

¹Formulation and quality control of cationic liposomes

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Abstract

Cationic liposomes are traditionally used for delivering macromolecules such as nucleic acids to mammalian and plant cells. This paper describes a novel simple and relatively inexpensive method for preparation of cationic liposomes using an ethanol injection/pressure extrusion method. The study also evaluated the utility of a colorimetric method for quantification of cationic liposomes. Binding of erythrosine dye to cationic liposomes resulted in a shift of the absorption maximum of the dye from 528nm to 549nm in a buffer at pH 4.25, allowing quantification of these vesicles. Colour development was completed in 5 to 10 minutes at room temperature, with only 10% decrease in absorbance observed in the following 2 hours. Divergent values were noted in the presence of interfering agents such as detergents and salts. The erythrosine method is sensitive down to 0.20µg/mL of cationic lipid and is linear to 3.13µg/mL. The erythrosine dye method for quantitation of cationic liposomes is valuable for the field of liposome technology. In addition, a relatively simple method for separation of nucleic acids complexed to cationic liposomes from unbound molecules is presented. This method utilises a Ficoll-based gradient centrifugation method. Laboratory-formulated liposomes were just as efficient in binding nucleic acids as commercially available types.

Keywords: plasmid, liposome, lipoplex, transfection, cationic, lipid

1 Introduction

Cationic liposomes have shown great utility in delivering nucleic-acid-based constructs such as plasmids into cells of both mammalian (Dass and Burton, 1999) and plant (Maccarone *et al.* 1992; Zhu *et al.* 1993) origin. These liposomes are valuable in attempts to attenuate gene expression within cells either by downregulating nuisance genes or upregulating beneficial genes. Such genetic manipulation, commonly referred to as 'genetic engineering' or 'biotechnology', is important in medicine, agriculture and other diverse disciplines such as aquaculture and bioremediation.

In agronomics, these liposomes offer a better method for transfer of genetic material since current techniques such as microparticle bombardment of genetic constructs (Kohli *et al.* 1999) or *Agrobacterium*-mediated genetic transfer (Yokoi and Toriyama, 2000) into rice require specialised skills and equipment. For rice bioengineering, such genes include *bar* (for herbicide resistance), *cht-2* and *cht-3* (for fungal resistance), and *RSV-CP* (for viral resistance). Another area that these liposomes may be used is marine biotechnology, which currently lacks efficient methods for delivering genetic constructs to microalgae, cyanobacteria, sponges and fish species (Chisti 2000). The commercial production of such products as essential fatty acids, antibiotics, antiviral agents, anticancer drugs, insecticides, pigments and colorants derived from marine organisms needs to be made more profitable by over-expressing such useful genes in these organisms. Selection of over-producing organism clones becomes a crucial step in the production pipeline.

Furthermore, with appropriate modification, cationic liposomes may also be used to transfer other biomolecules such as proteins and carbohydrates into cells. While, most cationic liposomes used in laboratory-based studies and even clinical trials rely on commercial formulations, they

may be produced in the laboratory using simple and inexpensive procedures such as ethanol injection (Campbell 1995) or probe sonication (Teifel and Friedl 1995). However, appropriate quality control of the liposome preparation, which includes determination of the final lipid concentration in the vesicle suspension, is essential.

Two methods of quantitation are phosphate analysis in which phospholipids are processed to release phosphorus, which is then quantitated, and the inclusion of radioactively labelled lipids in the preparation mixture is another method commonly used. These methods have several disadvantages. Phosphate analysis is labour-intensive, requires concentrated acids, and is prone to error in any one of the multiple steps involved. It also requires ashing of the phospholipid solution at high temperature. The radioactive method is potentially hazardous due to the escape of aerosolised lipids into the atmosphere during processes such as vortexing or sonication. A relatively easy method is herein described for the quantitation of cationic liposomes. This method is an extension of previous work by Andree and Soedjak (1994) who developed it for detection of anionic and neutral phospholipids using erythrosine dye. The method is simple, requires minimal preparation, uses non-sophisticated equipment, is non-hazardous, and is time-efficient. The method has great utility for liposome technologists, especially those involved in gene therapy using cationic liposomes. This paper also extends the method for separation of DNA from lipid vesicles (New 1994) to include separation of plasmid DNA from cationic liposomes.

2 Materials and methods

2.1 Preparation of cationic liposomes

Dimethyl dioctadecyl ammonium bromide (DDAB) and dioleoyl phosphatidylethanolamine (DOPE), 8.0mg of each (Sigma-Aldrich, NSW) were both dissolved in 1.0mL absolute ethanol. Sterile reverse-osmosis water (950 μ L) was dispensed into a 10mL sterile glass centrifuge tube. The water was vortexed on setting 6 for 10 seconds before 50 μ L of the ethanolic lipids was rapidly injected through a narrow bore pipette (over 0.5s) in the centre of the tube (Campbell 1995). Vortex was maintained for an additional 10 seconds before the liposome suspension was transferred to a sterile microfuge tube. DDAB:PtdChol (1:1 w/w) liposomes were prepared similarly. Following preparation, liposomes were sized by sequential passage through 3.0 μ m, 0.6 μ m and finally a 0.2 μ m polycarbonate membrane filter (Nucleopore, USA). Liposomes were stored at 4°C.

As a quality control check (as performed with commercial liposomes), each laboratory-prepared liposome was checked for microbial content using the following media (Oxoid, Victoria): Blood Agar and Chocolate Agar (with haemolysed blood) for fastidious organisms, Sabourad's Agar for yeasts and moulds, and Terrific Agar for general bacterial species. Briefly, 50 μ L of each liposome was diluted with 950 μ L sterile water. The diluted liposome suspensions (100 μ L) were plated on to the different agar media. Plates were divided into two groups and incubated both aerobically and anaerobically at 37°C. Plates were monitored daily for three days.

2.2 Spectrophotometric scans of liposome-dye complexes

This study was undertaken to establish the absorption maximum of the lipid-dye complex. A 10mg/L erythrosine (Sigma-Aldrich) solution was prepared in 0.1M Tris, pH 4.25. Liposomes were diluted to 40 μ g/mL in sterile reverse-osmosis purified water. 50 μ L of liposome suspensions were aliquoted into 950 μ L erythrosine solution, incubated for 10 minutes, and then spectrophotometrically scanned from 280 to 700nm. A water blank was used and a scan of dye alone was also performed.

2.3 Standard curves for liposomes

To determine the sensitivity of the assay for the different cationic liposomes, stock Lipofectamine, Lipofectin and Lipofectace liposome suspensions (Life Technologies, USA) were diluted to 500 μ g/mL. The 1:1 (w/w) laboratory formulations of DDAB:DOPE and DDAB:PtdChol were also prepared at 1,500 μ g/mL and 600 μ g/mL respectively. In addition, a 500 μ g/mL water solution of the cationic lipid DDAB was prepared. Serial dilutions in water were performed for each liposome suspension and the DDAB solution. 50 μ L of each dilution was then added to 950 μ L of 10mg/L dye solution and left for 10 minutes at room temperature. Samples were then assayed spectrophotometrically at 549nm.

2.4 Complex stability

This study was carried out to determine the optimum time for colour development due to the lipid forming complexes with the dye. 50 μ L of stock, 1,500 μ g/mL of DDAB:DOPE (1:1) and 600 μ g/mL of DDAB:PtdChol (1:1) were added to 950 μ L of dye solution and incubated for 10 minutes.

Additionally, these stocks were diluted 1/10 in water and the dilutions added to dye solution and incubated. At time intervals of 1, 2, 5, 10, 30, 60 and 120 minutes, the samples were assayed at 549nm.

2.5 Assay interference

This study was performed to examine the effect of buffering and other solutions anticipated to act as interferants on the erythrosine assay of cationic liposomes. Stock solutions of potential interferants were prepared at twice the concentration tabulated (Table 1) in reverse-osmosis water and 25 μ L of each were added to 25 μ L of either DDAB (800 μ g/mL) or DDAB:DOPE (1:1, 480 μ g/mL). Erythrosine dye (950 μ L) was added, mixtures were incubated for 10 minutes, and assayed spectrophotometrically at 549nm.

2.6 Loading of plasmids on to liposomes

This experiment was performed to compare the carrying-capacity of the various liposomes for two types of plasmids tested. pCMV-CAT plasmid is 4,233 base-pairs in length and pRcCMV-p53 plasmid is 7,344 base-pairs long. Plasmids were propagated in *Escherichia coli* strain JM109 bacterial cells and were purified using ethanol precipitation followed by size exclusion column (Pharmacia-Biotech, Sweden) chromatography. Liposomes (100 μ g) were dispensed into separate microfuge tubes containing 40 μ g of plasmid. The volume in each tube was made up to 500 μ L with PBS and the mixtures were incubated for 45 minutes at laboratory temperature (22°C) to form complexes known as 'lipoplexes'. Following incubation, 1mL of 30% Ficoll 400 (in PBS) was dispensed into each tube and thoroughly mixed. The lipoplexes were also visualised under light microscopy in separate studies with complexes prepared identically.

Each mixture was then pipetted into 10mL clear polypropylene ultracentrifuge tubes. Ficoll (10% in PBS, 3.0mL) was layered on the previous layer using a 23G needle attached to a 5.0mL syringe. On top of this layer, PBS was layered to within 2 - 3mm of the brim of each tube. The contents were centrifuged at 100000g at 18°C for 1 hour. Following centrifugation, the top two layers were removed and the quantity of unbound plasmid DNA in the bottom layer measured using spectrophotometry at 260nm. These readings were then compared to a plasmid DNA sample of 40 μ g/mL with 30% Ficoll blanks subtracted from each value. Quadruplicate samples were analysed.

3 Results

3.1 Spectrophotometric scans of liposome-dye complexes

The absorbance maxima of erythrosine at pH 4.25 post-binding to all the liposomes studied shifted from 528 to 549nm (Figure 1). At 549nm (shown with dotted grey line), absorbance of free dye was lower than when complexed to cationic liposomes. All liposome preparations were tested negative for microbial contamination.

3.2 Standard curves for liposomes

The standard curve for Lipofectamine was sensitive to 0.20 μ g/mL and to 0.39 μ g/mL for both Lipofectin and Lipofectace (Figure 2). Graphs were linear over the range

of 0 - 3.13 $\mu\text{g}/\text{mL}$ for all three commercial liposomes. The standard curve for both laboratory-prepared liposomes, namely, DDAB:DOPE (1:1) and DDAB:PtdChol (1:1), and that for DDAB alone was sensitive to 0.23 $\mu\text{g}/\text{mL}$ and was linear over 0 - 7.5 $\mu\text{g}/\text{mL}$ (Figure 3). Standard deviation error bars cannot be seen on the graphs since the assay method is very reproducible.

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3.3 Liposome-dye complex stability

The dye-liposome or dye-lipid colour development is completed essentially within 10 minutes (data not shown). Colour loss after two hours of incubation ranged from 7.3% for DDAB:PtdChol (dilute) to 15.8% for DDAB:PtdChol (stock). Even after 24h incubation, the maximum loss in absorbance was 20%.

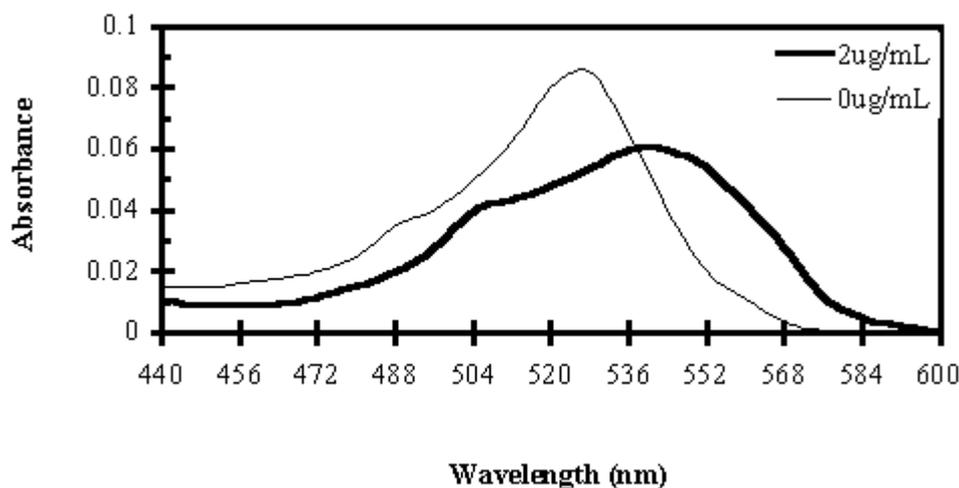


Figure 1. Absorption spectra of 1 $\mu\text{g}/\text{mL}$ erythrosine 0.1M Tris, pH 4.25 with 2 $\mu\text{g}/\text{mL}$ and 0 $\mu\text{g}/\text{mL}$ cationic liposomes. Samples were incubated at room temperature for 10 minutes prior to scanning. The shift is representative of three separate scans performed on each cationic liposome.

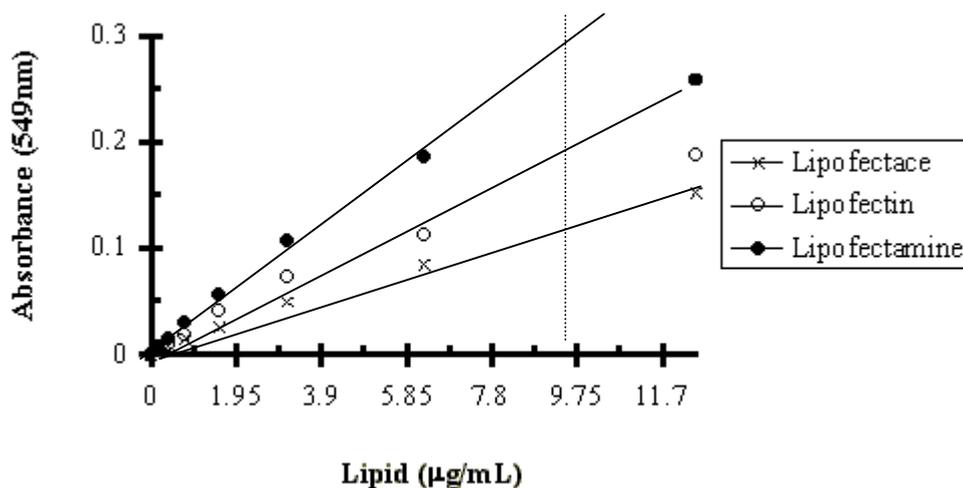


Figure 2. Standard curves of commercial liposomes. Samples were incubated for 10 minutes at room temperature and assayed spectrophotometrically at 549nm in triplicate.

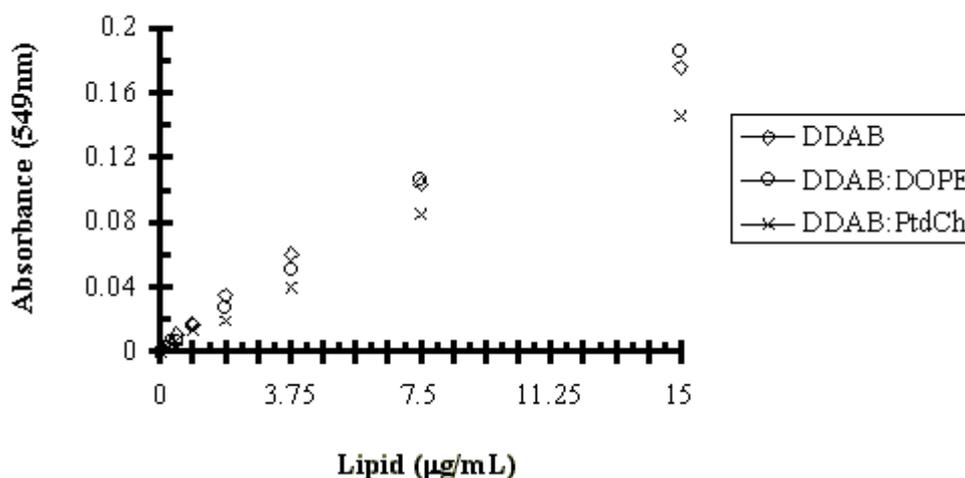


Figure 3. Standard curves of laboratory formulated liposomes. Samples were incubated for 10 minutes at room temperature and assayed spectrophotometrically at 549nm in triplicate.

3.4 Assay interference

Apart from 0.1M HCl, 3M urea and 20% propanol, all other agents evaluated interfered with the assay of DDAB lipid ($p < 0.05$, Table 1). Notable were the absorbance-suppressing effects of salts and buffers on the assay. In the assay of DDAB:DOPE liposomes, interference was not noted only with the sugars. Noteworthy also were the actions of detergents on the results. Sodium dodecyl sulphate (SDS) at both concentrations decreased

absorbance, while Triton X-100 elevated values. However, both salts and buffers had a lesser effect on the liposomal assay compared to assay of DDAB alone. Figure 4 is a depiction of how the three different bands of empty vesicles, liposome-plasmid complexes and unbound plasmids appear after ultracentrifugation. The bands are clearly visible and depend on the amount of liposomes and plasmids loaded on to the Ficoll gradient.

Table 1. Effect of interfering agents.

Agents*		DDAB found (\square g/mL)	DDAB:DOPE found (\square g/mL)
<i>H₂O</i>		20.0 \pm 0.2	12.0 \pm 0.2
<i>Acids/bases</i>	0.1M HCl	19.0 \pm 0.3	6.6 \pm 0.1
	1.0% trichloroacetic acid	6.3 \pm 0.2	4.5 \pm 0.2
	0.1M NaOH	8.8 \pm 0.3	5.4 \pm 0.1
<i>Salts</i>	1.0M NaCl	3.3 \pm 0.3	8.1 \pm 0.1
	1.0M MgCl ₂	3.3 \pm 0.1	7.8 \pm 0.1
	1.0M LiCl	3.1 \pm 0.2	7.5 \pm 0.1
	1.0M NH ₄ CH ₃ COO	7.5 \pm 0.2	7.8 \pm 0.1
	<i>Chelating agent</i>	0.1 EDTA	10.5 \pm 0.2
<i>Detergents</i>	0.25% Triton X-100	30.2 \pm 0.2	18.0 \pm 0.1
	0.01% Triton X-100	27.0 \pm 0.3	13.2 \pm 0.1
	0.10% SDS	2.8 \pm 0.2	1.2 \pm 0.1
	0.01% SDS	15.0 \pm 0.2	2.4 \pm 0.1
	<i>Denaturing/reducing agents</i>	3M Urea	19.5 \pm 0.2
4M Guanidium isothiocyanate		14.5 \pm 0.2	10.2 \pm 0.1
<i>Sugars</i>	1.0M Glucose	23.0 \pm 0.1	11.7 \pm 0.1
	1.0M Sucrose	26.8 \pm 0.2	11.4 \pm 0.1
<i>Buffers</i>	0.25M Tris, pH 7.5	15.0 \pm 0.1	6.9 \pm 0.1
	PBS, pH 7.2	4.2 \pm 0.1	7.3 \pm 0.1
	Tris(10mM)-EDTA(1mM), pH 8.0	7.9 \pm 0.2	7.5 \pm 0.1
<i>Solvents</i>	20% Ethanol	17.5 \pm 0.1	14.1 \pm 0.1
	20% Propanol	19.9 \pm 0.1	13.9 \pm 0.1

* Stock solutions (2x) of the potential interferants (25µL) were added to 25µL of either DDAB (800µg/mL) or DDAB:DOPE (1:1, 480µg/mL) and dye (950µL). Mixtures were incubated for 10 minutes, and assayed spectrophotometrically at 549nm in triplicate.

3.5 Loading of plasmids on to liposomes to form lipoplexes

Lipofectamine bound the most number of both plasmids (Table 2). The amount of plasmid bound on the formulated liposomes increased as the ratio of cationic lipid (DDAB) to neutral lipid (either DOPE or PtdChol) was increased. Increase was directly proportional to the amount of positive to neutral lipid ratio. For instance, when the ratio of DDAB:DOPE was increased from 1:2 to 1:1, the amount of additional plasmids bound was 50% of the additional numbers bound when the ratio was increased to 2:1. Similar results were obtained for the DDAB:PtdChol liposomes.

Also given in Table 2 is the cationic lipid quantity ($\times 10^{16}$ molecules) in each $100\mu\text{g}$ liposome formulation. Both Lipofectin and Lipofectace bound an identical number of plasmids. CAT plasmid-Lipofectamine complexes were observable under standard light microscopy and ranged in size from less than 300nm to $5\mu\text{m}$ in size (data not shown). Similar complexes were seen with the other liposomes, though the sizes were smaller. This discrepancy could be due to the ability of the polycationic liposome Lipofectamine to form bridges between two adjacent plasmid-liposome complexes, thus resulting in larger aggregates.

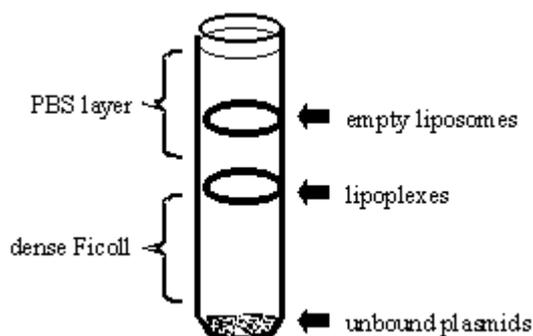


Figure 4. Separated bands of liposome-bound plasmids, empty liposomes, and unbound plasmids on a Ficoll gradient using ultracentrifugation.

Table 2. Plasmids bound by $100\mu\text{g}$ liposomes.

Liposome	CAT ($\times 10^{12}$)	p53 ($\times 10^{12}$)	Cationic species quantity ($\times 10^{16}$)
DDAB:PtdChol = 1:2	1.7	1.1	3.2
DDAB:PtdChol = 1:1	2.0	1.4	4.8
DDAB:PtdChol = 2:1	2.8	2.0	6.4
DDAB:DOPE = 1:2	1.8	1.2	3.2
DDAB:DOPE = 1:1	2.4	1.5	4.8
DDAB:DOPE = 2:1	2.9	1.7	6.4
Lipofectace	1.9	1.3	2.7
Lipofectin	1.9	1.3	4.5
Lipofectamine	5.4	4.1	3.1

Values represent the mean \pm standard deviation of triplicate experiments.

4 Discussion

Gene transfer technology is used in medicine, agriculture, marine culture and bioremediation, to cite a few uses. Techniques used for introducing genetic constructs into cells differ greatly, and one of the major shortcomings of this field of research is the cost attached to the 'tools of the trade'. The ethanol injection/pressure extrusion method for formulation of cationic liposomes presented herein is relatively simple, inexpensive and does not involve the use of specialised equipment. Liposomes were size-filtered to approximately 200nm in diameter. Other studies have confirmed that such sizing limits the diameter of lipid vesicles to within $\pm 10\%$ of the smallest pore size (unpublished results). Apart from comparable carrying capacity to commercial liposomes as seen in the present study, such vesicles have been tested in cell culture and *in vivo* and have been found to deliver the nucleic acid drug

load to target mammalian cells (Dass 1998). From experience, these vesicles should also be able to deliver nucleic acid constructs into other cell types including plant cells.

Shift of the absorbance maxima of erythrosine dye at pH 4.25 after complexing to all the cationic liposomes in the present study is similar to that with anionic and neutral phospholipids (Andree and Soedjak, 1994). There is a measurable difference between absorbance of free dye to that of dye complexed to liposomes at 549nm . For Lipofectamine, formulated from a polycationic lipid, the extinction coefficient equals $1.5 \times 10^{-2} \text{ mg}^{-1} \text{ L cm}^{-1}$ and detection limit was $0.20 \mu\text{g/mL}$. These values indicate that the assay is more sensitive with Lipofectamine than for both Lipofectin and Lipofectace (formulated from monocationic lipids). Detection limits for both DDAB:DOPE (1:1) and DDAB:PtdChol (1:1) were

0.23 μ g/mL, identical to that for DDAB alone. The low detection limit of free DDAB confirms that this assay is suitable for quantification of cationic lipids. Apart from its sensitivity and simplicity, the assay is very reproducible. Furthermore, the sample volume can be increased from 15 μ L to 150 μ L for detection of liposomes in more dilute suspensions. Alternatively, low assay volumes of 50 μ L may allow as low as 10ng of cationic lipid to be assayed. Furthermore, the dye-lipid colour development is completed essentially within 5 - 10 minutes.

Salts and buffers have a less interfering effect on the liposome assay compared to assay of DDAB alone. This could be due to greater accessibility of free cationic lipid for interaction with the anionic species in the salts/buffers, which would prevent it from complexing with dye. SDS caused a decrease in absorbance of free lipid. The decrease with liposomes however, was less significant. Again, this could be attributed to the easier accessibility of free DDAB to complex with dye compared to DDAB in the vesicle coat. In contrast, Triton X-100 caused an elevation of absorbance, with the increase being greater with free DDAB at the lower detergent concentration. This discrepancy could again be attributed to the greater accessibility of the free cationic lipid.

The centrifugational method for separation of plasmids is relatively simple and can be performed in any laboratory possessing an ultracentrifuge. This method has the advantage over separation using agarose gel electrophoresis in that plasmids not bound to liposomes may be recycled. Separated bands are readily visible and sample extraction from polypropylene tubes is not arduous. This separation method should lower the cost of nucleic-acid-based reagents used for attenuation of genes in both mammalian and plant cells.

Lipoplexes are heterogeneous in both size and shape (Dass and Burton, 1999), factors that may impair the transfection capacity of the particular liposome preparation. Such heterogeneity may also be responsible for variable lipofection *in vivo*. Ensuring that one batch of liposomes has the same amount of lipids compared to another may reduce such heterogeneity. Furthermore, it is important to quantitate the ability of liposomes to bind to other carrier agents such as microspheres used for drug targeting *in vivo* (Dass *et al.* 2000). The method for quantitation developed in the present study is simple, quick, inexpensive and non-hazardous. It detects cationic liposomes at very low limits and is reproducible. The

erythrosine dye also allows better visualisation of plasmid-liposome complexes by conventional light microscopy.

5 References

- Andree, H. A. M. and Soedjak, H. S. 1994. Colorimetric phospholipid determination with Erythrosin B. *Anal. Biochem.* **222**, 465 - 471.
- Campbell, M. J. 1995. Lipofection reagents prepared by a simple ethanol injection technique. *Biotechniques* **18**, 1027 - 1032.
- Chisti, Y. 2000. Marine biotechnology - a neglected resource. *Biotechnol. Adv.* **18**, 547 - 548.
- Dass, C. R. (1998). *Targeted gene therapy for cancer*. PhD thesis, Charles Sturt University, Wagga Wagga, Australia.
- Dass, C. R. and Burton, M. A. 1999. Lipoplexes and tumours. *J. Pharm. Pharmacol.* **51**, 755 - 770.
- Dass, C. R., Walker, T. L., Kalle, W. H. J. and Burton, M. A. 2000. A microsphere-lipoplex (microplex) vector for targeted gene therapy of cancer. II. *In vivo* biodistribution study in a solid tumour model. *Drug Delivery* **7**, 15 - 20.
- Kohli, A., Gahakwa, D., Vain, P., Lawrie, D. A. and Christou, P. 1999. Transgene expression in rice engineered through particle bombardment: Molecular factors controlling stable expression and transgene silencing. *Planta* **208**, 88 - 97.
- Maccarone, M., Dini, L., di Marzio, L., di Giulio, A., Rossi, A., Mossa, G. and Finazzi-Agro, A. 1992. Interaction of DNA with cationic liposomes: Ability of transfecting lentil protoplasts. *Biochem. Biophys. Res. Commun.* **186**, 1417 - 1422.
- New, R. R. C. 1994. Preparation of liposomes. In *Liposomes: A practical approach*, ed. R. R. C. New, Information Press: Oxford, England, 33 - 104.
- Teifel, M. and Friedl, P. 1995. New lipid mixture for efficient lipid-mediated transfection of BHK cells. *Biotechniques* **19**, 79 - 82.
- Yokoi, S. and Toriyama, K. 2000. Transgenic rice (*Oryza sativa*). In *Biotechnology in agriculture and forestry. Transgenic crops I*, vol. **46**. Bajaj, YPS (ed.). Berlin: Springer-Verlag, pp. 1 - 13.
- Zhu, Z., Sun, B., Liu, C., Xiao, G. and Li, X. 1993. Transformation of wheat protoplasts mediated by cationic liposome and recognition of transgenic plantlets. *Chin. J. Biotechnol.* **9**, 257 - 161.