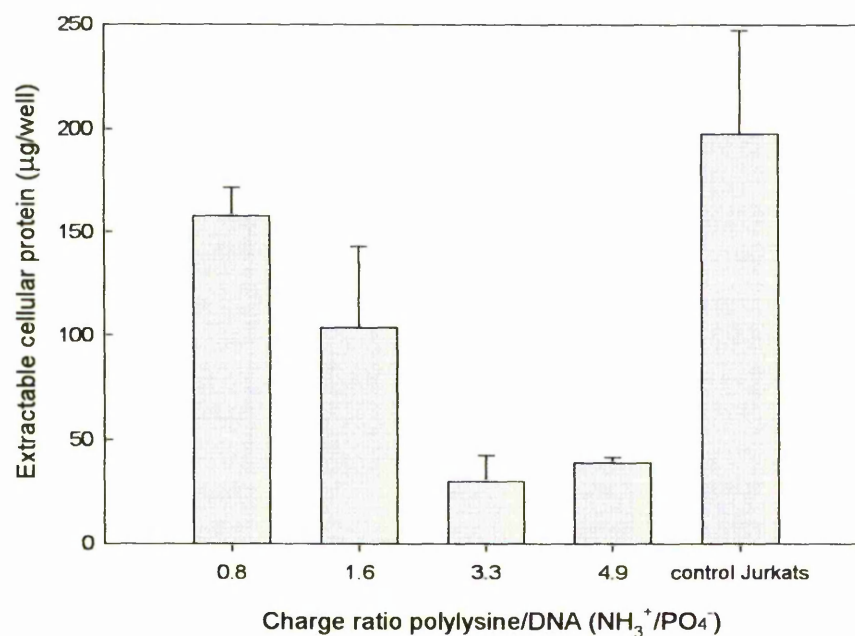


Figure 5.16 The effect of PLK_{99} -polyplex charge ratio on the extractable cellular protein content of polyplex-transfected Jurkat cell populations. Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with polyplexes. Data show mean \pm s.d. ($n=3$).

5.2.2 Secondary screen of liposome formulations for gene transfer activity

Three liposome formulations were used to transfect Jurkat cells with a further four DNA condensing agents (Table 5.2). Table 5.3 shows the condensing agents used, and the charge ratios chosen for the formation of the polyplexes.

Low \overline{M}_w poly-L-lysine (PLK₅) shows weaker DNA condensation properties in 10mM HEPES pH8.0/5% glucose than in diH₂O (Figure 5.17). Plank *et al.* (1999) state that poly-L-lysines of less than 30 residues have decreased DNA binding with an increase in salt concentration, but that PLK>30 residues are not affected. A polyplex with a seven-fold greater charge ratio is necessary to produce a 200nm particle in 10mM HEPES pH8.0/5% glucose compared to those formed in diH₂O (see Figure 3.2b). Therefore PLK₅ was used at three charge ratios producing not only a cationic complex, but also one with small particle size.

Table 5.2 Properties of anti-CD3 liposomes used in the secondary transfection screen

Liposome formulation (40:39.9:20:0.1 mol%)	Particle diameter (nm) ^a	Zeta potential (mV) ^b	Antibody coupling ratio (μ g/ μ mol)	Weight average Antibody molecules/liposome ^c	Liposome batch age (days) ^d
DOPE/cholesterol/oleic acid/-DSPE-PEG/anti-CD3	125.3 \pm 47.9	-60.8 \pm 0.9	34.2	30	4
DOPE/DOPC/oleic acid/DSPE-PEG/anti-CD3	86.9 \pm 34.1	-42.5 \pm 6.1	25.91	13	4
DMPE/cholesterol/oleic acid/DSPE-PEG/anti-CD3	105.6 \pm 33.5	-54.0 \pm 1.8	18.21	13	4

^a determined by PCS. \pm s.d. of unimodal distribution.

^b in 10mM HEPES pH8.0. Zeta potential calculated using $f(\kappa a)=1.3$. s.d. is for n=5.

^c calculated based on M_w of IgG=150,000 using D-LIPPRO computer program.

^d days stored at 4°C since preparation.

Liposomes of formulations DLPE/cholesterol/oleic acid/DSPE-PEG and DLPE/DLPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) were not used in the

secondary screen as they display no gene transfer activity using PLK₉₉ as a DNA condensing agent using each lipopolyplex formulation.

Sizes of the lipopolyplexes used in the transfections were not recorded.

Table 5.3 Properties of polyplexes used in the secondary transfection screen

DNA condensing agent	Polyplex aggregation point (NH ₃ ⁺ /PO ₄ ⁻) ^b	Charge ratio of anionic polyplexes (NH ₃ ⁺ /PO ₄ ⁻)	Charge ratios of cationic polyplexes (NH ₃ ⁺ /PO ₄ ⁻)	Lipid ratios (lipid/DNA, wt/wt) ^c
PLK ₅	~8.1	0.8	35.8, 39, 42.4	4, 8, 16, 32
PLK ₃₆	~1.63	0.8	2.5, 3.3, 4.9	4, 8, 16, 32
PLK ₅₃₁	<1.63	0.8	1.6, 3.3, 4.9	4, 8, 16, 32
PLR ₁₇ ^a	0.75-1	0.5	1.5, 2, 3	4, 8, 16, 32

^a poly-L-arginine 17a.a.

^b in 10mM HEPES pH8.0.

^c in lipopolyplexes.

5.2.2.1 PLK₅ DNA condensing agent

5.2.2.1.1 DOPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer

DOPE/cholesterol/oleic acid was the only liposome formulation tested to show gene transfer activity using PLK₅ as the DNA condensing agent in the lipopolyplex (Figure 5.18). The highest levels of β -galactosidase expression observed in transfected Jurkat cells were three-fold greater than those observed using PLK₉₉ as the DNA condensing agent. High levels of gene transfer were observed using lipopolyplexes where no particle aggregation was seen to occur prior to the transfection. No gene transfer activity was observed using lipopolyplexes having a PLK/DNA charge ratio of 0.8. Polyplexes of PLK₅ and plasmid DNA do not transfect Jurkat cells at each of the four PLK/DNA charge ratios tested (data not shown).

Jurkat cell populations transfected using lipopolyplexes having a PLK/DNA charge ratio of 0.8 have a similar extractable cellular protein content as the control Jurkat cell population. However, Jurkat cell populations transfected using lipopolyplexes having high PLK/DNA charge ratios have extractable cellular protein contents, on average 65% of that of the control population. Jurkat cell populations transfected using lipopolyplexes having these high PLK/DNA charge ratios display high levels of β -galactosidase expression. A higher extractable cellular protein content is not noted however, in Jurkat cell populations transfected using aggregated lipopolyplexes, also having high PLK/DNA charge ratios, where low β -galactosidase expression is observed (Figure 5.19). Jurkat cell populations transfected using PLK₅ polyplexes show similar extractable cellular protein contents at each PLK/DNA charge ratio (Figure 5.37).

5.2.2.1.2 DOPE/DOPC/oleic acid anti-CD3 liposome and DMPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer

These liposome formulations have no gene transfer activity in lipopolyplexes prepared using PLK₅ as the DNA condensing agent (data not shown). Extractable

cellular protein contents of all the transfected Jurkat cell populations are similar to those of the control population of Jurkat cells (Figures 5.20, 5.21).

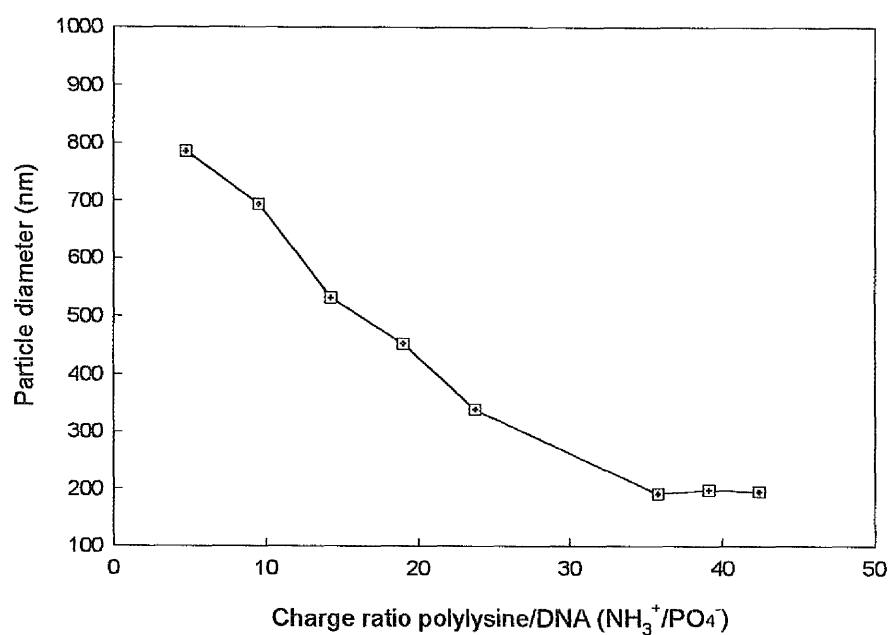


Figure 5.17 The effect of polylysine/DNA charge ratio on the diameter of **PLK₅**-polyplexes prepared in 10mM HEPES pH8.0/5% glucose.

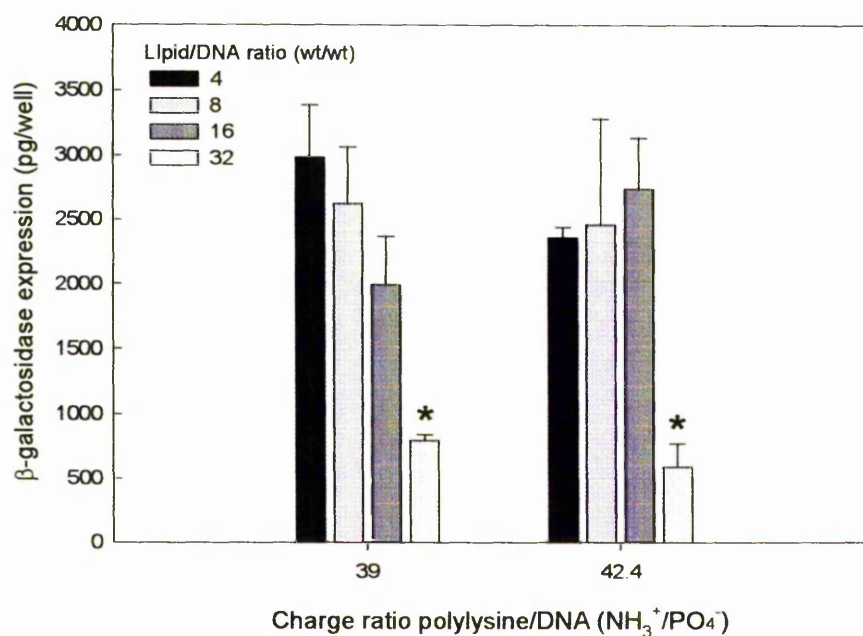
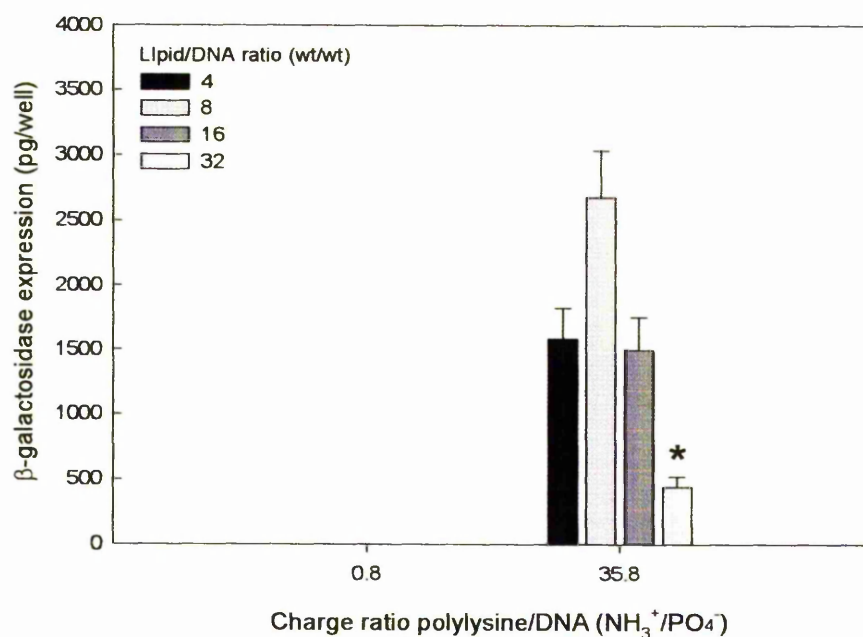


Figure 5.18 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₅-condensed pEGlacZ, on gene transfer activity in Jurkat cells (antibody coupling ratio 34.2 $\mu\text{g}/\mu\text{mol}$ lipid). β -galactosidase concentration in cell lysates was assayed 18h post-transfection.*- lipopolyplexes formed visible aggregates before addition to cells. Data show mean \pm s.d. (n=3).

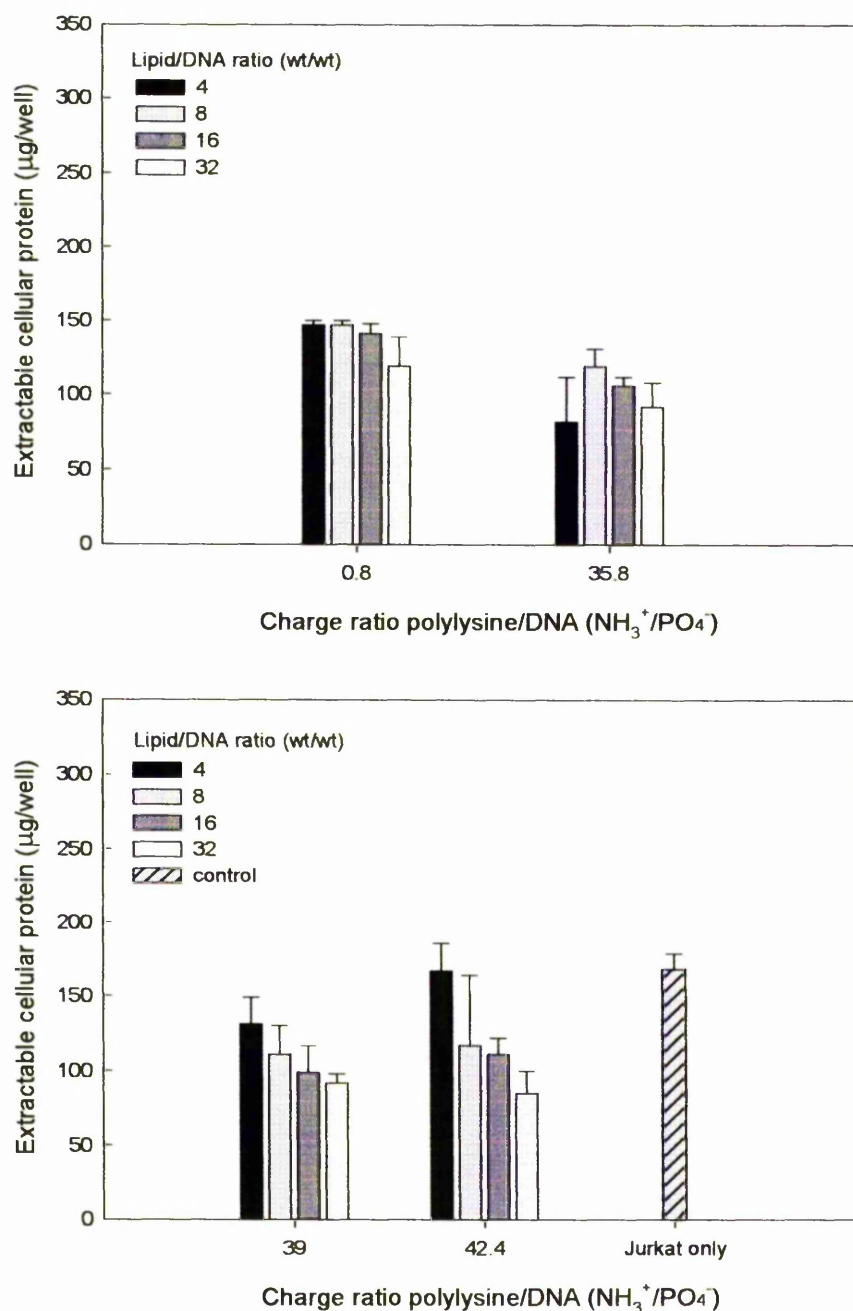


Figure 5.19 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₅-condensed pEGlacZ (see Figure 5.18). Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

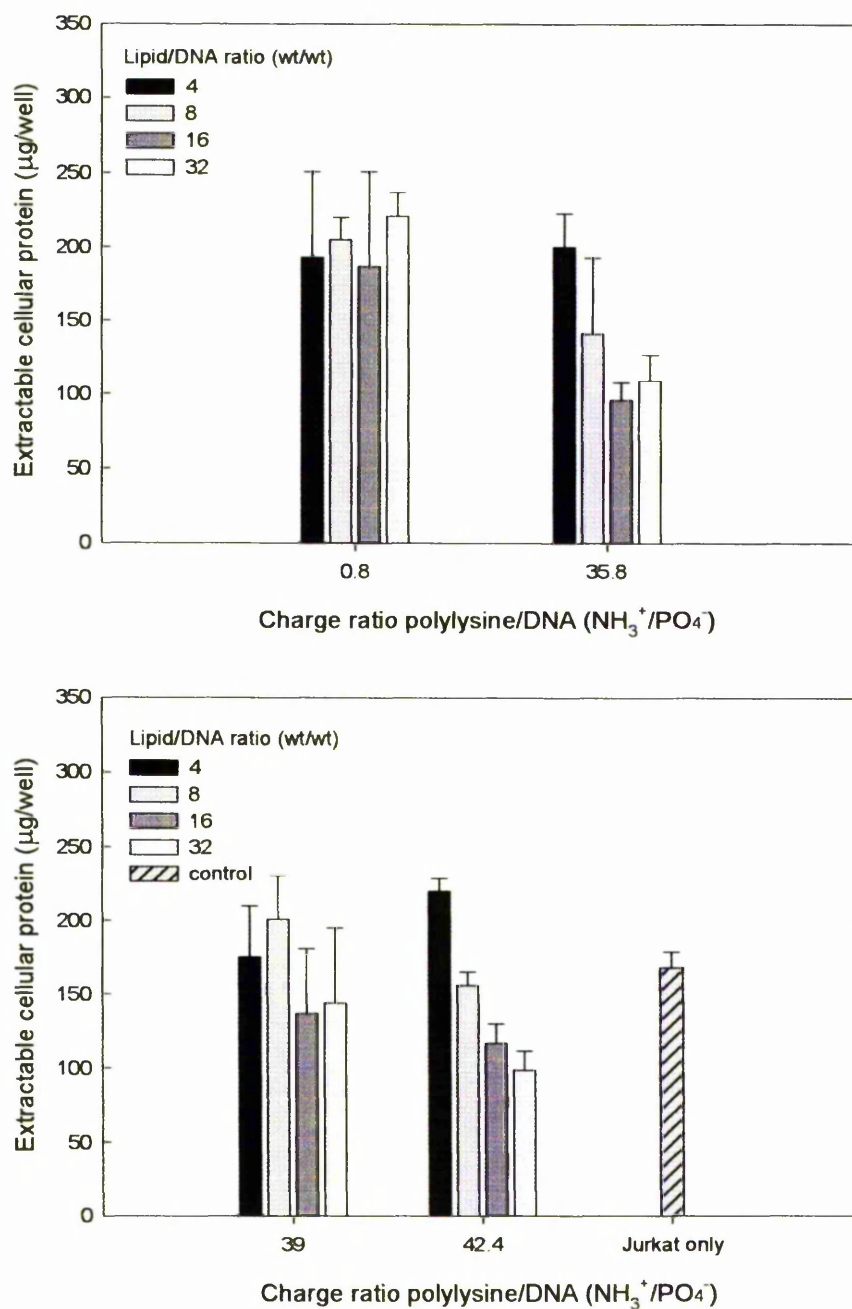


Figure 5.20 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₅-condensed pEGlacZ. Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

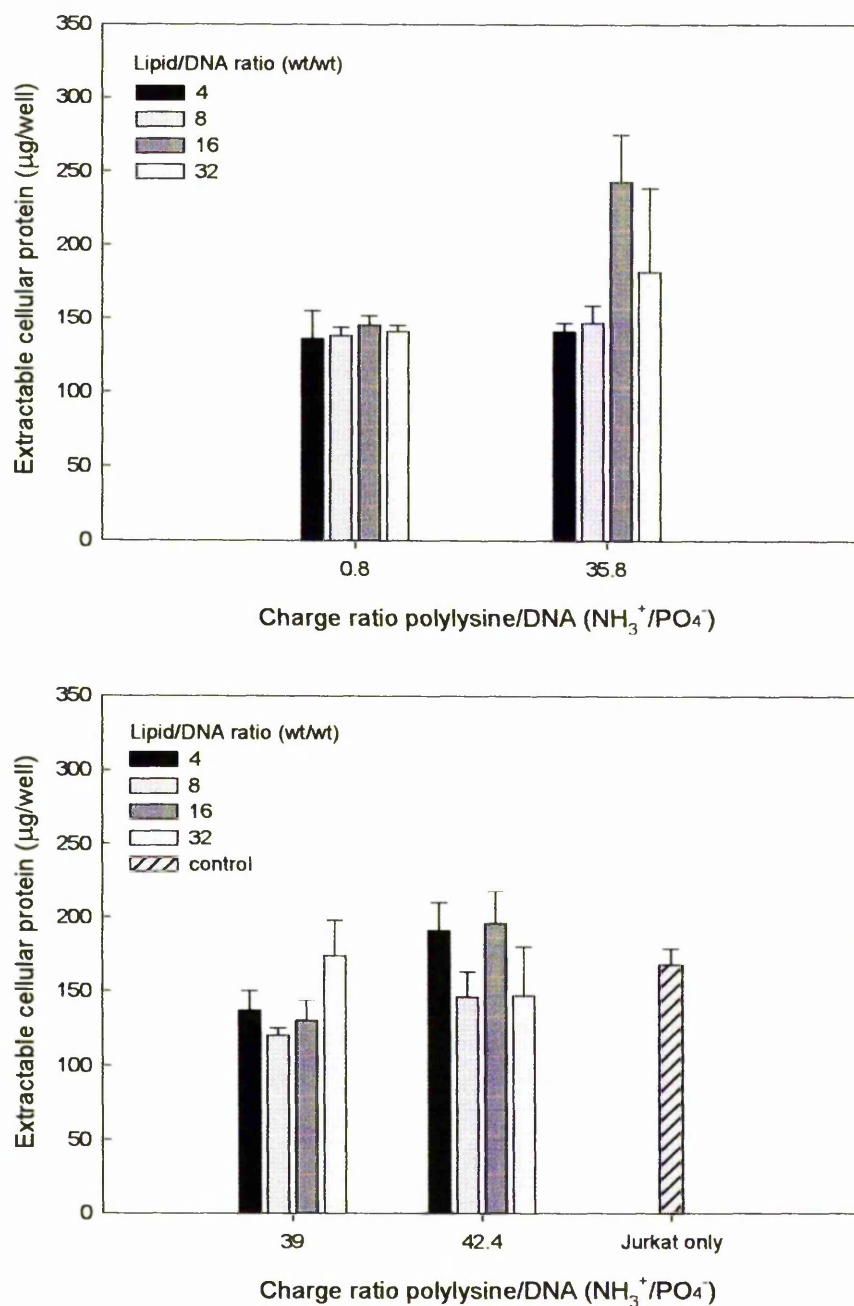


Figure 5.21 Extractable cellular protein content of Jurkat cell populations transfected using DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₅-condensed pEGlacZ. Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

5.2.2.2 PLK₃₆ DNA condensing agent

5.2.2.2.1 DOPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer

DOPE/cholesterol/oleic acid were the only liposome formulation tested to show gene transfer activity using PLK₃₆ as the DNA condensing agent in the lipopolyplex (Figure 5.22). Jurkat cells transfected using lipopolyplexes having a PLK/DNA charge ratio of 2.5, 3.3 or 4.9 display high levels of β -galactosidase expression. The highest levels of β -galactosidase expression were observed in Jurkat cells transfected using lipopolyplexes having a PLK/DNA charge ratio 4.9 and a lipid/DNA ratio of 4 or 8 (2953pg/well and 2854pg/well respectively). Maximum β -galactosidase expression is similar to the maximum expression observed using PLK₅ DNA condensing agent, and three-fold greater than the maximum using PLK₉₉ or PLK₅₃₁. At each lipopolyplex PLK/DNA charge ratio distinct peaks of high β -galactosidase expression are noted at a single lipid/DNA ratio (2 peaks at a lipopolyplex PLK/DNA charge ratio of 4.9). A similar trend can be seen using PLK₉₉ lipopolyplexes having a charge ratio of 1.6, and PLK₅ lipopolyplexes having a charge ratio of 35.8.

The extractable cellular protein content of a Jurkat cell population is lower when a lipopolyplex having a high PLK/DNA charge ratio is used for transfection (Figure 5.23). Jurkat cell populations transfected using PLK₃₆ polyplexes have similar extractable cellular protein contents at each charge ratio (Figure 5.38). The extractable cellular protein content of Jurkat populations transfected using lipopolyplexes having an identical PLK/DNA charge ratio, but different lipid/DNA ratios, are similar.

Jurkat cells transfected using PLK₃₆ polyplexes show no β -galactosidase expression at each PLK/DNA charge ratio, demonstrating that the lipid component is essential for gene transfer (data not shown). Jurkat cell populations transfected using PLK₃₆ polyplexes having a high charge ratio have a slightly lower extractable cellular protein content than those transfected with polyplexes having a low charge ratio (77% of the control population extractable cellular protein content at a charge ratio of 0.8, 68% of the control population extractable cellular protein content at a charge ratio of 4.9) (Figure 5.38). Jurkat cell populations transfected using each of the lipopolyplexes having a PLK/DNA charge of 0.8 have extractable cellular protein contents that are

not significantly different from those transfected using a PLK₃₆ polyplex also having a charge ratio of 0.8 (73% and 78% of the control cell population extractable cellular protein content respectively; ANOVA analysis Dunnett's test, $F=3.032$, $P>0.05$). Conversely, Jurkat cell populations transfected using each of the lipopolyplexes having a PLK/DNA charge of 3.3 or 4.9 (each displaying high β -galactosidase expression) have a lower extractable cellular protein content than those populations transfected using a PLK₃₆ polyplex having an identical charge ratio, though not statistically significant at all lipid ratios (Figure 5.23).

5.2.2.2.2 DOPE/DOPC/oleic acid anti-CD3 liposome and DMPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer

These liposome formulations have no gene transfer activity in lipopolyplexes formed using PLK₃₆ as the DNA condensing agent (data not shown). The extractable cellular protein content of each of the transfected Jurkat cell populations is similar to that of the control population of Jurkat cells (Figure 5.24, 5.25).

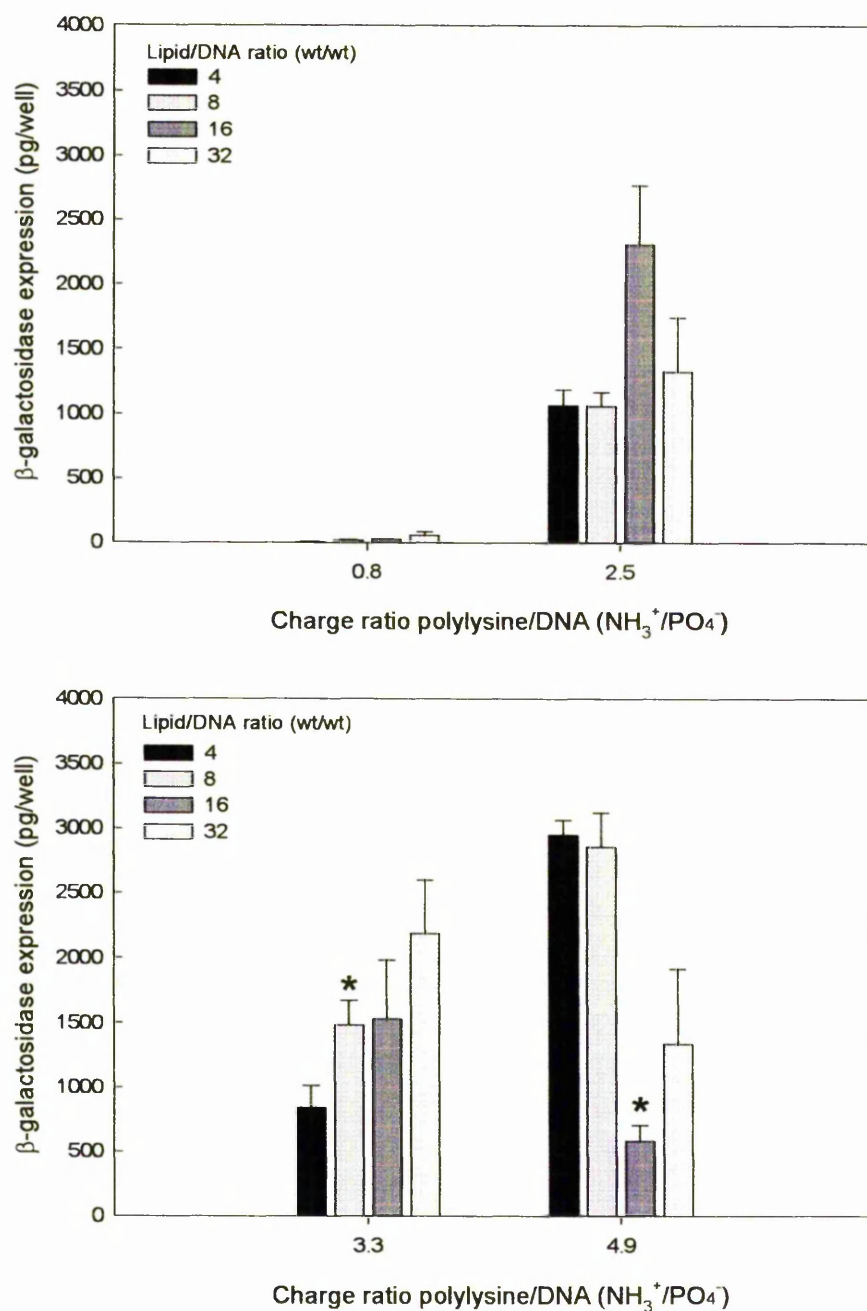


Figure 5.22 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₃₆-condensed pEGlacZ, on gene transfer activity in Jurkat cells (antibody coupling ratio 34.2 $\mu\text{g}/\mu\text{mol}$ lipid). β -galactosidase concentration in cell lysates was assayed 18h post-transfection.*- lipopolyplexes formed visible aggregates before addition to cells. Data show mean \pm s.d. ($n=3$).

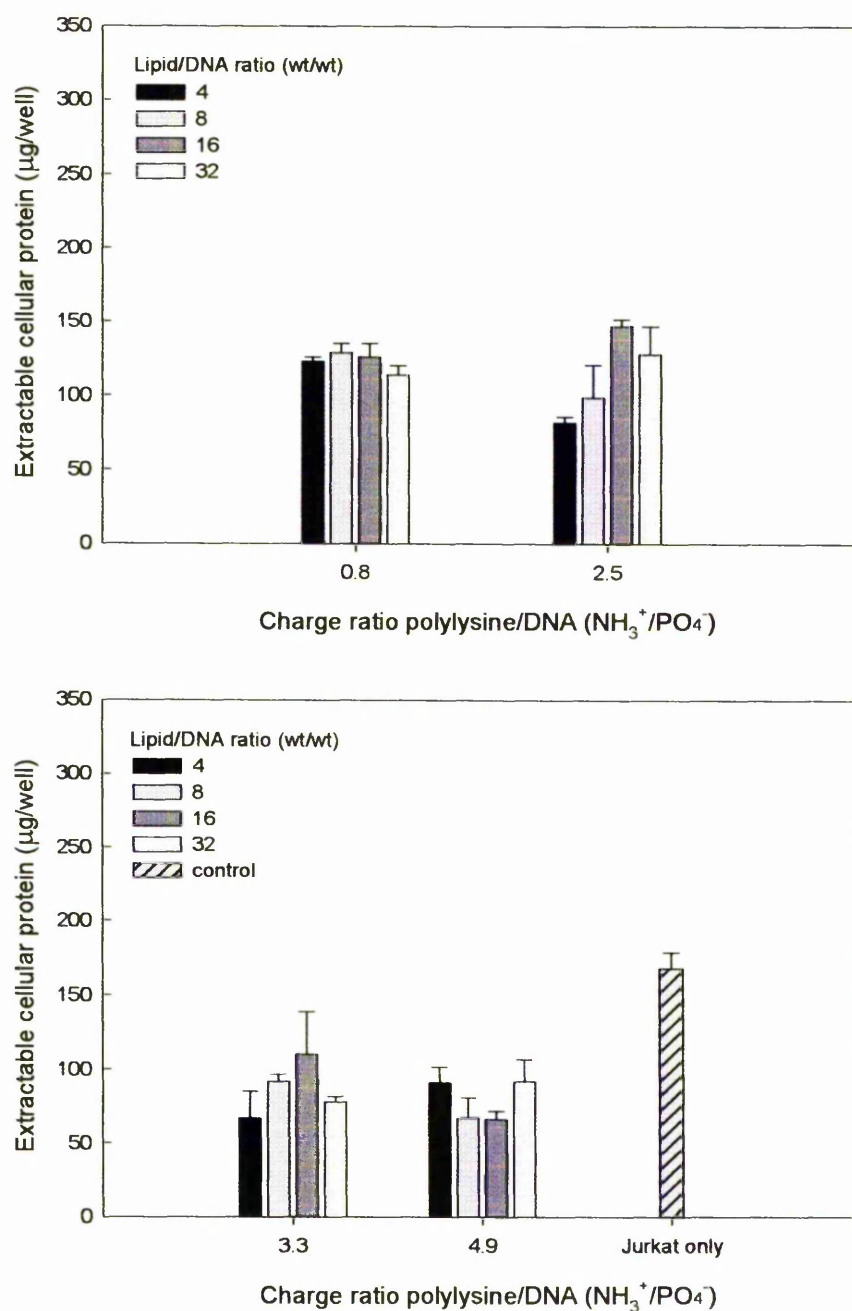


Figure 5.23 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₃₆-condensed pEGlacZ (see Figure 5.22). Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

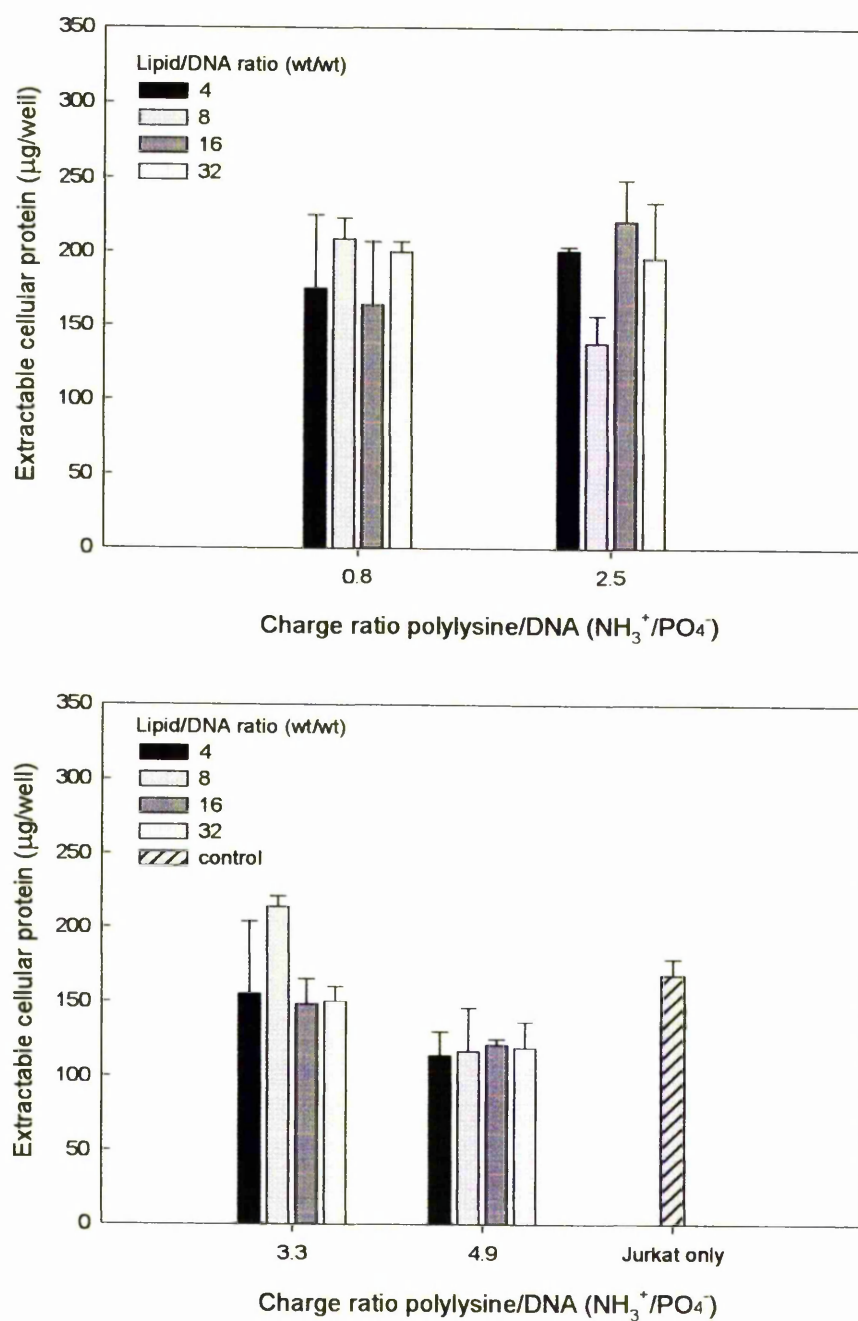


Figure 5.24 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₃₆-condensed pEGlacZ. Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

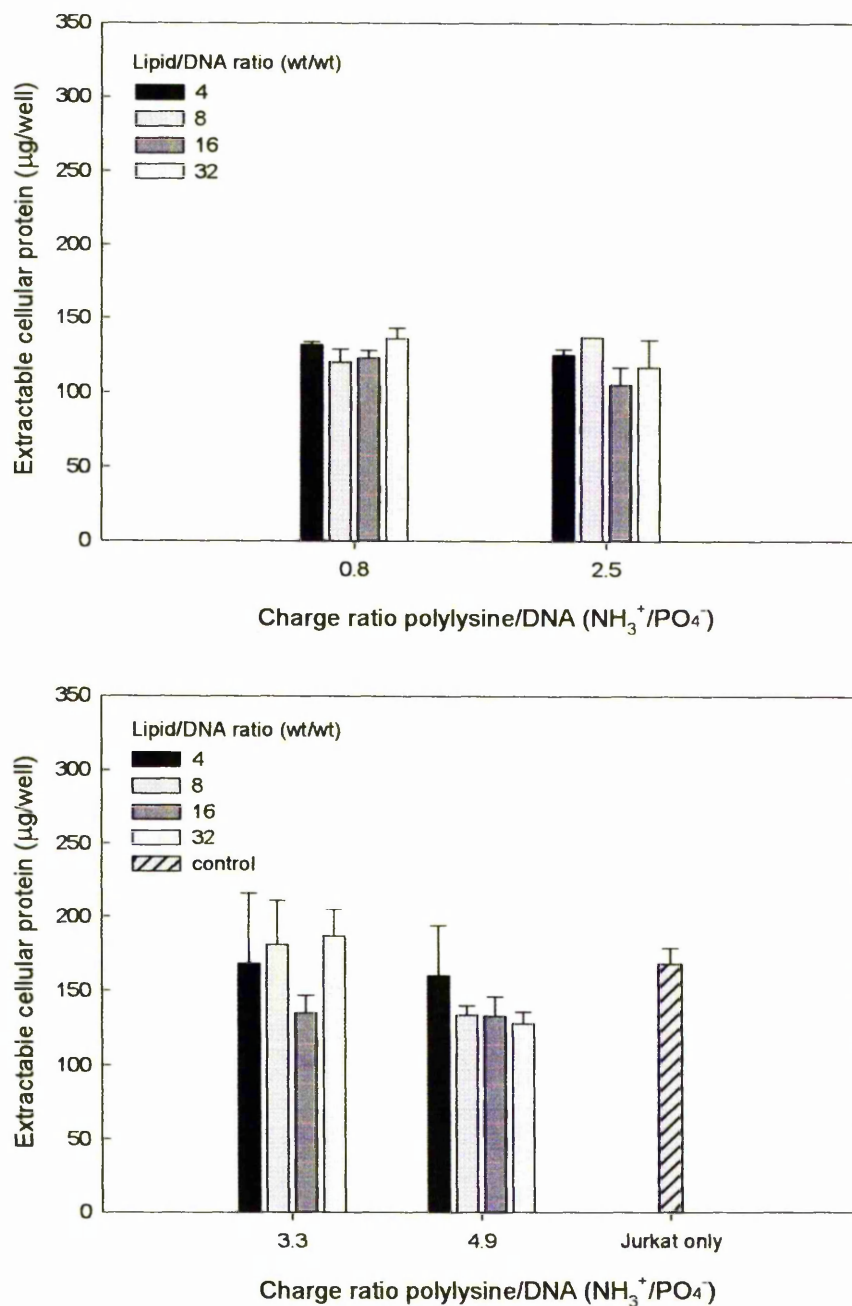


Figure 5.25 Extractable cellular protein content of Jurkat cell populations transfected using DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK₃₆-condensed pEGlacZ. Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

5.2.2.3 PLK₅₃₁ DNA condensing agent

5.2.2.3.1 DOPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer

Liposomes of the formulation DOPE/cholesterol/oleic acid have greater gene transfer activity than each of the other liposome formulations assayed using PLK₅₃₁ as the DNA condensing agent (Figure 5.26). Jurkat cells transfected using PLK₅₃₁ polyplexes display no β -galactosidase expression with polyplexes having a PLK/DNA charge ratio of 0.8, 1.6, or 3.3, but small amounts (13pg/well) with polyplexes having a charge ratio of 4.9 (data not shown). Jurkat cells transfected using lipopolyplexes having a PLK/DNA charge ratio of 0.8 display low levels of β -galactosidase expression at each lipid/DNA ratio. The highest levels of β -galactosidase were observed in Jurkat cells transfected using lipopolyplexes having a PLK/DNA charge ratio of 1.6, and a lipid/DNA ratio of 8 (771pg/well).

The extractable cellular protein content of Jurkat cell populations is lowest when transfected using lipopolyplexes having a high PLK/DNA charge ratio (Figure 5.27). Transfection with lipopolyplexes having a PLK/DNA charge ratio of 3.3 or 4.9 causes the extractable cellular protein content of the Jurkat cell population to approach 0 μ g/well. Jurkat cell populations transfected using PLK₅₃₁ polyplexes having a high PLK/DNA charge ratios also have low extractable cellular protein content (Figure 5.39).

5.2.2.3.2 DOPE/DOPC/oleic acid anti-CD3 liposome mediated gene transfer

Liposomes of the formulation DOPE/DOPC/oleic acid have maximum gene transfer activity in lipopolyplexes having a PLK/DNA charge ratio of 3.3 or 4.9, with little or no gene transfer activity at charge ratios of 0.8 or 1.6 (Figure 5.28).

Jurkat cell populations have significantly lower extractable cellular protein contents following transfection using lipopolyplexes having a PLK/DNA charge ratio of 3.3 or 4.9, in comparison with a control population (except 3.3, 32 lipopolyplex) (3.3 charge ratio ANOVA analysis, Dunnett's test, $F=34.799$, all lipid ratios $P<0.05$; 4.9 charge

ratio ANOVA analysis, Dunnett's test, $F=12.361$, all lipid ratios $P<0.05$; Figure 5.29). The extractable cellular protein content of Jurkat cell populations transfected using lipopolyplexes is similar to that of an equivalent cell population transfected using a polyplex having an identical charge ratio (Figure 5.39).

5.2.2.3.3 DMPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer

The maximum β -galactosidase expression was in Jurkat cells transfected using lipopolyplexes having a PLK/DNA charge ratio of 3.3 or 4.9. The maximum level of β -galactosidase expression is similar to that observed in Jurkat cells transfected using DOPE/DOPC/oleic acid liposomes using PLK₅₃₁ DNA condensing agent, but 10-fold less than when using DOPE/cholesterol/oleic acid liposomes (cf. Figure 5.30 and Figure 5.26).

The extractable cellular protein content is significantly lowered in Jurkat cell populations transfected using lipopolyplexes having a PLK/DNA charge ratio of 3.3 or 4.9, in comparison with a control population (except 3.3, 32 lipopolyplex) (3.3 charge ratio ANOVA analysis, Dunnett's test, $F=70.237$, all lipid ratios $P<0.05$; 4.9 charge ratio ANOVA analysis, Dunnett's test, $F=117.744$, all lipid ratios $P<0.05$; Figure 5.31).

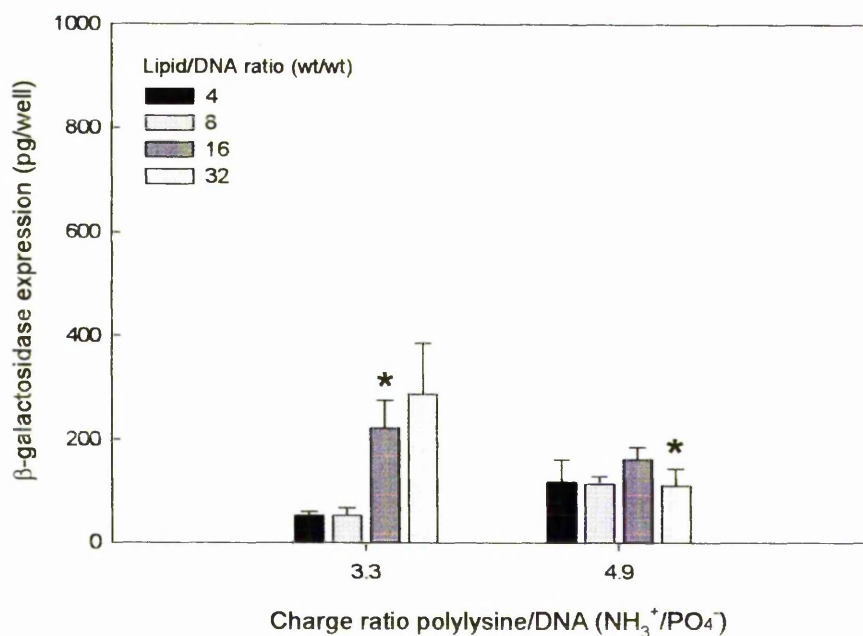
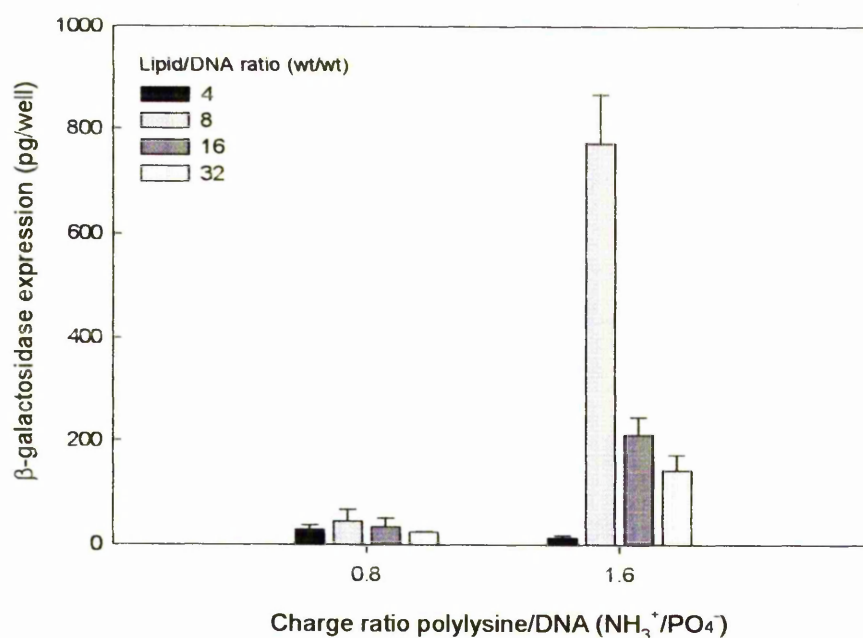


Figure 5.26 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of **DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** prepared using **PLK₅₃₁-condensed pEGlacZ**, on gene transfer activity in Jurkat cells (antibody coupling ratio 34.2 $\mu\text{g}/\mu\text{mol}$ lipid). β -galactosidase concentration in cell lysates was assayed 18h post-transfection.*- lipopolyplexes formed visible aggregates before addition to cells. Data show mean \pm s.d. (n=3).

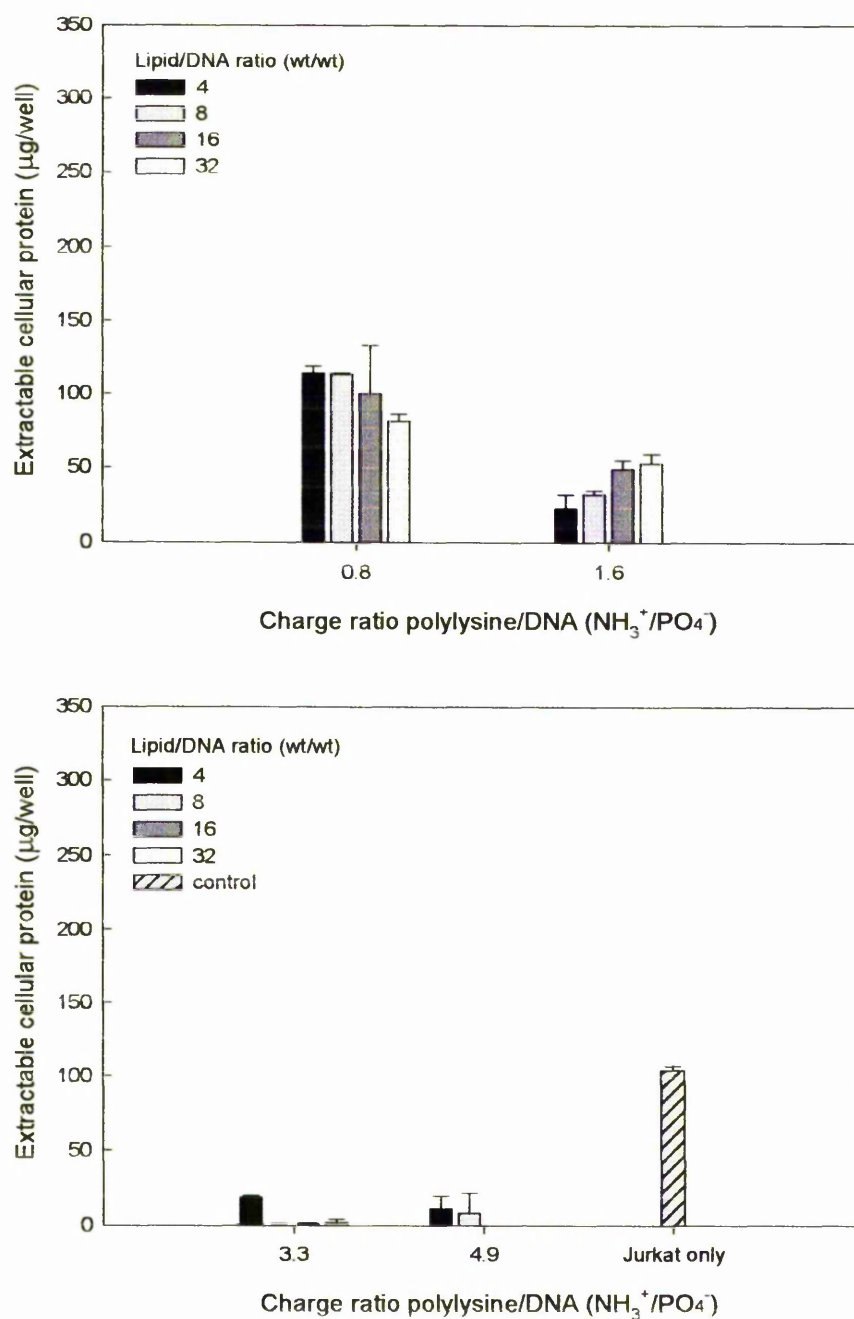


Figure 5.27 Extractable cellular protein content of Jurkat cell populations transfected using **DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** prepared using **PLK₅₃₁-condensed pEGlacZ** (see Figure 5.26). Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

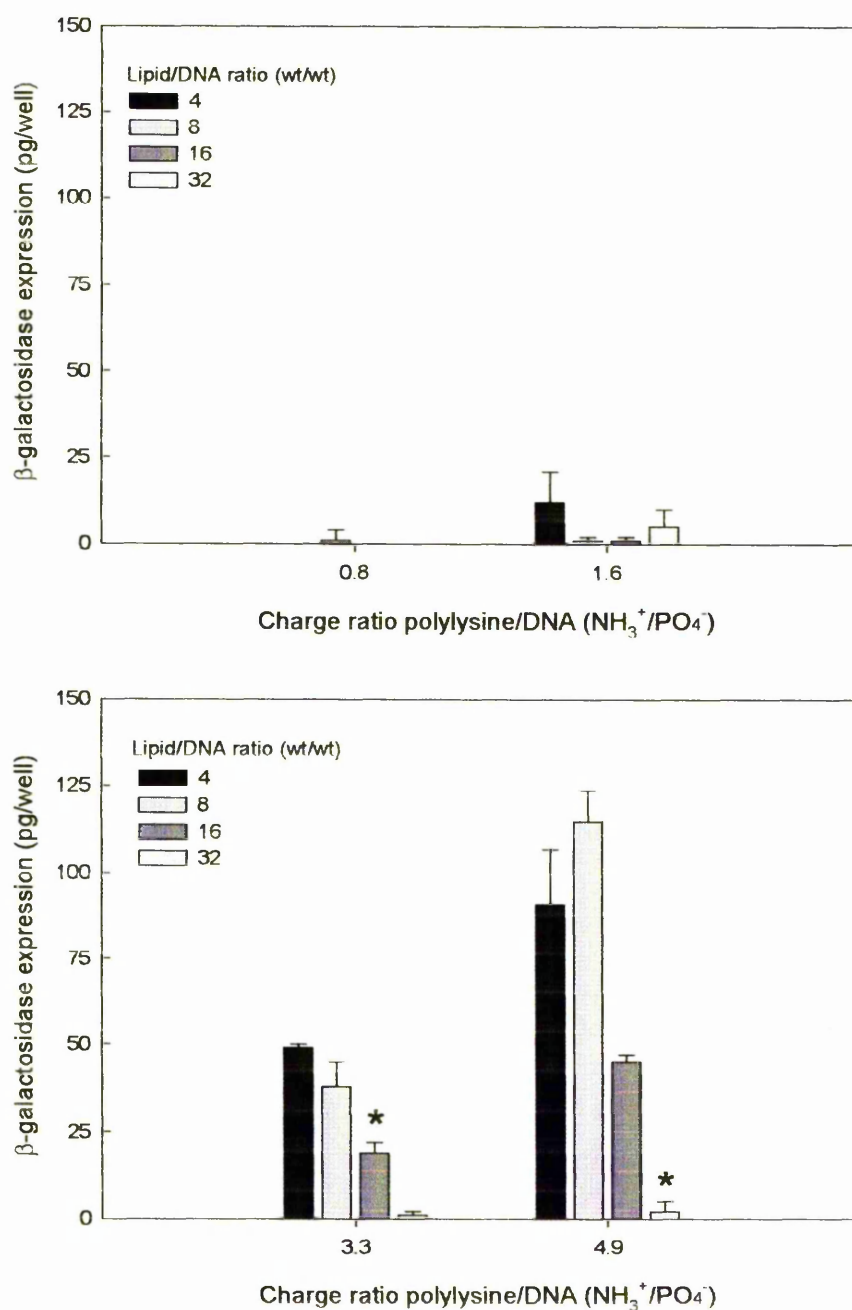


Figure 5.28 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of **DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** prepared using **PLK₅₃₁-condensed pEGlacZ**, on gene transfer activity in Jurkat cells (antibody coupling ratio 25.91 $\mu\text{g}/\mu\text{mol}$ lipid). β -galactosidase concentration in cell lysates was assayed 18h post-transfection.*- lipopolyplexes formed visible aggregates before addition to cells. Data show mean \pm s.d. (n=3). Note change of scale of y-axis compared to Figure 5.26.

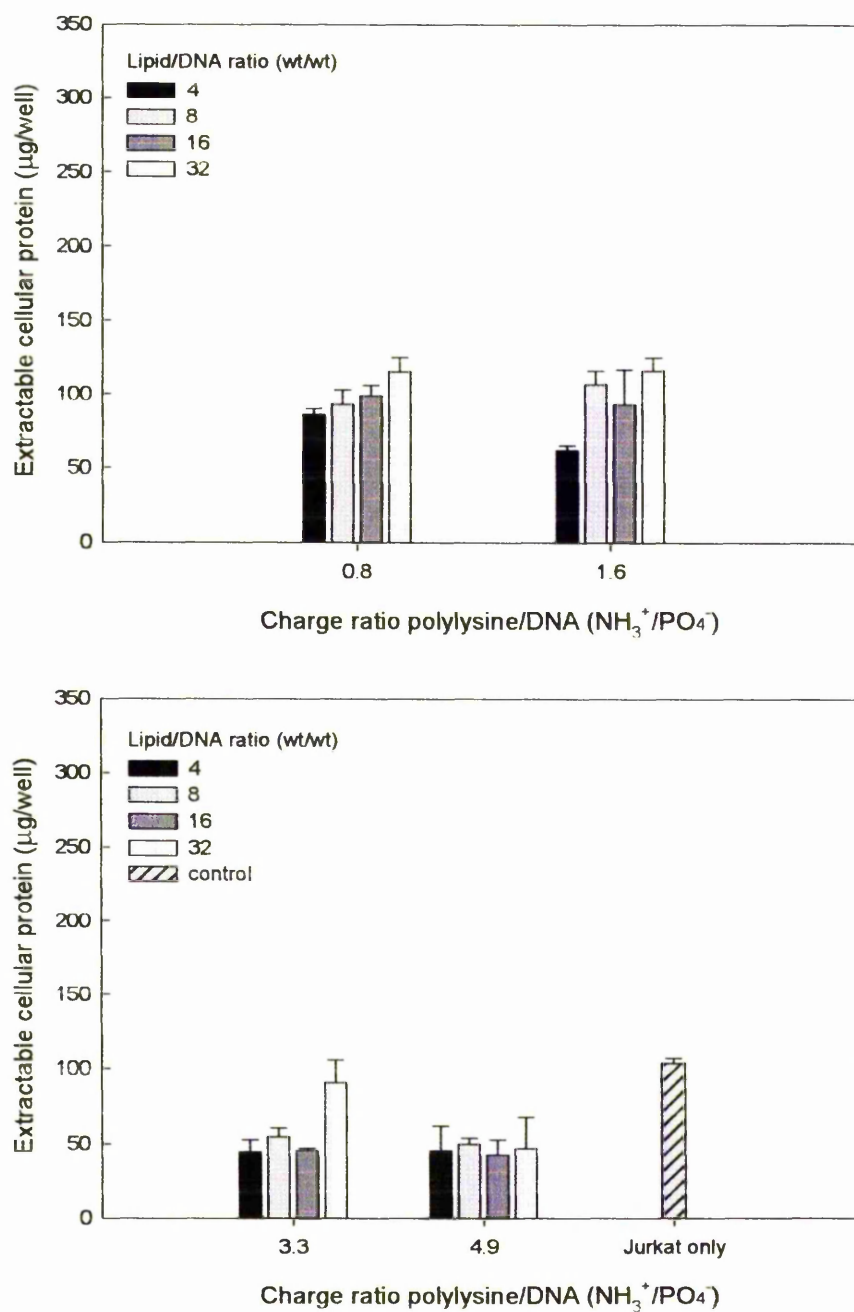


Figure 5.29 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLK_{S31}-condensed pEGlacZ (see Figure 5.28). Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

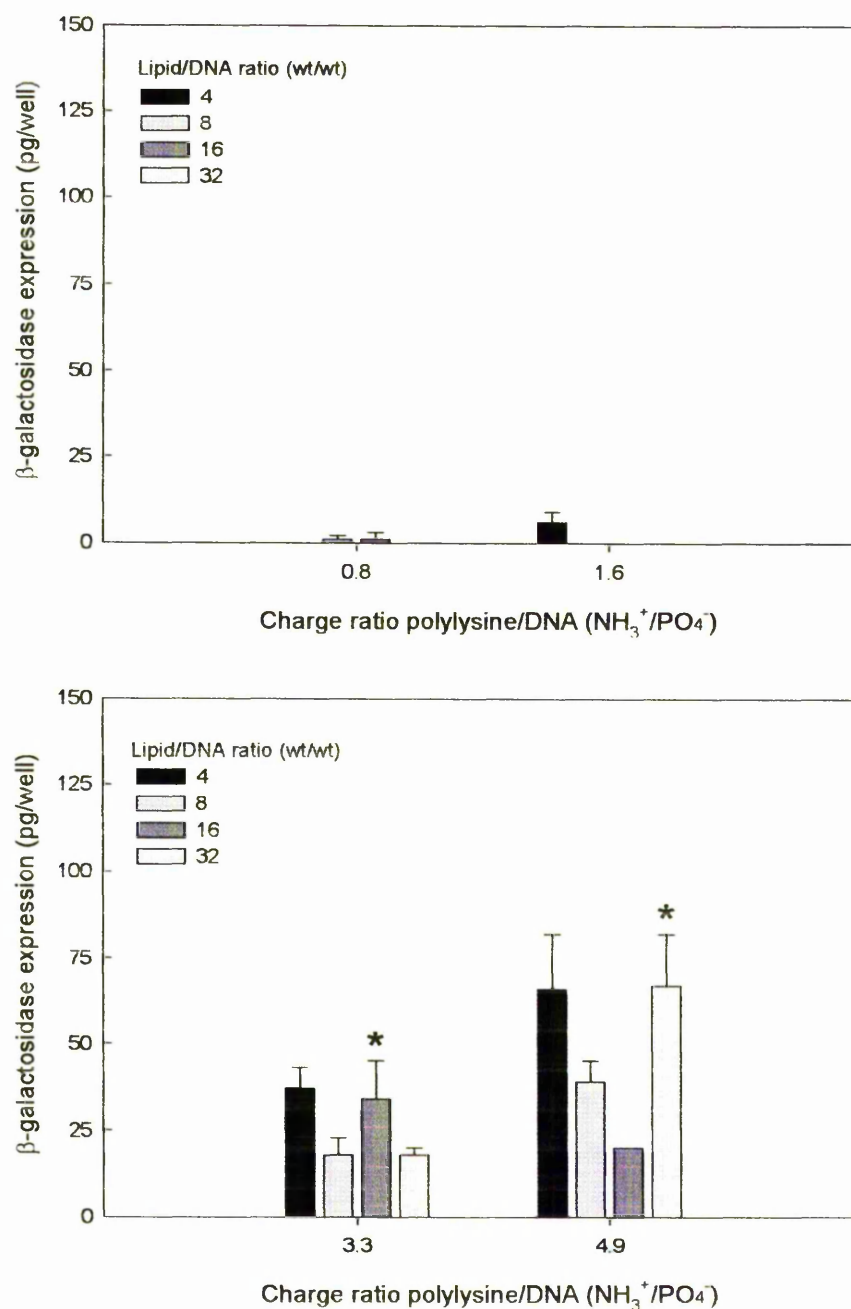


Figure 5.30 The effect of PLK/DNA charge ratio and lipid/DNA weight ratio of **DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** prepared using **PLK₅₃₁-condensed pEGlacZ**, on gene transfer activity in Jurkat cells (antibody coupling ratio 18.21 $\mu\text{g}/\mu\text{mol}$ lipid). β -galactosidase concentration in cell lysates was assayed 18h post-transfection.*- lipopolyplexes formed visible aggregates before addition to cells. Data show mean \pm s.d. (n=3). Note change of scale of y-axis compared to Figure 5.26.

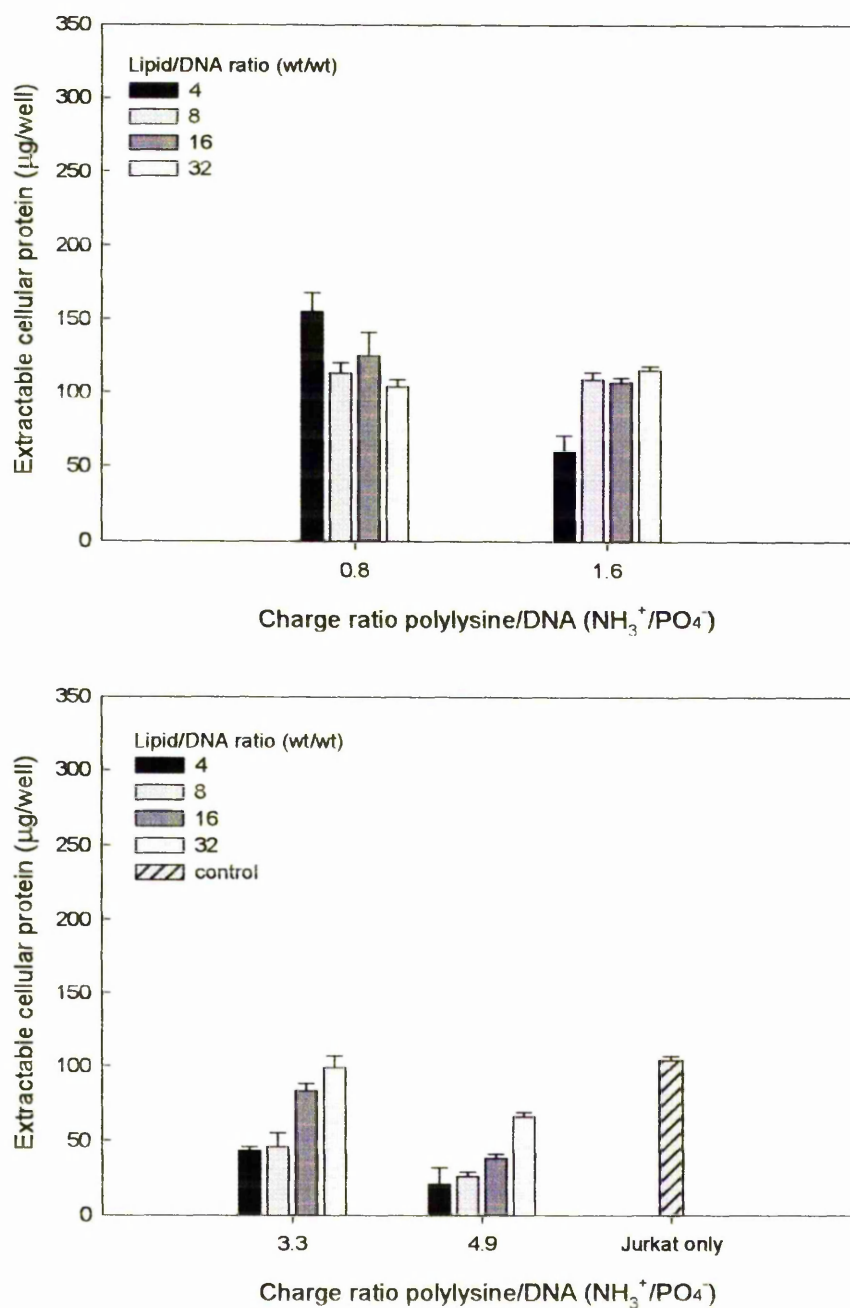


Figure 5.31 Extractable cellular protein content of Jurkat cell populations transfected using **DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** prepared using **PLK₅₃₁-condensed pEGlacZ** (see Figure 5.30). Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

5.2.2.4 PLR₁₇ DNA condensing agent

5.2.2.4.1 DOPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer

Jurkat cells display high levels of β -galactosidase expression following transfection using lipopolyplexes having a PLR/DNA charge ratio of 1.5, 2 or 3 (Figure 5.32). The highest levels of β -galactosidase expression are in Jurkat cells transfected using lipopolyplexes having a PLR/DNA charge ratio of 1.5, and a lipid/DNA ratio of 8 (3179pg/well).

The extractable cellular protein content of lipopolyplex-transfected Jurkat cell populations displaying high β -galactosidase expression is considerably lower than in non- β -galactosidase expressing populations (Figure 5.33).

5.2.2.4.2 DOPE/DOPC/oleic acid anti-CD3 liposome mediated gene transfer

Liposomes of the formulation DOPE/DOPC/oleic acid have minimal gene transfer activity using each of the assayed lipopolyplex compositions (Figure 5.34).

The extractable cellular protein contents of each of the lipopolyplex-transfected Jurkat cell populations are similar to those of Jurkat cell populations transfected with polyplexes of an equivalent PLR/DNA charge ratio (cf. Figure 5.35 and Figure 5.40). Jurkat cells transfected using PLR₁₇ polyplexes display no β -galactosidase expression (data not shown).

5.2.2.4.3 DMPE/cholesterol/oleic acid anti-CD3 liposome mediated gene transfer

Jurkat cells transfected using DMPE/cholesterol/oleic acid lipopolyplexes having PLR₁₇ condensed DNA show no β -galactosidase expression (data not shown).

The extractable cellular protein contents of the lipopolyplex transfected Jurkat cell populations are similar to those of the control cell population when the PLR/DNA charge ratio is 0.5 or 1.5 (Figure 5.36).

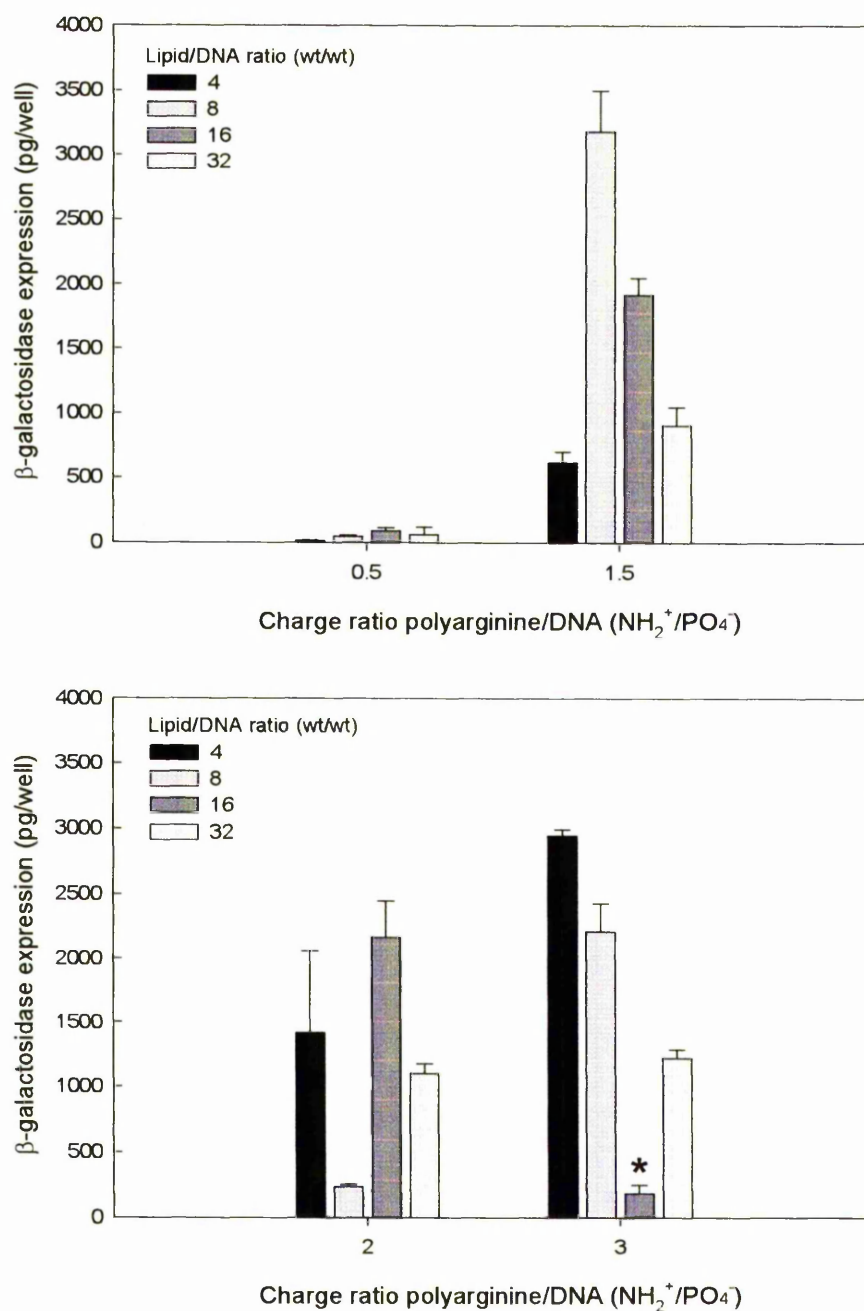


Figure 5.32 The effect of PLR/DNA charge ratio and lipid/DNA weight ratio of **DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** prepared using **PLR₁₇-condensed pEGlacZ**, on gene transfer activity in Jurkat cells (antibody coupling ratio 34.2 $\mu\text{g}/\mu\text{mol}$ lipid). β -galactosidase concentration in cell lysates was assayed 18h post-transfection.*- lipopolyplexes formed visible aggregates before addition to cells. Data show mean \pm s.d. (n=3).

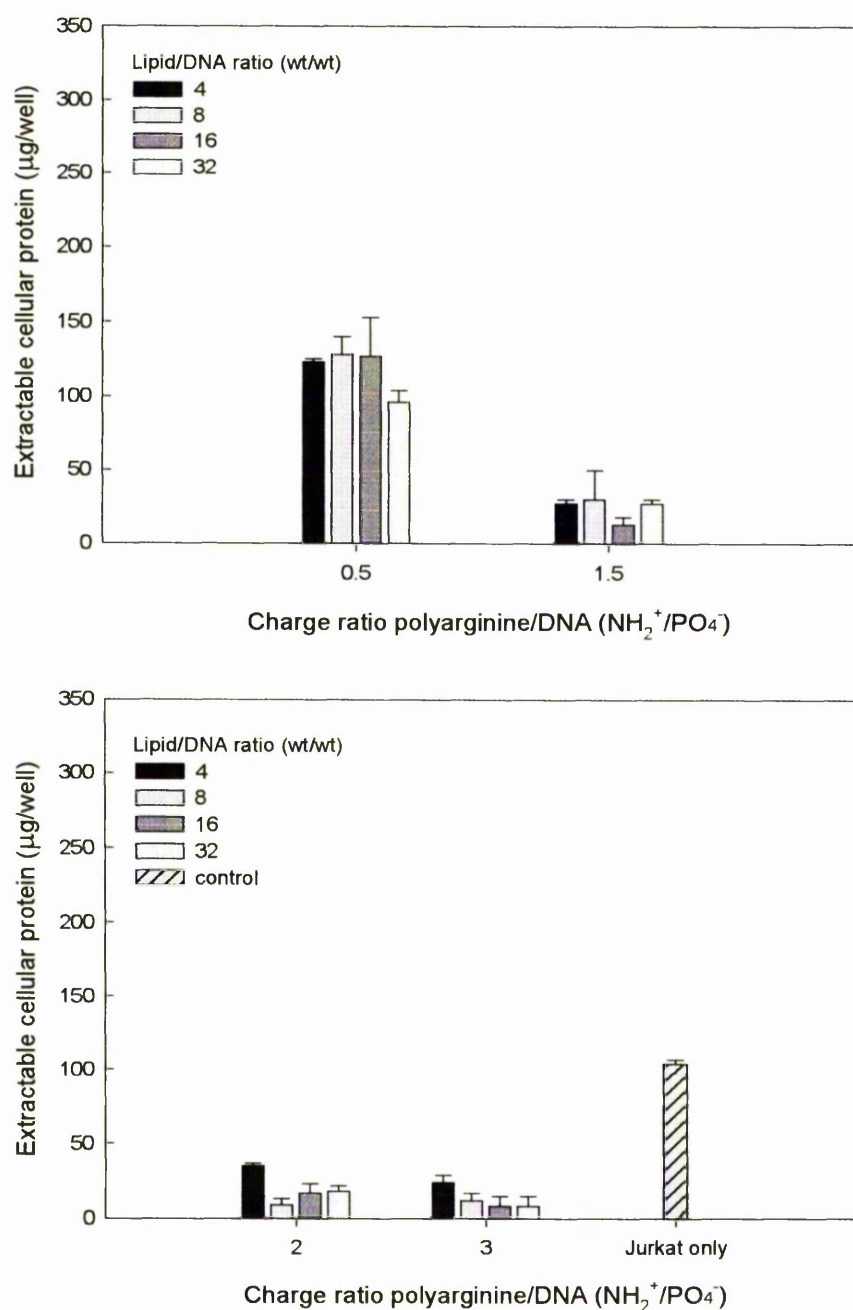


Figure 5.33 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLR₁₇-condensed pEGlacZ (see Figure 5.32). Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

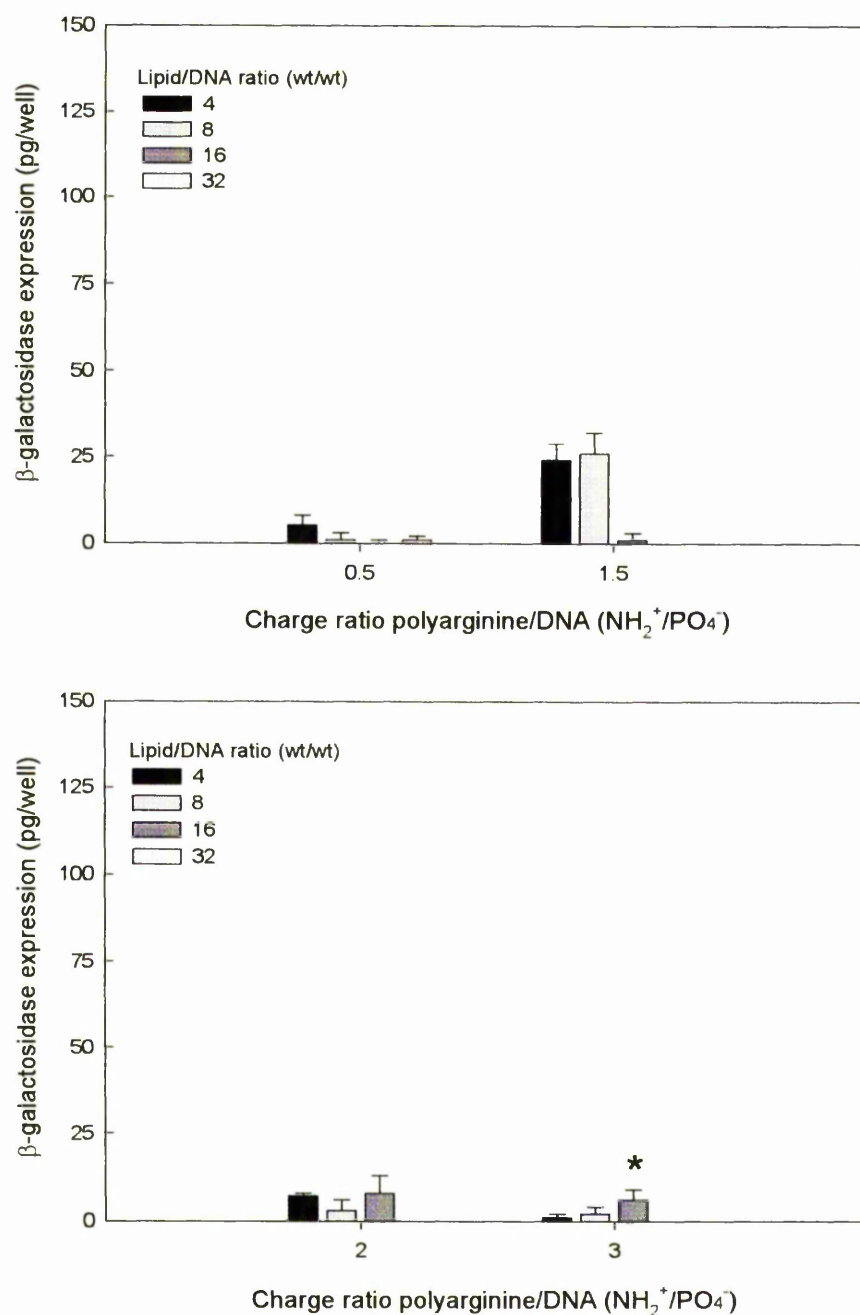


Figure 5.34 The effect of PLR/DNA charge ratio and lipid/DNA weight ratio of **DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** prepared using **PLR₁₇-condensed pEGlacZ**, on gene transfer activity in Jurkat cells (antibody coupling ratio 25.91 $\mu\text{g}/\mu\text{mol}$ lipid). β -galactosidase concentration in cell lysates was assayed 18h post-transfection.*- lipopolyplexes formed visible aggregates before addition to cells. Data show mean \pm s.d. (n=3). Note change of scale of y-axis compared to Figure 5.32.

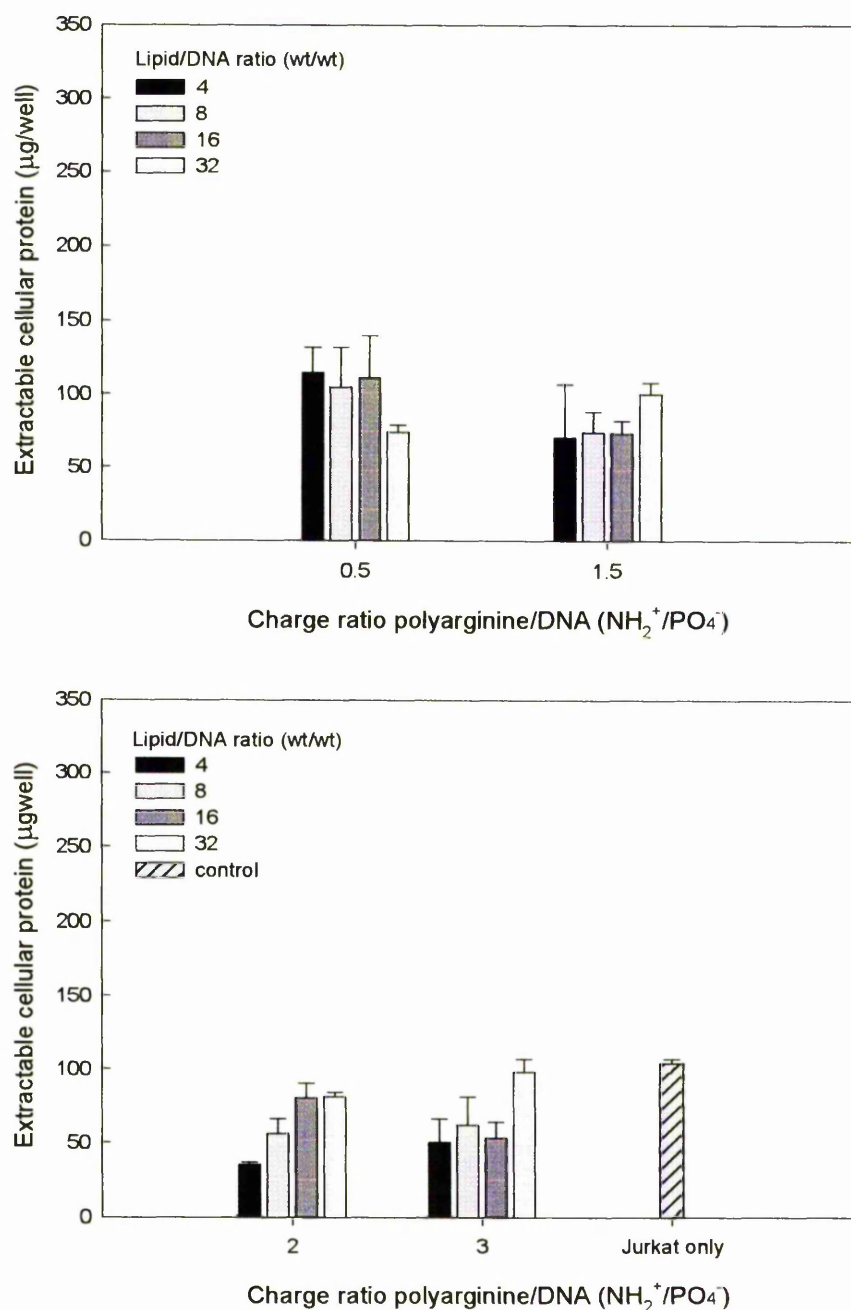


Figure 5.35 Extractable cellular protein content of Jurkat cell populations transfected using DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLR₁₇-condensed pEGlacZ (see Figure 5.34). Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

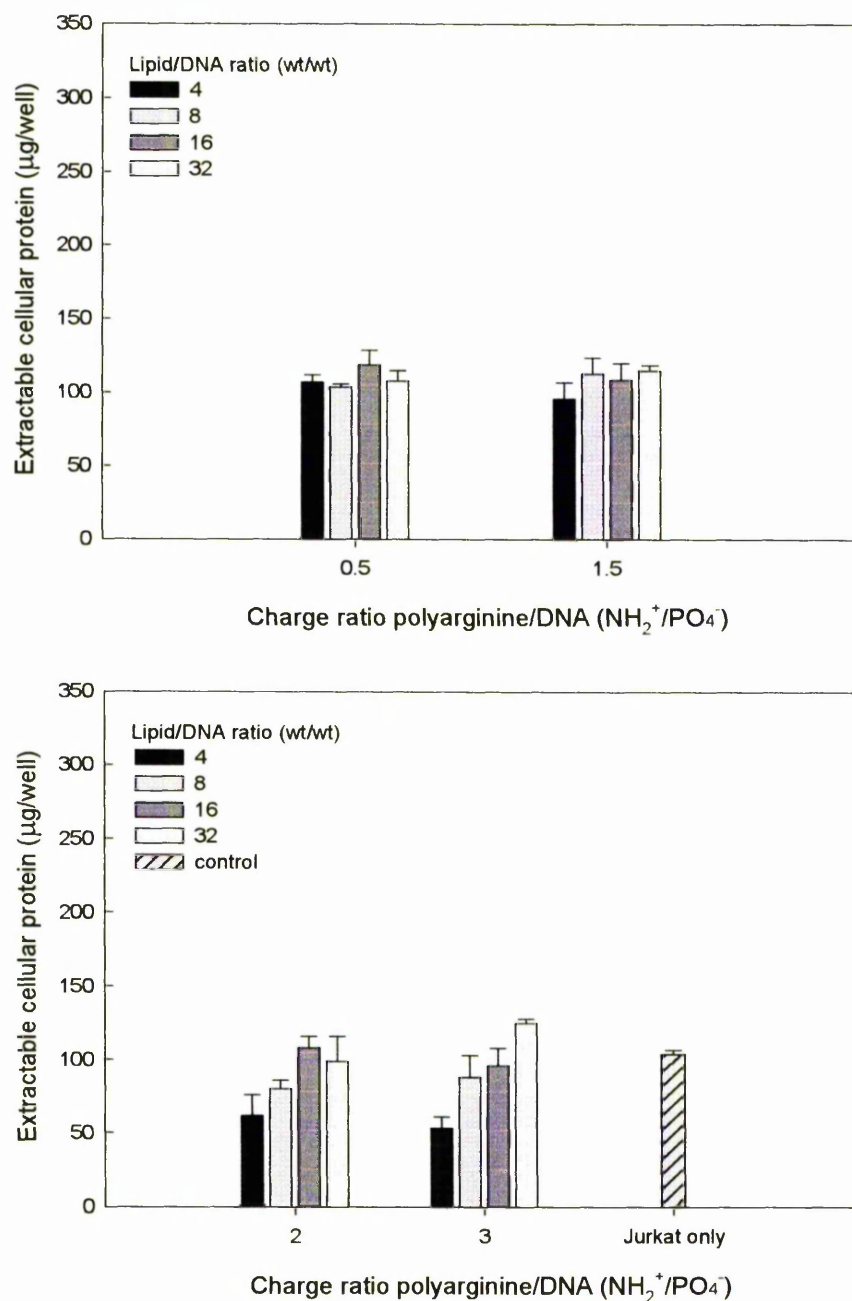


Figure 5.36 Extractable cellular protein content of Jurkat cell populations transfected using DMPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes prepared using PLR₁₇-condensed pEGlacZ. Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

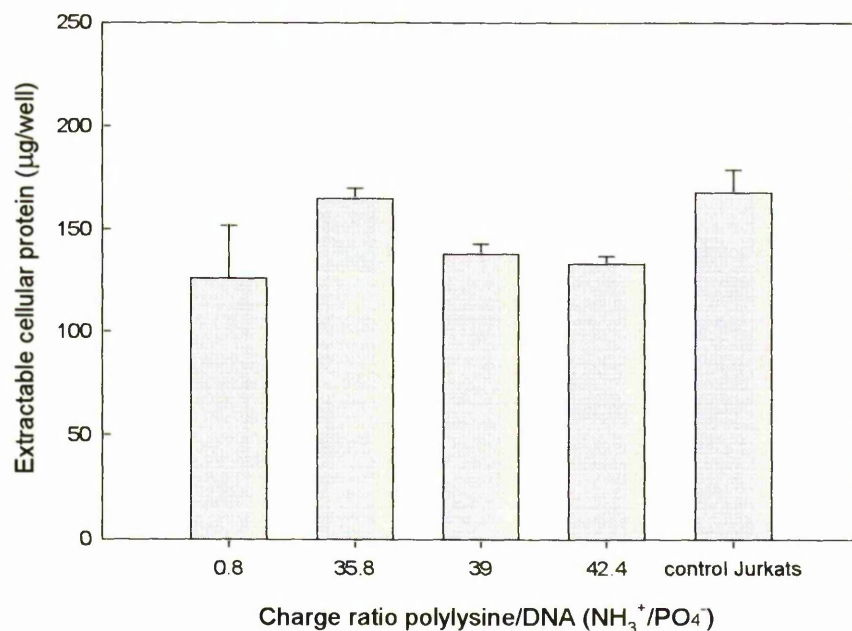


Figure 5.37 The effect of PLK_5 -polyplex charge ratio on the extractable cellular protein content of polyplex-transfected Jurkat cell populations. Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with polyplexes. Data show mean \pm s.d. (n=3).

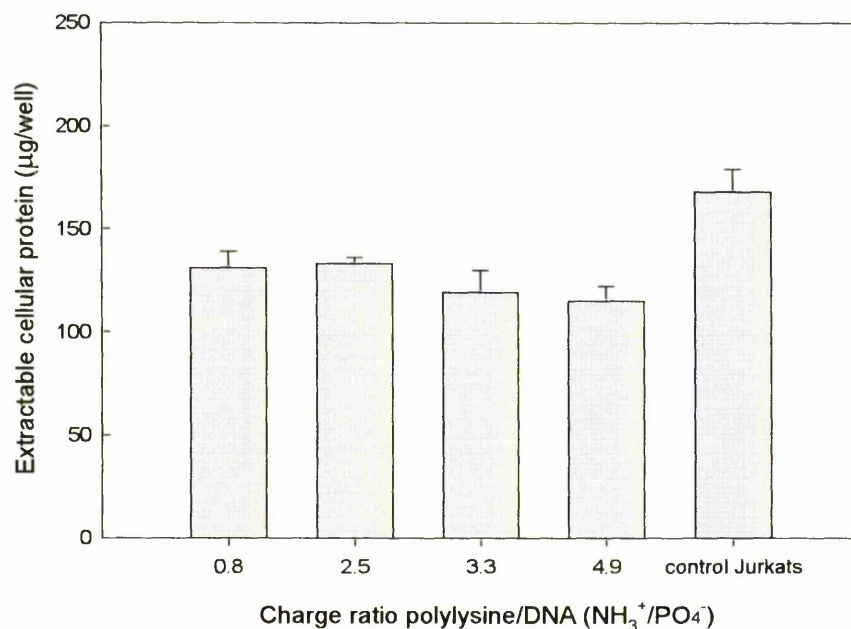


Figure 5.38 The effect of PLK_{36} -polyplex charge ratio on the extractable cellular protein content of polyplex-transfected Jurkat cell populations. Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with polyplexes. Data show mean \pm s.d. (n=3).

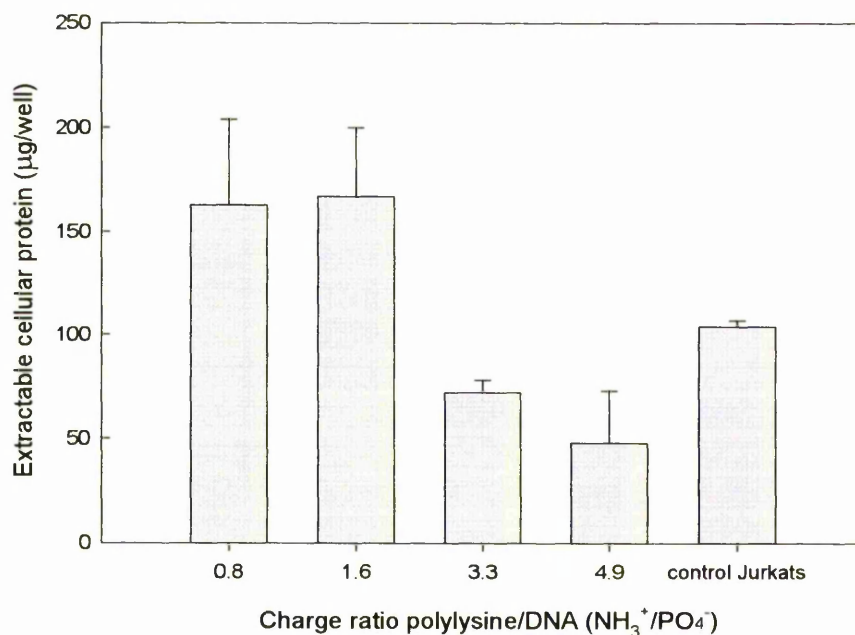


Figure 5.39 The effect of PLK_{531} -polyplex charge ratio on the extractable cellular protein content of polyplex-transfected Jurkat cell populations. Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with polyplexes. Data show mean \pm s.d. (n=3).

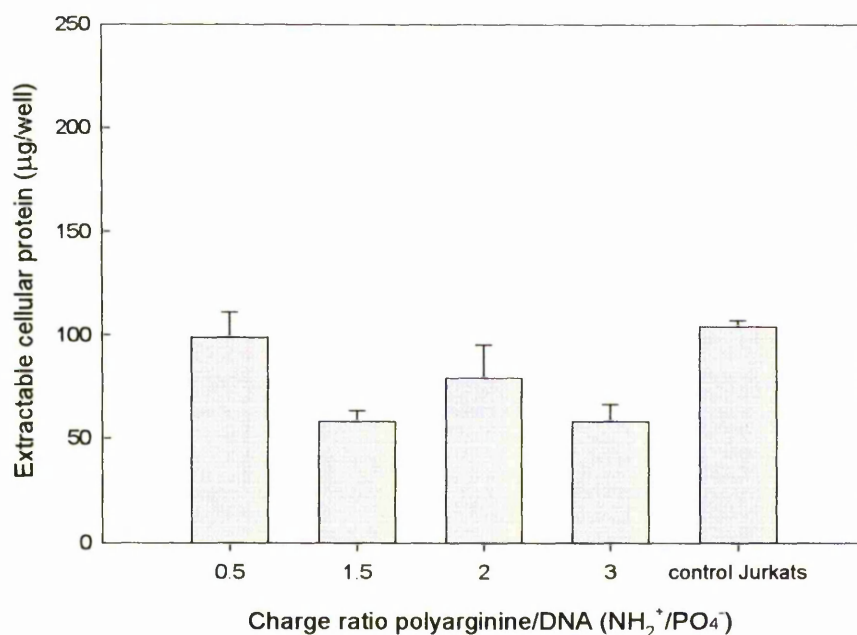


Figure 5.40 The effect of PLR_{17} -polyplex charge ratio on the extractable cellular protein content of polyplex-transfected Jurkat cell populations. Jurkat cell population pre-transfection 1×10^6 cells/well. Extractable cellular protein content was assayed 18h post-transfection from cell lysates. Control Jurkat cell population was not challenged with polyplexes. Data show mean \pm s.d. (n=3).

5.2.3 Targeted transfection of Jurkat cells

The transfections performed in the primary and secondary screens (5.2.1, 5.2.2) used liposomes bearing anti-CD3 antibodies. A further series of experiments determined if any transfection activity observed was specific to the presence of the targeting antibody. Liposomes of identical compositions were prepared, bearing either anti-CD3 antibodies or a non-CD3 specific antibody (Table 5.4). Lipopolyplexes were prepared from these liposomes to transfect Jurkat cells.

The highest levels of β -galactosidase expression have been observed in Jurkat cells transfected with DOPE/cholesterol/oleic acid liposomes. DNA condensing agents PLK₅, PLK₃₆, and PLR₁₇ gave similar maximum β -galactosidase expression levels in Jurkat cells of ~3000pg/well when complexed to this liposome formulation.

Table 5.4 Properties of liposomes used for the targeted transfection of Jurkat cells

Liposome formulation (40:39.9:20:0.1 mol%)	Particle diameter (nm) ^a	Zeta potential (mV) ^b	Antibody coupling ratio (μ g/ μ mol)	Weight average Antibody molecules/liposome ^c	Liposome batch age (days) ^d
DOPE/cholesterol/oleic acid/DSPE-PEG/anti-CD3	125.3 \pm 47.9	-60.8 \pm 0.9	34.2	30	12
DOPE/cholesterol/oleic acid/DSPE-PEG/non-CD3 specific	118.4 \pm 41.9	-57.4 \pm 3.4	22.9	21	70
DOPE/DOPC/oleic acid/DSPE-PEG/anti-CD3	86.9 \pm 34.1	-42.5 \pm 6.1	25.91	13	12
DOPE/DOPC/oleic acid/DSPE-PEG/non-CD3 specific	108.6 \pm 20	-65.1 \pm 1.5	18.3	13	70
DMPE/cholesterol/oleic acid/DSPE-PEG/anti-CD3	105.6 \pm 33.5	-54.0 \pm 1.8	18.21	13	12
DMPE/cholesterol/oleic acid/DSPE-PEG/non-CD3 specific	134.9 \pm 49.3	-48.6 \pm 0.7	0.26	0	60

^a determined by PCS. \pm s.d. of unimodal distribution.

^b in 10mM HEPES pH8.0. Zeta potential calculated using $f(\kappa a)=1.3$. s.d. is for n=5.

^c calculated based on M_w of IgG=150,000 using D-LIPPRO computer program.

^d days stored at 4°C since preparation.

Lipopolyplexes containing PLK₃₆ at a PLK/DNA charge ratio of 4.9, and a lipid/DNA ratio of 8 (4.9, 8 lipopolyplexes) were chosen to show targeted transfection of Jurkat cells. A lipopolyplex having a charge ratio of 3.3 and a lipid/DNA ratio of 32 (3.3, 32 lipopolyplex), was also chosen. This lipopolyplex composition also displayed high levels of gene transfer activity in Jurkat cells, but was thought to have different surface charge properties to a 4.9, 8 lipopolyplex. Targeted transfection was also attempted using the DOPE/DOPC/oleic acid and DMPE/cholesterol/oleic acid liposome formulations using the lipopolyplex formulations described.

Lipopolyplexes were prepared using three liposome formulations. Each liposome formulation had populations bearing either an anti-CD3 antibody, or a non-CD3 specific antibody (Table 5.4). Zeta potential measurements determined using a DMPE/cholesterol/oleic acid liposome preparation, a 3.3, 32 lipopolyplex has a zeta potential of -42.8 ± 1.1 mV, whereas that of a 4.9, 8 lipopolyplex is $+19.5 \pm 1.8$ mV (Table 5.5). Lipopolyplexes prepared using DOPE/cholesterol/oleic acid and DOPE/DOPC/oleic acid liposomes have similar properties (data not shown).

Table 5.5 Properties of lipopolyplexes used for targeted transfection of Jurkat cells

Lipopolyplex component	Zeta potential (mV) ^b	Particle diameter (nm) ^c
DMPE/cholesterol/oleic acid/DSPE-PEG/anti-CD3 liposomes	-54.0 ± 1.8	105.6 ± 33.5
PLK ₃₆ polyplex charge ratio 4.9 (NH ₃ ⁺ /PO ₄ ⁻)	$+21.5 \pm 2.9$	117.7 ± 32.5
PLK ₃₆ polyplex charge ratio 3.3 (NH ₃ ⁺ /PO ₄ ⁻)	$+20.3 \pm 0.9$	338.4 ± 70.9
3.3, 32 lipopolyplex ^a	-42.8 ± 1.1	119.9 ± 35.5
4.9, 8 lipopolyplex ^a	$+19.5 \pm 1.8$	357.6 ± 27.6

^a for definition see Section 5.2.3.

^b in 10mM HEPES pH8.0. Zeta potential calculated using $f(\kappa a)=1.3$. s.d. is for n=5.

^c determined by PCS. \pm s.d. of unimodal distribution.

5.2.3.1 Targeted transfection of Jurkat cells in serum-free media

DOPE/cholesterol/oleic acid anti-CD3 lipopolyplexes have high gene transfer activity in Jurkat cells. Lipopolyplexes bearing a non-specific antibody have significantly lower gene transfer activity, 69% (4.9, 8 lipopolyplexes; t-test, $t=6.596$, $P<0.01$) and 76% (3.3, 32 lipopolyplexes; t-test, $t=8.294$, $P<0.01$) of that of equivalent lipopolyplexes carrying a specific anti-CD3 antibody (Figure 5.41a).

The extractable cellular protein content of anti-CD3 lipopolyplex transfected Jurkat cell populations is lower than that of non-specific lipopolyplex transfected cell populations, though 3.3, 32 lipopolyplexes not significantly so (4.9, 8 lipopolyplexes t-test, $t=-13.276$, $P<0.001$; 3.3, 32 lipopolyplexes t-test, $t=-2.215$, $P=0.091$; Figure 5.41b). Despite having similar levels of β -galactosidase expression, Jurkat cell populations transfected using 4.9, 8 anti-CD3 lipopolyplexes have a significantly lower extractable cellular protein content than those transfected using 3.3, 32 anti-CD3 lipopolyplexes (t-test, $t=-11.804$, $P<0.001$).

No transfection activity was noted in Jurkat cells transfected using lipopolyplexes of DOPE/DOPC/ oleic acid and DMPE/cholesterol/oleic acid liposome formulations (data not shown).

5.2.3.2 Targeted transfection of Jurkat cells in the presence of serum

Targeted transfection of Jurkat cells was attempted with the presence of 10% foetal calf serum (FCS) in the transfection medium (DMEM, 10% FCS, see method, 2.2.12). Lipopolyplexes were prepared as a single batch and used for both targeting in the presence of, and in the absence of serum (5.2.3.1).

5.2.3.2.1 DOPE/cholesterol/oleic acid liposome mediated gene transfer

Expression of β -galactosidase was seen only in Jurkat cells transfected using 4.9, 8 anti-CD3 lipopolyplexes. Non-CD3-specific 4.9, 8 lipopolyplex transfected Jurkat cells display little β -galactosidase expression above background levels (Figure 5.42a).

The specificity of transfection was determined by the presence of anti-CD3 antibody in the lipopolyplex (t-test, $t=361.889$, $P<0.001$). Jurkat cells transfected using 3.3, 32 lipopolyplexes display no β -galactosidase expression with or without the presence of anti-CD3 targeting antibody. The level of β -galactosidase expression in 4.9, 8 anti-CD3 lipopolyplex-transfected Jurkat cells is four-fold lower than in those transfected in the absence of serum using the same lipopolyplex (t-test, $t=-18.367$, $P<0.001$; Figure 5.41a). Polyplexes using PLK₃₆ having PLK/DNA charge ratios of 3.3 and 4.9, show no gene transfer activity in serum containing media (data not shown).

The extractable cellular protein content of the Jurkat cell populations transfected using either 4.9, 8 lipopolyplex is significantly lower than that of the control population of Jurkat cells (ANOVA analysis, Dunnett's test, $F=3.944$, \pm antibody $P<0.05$; Figure 5.42b). Jurkat cell populations transfected using 4.9, 8 anti-CD3 lipopolyplexes in the absence of serum have a lower extractable cellular protein content (t-test, $t=9.73$, $P<0.001$; cf. Figure 5.41a). Jurkat cell populations transfected with PLK₃₆ polyplexes have extractable cellular protein contents similar to that of the control cell population (98%). Jurkat cell populations transfection using 3.3, 32 lipopolyplexes have no reduction in extractable cellular protein content compared to the control population.

Jurkat cells transfected in the presence of serum have similar morphology and growth characteristics (i.e. clumping at high cell concentrations) to non-transfected cells when examined under a light microscope. Jurkat cells transfected in the absence of serum often appeared less rounded, and were less prone to clumping when compared to cells transfected with the same lipopolyplexes.

5.2.3.2.2 DOPE/DOPC/oleic acid liposome and DMPE/cholesterol/oleic acid liposome mediated gene transfer

Liposomes of these compositions were tested for gene transfer activity to Jurkat cells in the presence of serum. This would determine if their lipid composition conferred greater serum resistance than that of DOPE/cholesterol/oleic acid liposomes.

No β -galactosidase expression was noted in Jurkat cells transfected using lipopolyplexes containing these liposome formulations in the presence of 10% FCS (data not shown).

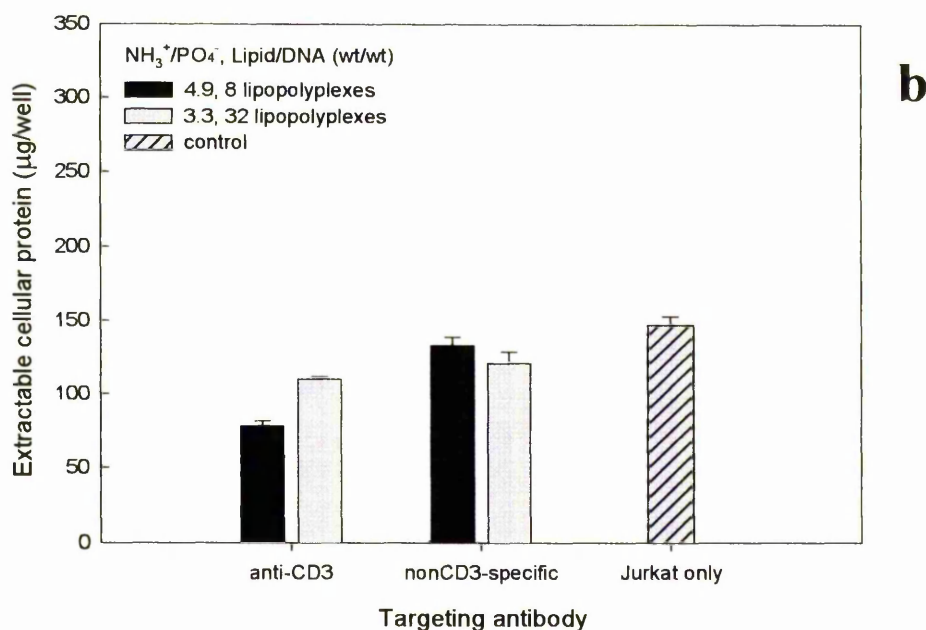
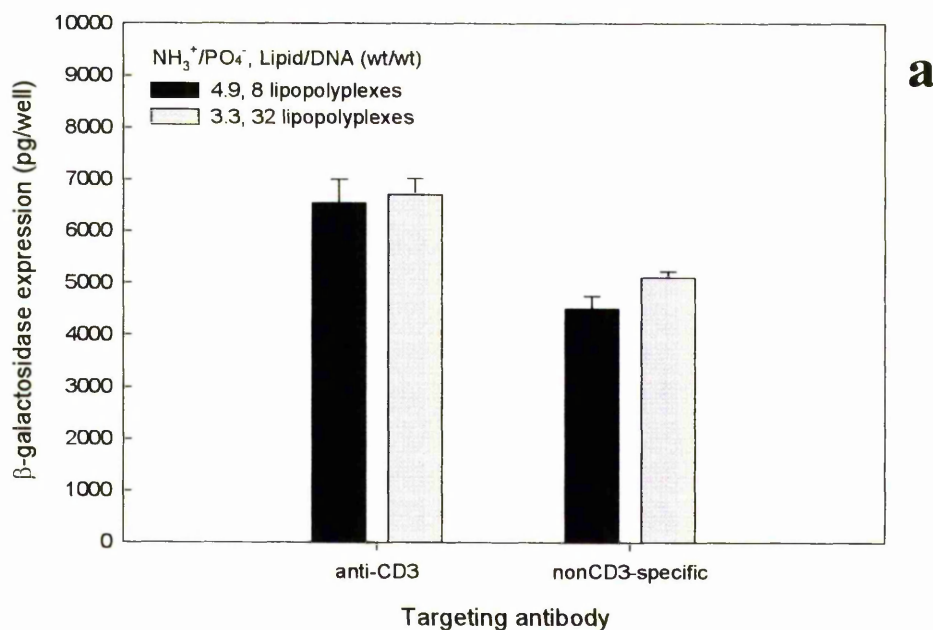


Figure 5.41 Targeted transfection of Jurkat cells using **DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** in serum-free media (a) β-galactosidase expression (b) extractable cellular protein content. **PLK₃₆**-condensed pEGlacZ. Antibody coupling ratios, anti-CD3 34.2μg/μmol lipid, non-CD3 specific 22.9μg/μmol lipid. Jurkat cell population pre-transfection 1×10⁶ cells/well. β-galactosidase concentration and extractable cellular protein content in cell lysates were assayed 18h post-transfection. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean ± s.d. (n=3).

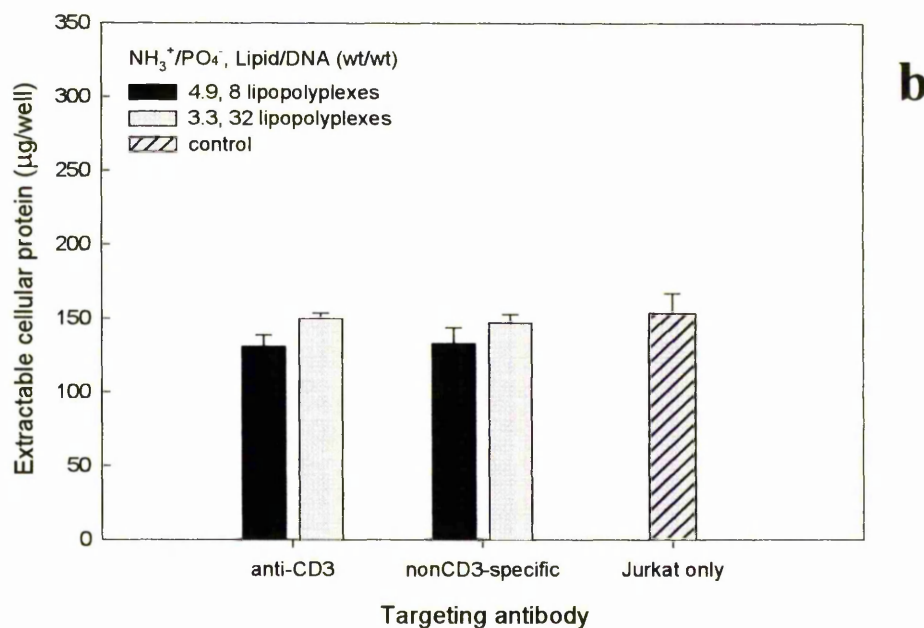
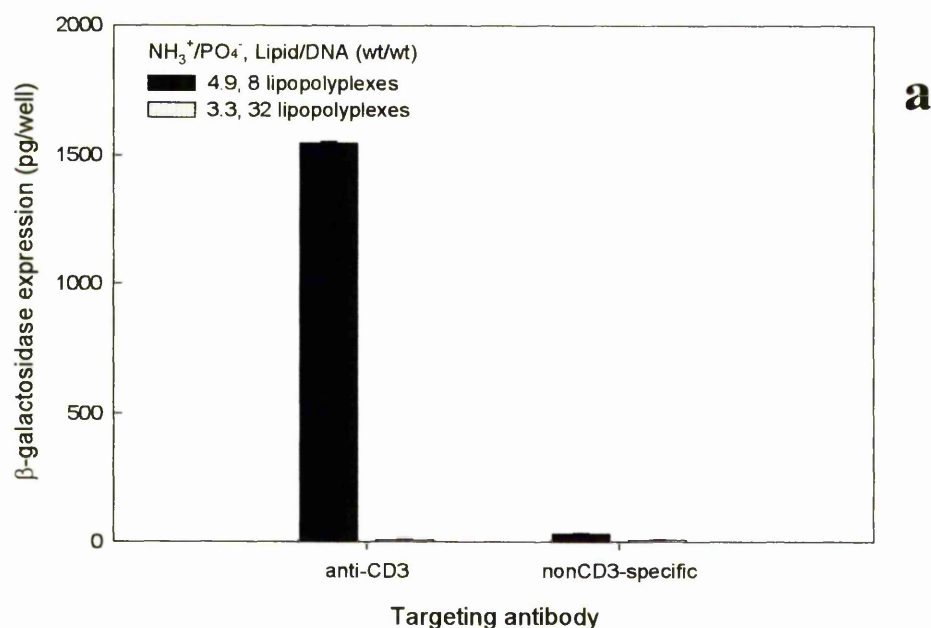


Figure 5.42 Targeted transfection of Jurkat cells using **DOPE/cholesterol/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** in the presence of 10% FCS. (a) β -galactosidase expression (b) extractable cellular protein content. **PLK₃₆-condensed pEGlacZ**. Antibody coupling ratios, anti-CD3 34.2 $\mu\text{g}/\mu\text{mol}$ lipid, non-CD3 specific 22.9 $\mu\text{g}/\mu\text{mol}$ lipid. Jurkat cell population pre-transfection 1×10^6 cells/well. β -galactosidase concentration and extractable cellular protein content in cell lysates were assayed 18h post-transfection. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3). Note change of scale of y-axis of (a) compared to Figure 5.41(a).

5.2.3.3 DOPE/DOPC/oleic acid liposome targeted transfection of Jurkat cells

DOPE/DOPC/oleic acid anti-CD3 lipopolyplexes have gene transfer activity when complexed to PLK₉₉ condensed DNA at a PLK/DNA charge ratio of 4.9 (Figure 5.4). To determine the specificity of this activity to the presence of the anti-CD3 antibody, Jurkat cells were transfected using identical lipopolyplexes, bearing either non-CD3 specific antibody or anti-CD3 specific antibody (Table 5.6).

Table 5.6 Properties of DOPE/DOPC/oleic acid liposomes used for the targeted transfection of Jurkat cells

Liposome formulation (40:39.9:20:0.1 mol%)	Particle diameter (nm) ^a	Zeta potential (mV) ^b	Antibody coupling ratio ($\mu\text{g}/\mu\text{mol}$)	Weight average Antibody molecules/ liposome ^c	Liposome batch age (days) ^d
DOPE/DOPC/oleic acid/DSPE-PEG/anti-CD3	108.7 ± 39.9	-44.3 ± 3.6	21.7	13	36
DOPE/DOPC/oleic acid/DSPE-PEG/non-CD3 specific	108.6 ± 20	-65.1 ± 1.5	18.3	13	36

^a determined by PCS. \pm s.d. of unimodal distribution.

^b in 10mM HEPES pH8.0. Zeta potential calculated using $f(\kappa a)=1.3$. s.d. is for $n=5$.

^c calculated based on M_w of IgG=150,000 using D-LIPPRO computer program.

^d days stored at 4°C since preparation.

Jurkat cells transfected using anti-CD3 lipopolyplexes display β -galactosidase expression levels that are greater at high lipid/DNA ratios (cf. lipid ratios of 4 and 32, t-test, $t=-9.28$, $P<0.001$; Figure 5.43a). Jurkat cells transfected using non-CD3 specific lipopolyplexes display little β -galactosidase expression.

The extractable cellular protein contents of anti-CD3 transfected Jurkat cell populations are lower than those of Jurkat cells transfected using non-CD3 specific lipopolyplexes (all lipid ratios, t-test, $P<0.05$; Figure 5.43b).

Lipopolyplex size, of both anti-CD3 and non-anti-CD3 specificity, is larger at high lipid/DNA ratios. Lipopolyplexes of equivalent lipid/DNA ratio are of similar size (Figure 5.44).

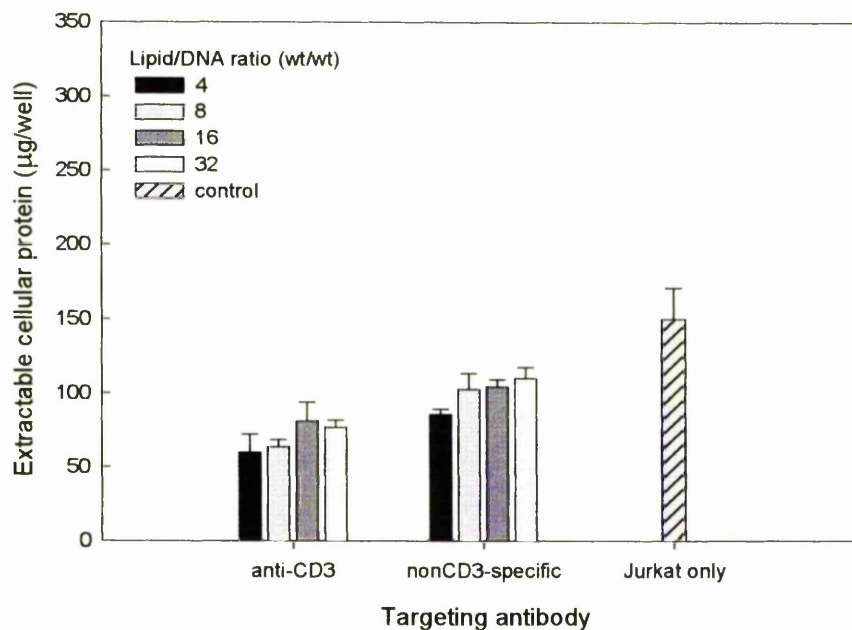
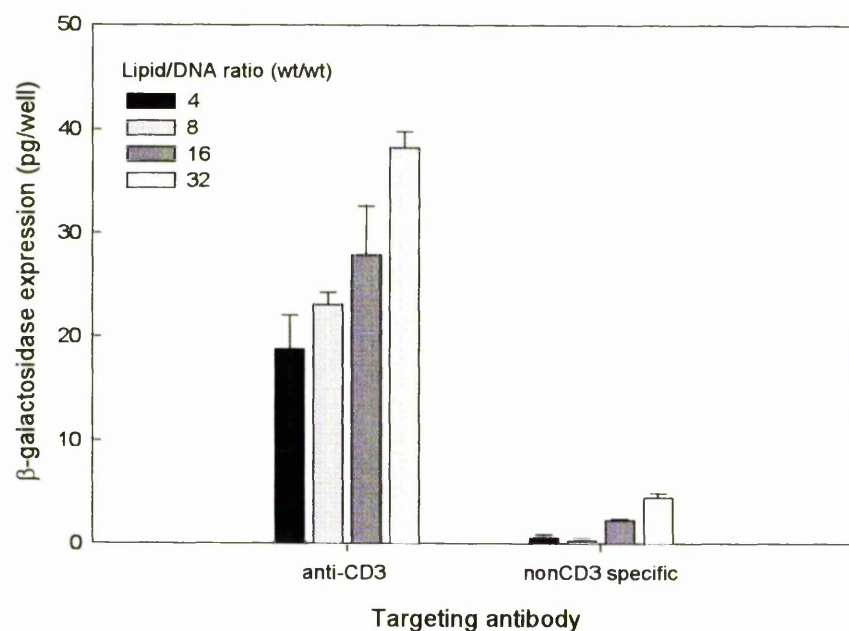


Figure 5.43 Targeted transfection of Jurkat cells using **DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) anti-CD3 lipopolyplexes** in serum-free media. (a) β -galactosidase expression (b) extractable cellular protein content. **PLK₉₉**-condensed pEGlacZ having a PLK/DNA charge ratio of 4.9. Antibody coupling ratios, anti-CD3 21.7 $\mu\text{g}/\mu\text{mol}$ lipid, non CD3-specific 18.3 $\mu\text{g}/\mu\text{mol}$ lipid. Jurkat cell population pre-transfection 1×10^6 cells/well. β -galactosidase concentration and extractable cellular protein content in cell lysates were assayed 18h post-transfection. Control Jurkat cell population was not challenged with lipopolyplexes. Data show mean \pm s.d. (n=3).

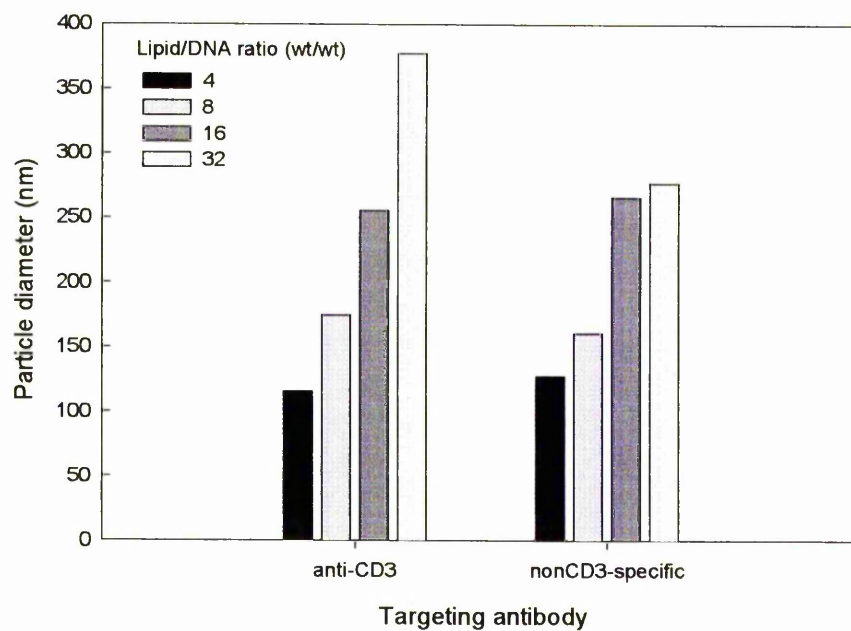


Figure 5.44 Particle diameters of **DOPE/DOPC/oleic acid/DSPE-PEG 40:39.9:20:0.1 mol%** anti-CD3 lipopolyplexes used for the targeted transfection of Jurkat cells (see Figure 5.43). Size determined by PCS. Diameter of component liposomes-anti-CD3 86.9nm, non-CD3 specific 108.6nm.

5.3 Discussion

5.3.1 Analysis of transfection data

The success of any transfection experiment is usually determined by the detection of a distinctive phenotype, conferred by the transfected gene, in the target cell. In 1944, when Avery demonstrated DNA was the transforming agent, transformed Pneumococci (*Streptococcus pneumoniae*) were detected using their distinct colony morphology when compared to that of non-transformed bacteria (Avery *et al.*, 1944).

Today, the gene that reports successful mammalian cell transfection usually expresses either β -galactosidase or fire fly luciferase enzymes. Using either gene however, the method by which the efficiency of transfection is reported varies.

The most straightforward analysis of transfection efficiency is to determine the number of cells in a transfected population expressing the transfected gene. This figure is usually expressed as a percentage of the total population of the cells transfected. If the figure of % cells transfected is used in conjunction with the total amount of gene expression present, it can be determined if only a small number of transfected cells are producing large amounts of gene product, or if all the cells are transfected and each are producing lesser amounts.

A second measure of the transfection efficiency is to determine the total amount of transfected gene product produced by the transfected cell population. By comparing total gene expression, any effect of cell growth or cell death in the transfected population is disregarded. The total gene-product produced by what is an unquantified cell population determines a successful transfection. This technique does however, rely upon high efficiency cell harvesting and lysis procedures to enable comparison between different transfected populations. The results of the transfection are usually expressed in terms of e.g. pg β -galactosidase/volume of cell lysates (e.g. McLachlan *et al.*, 1995) or pg β -galactosidase/cell, where cell number is taken as that of the cell population pre-transfection (e.g. Wheeler *et al.*, 1999).

By measuring the total extractable cellular protein of the transfected cell population, gene expression can be standardised per mg of cell protein. This technique allows comparison of transfected cell populations in which cell number post-transfection may have changed (Caplen *et al.*, 1995). The figure generated implies a certain

amount of transgene product is produced by each cell, however, no indication as to the proportion of cells transfected can be determined. This technique does not rely upon 100% lysis and harvesting efficiencies of the transfected cells, as the ratio of gene product/total cell protein should remain constant.

By standardising gene expression in terms of total cell protein, anomalies in the analysis of transfection efficiency can occur under certain transformation conditions. Under normal transfection conditions, pg/mg protein gives an indication not only of gene expression per cell, but the relative totals of gene-product produced by each transfected population. However, if the transfection agent is highly cytotoxic, the number of cells can be greatly reduced with a subsequent lowering of the total amount of extractable cellular protein. If the transfected gene is expressed by this population, the figure of pg expression /mg protein data may indicate a high level of transfection. As the total amount of extractable cellular protein is greatly reduced, the total amount of gene expression is very small, compared to a healthy transfected population displaying a similar pg/mg protein expression (Figure 5.45). It could be said however, that the small number of transfected cells in the unhealthy population are expressing large amounts of gene product, and that is the purpose of the transfection.

In the present studies, a number of the DNA condensing agents display high levels of cytotoxicity. An example of how this skews the apparent efficiency of transfection is described below.

A population of Jurkat cell transfected using a 4.9, 32 lipopolyplex having PLK₅₃₁ as the DNA condensing agent produces 110pg β -galactosidase/well (Figure 5.26). A similar Jurkat cell population transfected using a 2.5, 8 lipopolyplex having PLK₃₆ as the DNA condensing agent produces 1061pg β -galactosidase/well (Figure 5.22). However, as very few cells are present in the former transfection population (extractable cellular protein content <10 μ g/well, Figure 5.27) compared to the latter (99 μ g/well, Figure 5.23) both display similar transfection efficiencies in terms of pg β -galactosidase/mg protein (10,953pg/mg and 10,907pg/mg respectively).

It was therefore decided to analyse the transfection data in terms of pg β -galactosidase per well (i.e. per total cell population). To enable direct comparison of each transfection reaction. The total extractable cellular protein of the transfected cell population was measured to give an indication as to the cytotoxic effect of the lipopolyplex preparations.

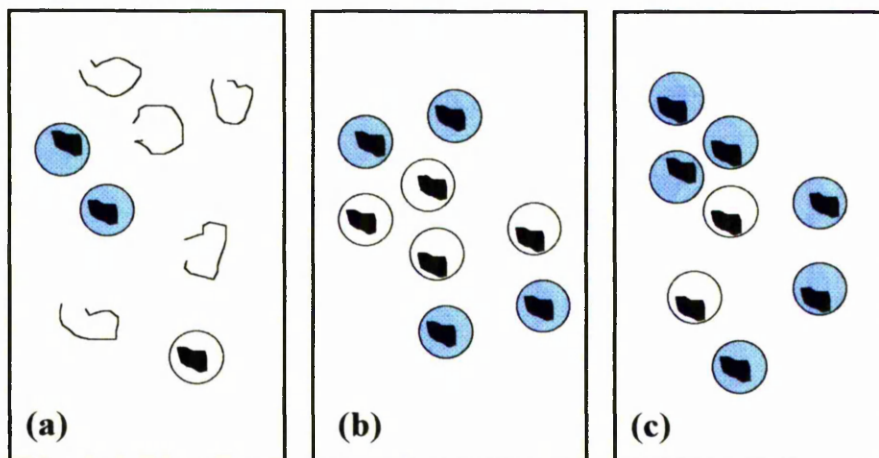


Figure 5.45 The effect of cell viability on the interpretation of experimental transfection data. White spheres=viable non-transfected cells, high protein content. Blue spheres=viable transfected cells each expressing an equal amount of β -galactosidase, high protein content. Open lines=non-viable cells containing no extractable protein. Analysis of the transfection in terms of pg β -galactosidase/total cell population would show the efficiency of (c)>(b)>(a). Analysis in terms of pg β -galactosidase/mg extractable protein (per viable cell) would show efficiency (c) \geq (a)>(b). Transfection efficiency of (a) is skewed upwards with respect to (b) and (c) when analysed using pg/mg protein despite the total amount of β -galactosidase expressed by (a) being far lower. Population (c) shows higher transfection efficiency than (b) using either analysis. Proportions of each cell type are representative of the entire transfected cell population

5.3.2 Primary transfection screen

5.3.2.1 Transfection of Jurkat cells using PLK₉₉ polyplexes

Jurkat cells cannot be successfully transfected using PLK₉₉ polyplexes with the current transfection protocol. This is true of both anionic polyplexes (PLK/DNA charge ratio 0.8) and cationic polyplexes (PLK/DNA charge ratio 1.6, 3.3, and 4.9) (data not shown). Any transfection activity seen in Jurkat cells transfected using lipopolyplexes can be assumed to be conferred by the liposome (lipid) component of the vector. Thurnher *et al.* (1994) successfully transfected Jurkat cells using anti-CD3 polyplexes, but it was necessary for the endosmolytic agent chloroquine, to be present in the transfection medium. The presence of an anti-CD3 antibody enabled the polyplexes to be taken up by the cell via receptor-mediated endocytosis. Chloroquine caused the rupture of the endosomal membrane releasing intact polyplexes into the cytoplasm. The polyplexes could then be transported to the nucleus of the cell leading to successful transfection. In the absence of an endosmolytic agent, polyplexes are retained in the endosomal compartment and further processed by lysosomes (Wagner, 1998). This suggests that the lipid component of the lipopolyplex has to provide an endosome escape mechanism for its associated condensed DNA (a modified polyplex), if successful gene transfer is to occur.

Polyplexes containing PLK₉₉ display a cytotoxic effect toward Jurkat cells (Figure 5.16). Polyplexes having a high PLK/DNA charge ratio (high PLK content) have high cytotoxicity (19% of the extractable cellular protein content of untreated control cells, PLK/DNA charge ratio of 4.9), whereas polyplexes having a charge ratio of 0.8 have minimal cytotoxicity. The high levels of cytotoxicity are not related to high levels of observed transfection (β -galactosidase expression).

Poly-L-lysine has been shown to be cytotoxic to a number of cell types, including endothelial cells (Morgan *et al.*, 1988 and 1989), and has shown antitumour activity *in vivo* (Arnold *et al.*, 1979). Poly-L-lysine is thought to mediate its cytotoxic effect by binding to the cell surface (via phospholipids) and altering the membrane fluidity of the affected cell. This alteration of membrane fluidity leads to membrane rupture and loss of intracellular contents. Cell number does not necessarily decrease, but affected cells may lose much of their intracellular protein content (Morgan *et al.*, 1988). The

toxicity of poly-L-lysine is dose, time, and molecular weight dependent. Polymers of high molecular weight display greater cytotoxicity, whereas polymers of $\overline{M}_w < 25,000$ have no cytotoxic effect (Morgan *et al.*, 1998). This would suggest that the poly-L-lysine used in the present studies PLK₉₉, which has an average molecular weight of 20,700 Da, will have low cytotoxicity. However, Sigma Chemicals own analysis of PLK₉₉ shows it to be very heterogeneous, 10% of the polymers in the preparation having a M_w greater than 49,000 Da. The presence of these high \overline{M}_w polymers in the polyplex preparation could cause the cytotoxic effect observed in the Jurkat cells.

All studies of the cytotoxicity of poly-L-lysine do however, employ solutions of free poly-L-lysine, and not poly-L-lysine bound to DNA as is the case here. This suggests that either the polyplex preparation contains “free” poly-L-lysine polymers not bound up in polyplexes, which confer the cytotoxicity, or that poly-L-lysine has a greater affinity for the cell surface than the DNA in the polyplex, leading to polyplex dissociation and cell surface binding of the released poly-L-lysine. Zeta potential analysis of PLK₉₉ polyplexes supports the former analysis that “free” poly-L-lysine is present in polyplex preparations. Above a PLK/DNA charge ratio of 1, polyplexes carry a zeta potential of +40mV, a value that does not increase at higher charge ratios. This suggests that the polyplex has become saturated with poly-L-lysine, as any further addition does not confer a greater, more positive zeta potential. An equilibrium is set up, with any excess poly-lysine added being present unbound in solution (Figure 3.4b). Conversely, when increasing the PLK/DNA charge ratio of the polyplex from 0 to 1, its zeta potential becomes less negative with each addition of poly-L-lysine, suggesting all poly-L-lysine can bind to unoccupied sites on the polyplex (implicit in Pouton *et al.*, 1998).

Polyplexes having high PLK/DNA charge ratios would therefore have a greater proportion of their poly-L-lysine content free in solution, and be able to exert a greater cytotoxic effect. Intact polyplexes may bind to the cell surface in conjunction with free poly-L-lysine and exert their own cytotoxic effect. However, as polyplexes having charge ratios of 1.6 and 4.9 probably contain similar amounts of poly-L-lysine, the extra cytotoxicity observed from a polyplex having a charge ratio of 4.9 is probably largely due to free poly-L-lysine.

Summary

- PLK₉₉-DNA polyplexes in isolation appear to lack the necessary properties for successful gene transfer to Jurkat cells.
- PLK₉₉-DNA polyplexes are cytotoxic to Jurkat cells at high charge ratios, perhaps due to the presence of unbound poly-L-lysine in the polyplex preparation.

5.3.2.2 Gene transfer properties of DOPE/cholesterol/oleic acid liposomes

Lipopolyplexes prepared from DOPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 (40:39.9:20:0.1 mol%) liposomes and PLK₉₉ condensed DNA transfect Jurkat cells. The lack of gene transfer activity of lipopolyplexes having a PLK/DNA charge ratio of 0.8 suggests electrostatic interaction between anionic liposome and polyplex is required for successful transfection. All lipopolyplexes formed by the interaction of cationic polyplexes (charge ratios of 1.6, 3.3, and 4.9) and anionic liposomes have gene transfer activity (Figure 5.1).

The transfecting lipopolyplexes are thought to bind to their CD3 ligand and become internalised via receptor-mediated endocytosis. The clathrin coated vesicles (CCVs) formed by this process are generally processed to lysosomes via endosomes, where their contents are degraded by hydrolytic enzymes (Maurer *et al.*, 1999). Polyplexes that enter the cell via endocytosis are probably degraded in this manner and therefore have low gene transfer activity. However, DOPE/cholesterol/oleic acid lipopolyplexes have high gene transfer activity, so their DNA must avoid the deleterious effects of lysosomal processing.

DOPE/cholesterol/oleic acid (40:40:20 mol%) liposomes have been shown to destabilise at pH values of less than 6, a similar pH to that found in early endosomes and lysosomes (Wang and Huang, 1987). These liposomes are pH-sensitive, the fall in pH causes them to fuse with the endosomal membrane, and release any liposome-associated contents into the cytoplasm. This would cause the release of lipopolyplex associated DNA into the cytoplasm without any degradative enzyme processing, and increases the chance of successful transfection.

The condensed DNA particle in the lipopolyplex is not thought to be free in the aqueous core of the liposome, but adsorbed onto the liposomes external leaflet via electrostatic interactions (4.3.1). Therefore a liposome/endosome fusion event would

not directly lead to the translocation of the DNA particle into the cytoplasm. It has been suggested that endosome destabilisation occurs following the fusion event allowing DNA delivery into the cytoplasm (Litzinger and Huang, 1992).

Lee and Huang (1996) used similar lipopolyplexes to transfect a KB (epidermal) cell line. Polyplexes of PLK₁₂₂ having a charge ratio of 1.1 were associated with anionic liposomes of the formulation DOPE/cholesteryl hemisuccinate/folate-PEG-DOPE (6:4:0.01 mol/mol). They noted a peak in transfection using lipopolyplexes having a lipid/DNA ratio of 12, with transfection activity falling at higher lipid ratios. Minimal transfection activity was noted from lipopolyplexes that had aggregated before addition to the cells (lipid/DNA ratio of 8). The pattern of transfection activities is similar to those shown in the present study by the series of lipopolyplexes having a PLK/DNA charge ratio of 1.6. Here, peak transfection activity is also noted at a lipid/DNA ratio greater than that at which lipopolyplexes aggregate, with transfection activity falling at higher lipid/DNA ratios. It was suggested by Lee and Huang that this effect may be due to excess "empty" liposomes inhibiting successful transfection.

The lower transfection activities observed using lipopolyplexes having a PLK/DNA charge ratio of 3.3 or 4.9 appears to be due to the cytotoxicity of the complexes to the targeted cells. Lipopolyplexes having these charge ratios have similar cytotoxicities as polyplexes having the same charge ratio (Figures 5.2, 5.16). Cytotoxicity does not appear to be related to total lipid content of the lipopolyplex, as cytotoxicity does not increase at high lipid/DNA ratios. The cytotoxicity observed may prevent successful transfection of the target cells. Alternatively, the lipid/DNA ratios tested may not be optimal. Lee and Huang (1996) suggest that the optimal transfecting lipopolyplex has negative charge from the liposome component in excess of the positive charge from the condensed DNA. For polyplexes at PLK/DNA charge ratios of 3.3 and 4.9, addition of liposomes to form lipopolyplexes having neutral overall charge (aggregating lipopolyplexes) have lipid/DNA ratios of 16 and 32 respectively. Therefore, only one and zero lipid/DNA ratios respectively, were tested where the lipopolyplex had an overall negative charge, and perhaps the optimum formulation was not tested.

Cytotoxicity of all lipopolyplexes was similar to that of polyplexes having an identical PLK/DNA charge ratio. No additional cytotoxicity was noted in the transfected Jurkat cell populations expressing high levels of β -galactosidase. This suggests that

successful transfection by the lipopolyplex and any subsequent gene expression is not detrimental to the cell (Figure 5.2).

Size of the lipopolyplex is not related to transfection activity when the particles are colloidal. The measured lipopolyplex diameter is greater at lipid/DNA ratios when the resultant complex has low overall surface charge (lipid/DNA ratios close to aggregation point). Whether this indicates the formation of larger individual complexes, or simply a population of the smaller complexes aggregating is unknown. Lipopolyplexes having a PLK/DNA charge ratio of 0.8 do not aggregate at any of lipid/DNA ratios assayed (Figure 5.3). The smaller particle diameter of lipopolyplexes having a high lipid/DNA ratio could be due to the lipopolyplex preparation containing a greater proportion of liposomes (124.1nm) to polyplexes (257.6nm), giving an average diameter of ~150nm.

Contradictions in the literature exist with regard to the influence of transfection complex size on transfection efficiency. Ross and Hui (1999) found that lipoplexes of large size (>2000nm) were more effective in transfecting CHO cells *in vitro*. Nagoyosu *et al.* (1999) add that vesicles of 100nm become trapped in the interstitial spaces of tumours, an important factor to be considered for *in vivo* applications. The lipopolyplexes used in the present study conferring maximum transfection have a diameter of 168.4 ± 44.3 nm. Complexes of this size appear to be readily endocytosed by Jurkat cells.

Summary

- DOPE/cholesterol/oleic acid liposomes complexed with PLK₉₉-condensed DNA transfect Jurkat cells most efficiently using lipopolyplexes having a charge ratio of 1.6.
- Gene transfer activity is probably related to the ability of DOPE/cholesterol/oleic acid liposomes to rupture the endosomal compartment and release undegraded DNA into the cytoplasm.
- Lipopolyplexes having high charge ratios confer low levels of transfection perhaps due to their high cytotoxicity.
- Cytotoxicity of lipopolyplexes appears to be conferred by the poly-L-lysine component.

- Non-colloidal aggregates of lipopolyplexes having low surface charge have little gene transfer activity.

5.3.2.3 Gene transfer properties of DOPE/DOPC/oleic acid liposomes

Jurkat cells transfected using DOPE/DOPC/oleic acid/DSPE-PEG-anti-CD3 lipopolyplexes display little transgene expression (Figure 5.4). Lipopolyplexes formed using this liposome formulation are of similar size to those prepared from DOPE/cholesterol/oleic acid liposomes at equivalent PLK/DNA and lipid/DNA ratios (Figures 5.3, 5.6). The type of interaction between the condensed DNA and the liposome in each system is somewhat similar (4.3.2). The two liposome formulations also bear similar numbers of anti-CD3 antibodies, and have similar size and zeta potential measurements (Table 5.1), and can be assumed to interact with Jurkat cells in a similar CD3-dependent manner. The reduction in transfection efficacy appears to be caused by the substitution of cholesterol for dioleoylphosphatidylcholine (DOPC) in the associated liposome.

It has been noted that DOPE/palmitoylhomocysteine (80:20 mol%) liposomes undergo a pH-induced fusion at pH5, whereas liposomes substituted with DOPC do not undergo a similar fusion event (Connor *et al.*, 1984). Furthermore, DOPC/cholesterol/hemisuccinate (6:4 mol/mol) liposomes do not transfect KB epidermal cells, whereas a DOPE substituted formulation deliver high levels of transfection (Lee and Huang, 1996).

DOPC containing liposomes are not pH-sensitive, and cannot release their contents from endosomal vesicles when their external pH is lowered (Chu and Szoka, 1994). Unlike the cone-shaped lipid DOPE, DOPC is a cylindrical-shaped lipid and does not readily form the H_{II} phases involved in membrane fusion under physiological conditions (Boni and Hui, 1983). A DOPE liposome at neutral pH can be stabilised in the bilayer phase using, in the present study, oleic acid. A fall in pH, for example in an endosomal compartment, leads to the protonation of the acid head group which is then unable to repulse intermolecular interactions between DOPE molecules. The DOPE lipid reverts to a H_{II} (inverted hexagonal) phase causing a membrane fusion event. In a transfection particle the fusion event would release DNA into the cytoplasm and increase the probability of successful transfection. A DOPC/oleic acid

liposome would similarly become protonated at the low pH in an endosome. However, it would remain in the bilayer phase as it has no propensity to form H_{II} phase structures. Any DNA associated with a DOPC/oleic acid liposome is likely to be processed by lysosomal enzymes as it has no mechanism to escape the endosomal compartment with high efficiency. Few intact DNA particles would be released into the cytoplasm to enable successful transfection.

It would appear that the liposomes used in the present study of the formulation DOPE/DOPC/oleic acid/DSPE-PEG (40:39.9:20:0.1 mol%) are not pH sensitive despite having a DOPE content of 40 mol%. Tilcock *et al.* (1982) have shown that as little as 20 mol% of a PC lipid in a PE lipid suspension is enough to stabilise it in the bilayer phase at 40°C. It would appear that the DOPE lipid in the DOPE/DOPC/oleic acid liposome formulation is unable to undergo a bilayer to hexagonal (H_{II}) phase transition at low pH due to its stabilisation in the bilayer phase by DOPC.

Despite the lack of pH-sensitivity of this liposome formulation, low levels of transfection are observed using each lipopolyplex formulation.

Why would these lipopolyplex-transfected cells display higher levels of transfection than polyplex transfected cells if both are subject to lysosomal degradation? It is perhaps a matter of the increased probability of release of undamaged DNA from lysosomes using lipopolyplex transfection. Polyplexes bear no targeting ligand, and therefore the non-specific binding of these complexes to the cell surface probably causes little uptake via receptor-mediated endocytosis into endosomal vesicles. Of the small number of polyplexes taken up by the cell very few of these will escape endosomal degradation to enable successful transfection. In contrast, lipopolyplexes are targeted to the CD3 ligand and would therefore be taken up by the cell via receptor-mediated endocytosis in great numbers. If a similar proportion of lipopolyplex associated DNA were to escape from the lysosomal compartment intact as do from polyplexes, a greater level of transfection would be observed. Resistance to lysosomal degradation is perhaps facilitated by the DNA being condensed using poly-L-lysine, such condensation has been shown to have nuclease resistant properties (Zauner *et al.*, 1998).

DOPE/DOPC/oleic acid lipopolyplexes that have aggregated have similar gene transfer activity as colloidal particles (contra Lee and Huang, 1996). Lipopolyplex aggregation has been shown to reduce gene transfer activity markedly when using DOPE/cholesterol/oleic acid lipopolyplexes (Figure 5.1). It may be possible that in

aggregated lipopolyplex preparations there are small numbers of “mono-lipopolyplexes” able to confer transfection. It is unlikely that large aggregates can be taken up by a Jurkat cell via receptor-mediated endocytosis (Maurer *et al.*, 1999).

Interestingly, lipopolyplexes having a PLK/DNA charge ratio of 0.8 have similar gene transfer activity to complexes having higher charge ratios (Figure 5.4). It is thought that lipopolyplex preparations having a charge ratio of 0.8 exist as separate particles, of anionic condensed DNA and anionic liposomes, due to charge repulsion. For successful transfection to occur using this lipopolyplex, the DNA particle would have to be co-endocytosed with the anti-CD3 liposome and escape lysosomal degradation.

The lipid component does not appear to be cytotoxic, and therefore caused no apparent reduction in transfection activity.

Summary

- DOPE/DOPC/oleic acid liposomes lack the majority of the gene transfer activity of DOPE/cholesterol liposomes.
- The DOPC lipid probably renders the liposome formulation non-pH-sensitive and unable to rupture the endosomal compartment at low pH.
- All observed transfection activity is probably conferred by the nuclease resistant properties of the poly-L-lysine condensed DNA delivered to the cells by the anti-CD3 liposomes in large amounts.

5.3.2.4 Gene transfer properties of DMPE/cholesterol/oleic acid liposomes

Lipopolyplexes prepared using DMPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 liposomes have low levels of gene transfer activity in Jurkat cells (Figure 5.7). This suggests that DMPE/cholesterol/oleic acid lipopolyplexes cannot rupture the endosomal compartment, and that their associated DNA is subject to degradation in the lysosomes (Wagner, 1998). Felgner *et al.* (1994) used a series of cationic liposome preparations containing neutral helper lipids (PE lipids) of different acyl chain lengths, to transfect Cos7 cells. They observed that a cationic formulation containing the helper lipid DMPE had a seven-fold reduction in transfection activity compared to that of a DOPE containing preparation.

By examining bilayer L_{α} - H_{II} inverted hexagonal phase transition properties of each of the lipids, a possible explanation for the loss of transfection activity can be proposed. The lamellar bilayer-inverted hexagonal phase transition temperature (T_h) of PE lipids is affected by a number of factors including acyl chain length and saturation. Generally, PE lipids of shorter acyl chain length and greater saturation have a higher T_h (Lewis *et al.*, 1989). The T_h of an aqueous suspension of DMPE lipid is $>150^{\circ}\text{C}$, whereas that of DOPE is $+10^{\circ}\text{C}$ (Seddon *et al.*, 1983). Therefore, under physiological conditions (37°C , low ionic strength) DMPE favours the lamellar bilayer phase, whereas DOPE favours the inverted hexagonal phase. A liposome composed of DMPE/cholesterol/oleic acid exposed to the low pH in the endosome will be in a stable bilayer phase despite oleic acid protonation, and will not form H_{II} phase structures involved in membrane fusion. A similar series of events to those observed using DOPE/DOPC/oleic acid liposome preparations will lead to lysosomal degradation of the majority of lipopolyplex associated DNA and little observed transfection.

Little additional cytotoxicity is noted from DMPE/cholesterol/oleic acid lipopolyplexes when compared to PLK₉₉ polyplexes having an equivalent charge ratio (Figure 5.8). This adds to the evidence obtained so far that the majority of cytotoxicity is conferred by the DNA/poly-L-lysine component of the vector. Again, no correlation is observed between lipopolyplex size and transfection activity (Figure 5.9).

Summary

- DMPE/cholesterol/oleic acid liposomes have low gene transfer activity, probably due to their lack of pH-sensitive function.

5.3.2.5 Gene transfer properties of DLPE/cholesterol/oleic acid liposomes and DLPE/DLPC/oleic acid liposomes

Lipopolyplexes prepared using DLPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 liposomes have minimal gene transfer activity in Jurkat cells (Figure 5.10), DLPE/DLPC/oleic acid/DSPE-PEG-anti-CD3 lipopolyplexes have no gene transfer activity (data not shown). DLPE does not favour the H_{II} phase under physiological

conditions ($T_h = >150^\circ\text{C}$, Seddon *et al.*, 1983), therefore DLPE containing "pH-sensitive" liposome preparations are unlikely to fuse with endosomal vesicles at low pH. This total lack of transfection is anomalous compared to the low levels of transfection observed using DOPE/DOPC/oleic acid and DMPE/cholesterol/oleic acid lipopolyplexes. These formulations are similarly thought to lack endosomal fusion properties, but they still show residual transfection activity using each of the lipopolyplex preparations. This transfection was suggested to be due to the large amounts of DNA being trafficked into the endosomal compartment, increasing the probability of a small amount of intact plasmid DNA escaping lysosomal degradation. Using DLPE containing lipopolyplexes for transfection this does not appear to be the case. DLPE/cholesterol/oleic acid lipopolyplexes have no additional cytotoxicity in comparison to the other liposome preparations used for transfection. Lipopolyplex particle size is similar to that found using each of the other liposome preparations. In addition, the DLPE/cholesterol/oleic acid liposomes bear a similar amount of anti-CD3 antibody to both the DOPE/DOPC/oleic acid and DMPE/cholesterol/oleic acid liposome preparations. This suggests a similar number of lipopolyplexes should be endocytosed by the Jurkat cells into endosomal vesicles.

The lower observed gene transfer activity could be due to a number of factors. Plasmid DNA may be more efficiently protected from lysosomal degradation by DOPE/DOPC/oleic acid and DMPE/cholesterol/oleic acid liposomes, although there is little evidence to suggest that their lipopolyplexes have different structures (4.3.2). DNA may be bound to DLPE/cholesterol/oleic acid liposomes in such a manner that prevents its release from the lipid membrane upon endosomal rupture. It is perhaps more likely that DOPE/DOPC/oleic acid and DMPE/cholesterol/oleic acid liposomes retain some residual membrane fusion activity, and allow small amounts of non-degraded DNA to be released from the endosomal compartment. Felgner *et al.* (1994) imply that cationic liposomes incorporating DMPE are able to escape endosomal compartments but at a low rate.

Summary

- DLPE containing liposomes lack any gene transfer activity, probably due to their lack of pH-sensitive function.

5.3.3 Secondary transfection screen

The secondary transfection screen determined the effect the degree of polymerisation of the poly-L-lysine chain has on lipopolyplex gene transfer activity, with comparison to PLK₉₉ lipopolyplexes used in the primary transfection screen. A poly-L-arginine of defined degree of polymerisation PLR₁₇, was also tested as a DNA condensing agent with different chemical properties.

It has been suggested that polyplex receptor-mediated transfection is aided by poly-L-lysine of high \overline{M}_w (Zauner *et al.*, 1999; Pouton *et al.*, 1998; Ziady *et al.*, 1999; Xu *et al.*, 1998). Cationic lipid-mediated transfection has been shown to be enhanced by poly-L-lysines of \overline{M}_w 3,400, 25,600 and 200,000 (Gao and Huang, 1996). Few ternary systems using anionic liposomes have used poly-L-lysine as the DNA condensing agent (e.g. Lee and Huang, 1996).

5.3.3.1 Transfection of Jurkat cells using lipopolyplexes containing PLK₅- and PLK₃₆-condensed DNA

In the present study, lipopolyplexes prepared using PLK₅ and DOPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 liposomes show enhanced gene transfer activity compared to PLK₉₉ lipopolyplexes (Figure 5.18). The maximum transfection activity observed using PLK₅ lipopolyplexes is three-fold greater than the maximum levels obtained using PLK₉₉ lipopolyplexes. The higher gene transfer activity of PLK₅ lipopolyplexes could be due to the low cytotoxicity of PLK₅. Polyplexes prepared using PLK₅ show no gene transfer activity, but show little cytotoxicity to Jurkat cells even at high PLK/DNA charge ratios (Figure 5.37). Small poly-L-lysine molecules such as PLK₅ have been shown to have little cytotoxicity toward endothelial cells (Morgan *et al.*, 1988). As was shown using PLK₉₉ lipopolyplexes, most of the cytotoxicity of the vector was conferred by the poly-L-lysine condensed DNA (Figure 5.19). It is possible that the lower cytotoxicity of PLK₅ lipopolyplexes allows a greater number of viable cells to be successfully transfected.

The slight increase in cytotoxicity shown by Jurkat cells transfected using lipopolyplexes having a high PLK/DNA charge ratio appears to be due to their high levels of β -galactosidase expression. Higher β -galactosidase expression probably

indicates delivery of greater amounts of plasmid DNA. Bacterially produced plasmid DNA has potent immunostimulatory activity in mammalian systems. Structural motifs such as unmethylated CpG dinucleotides found in bacterial DNA are uncommon in mammalian genomes and act as immunostimulatory sequences (ISSs) triggering innate immunity (Krieg *et al.*, 1995). Bacterial promoters and antibiotic resistance genes contain many such sequences. Delivery of large quantities of plasmid DNA to T lymphocytes may cause a reduction in cell viability. In addition, it has been observed that a cytotoxic T lymphocyte response is activated by “bacterial” β -galactosidase expression *in vivo*, where cells expressing the protein are preferentially targeted and destroyed (Lai *et al.*, 1989). The targeting of these cells indicates a somewhat detrimental effect on the cell when β -galactosidase is expressed.

A remarkably similar trend is shown when using PLK₃₆ lipopolyplexes prepared from DOPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 liposomes. Polyplexes of PLK₃₆ having high PLK/DNA charge ratios show a lower level of cytotoxicity than equivalent PLK₉₉ polyplexes (Figure 5.38). Maximum transfection levels are similar to those obtained using PLK₅ lipopolyplexes (Figure 5.22). Again, additional cytotoxicity observed in transfected cells appears to be caused by high levels of β -galactosidase expression (Figure 5.23). It appears that efficient transfection using lipopolyplexes having high PLK/DNA charge ratios is possible using DNA condensing agents such as PLK₅ and PLK₃₆ that confer low cytotoxicity. Indeed, lipopolyplexes prepared using PLK₃₆ at a PLK/DNA charge ratio of 4.9, and low lipid/DNA ratios, confer greater transfection activity than lipopolyplexes having charge ratios of 2.5 and 3.3 (contra Pouton *et al.*, 1998).

Previous studies have shown that polyplexes prepared using low \overline{M}_w poly-L-lysines have low transfection activity *in vitro* in comparison to high \overline{M}_w polymers (Ziady *et al.*, 1999; Xu *et al.*, 1998). This apparent contradiction with the present study may be related to the role of the poly-L-lysine in each of the different types of transfection procedure. Most studies of poly-L-lysine-based transfection use polyplexes targeted to the cell surface via a specific receptor. These polyplexes are taken up by the cell via receptor-mediated endocytosis and processed to endosomal vesicles, from which they must escape if transfection is to be successful (Wagner, 1998). DNA in polyplexes incorporating a high \overline{M}_w poly-L-lysine has been shown to be resistant to serum nuclease degradation (Kwoh *et al.*, 1999) and attack from DNase enzymes (Ziady *et al.*, 1999). This enables intact DNA to escape lysosomal degradation. Small poly-L-

lysines tend to form larger polyplexes that are less resistant to enzymic degradation and often confer low transfection activity. Poly-L-lysine in this case serves to stabilise the DNA during endosomal processing in addition to its DNA condensing and ligand targeting roles.

The lack of enzymic protection of DNA by PLK₅ and PLK₃₆ can be inferred by studying the levels of transfection observed using DOPE/DOPC/oleic acid and DMPE/cholesterol/oleic acid lipopolyplexes prepared using each agent.

A residual level of gene transfer is shown by PLK₉₉ lipopolyplexes prepared using each of these liposome formulations. This gene transfer activity was thought to be conferred by small amounts of intact plasmid DNA escaping lysosomal degradation following receptor-mediated endocytosis. No gene transfer activity is observed using PLK₅ and PLK₃₆ lipopolyplexes prepared using each of these liposome formulations, with little associated cytotoxicity (Figures 5.20, 5.21, 5.24, 5.25). This suggests that the condensed DNA particle associated with the lipopolyplex is more susceptible to lysosomal degradation (and cytosolic nucleases) than that in a PLK₉₉ lipopolyplex.

Lipopolyplex formulations which can escape the endosomal compartment do not require the enzymic resistance properties of high \overline{M}_w poly-L-lysine condensed DNA as their sole mechanism for successful cytoplasmic delivery. The function of the poly-L-lysine in this system is to condense the DNA into a small, positively charged particle that can interact with the anionic liposome. If the poly-L-lysine has low toxicity to the targeted cell, it may ultimately enable a higher transfection efficiency as a greater number of cells can be transfected. Further roles for poly-L-lysine in gene transfer have also been suggested. Zauner *et al.* (1999) suggest that poly-L-lysine may have a role in the targeting of condensed DNA particles to the nucleus. This is based on the observation that cytoplasmically injected poly-L-lysine-condensed DNA is transported to the nucleus to a greater extent than naked DNA. However, as demonstrated by Lechardeur *et al.* (1999) plasmid DNA is inherently unstable in the cytosol. Poly-L-lysine may therefore be acting to protect the DNA from the action of cytosolic nucleases. Ziady *et al.* (1999) suggest that DNA condensed using low \overline{M}_w poly-L-lysine may be more readily accessible to RNA polymerase, aiding gene expression.

Summary

- PLK₅ and PLK₃₆ DNA condensing agents show enhanced gene transfer activity compared to PLK₉₉ when complexed to DOPE/cholesterol/oleic acid liposomes.
- Enhanced gene transfer activity may be related to the low cytotoxicity of PLK₅ and PLK₃₆.
- High levels of transfection in Jurkat cells appear to be “cytotoxic”
- DNA condensed using PLK₅ and PLK₃₆ appears to be susceptible to lysosomal/cytoplasmic nucleases.

5.3.3.2 Transfection of Jurkat cells using lipopolyplexes containing PLK₅₃₁—condensed DNA

Polyplexes prepared using PLK₅₃₁ show small amounts of gene transfer activity when prepared at a PLK/DNA charge ratio of 3.3 (4pg β -galactosidase/well) and 4.9 (13pg/well). Polyplexes prepared using PLK₅, PLK₃₆ and PLK₉₉ do not transfect Jurkat cells using the current transfection protocol. It would appear that PLK₅₃₁ polyplexes having high PLK/DNA charge ratios are able to overcome cellular barriers that inactivate polyplexes prepared using smaller poly-L-lysines. A similar trend was observed by Wagner *et al.* (1990) when transfecting erythroblast cells using transferrin-modified poly-L-lysine polyplexes. They found that a high \overline{M}_w poly-L-lysine (PLK₂₇₀) was more efficient for transfection than one having a lower \overline{M}_w (PLK₉₀). In addition, Xu *et al.* (1998) have shown that polyplexes prepared using poly-L-lysine substituted with epidermal growth factor have highest transfection activity when prepared using PLK₁₁₁₆, but little transfection activity when prepared using PLK₁₁₄ or PLK₁₉.

It has been shown that PLK polyplexes prepared using high \overline{M}_w poly-L-lysine are more resistant to nuclease degradation (Ziady *et al.*, 1999). This may increase the probability that the PLK₅₃₁ polyplexes used in this study escape lysosomal degradation following endosomal uptake.

Polyplexes prepared using PLK₅₃₁ have similar cytotoxicities to Jurkat cells as PLK₉₉ polyplexes prepared at identical charge ratios (Figure 5.39). It could be expected that a poly-L-lysine of \overline{M}_w 111,000 (PLK₅₃₁) would be more cytotoxic than of \overline{M}_w 20,700 (PLK₉₉) (Choksakulnimitr *et al.*, 1995). However, Morgan *et al.* (1988) show that poly-L-lysines of \overline{M}_w 111,000 and 20,700 have similar cytotoxicities up to a

concentration 800nM. The poly-L-lysine concentration in a Jurkat cell population transfected using PLK₉₉ polyplex having a charge ratio of 4.9 is 854nM. However, most of which it is assumed is bound to the DNA and not free in solution. Therefore, the free poly-L-lysine concentration in PLK₉₉ and PLK₅₃₁ polyplexes is lower than that required to show differential cytotoxicities.

It is likely that the Jurkat cells have different susceptibility to poly-L-lysine cytotoxicity than the endothelial cells used by Morgan *et al.* (1988). Choksakulnimitr *et al.* (1995) show that small poly-L-lysines are more cytotoxic to endothelial cells than macrophages. It must also be noted that the untreated Jurkat cells in the present transfection have a lower extractable cellular protein content than those used in previous transfections (104µg/well, compared to 197µg/well (Figure 5.16) and 169µg/well (Figure 5.37, Figure 5.38)). The lowering of extractable cellular protein may have changed the Jurkat cells' susceptibility to poly-L-lysine cytotoxicity.

Lipopolyplexes prepared using PLK₅₃₁ and DOPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 liposomes have similar gene transfer activity as those prepared using PLK₉₉. The highest transfection activity was observed in Jurkat cells transfected using lipopolyplexes having a charge ratio of 1.6 and a lipid/DNA ratio of 8 (Figure 5.26). It would appear that unlike a reduction in \overline{M}_w of the poly-L-lysine compared to PLK₉₉, which gives an increase in transfection activity, an increase in the \overline{M}_w of the poly-L-lysine does not cause a decrease in transfection activity.

The cytotoxicity of the lipopolyplexes does however appear to change. Lipopolyplexes prepared using PLK₅₃₁ having a PLK/DNA charge ratio of 3.3 or 4.9 are very cytotoxic (Figure 5.27). The extractable cellular protein content of Jurkat cell populations transfected using these lipopolyplexes is between 0-19µg/well (control population 104µg/well). This extra cytotoxicity does not appear to be related to increased observed transfection activity, as Jurkat cells transfected using lipopolyplexes having a charge ratio of one show higher levels of β -galactosidase expression and greater extractable cellular protein. It can be observed that polyplexes having a charge ratio of 0.8 or 1.6 have similar cytotoxicities, and that in comparison Jurkat cells challenged with polyplexes having a charge ratio of 3.3 or 4.9 have ~66% less extractable cellular protein. The extra poly-L-lysine content of lipopolyplexes having charge ratios of 3.3 or 4.9 may give such high levels of cytotoxicity that few cells in the population are able to remain viable. Interestingly, lipopolyplexes prepared using DOPC and DMPE containing liposomes having charge ratios of 3.3 or

4.9 have only slightly greater cytotoxicity than those having a lower charge ratio, suggesting a possible intracellular aspect to the toxicity of DOPE lipopolyplexes.

The low extractable cellular protein content of Jurkat cell populations transfected using lipopolyplexes having charge ratios of 3.3 or 4.9 suggests only a small number of viable cells remain. The total amount of β -galactosidase expressed by each of these populations in terms of pg β -galactosidase/well is relatively low. However, when expressed as pg β -galactosidase/mg protein the efficiency of transfection appears very high. It would appear that the small number of viable cells remaining are expressing large amounts of β -galactosidase.

DOPE/DOPC/oleic acid/DSPE-PEG-anti-CD3 and DMPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 liposomes have little gene transfer activity when associated with PLK₉₉ condensed DNA, and no transfection activity using PLK₅ and PLK₃₆. This was suggested to be related to the relative resistance to lysosomal degradation afforded by each of the poly-L-lysine species to its DNA (Kwoh *et al.*, 1999). Indeed, lipopolyplexes prepared with each of these liposomal formulations with PLK₅₃₁ condensed DNA confer higher transfection activity than those prepared from PLK₉₉ condensed DNA. It has been shown that poly-L-lysines of high \overline{M}_w such as PLK₅₃₁ confer greater resistance to DNase degradation on plasmid DNA than poly-L-lysines of low \overline{M}_w (Ziady *et al.*, 1999). DOPE/DOPC/oleic acid lipopolyplexes with PLK₅₃₁ show little gene transfer activity having a charge ratio of 0.8 or 1.6, with greater gene transfer activity at a charge ratio of 3.3, and maximal at a charge ratio of 4.9. Similarly, DMPE/cholesterol/oleic acid lipopolyplexes only have gene transfer activity at charge ratios of 3.3 or 4.9. The large amount of condensing poly-L-lysine could assist nuclease resistance, or enable efficient delivery to the nucleus of the transfected cell (Zauner *et al.*, 1999). Pouton *et al.* (1998) note that PLK₂₁₉ polyplexes transfected murine melanoma cells more efficiently at a charge ratio of 1.5 than at lower ratios.

Summary

- Lipopolyplexes of DOPE/cholesterol/oleic acid and PLK₅₃₁ condensed DNA confer high levels of transfection only at low charge ratios.
- PLK₅₃₁ lipopolyplexes are highly cytotoxic.

- PLK₅₃₁ condensed DNA appears to be more resistant to nuclease attack than that condensed using PLK₅, PLK₃₆ and PLK₉₉.

5.3.3.4 Transfection of Jurkat cells using lipopolyplexes containing PLR₁₇-condensed DNA

Lipopolyplexes prepared using PLR₁₇ condensed DNA and DOPE/cholesterol/oleic acid anti-CD3 liposomes have maximum gene transfer activities similar to those obtained using PLK₅ and PLK₃₆ DNA condensing agents. This activity is enhanced over that obtained using PLK₉₉ and PLK₅₃₁ (Figure 5.32). High levels of transfection in Jurkat cells were observed using lipopolyplexes having charge ratios of 1.5, 2 and 3. Similarly, high transfection at all cationic charge ratios was also noted using PLK₅ and PLK₃₆ DNA condensing agents. This suggests that polymer content of the polyplex is less important than its net charge for maximum transfection activity. The charge ratio, lipid/DNA ratio combination giving maximum transfection activity is however different at each charge ratio.

Few studies have examined the relative gene transfer activities of polyplexes prepared using poly-L-lysine and poly-L-arginine. Plank *et al.* (1999) prepared a series of branched peptides containing 5-9 cationic residues of either lysine or arginine. They found that at all charge ratios and chain lengths of polymer an arginine containing polymer delivered better transfection than one containing lysine, and that a polymer containing 6 arginines and one lysine was optimal. They also observed that arginine containing polymers bind more tightly to DNA than those containing lysine. Conversely, Pouton *et al.* (1998) noted that a high \overline{M}_w poly-L-arginine condensing agent conferred half the transfection activity of a similar sized poly-L-lysine in murine melanoma cells (in the presence of chloroquine).

It would appear that PLR₁₇ polyplexes are cytotoxic to Jurkat cells, but the cytotoxicity does not appear to increase with polyplexes having a greater polymer content (Figure 5.40). The additional cytotoxicity shown by PLR₁₇ lipopolyplexes having charge ratios of 1.5, 2 and 3 appears to be conferred by the success of the transfection with high levels of β -galactosidase expression (Figure 5.33). As has been noted in the transfection using PLK₅₃₁ condensing agent, the Jurkat cells used in the transfection may be more susceptible to cytotoxicity due to their low extractable cellular protein content (Figure 5.33, Figure 5.27). The cytotoxicity observed using

PLK₅₃₁ lipopolyplexes appears related to their poly-L-lysine content, as most cytotoxicity is observed at high charge ratios and not necessarily in those cells displaying the highest levels of transfection (Figure 5.26, Figure 5.27). Cytotoxicity of PLR₁₇ lipopolyplexes is uniform at charge ratios 1.5, 2 and 3 suggesting independence from polymer content. It has been noted that using poly-L-lysine condensing agents, Jurkat cells displaying high cytotoxicity have low β -galactosidase expression (e.g. Figures 5.1, 5.26). Using PLR₁₇ condensing agent, cell populations that are apparently very sick are expressing large amounts of β -galactosidase. This suggests that a small number of cells are expressing large amounts of β -galactosidase and that PLR₁₇ is a more efficient transfection agent (expressing pg β -galactosidase/mg protein) than PLK₅ and PLK₃₆. The reduction in extractable cellular protein content may be related to a cytostatic effect of successful transfection (giving lower protein content at harvesting due to lower cell number) rather than a reduction in cell number due to a cytotoxic effect.

The endocytotic uptake of a large number of transfection complexes may upset cellular homeostasis by saturating particle uptake mechanisms and their subsequent processing procedures. This effect can also be observed in Jurkat cells transfected using PLK₅ and PLK₃₆, the lower overall cytotoxicity observed may be related to the higher viability of the Jurkat cell population used in the transfection (Figure 5.23; control population extractable cellular protein 168 μ g/well compared to 104 μ g/well used in PLR₁₇ transfection).

DOPE/DOPC/oleic acid anti-CD3 and DMPE/cholesterol/oleic acid anti-CD3 liposomes have little gene transfer activity when associated with PLR₁₇ condensed DNA (Figure 5.34). These liposome formulations appear to require the associated DNA to be highly condensed using a polymer of high \overline{M}_w to confer transfection (5.3.3.2), as the liposomes cannot rupture the endosomal compartment efficiently. PLR₁₇ condensed DNA does not appear to confer these properties as observed using the small polymers PLK₅ and PLK₃₆. The lipopolyplexes do not appear to be cytotoxic to Jurkat cells (Figure 5.35, Figure 5.36).

Summary

- PLR₁₇ DNA condensing agent shows enhanced gene transfer activity compared to PLK₉₉ when complexed to DOPE/cholesterol/oleic acid liposomes.

- Cytotoxicity observed in transfected Jurkat cells may be related to the efficient nature of the transfection rather than a toxic component of the lipopolyplexes.

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5.3.4 Targeted transfection of Jurkat cells

Lipopolyplexes prepared using PLK₃₆ condensed DNA and DOPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 liposomes have high gene transfer activity in Jurkat cells when prepared as 3.3, 32 lipopolyplexes and 4.9, 8 lipopolyplexes (Figure 5.41a). However, non-CD3 specific 3.3, 32 and 4.9, 8 lipopolyplexes also have high gene transfer activities (76% and 68% respectively of that shown by anti-CD3 lipopolyplexes). This suggests that not all gene transfer activity is due to the presence of the specific targeting antibody. It is possible that the lipopolyplexes are interacting in a non-specific manner with the target cells before uptake, possibly by receptor-mediated endocytosis. Cationic liposomes are thought to mediate transfection by binding to the negatively charged surface of the mammalian cell in a non-specific manner. It is this non-specific electrostatic interaction that makes the targeting of cationic lipid-mediated transfection problematic.

If the lipopolyplexes used in the current transfection had a component/composition that could interact with the cell surface in a non-specific manner, this could cause non-targeted gene transfer. Lee and Huang (1996) also used a ternary complex of anionic liposomes and poly-L-lysine condensed DNA (lipopolyplex) to transfect KB epidermal cells. They determined that overall lipopolyplex surface charge influenced the specificity of gene transfer. Cationic lipopolyplexes (PLK charges in excess) transfected KB cells equally efficiently with or without targeting ligand. Anionic lipopolyplexes (lipid charges in excess) transfected the KB cells to a greater extent, but only with the presence of targeting ligand on the complex.

The lipopolyplexes used in this study to transfect Jurkat cells were characterised in terms of their overall surface charge (zeta potential) (Table 5.5). A positive zeta potential is possessed by 4.9, 8 lipopolyplexes. It is possible that these complexes interact with the anionic cell surface in a non-CD3 specific manner and lead to successful transfection. A 3.3, 32 lipopolyplex has a strongly negative zeta potential. Similar anionic lipopolyplexes used by Lee and Huang (1996) transfected in a ligand-dependent manner. However, non-CD3 specific 4.9, 32 lipopolyplexes have high gene transfer activity.

It is possible that a structural aspect of the lipopolyplex confers the non-CD3 specific gene transfer activity of these anionic complexes. As has been suggested previously (Chapter 4) the lipopolyplex may consist of a cationic poly-L-lysine condensed DNA

particle attached to the outer surface of an anionic liposome. The zeta potential measurement suggests that the particle has excess negative charges on its surface. On the lipopolyplex surface there would be an area of positive charge conferred by the condensed DNA particle giving a "bipolar complex". This area of positive charge could readily interact with the cell surface in a non-specific manner.

Non-CD3 specific 4.9, 8 lipopolyplexes have a significantly lower cytotoxic effect on Jurkat cells than specifically targeted lipopolyplexes (t-test, $t=-13.276$, $P<0.001$; Figure 5.41b). The additional cytotoxicity of anti-CD3 lipopolyplexes appears to be correlated with the increased level of β -galactosidase expression observed following transfection.

Summary

- Cationic and anionic lipopolyplexes confer high levels of transfection in Jurkat cells.
- Lack of specificity of gene transfer may be due to non-specific binding of non-CD3-specific lipopolyplexes to the Jurkat cell surface.
- It is possible that the physical structure of the lipopolyplexes may allow non-specific electrostatic interactions to occur with the cell.

5.3.5 Targeted transfection of Jurkat cells in the presence of serum

The specificity of gene transfer was also determined in the presence of 10% foetal calf serum (FCS) in the transfection medium. This would determine not only if specificity of gene transfer could be obtained in the presence of serum, but also if the lipopolyplex composition was able to resist serum degradation and successfully transfect Jurkat cells.

Gene transfer specific to the CD3 epitope was obtained using 4.9, 8 lipopolyplexes prepared using DOPE/cholesterol/oleic acid liposomes. Jurkat cells transfected using a CD3-specific 4.9, 8 lipopolyplex displayed high levels of β -galactosidase expression, whereas non-CD3 specific complexes did not transfect Jurkat cells (Figure 5.42a). The level of β -galactosidase expression is four-fold lower than that observed using an anti-CD3 4.9, 8 lipopolyplex in the absence of serum.

A 3.3, 32 lipopolyplex did not transfect Jurkat cells in the presence of 10% serum, even with the presence of targeting ligand.

The composition of liposomes has been shown to affect both their viability and gene transfer activity in the presence of serum. Semple *et al.* (1996) show that the presence of 30mol% cholesterol in a neutral liposome formulation increases circulation time *in vivo*. They suggest that cholesterol lowers the amount of serum proteins that bind to the liposome surface. This is suggested by the observations of Crook *et al.* (1998) where the incorporation of cholesterol into cationic liposome preparation allows efficient transfection in the presence of up to 80% serum. The lack of gene transfer activity of liposome compositions lacking cholesterol was thought to be due to reduced levels of cell binding of the transfection complexes.

As the 4.9, 8 lipopolyplex is transfection competent this would suggest the liposome component is not greatly affected by serum as it can bind to the cell surface and apparently undergo a pH-sensitive destabilisation in the endosome compartment. The 39.9mol% cholesterol used in the liposome formulation may reduce serum inactivation to such a degree that adequate cell interaction can still occur and retain the liposomes functionality. Serum components such as serum albumin have been shown to extract oleic acid from DOPE/oleic acid liposomes and insert into the bilayer (Liu and Huang, 1994). The interaction of serum albumin removes the pH-sensitive function of the liposomes. Cholesterol may inhibit such deleterious

interactions. High-density lipoprotein and low-density lipoprotein can bind to liposomes *in vivo* and cause release of encapsulated solutes (Semple *et al.*, 1998).

If the liposome is protected from serum it would suggest that the DNA in a 3.3, 32 lipopolyplex cannot be delivered to the cell intact. It has been determined that the condensed DNA particle in the lipopolyplex is exposed on the liposome surface and not contained within its aqueous core. This probably renders the DNA accessible to serum nucleases. It has been shown that such poly-L-lysine condensed DNA is resistant to nuclease degradation, but only if high \overline{M}_w poly-L-lysine is used for condensation (Ziady *et al.*, 1999). Poly-L-lysine of \overline{M}_w 7, 500 used to condense the DNA in the lipopolyplex used here would confer relatively little nuclease resistance. At the PLK/DNA charge ratio of 4.9 the condensed particle is smaller than at a charge ratio of 3.3 (Table 5.5) and may be more resistant to serum nucleases.

The various components of the lipopolyplex have different cytotoxicities when incubated with Jurkat cells in the presence of 10% FCS. Polyplexes having a PLK/DNA charge ratio of 3.3 or 4.9 have no cytotoxic effect on Jurkat cells (98% extractable cellular protein of control cell population, compared to 71% and 68% respectively in the absence of serum). This suggests the cytotoxic agent is rendered inert by serum. Similarly, 3.3, 32 lipopolyplexes have little cytotoxicity in the presence of 10% serum (Figure 5.42b). A slightly increased cytotoxic effect is noted when 4.9, 8 lipopolyplexes are used for transfection, but again less than that observed in the absence of serum. Jurkat cells transfected using 4.9, 8 anti-CD3 lipopolyplexes show high β -galactosidase expression display no greater cytotoxicity than non-CD3 specific lipopolyplex transfected cells. As has been suggested previously (5.3.1) the poly-L-lysine component of the lipopolyplex vector appears to confer most of the observed cytotoxicity. Poly-L-lysine has been shown to readily interact with anionic serum components (e.g. Dash *et al.*, 1999) and the presence of serum has been shown to reduce PLK cytotoxicity (Wolfert and Seymour, 1996). However, the latter effect is only noted using poly-L-lysines of low \overline{M}_w . The “mopping-up” of free poly-L-lysine may contribute to the lower cytotoxicity of the vectors in the presence of serum. Conversely, free poly-L-lysine may mop-up (neutralise) serum components that could otherwise interact with the vector and inactivate it.

Summary

- Targeted transfection of Jurkat cells was obtained in the presence of 10% FCS using DOPE/cholesterol/oleic acid liposomes and PLK₃₆-condensed DNA.
- Cationic lipopolyplexes having a high charge ratio are partly resistant to the action of serum.
- Anionic lipopolyplexes having a low charge ratio appear susceptible to the action of serum.
- Polyplexes and lipopolyplexes have little cytotoxicity in the presence of serum.

5.3.6 Targeted transfection using DOPE/DOPC/oleic acid/DSPE-PEG-anti-CD3 liposomes

No gene transfer activity is noted from lipopolyplexes prepared using PLK₃₆ condensed DNA and DOPE/DOPC/oleic acid liposomes used in the previous screen for gene transfer specificity. However, small amounts of gene transfer activity are noted from lipopolyplexes prepared using PLK₉₉ condensing agent (Figure 5.4). The specificity of gene transfer to either presence of the CD3 targeting antibody was determined. Using lipopolyplexes having a PLK/DNA charge ratio of 4.9, gene transfer is CD3-specific at each lipid/DNA ratio tested (Figure 5.43a). Gene transfer activity appears to be greater at high lipid/DNA ratios, which correlates with an increase in lipopolyplex size (Figure 5.44). As these lipopolyplexes have a similar structure to the lipopolyplexes prepared using PLK₃₆ and DOPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 liposomes, it is unknown why so little non-specific gene transfer is observed.

It appears a greater cytotoxic effect is shown by Jurkat cells transfected using anti-CD3 lipopolyplexes, these cells displaying higher levels of β -galactosidase expression. It has been suggested that this extra cytotoxicity could be due to β -galactosidase expression, or more simply the process of successful transfection (see 5.3.3.1).

Summary

- Targeted gene transfer can be obtained using DOPE/DOPC/oleic acid liposomes using PLK₉₉ DNA condensing agent.

Chapter 6

Discussion and Conclusion

6.1 Discussion and Conclusion

6.1.1 Characterisation studies

DNA-poly-L-lysine polyplexes have been characterised in terms of their morphology, size and zeta potential. The 7.6kb plasmid pEGlacZ was condensed using PLK₉₉ into heterogeneous structures as observed by electron microscopy. At a final DNA concentration of 60µg/ml the majority of the structures have a rod-like morphology, varying in length from 53-160nm. It can be estimated that the rods contain a number of individual plasmid molecules condensed (aggregated) into a single structure. Smaller, spherical structures (possibly torroids), similar to those observed by other groups, were also observed. These spherical particles possibly contain a single plasmid molecule. The generation of a heterogeneous population of polyplexes may affect the reproducibility of gene transfer of lipopolyplexes prepared from such samples.

Plasmid DNA was shown to have a strongly negative zeta potential in deionised water. This charge can be readily neutralised by the addition of poly-L-lysine forming small, light-scattering particles. At charge ratios between 0 and ~1 small particles (polyplexes) are formed that carry a negative zeta potential. Step-wise addition of poly-L-lysine to plasmid DNA causes the zeta potential of the resultant particles to become increasingly less negative. At a charge ratio of ~1, large aggregates of particles are formed. These aggregates carry a low zeta potential. Particles having a low zeta potential form unstable suspensions and therefore aggregate; particles with the lowest zeta potential forming the largest aggregates. Small, positively charged particles are formed when the polyplexes have a calculated charge ratio greater than 1-1.25 depending on the poly-L-lysine species used for condensation. Plasmid DNA appears to become saturated with poly-L-lysine at high charge ratios, possibly leading to "free" poly-L-lysine being present in solution. A positive zeta potential is possibly conferred by a number of the condensing poly-L-lysine molecules being partly bound to the DNA-phosphate backbone, and partly free in the surrounding milieu. A number of high \overline{M}_w poly-L-lysines generate particles of similar size and zeta potential at each charge ratio. Low \overline{M}_w poly-L-lysine (PLK₅) does not form stable polyplexes that carry a negative zeta potential. Stable particles could only be formed carrying a positive zeta potential (at a charge ratio of >1.5). Low \overline{M}_w poly-L-lysines may bind to

plasmid DNA with low affinity, as there will be less cooperative binding of the polymer compared to those having a high \overline{M}_w . Zeta potential and PCS analysis of polyplexes allows their rapid characterisation. The calculated charge ratio gives an indication of particle surface potential at high and low charge ratios. PCS analysis gives an accurate determination of particle size, comparable with that observed by electron microscopy.

Agarose gel electrophoresis of polyplexes is an unsuitable technique for determining polyplex surface potential. The high ionic strength of the electrophoresis buffer possibly causes particle aggregation in the gel loading well, if the polyplexes carry a low zeta potential. Retardation of polyplexes in the gel loading well does not always indicate charge neutralisation by the condensing peptide. Highly condensed DNA particles having strongly positive zeta potentials do not appear to enter the gel matrix possibly due to their structural properties.

Several anionic liposome formulations were produced, and their interaction with DNA-poly-L-lysine polyplexes studied. The liposomes differed in the structure of their PE lipid and the presence of either cholesterol or a PC lipid.

The structure of lipopolyplexes appears to be independent of the lipid composition of the component liposomes. Liposomes retain their structural integrity of their membrane throughout their interaction with polyplexes. All the liposome formulations contain the same amount of oleic acid, which probably contributes the majority of the electrostatic interaction with the polyplex. The interaction of liposome with peptide-condensed DNA appears to be a more "gentle" interaction than that of cationic liposomes and uncondensed-DNA where radical topological changes occur. The retention of structural integrity of the liposome suggests that the polyplex particle is located on the outer surface of the liposome and not internalised within its aqueous core. Further evidence is supplied by the observation that polyplex DNA is still susceptible to pronase enzyme degradation when associated with anionic liposomes. A small number of lipopolyplexes may contain condensed-DNA that is inaccessible to the pronase enzyme. Cryo electron microscopy of lipopolyplexes could confirm the specific location of the condensed DNA, or the presence of a heterogeneous population of lipopolyplexes.

Each of the liposomes tested were shown to have different permeabilities to an entrapped solute. A method was devised to determine the permeability of lipopolyplexes prepared from each of the lipid compositions. Lipopolyplexes of all

lipid compositions maintain a permeability barrier during their interaction with polyplexes, but all become more permeable. The extent of the increase in permeability appears to be related to the initial permeability of the component liposomes. Low permeability liposomes show the greatest increase in observed permeability upon their interaction with polyplexes. The mechanism of increased permeability is unknown, but may be due to the interaction of cationic poly-L-lysine with the lipid membrane. The retention of the membrane permeability barrier may allow co-delivery of transfection enhancing molecules with the condensed DNA during gene transfer.

6.1.2 Gene transfer studies

CD3⁺ T lymphocytes (Jurkat cells) have been successfully transfected using a lipopolyplex gene transfer vector. A number of lipopolyplex properties have been studied and their effect on gene transfer efficiency determined.

DNA condensed using polypeptides (forming polyplexes) confers little, if any transfection activity in Jurkat cells. The lack of gene transfer activity is probably due to non-specific binding of the complex to the cell surface, and therefore low uptake into the cellular endocytic system by receptor-mediated endocytosis. The majority of the condensed DNA taken up in this manner is probably trafficked to lysosomes where it is degraded. DNA condensed using high \overline{M}_w poly-L-lysine having high charge ratios appears partly protected from these degradative processes and has a low level of gene transfer activity. Polyplexes containing high \overline{M}_w poly-L-lysine are cytotoxic to Jurkat cells. This cytotoxic effect is magnified when the polyplexes have a high charge ratio.

In this study, the lipid composition of the lipopolyplex has a greater influence than the DNA-condensing peptide on its total gene transfer activity (Table 6.1). DOPE/cholesterol/oleic acid (40:40:20 mol%) liposomes have been shown to be pH-sensitive (Wang and Huang, 1987). Liposomes of this formulation deliver the highest levels of transfection in Jurkat cells. These liposomes can probably rupture the endosomal membrane and release their associated-DNA into the cytoplasm. Optimum transfection of Jurkat cells was obtained when these liposomes were associated with DNA condensed using low \overline{M}_w peptides. It has not been determined that these peptides actually enhance transfection, but they appear to confer low levels of cytotoxicity, which may enable greater levels of transgene expression. Low \overline{M}_w poly-

L-lysine appears to function mainly to confer a positive charge to the DNA particle and aid its association with the anionic liposome. The small size of such condensed particles and possible nuclease resistance/nuclear targeting properties may aid gene transfer.

Table 6.1 Optimum lipopolyplex formulations for transfection of Jurkat cells

DNA condensing agent	Lipopolyplex Charge ratio	Lipopolyplex Lipid/DNA ratio	β -galactosidase expression (pg/well)	β -galactosidase expression (pg/mg protein)
<i>DOPE/cholesterol/oleic acid anti-CD3 liposomes</i>				
PLK ₅	39	4	2982 \pm 407	22700 \pm 87
PLK ₃₆	4.9	4	2953 \pm 111	32900 \pm 4100
	4.9	8	2854 \pm 269	44000 \pm 9300
PLK ₉₉	1.6	8	871 \pm 81	11700 \pm 3000
PLK ₅₃₁	1.6	8	771 \pm 95	24000 \pm 1000
PLR ₁₇	1.5	8	3179 \pm 318	127000 \pm 52000
<i>DOPE/DOPC/oleic acid anti-CD3 liposomes</i>				
PLK ₅	*	*	*	*
PLK ₃₆	4.9	4	4 \pm 3	36 \pm 28
PLK ₉₉	1.6	4	7 \pm 2	46 \pm 10
	4.9	4	7 \pm 1	194 \pm 25
PLK ₅₃₁	4.9	8	115 \pm 9	2283 \pm 66
PLR ₁₇	1.5	8	26 \pm 6	371 \pm 139
<i>DMPE/cholesterol/oleic acid anti-CD3 liposomes</i>				
PLK ₅	*	*	*	*
PLK ₃₆	*	*	*	*
PLK ₉₉	3.3	16	12 \pm 4	120 \pm 71
	4.9	4	12 \pm 1	806 \pm 288
PLK ₅₃₁	4.9	32	67 \pm 15	1022 \pm 200
PLR ₁₇	*	*	*	*

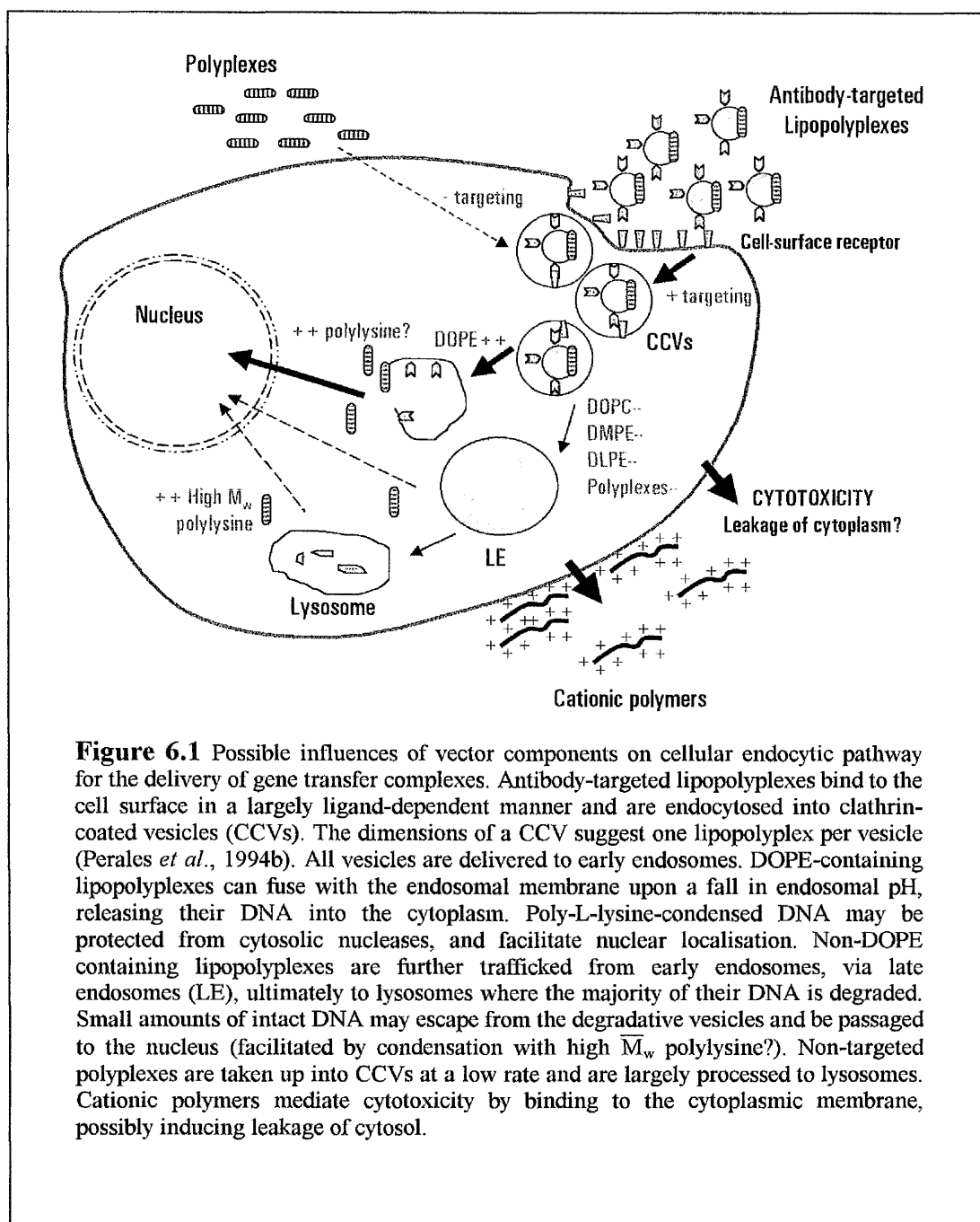
* no transfection observed using this condensing agent with each formulation

Lipopolyplexes appear to confer cytotoxicity to Jurkat cells to some degree, the majority of which appears to be conferred by the DNA-condensing agent. High \overline{M}_w poly-L-lysines confer lower gene transfer activity when used as a DNA-condensing agent when associated with DOPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 liposomes. This effect appears to be related to the cytotoxicity of such lipopolyplexes, the effect being most notable using lipopolyplexes containing the greatest amounts of poly-L-lysine. Additional cytotoxicity may be conferred by the transfected DNA itself, which due to its bacterial origins may have immunostimulatory properties.

It was noted that non-colloidal aggregates of lipopolyplexes have little gene transfer activity. Not only are such aggregates probably too large to be endocytosed by Jurkat cells, they readily fall out of suspension and would have little contact time with suspension cell lines. Observations by Keller *et al.* (1999) show that that hematopoietic cells can be more readily transfected using a cationic liposome vector when attached to an adherent cell monolayer (ACM). As cationic liposome/DNA often form large aggregates, this may co-localise cells and complexes enabling enhanced transfection. This may help explain why adherent cell lines are more readily transfected by cationic liposome complexes. Alternatively, Labat-Moleur *et al.* (1996) suggest that Ca^{2+} -mediated cell anchoring components are most important for cationic liposome/cell binding, these being lacking in suspension cell lines. They do not show however if cationic liposome complexes bind to suspension cell lines associated with a solid surface allowing sedimentation of the complexes onto the cells.

Liposomes that lack an apparent pH-sensitive function confer low levels of gene transfer activity. The majority of the gene transfer activity of these vectors is probably conferred by the DNA-condensing agent rather than the lipid component (cf. DOPE/cholesterol/oleic acid). Substitution of cholesterol in the liposome formulation DOPE/cholesterol/oleic acid/DSPE-PEG with DOPC abolishes most of its gene transfer activity regardless of DNA-condensing agent. DOPC probably renders the liposome formulation non-pH-sensitive and unable to rupture the endosome/lysosome compartments. Similarly, DMPE and DLPE containing vectors probably lack pH-sensitive function due to the short acyl chain length and saturation of the PE lipid. All non-pH-sensitive vectors confer the greatest levels of gene transfer activity when the associated DNA is condensed using high \overline{M}_w poly-L-lysine at a high charge ratio. Such DNA is probably able to withstand lysosomal degradation.

The influence of individual vector components on each stage of gene transfer is shown in Figure 6.1.



It was shown that both cationic and anionic lipopolyplexes confer high levels of transfection in Jurkat cells. The lack of specificity of the majority of the gene transfer may be due to the non-specific binding of the non-CD3-specific lipopolyplexes to the Jurkat cell surface. The extra-liposomal localisation of the condensed DNA particle in the lipopolyplex may be responsible for the non-specific interaction.

Targeted transfection of Jurkat cells was observed in the presence of 10% FCS using DOPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 liposomes and PLK₃₆-condensed DNA (4.9, 8 lipopolyplexes). The absolute level of transfection is lower than that observed in the absence of serum. The cytotoxicity of the vector is reduced in the presence of serum. Binding of serum proteins to the vector may prevent non-specific interactions with the cell surface hence anti-CD3 antibodies provide a specific binding point. Serum components appear to deactivate vectors having DNA condensed at low peptide/DNA charge ratios.

DOPE/DOPC/oleic acid lipopolyplexes can be targeted to Jurkat cells in a CD3-specific manner to give low levels of transfection.

Puls and Minchin (1999) demonstrated targeted CD4-specific transfection of T lymphocytes with a poly-L-lysine-condensed DNA vector with conjugated anti-CD4 antibody. The vector has a calculated overall negative charge, which probably reduces non-CD4 specific interactions with the cell surface. Unfortunately, little transfection is observed without the cells being stimulated with phorbol 12-myristate 13-acetate (PMA). Cell stimulation is required for increased uptake of the CD4 surface receptor, which is naturally endocytosed at a low rate. No endosomal release agent appears to be required by the vector.

Buschle *et al.* (1995) transfected Jurkat cells in a CD3-specific manner using poly-L-lysine condensed DNA particles with conjugated anti-CD3 antibodies. Transfection was greatly enhanced using complexes with conjugated adenovirus particles.

It can be seen in Table 6.1 that the majority of the lipopolyplex formulations using DOPE/cholesterol/oleic acid/DSPE-PEG-anti-CD3 liposomes giving optimum transfection in Jurkat cells have a lipid/DNA ratio of 8. At a lipid/DNA ratio of 8 each transfection reaction contains 32µg of lipid. The DOPE/cholesterol/oleic acid/DSPE-PEG liposome used in the secondary transfection screen has an average diameter of 125.3 ± 47.9 nm (Table 5.2). Each of these liposomes contains an average of 175,088 lipid molecules (0.5nm^2 /lipid molecule, bilayer thickness 7.5nm). The average lipid molecular weight in the liposome formulation is 511Da, so 32µg of lipid contains

2.154×10^{11} liposomes. Assuming that the $4\mu\text{g}$ of plasmid DNA per transfection is found in polyplexes of 1 plasmid/complex there are 4.174×10^{11} polyplexes per transfection, and 2.2 polyplexes/liposome (in each lipopolyplex). This number appears to be optimal for transfection. As each polyplex probably contains >1 plasmid this ratio is the highest estimate. It is interesting to note that in each transfection reaction there are 4.74×10^5 plasmids per targeted cell.

6.2 Future work

Optimisation of the lipopolyplex vector for transfection should be the priority of any additional studies. It has been shown that a liposome of the formulation DOPE/cholesterol/oleic acid (40:40:20 mol%) confers the highest levels of transfection in Jurkat cells. It is not known if either the choice of PE helper lipid, the choice of titratable anionic lipid or the molar ratios of the individual components are optimal.

A greater range of PE helper lipids should be investigated for their gene transfer activities. The transfection activity of DOPE/cholesterol/oleic acid liposomes is probably related to the membrane fusion activity of DOPE (dioleoylphosphatidylethanolamine, 18:1 *c*Δ9-PE) when it forms the H_{II} (inverted hexagonal) lipid phase. The tendency of a lipid to form the H_{II} phase at a certain temperature is given by the H_{II} phase transition temperature (T_h). As the T_h of DOPE is 10°C, at 37°C found in a transfection experiment the lipid will form H_{II} phase unless it is physically stabilised. This stabilisation is performed by the deprotonated oleic acid head-group at physiological pH, but upon protonation at low pH in the endosome is unable to prevent DOPE forming H_{II} phase.

Other lipids such as DMPE and DLPE have a T_h of >150°C and are therefore unlikely to form H_{II} phase at 37°C. This study has shown a possible correlation between the T_h of a PE lipid and transfection activity.

Other lipids such as dilinoleoylphosphatidylethanolamine (C18:2 *c,c*Δ9,12-PE) and dilinolenoylphosphatidylethanolamine (C18:3 *c,c,c*Δ9,12,15-PE) have a lower T_h than DOPE (-15°C and -30°C respectively). Liposomes formed from these lipids may be less stable at low pH found in endosomes and have greater membrane fusion activity than DOPE containing liposomes. It can be predicted that these lipids will have greater gene transfer activity in an identical liposome formulation. Transfection studies by Fasbender *et al.* (1997) show that C18:2 *c,c*Δ9,12-PE and C18:3 *c,c,c*Δ9,12,15-PE-containing cationic liposomes have greater gene transfer activity than a 18:1 *c*Δ9-PE (DOPE) containing formulation. Their data can be analysed to show a correlation between an increase in gene transfer activity and a decrease in T_h of the PE helper lipid. This can be compared with the T_h values of other inefficient helper lipids used in the present study (Table 6.2).

Table 6.2 Phase transition temperatures of phosphatidylethanolamine (PE) helper lipids and their relationship to observed transfection activities

Lipid	Chemical structure	T_m (°C) ^a	T_h (°C) ^b	Relative transfection activity (Fasbender <i>et al.</i> , 1997) ^c	Reference ^d
1,2-Dielaidoyl- <i>sn</i> -glycero-3-phosphoethanolamine	18:1 <i>t</i> Δ9-PE	38.3	63.5	18%	1
1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine	18:1 <i>c</i> Δ9-PE	-16	10	100%	1
1,2-dilinoleoyl- <i>sn</i> -glycero-3-phosphoethanolamine	18:2 <i>c, c</i> Δ9,12-PE	-40	-15	200%	1
1,2-dilinolenoyl- <i>sn</i> -glycero-3-phosphoethanolamine	18:3 <i>c, c, c</i> Δ9,12,15-PE	-	-30	200%	2
1-palmitoyl, 2-oleoyl- <i>sn</i> -glycero-3-phosphoethanolamine	16:0/18:1 <i>c</i> Δ9-PE	24.4	70.8	~5%	2
1,2-Dipalmitoleoyl- <i>sn</i> -glycero-3-phosphoethanolamine	16:1 <i>c</i> Δ9-PE	-33	43.4	-	3
1,2-Dipalmitoyl- <i>sn</i> -glycero-3-phosphoethanolamine	16:0-PE	64	123	-	4
1,2-Dimyristoyl- <i>sn</i> -glycero-3-phosphoethanolamine	14:0-PE	49.5	>150	-	4
1,2-Dilauroyl- <i>sn</i> -glycero-3-phosphoethanolamine	12:0-PE	30.5	>150	-	4

^a Lamellar gel/Liquid-Crystalline phase transition temperature.

^b Liquid-Crystalline/Reverse Hexagonal phase transition temperature.

^c Transfection activity of lipid in COS-1 cells using cationic liposome formulation with DC-Chol (69:31 wt/wt). Activity observed using 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) taken as 100%.

^d Phase transition temperature measurement references. (1) Lewis *et al.*, 1989. (2) Dekker *et al.*, 1983. (3) Epanand and Leon, 1992. (4) Seddon *et al.*, 1983.

The current liposome composition of PE/cholesterol/oleic acid (40:40:20 mol%) should be optimised using these additional helper lipids. The dependence of T_h on the transfection activity of DOPE could be confirmed using its *trans* isomer dielaidoyl-phosphatidylethanolamine (18:1 Δ^9 -PE) that has a measured T_h of 63.5°C.

Additionally, it must be determined if 40mol% PE lipid is optimal, and if the PE/oleic acid ratio of 2:1 needs to be changed. It must be demonstrated if the cholesterol component of the liposome is essential for gene transfer. Bennett *et al.* (1995) suggest that cholesterol enhances cationic liposome-mediated transfection. Cholesterol has been shown to modify the T_h of PE lipids (Takahashi *et al.*, 1996), this may affect the membrane fusion properties of liposomes containing both cholesterol and PE.

A further priority is to determine which factors confer transfection activity in the presence of serum. Importantly, the properties of a 4.9, 8 lipopolyplex prepared from DOPE/cholesterol/oleic acid liposomes render it more serum resistant than a 3.3, 32 lipopolyplex of the same lipid composition. Is this related simply to the overall surface charge of the complex, or does the charge ratio of the condensed DNA confer different serum resistance properties? Dash *et al.* (1999) have shown that polyplexes prepared at high charge ratios bind less serum albumin, which may affect cell interaction and contribute to DNA degradation.

Many modifications can be suggested for the preparation of polyplexes. Most importantly, defined size peptides of lysine must be used. This would remove any variability in polyplex properties due to polydispersity of the peptide preparation. This would remove the possibility of any cytotoxic high \overline{M}_w poly-L-lysines being present in low \overline{M}_w preparations.

It would be of interest to determine how much (if any) unbound poly-L-lysine is present in polyplex preparations at each charge ratio, and if so can it be removed without affecting the polyplexes. Any unbound poly-L-lysine may influence liposome stability and ultimately transfection (in serum especially).

The effect of charge ratio on polyplex properties needs further investigation. Do polyplexes having high charge ratios contain more poly-L-lysine than those having a lower charge ratio? Are polyplexes prepared at high charge ratios more stable to e.g. serum degradation? Is the DNA more highly condensed as size measurements possibly indicate? It is important to determine if polyplexes prepared at different charge ratios bind different amounts/types of serum proteins as observed by Dash *et*

al. (1999). Most importantly, do polyplexes containing poly-L-lysine activate the complement system or elicit other immune responses (e.g. Plank *et al.*, 1996). Trubetskoy *et al.* (1999b) have developed a quantitative assay for the determination of DNA condensation in peptide/DNA particles. Similar experiments could be carried out using poly-L-lysine of different chain lengths. When poly-L-lysine has low toxicity, charge ratio appears a less important factor in determining the gene transfer activity of the lipopolyplex.

Despite its apparent simplicity, the current method for polyplex preparation produces small particles that are highly efficient at gene transfer. The system could be further optimised, although this may be counter-balanced by increases in the complexity of the method. Not all polyplexes appear to contain a single or uniform number of plasmid molecules. This may be due to the relatively high DNA concentration used in the preparations, causing aggregation of a number of plasmids into each polyplex. If the DNA is further diluted, physical analysis of the particles by electron microscopy and light scattering would become problematic. In addition, sample volumes would become unwieldy for transfection experiments.

The fine structure of lipopolyplexes needs to be confirmed by cryo-TEM. This might determine if lipopolyplexes are a heterogeneous population of “empty” liposomes and DNA-containing complexes, and if free polyplexes exist in solution.

It has been suggested that liposome binding of polyplexes may affect the overall condensation of the DNA. If this can be determined, it may be possible to compare the serum resistance properties of unbound polyplexes and those bound to liposomes. Importantly, the mechanism for the increased permeability of lipopolyplexes should be determined. The importance of lipid composition should be determined. Use should be made of the trapped volume in lipopolyplexes to encapsulate molecules useful in enhancing the transfection process.

The factors that determine efficient transfection should be further characterised. The stage of transfection at which DLPE, DMPE and DOPC containing vectors are inefficient could be determined. Transfection in the presence of endosmolytic agents such as chloroquine would show if endosomal escape is the rate-limiting step.

A more quantitative method for determining cell viability before and after exposure to lipopolyplexes needs to be determined. The relative toxicities of the vector components to Jurkat cells could be determined using a tetrazolium-based assay

(Sgouras and Duncan, 1990). This should confirm that the poly-L-lysine component of the vector confers most of the cytotoxicity observed in transfected Jurkat cells.

The proportion of the cell population transfected by each lipopolyplex preparation needs to be determined. At present cell transfection activities are standardised for the whole cell population. As extractable cellular protein appears to be affected by certain transfection agents, gene expression needs to be standardised to an unrelated factor that is related directly to cell number. This would allow meaningful comparison of all transfection data.

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Appendices

Appendix 1. Bacteriological Medium

LB (Luria Bertani)

Bacto-Tryptone **10g**

Yeast extract **5g**

NaCl **10g**

Made up to 1L in deionised water.

The medium was solidified by the addition of 1.5% (w/v) bacteriological agar.

Kanamycin solutions were filter-sterilised using a Millex 0.2µm disc filter and stored at -20°C.

Appendix 2. Calculating the number of antibody molecules per liposome

The computer program "D-LIPPRO" written for BBC Basic calculates the weight average diameter (\bar{d}_w) and the number of molecules per liposome for a population of vesicles based on the output from the Zetasizer 3000 or Coulter N4 Plus Submicron Particle Sizer.

```

580 PRINT " MEAN (DM)  NUMBER OF PROTEINS PER LIPOSOME = "PM
590 PRINT " MEAN (DZ)  NUMBER OF PROTEINS PER LIPOSOME = "PZ
600 PRINT " MEAN (DW)  NUMBER OF PROTEINS PER LIPOSOME = "PW
610 PRINT " MEAN (DN)  NUMBER OF PROTEINS PER LIPOSOME = "PN
620 PRINT " NTM  =      "NTM
630 PRINT " NTZ  =      "NTZ
640 PRINT " NTW  =      "NTW
650 PRINT " NTN  =      "NTN
660 VDU3
670 STOP
>LIST
10 REM D-LIPPRO
20 CLS
30 INPUT "ENTER THE Z-AVERAGE NUMBER ";DZ
40 INPUT "ENTER THE PEAK SIZE OF THE DISTRIBUTION ";DM
50 INPUT "ENTER THE STANDARD DEVIATION OF THE DISTRIBUTION ";S
60 INPUT "ENTER THE PROTEIN:LIPID MOLAR RATIO ";RHO
70 INPUT "ENTER THE LOWER LIMIT OF THE DISTRIBUTION ";LL
80 INPUT "ENTER THE UPPER LIMIT OF THE DISTRIBUTION ";UL
90 CLS
100 FOR I=LL TO UL
110 Y= (1/ (S*2.5066)) * (EXP( - ((I-DM) ^2) / (2*S^2)))
120 N=Y/ (I^3)
130 SUMYD=SUMYD+ (Y*I)
140 SUMY=SUMY+Y
150 SUMND=SUMND+ (N*I)
160 SUMN=SUMN+N
170 R= (I/2) *1.0E-9
180 NO= (4*3.142* (R) ^2) /50E-20
190 NI= (4*3.142* (R-7.5E-9) ^2) /50E-20
200 NT=NO+NI
210 P=NT*RHO
220 SUMYP=SUMYP+ (P*Y)
230 SUMNP=SUMNP+ (P*N)
240 NEXT I
250 DW=SUMYD / SUMY
260 DN=SUMND / SUMN
270 NOM= (4*3.142* ((DM*1.0E-9) /2) ^2) /50E-20
280 NIM= (4*3.142* (((DM*1.0E-9) /2)-7.5E-9) ^2) /50E-20
290 NTM=NOM+NIM
300 PM=NTM*RHO
310 NOZ= (4*3.142* ((DZ*1.0E-9) /2) ^2) /50E-20
320 NIZ= (4*3.142* (((DZ*1.0E-9) /2)-7.5E-9) ^2) /50E-20
330 NTZ=NOZ+NIZ
340 PZ=NTZ*RHO

```

```

350  NOW= (4*3.142* ((DW*1.0E-9) /2) ^2) /50E-20
360  NIW= (4*3.142* (((DW*1.0E-9) /2)-7.5E-9) ^2) /50E-20
370  NTW=NOW+NIW
380  PW=NTW*RHO
390  NON= (4*3.142* ((DN*1.0E-9) /2) ^2) /50E-20
400  NIN= (4*3.142* (((DN*1.0E-9) /2)-7.5E-9) ^2) /50E-20
410  NTN=NON+NIN
420  PN=NTN*RHO
430  *FX6 , 0
440  VDU2
450  PRINT"Z-AVERAGE NUMBER = ";DZ
460  PRINT"PEAK SIZE = ";DM
470  PRINT"STANDARD DEVIATION = ";S
480  PRINT"PROTEIN:LIPID MOLAR RATIO = ";RHO
490  PRINT"LOWER LIMIT = ";LL

```

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