Liposomal Delivery of Drugs and Biologicals:

Development and Characterisation

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ABSTRACT

Liposomes, or bilayer lipid vesicles, are ideal models of cells and biomembranes. Furthermore, they are being used by the pharmaceutical, cosmetic and food industries as micro- and nano-carrier systems for the protection and delivery of bioactive agents. They are composed of one or more concentric lipid and/or phospholipid bilayers and can contain other molecules such as proteins or polymers in their structure. There are numerous lab-scale and a few large-scale techniques for liposome preparation. However, most of these techniques are not suitable for the encapsulation of sensitive substances because of their exposure either to mechanical stresses or potentially harmful chemicals during the preparation. The majority of liposome preparation techniques involve the application of volatile organic solvents (mainly chloroform, ether, or methanol), as a first step, to dissolve or solubilise the lipids. These solvents not only affect the chemical structure of the entrapped substance but will also remain in the final liposome formulation and contribute to toxicity and influence the stability of the vesicles.

In this thesis a new method for the production of liposomes without the use of any hazardous chemical or process is described. This method involves the heating of the liposome components, in the presence of glycerol. Glycerol is a water-soluble and physiologically acceptable chemical with the ability to increase the stability of the lipid vesicles and does not need to be removed from the final liposomal product. Since heating is the main step in this methodology it is termed the '*Heating Method*' and the resultant liposomes are referred to as '*HM-liposomes*'.

The '*Heating Method*' enabled preparation of stable liposomes with ease, good reproducibility and different size distributions. Physicochemical and biological characteristics of the HM-liposomes were compared with that of the conventional liposomes. Different types of liposomes (heterogenously sized multilamellar vesicles and unilamellar vesicles of 100nm and 400nm size) were prepared by conventional techniques (thin-layer hydration, bath sonication and extrusion methods) as well as the heating method. Liposomes produced were characterised in terms of their size, polydispersity, morphology and stability using light scattering instrument in addition to optical microscopy, Nikon eclipse microscopy, freeze

fracture scanning electron microscopy, transmission electron microscopy, scanning tunnelling microscopy and atomic force microscopy. Comparison of the change in the transition temperature of the vesicles was performed by differential scanning calorimetry. HM-liposomes were completely non-toxic when tested in a human lung epithelial cell line (HBE) using two different toxicity assays. The antineoplastic drug 5-FU and the antioxidant agent GSH were encapsulated in the HM-liposomes with high entrapment efficiencies. HM-liposomes sustained the release of these drugs for prolonged periods and reduced the cytotoxicity of the 5-FU towards the HBE cells.

A new non-viral, and non-cationic gene transfer vector was constructed by using the HM-liposomes. Incorporation of DNA to the HM-liposomes was achieved with high entrapment efficiency through a very mild procedure, which does not involve any potential harm to the DNA structure/function. Anionic HM-liposomes were complexed with plasmid DNA by the mediation of calcium ions at room temperature. High-resolution electron and scanning probe microscopic studies revealed either one huge vesicle or two to four aggregated/semi-fused vesicles for HM-liposomes of both multilamellar and unilamellar types as a result of their complexation with DNA and calcium. The HM-liposomes were detected to be able to protect the incorporated DNA from enzymatic degradation. Moreover, the vector was detected to be able to transfect cultured HBE cells, in the presence of serum, probably by a mechanism involving fusion of the ternary complex to the plasma membrane and/or the endosomal membrane.

DECLARATION

I declare that while registered as a candidate for the degree of Doctor of Philosophy I have not, in the duration of this research programme, been registered as a candidate for another award from any other academic or professional institution.

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(M. Reza Mozafari)

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I dedicate this thesis to my beloved parents, my sister and brother, my wife and two lovely children.

I hope one day true science helps us to overcome the barriers of race, ethnicity, nationality, religion and ideology towards establishing peace and respect among all who live in our planet.

ABBREVIATIONS

5FU	5-fluorouracil
AFM	atomic force microscopy
Ca^{2+}	calcium ion
CHOL	cholesterol
°C	degree Celsius
DCP	dicetylphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease enzyme
DPPC	dipalmitoylphosphatidylcholine
DSC	differential scanning calorimetry
et al	'and others': et alii (masculine plural), et aliae (feminine plural), et alia (neutral plural); also means 'and elsewhere'
EtBr	ethidium bromide
GSH	glutathione
HBE	human bronchial epithelial cells
HM	heating method
HM-liposomes	lipid vesicles prepared by the heating method
LUV	large unilamellar vesicles
MLV	multilamellar vesicles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nm	nanometer
NRU	neutral red uptake assay
PBS	phosphate-buffered saline
PC	phosphatidylcholine
SEM	scanning electron microscopy
STM	scanning tunneling microscopy
SUV	small unilamellar vesicles
T_c	phase transition temperature
TEM	transmission electron microscopy
TLC	thin layer chromatography

RELATED PUBLICATIONS AND PRESENTATIONS IN CONJUNCTION WITH THE RESEARCH PROGRAMME

The present PhD study have resulted in nine peer-reviewed publications and one review article submitted with more papers in preparation in addition to one patent application, a complete list of which is provided at the end of this thesis. Furthermore, during this research programme I have attended conferences and symposia and presented my work as follows:

1. Mozafari, M.R., Reed, C.J., Rostron, C., Martin, D.S., Kocum, C. & Piskin, E. (2004) Scanning probe microscopy investigations of nano and microscale bioactive carrier complexes. *UKSPM 2004, UK Annual Scanning Probe Microscopy Meeting,* Nottingham, England.
2. Mozafari, M.R., Reed, C.J., Rostron, C., & Martin, D.S. (2003) Second Generation of Liposome/Ca^{2+}/DNA Complexes: A Microstructural Study Using Atomic Force Microscopy. *6th International Conference, Liposome Advances, Progress in Drug and Vaccine Delivery*, London, England, pp 114.
3. Mozafari, M.R., Reed, C.J., Rostron, C., & Martin, D.S. (2003) Transfection of Human Airway Epithelial Cells Using a Lipid-Based Vector Prepared by the Heating Method. *14th Drug Delivery to the Lungs Conference,* London, England, pp 54-58. ISBN 0-0544567-1-8.
4. Mozafari, M.R., Reed, C.J. & Rostron, C. (2003) 5-Fluorouracil Encapsulation in Colloidal Lipid Particles: Entrapment, Release and Cytotoxicity Evaluation in an Airway Cell Line. *14th Drug Delivery to the Lungs Conference,* London, England, pp 180-183. ISBN 0-0544567-1-8.
5. Mozafari, M.R., Reed, C.J., Rostron, C., Kocum, C. & Piskin, E. (2002) Pharmaceutical Applications of Nanotechnology- Implications in Drug Targeting and Gene Therapy. *Proceedings of Pharmacy & Pharmacology, 9th Iranian Students Seminar in Europe (ISS-2002)*, Birmingham, England, pp 7.
6. Mozafari, M.R., Reed, C.J., Rostron, C., Kocum, C. & Piskin, E. (2002) Formation and Characterisation of Non-Toxic Anionic Liposomes for Delivery of Therapeutic Agents to the Pulmonary Airways. *9. Liposomes. From Models to Applications*, Poland.
7. Mozafari, M.R., Reed, C.J. & Rostron, C. (2002) Safety and Efficacy of Anionic Liposome Mediated Gene Transfer to the Human Airway Epithelial Cells. *Genes as Medicines,* London, England.
8. Mozafari, M.R., Reed, C.J. & Rostron, C. (2002) Cystic Fibrosis Gene Therapy by Liposomes: Prospects and Problems. *The Ian Ward Members Papers Evening, The Association of Clinical Biochemists North West Region*, Liverpool, England.
9. Mozafari, M.R., Reed, C.J. & Rostron, C. (2001) Reduced Cytotoxicity of Anionic Liposome/Calcium/DNA Complexes Prepared by the Heating Method. *British Pharmaceutical Conference Science Proceedings 2001*, pp 100 (Pharmaceutical Press, London, UK). ISBN 0 85369 512 1.
10. Mozafari, M.R., Reed, C.J. & Rostron, C. (2001) Non-Toxic Liposomal Formulations for Gene and Drug Delivery to the Lung. *8th Biomedical Science and Technology Symposium*, Ankara, Turkey, pp O16.
11. Mozafari, M.R., Reed, C. & Rostron, C. (2001) The Influence of the Preparation Method on the Toxicity of Anionic Liposomes. *Proceedings of Pharmacy & Pharmacology, 8th Iranian Students Seminar in Europe (ISS-2001)*, UMIST, Manchester, England, pp 3.

CONTENTS

1: GENERAL INTRODUCTION

1.1. A Historical Perspective of Liposomes

The simplest way to introduce liposomes may be to define them as "lipid vesicles enclosing aqueous space(s)" (New 1990). In another words liposomes are simplified forms of living cells (Figure 1.1.). Their formation does not require the inclusion of surfactants or emulsifiers; they may be single- or multi-lamellar and vary according to lipid content, surface charge, size, and method of preparation. The properties of these vesicles have been investigated extensively, and ingenious ways have been found to manipulate them to impart behavioural characteristics such as sensitivity to light, heat, pH and magnetic field. Few other areas of research can routinely bring into realisation such a wide range of phenomena. The extensive and ever increasing literature covering the field of liposomology written by researchers with diverse interests is an indication of liposomes being a ubiquitous biochemical tool. Applications of liposomes in the areas of immuno-modulation, diagnosis, drug delivery, cosmetics, genetic engineering and investigations into the origin of life (Pozzi et al 1996; Nomura et al 2001) have been identified and developments are ongoing. In addition, the resemblance of liposomes to cell membranes have made them an ideal system for studying the characteristics of biological membranes.

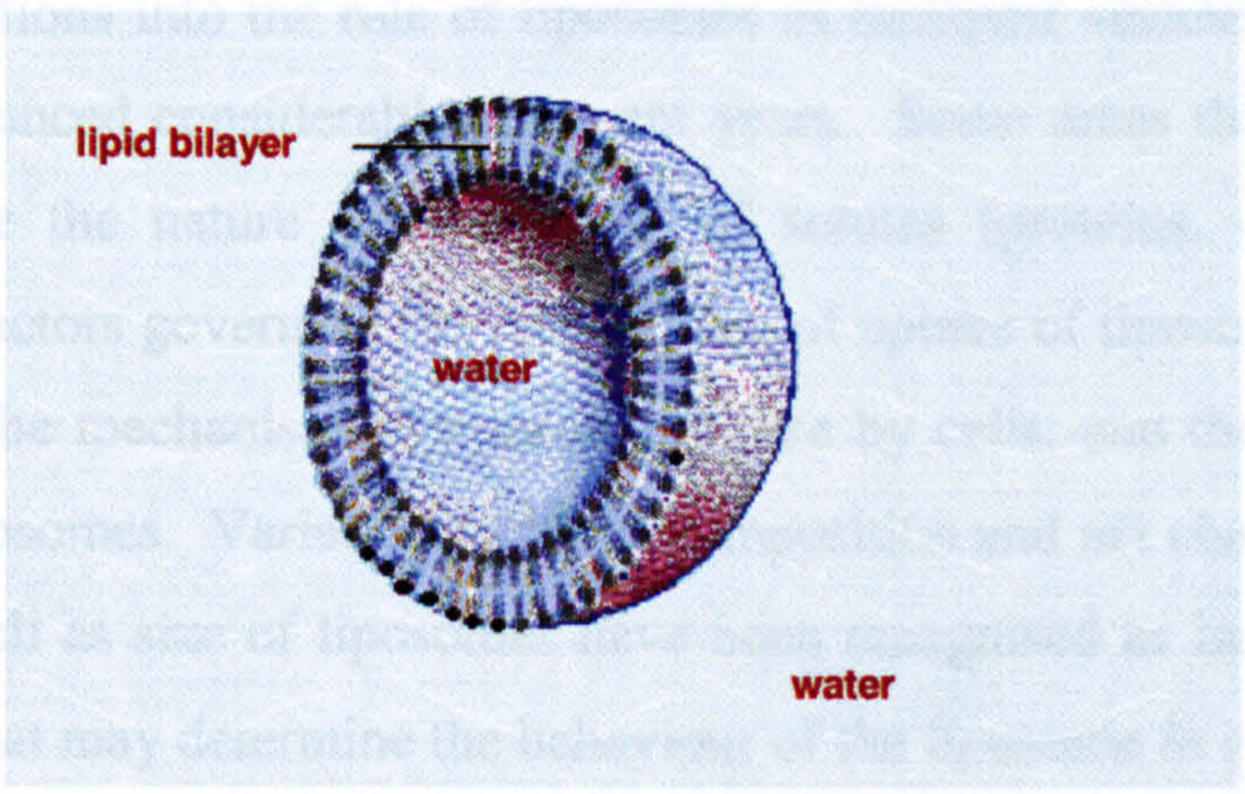


Figure 1.1. Cross sectional view of a unilamellar liposome.

The concept that lamellar structures composed of lipid and water systems might be useful as models for cell membranes has a long history (e.g. see Teitel Bernard 1945-47, also see Chapman and Arrondo 1981 for a review). However, it was in the early 1960s that much more emphasis was placed upon the study of the properties of lipid-water systems as model biomembranes. Initially named `Bangosomes`, after being brought to the attention of the scientific world by A. D. Bangham (Bangham et al 1965), they were later termed `Liposomes`. Bangham himself, however, proposed the name `Amphisome` (Bangham 1981) as a more appropriate term since membrane molecules are characteristically amphiphiles.

Pioneers in the field of model biomembranes, Bangham, Chapman, Dervichian, and Luzzati, studied these systems from different view points. The study of the dynamic and sub-microscopic properties of these model membranes, such as fluidity (Chapman et al 1966), and change to other mesomorphic structures, and phase transitions, was for some time carried out distinctively and separately from the study of more macroscopic properties such as their ion trapping and release characteristics (Bangham et al 1965). Some of these investigations have been useful for providing insight into the dynamic structure of biomembranes, such as the fluidity of the lipid matrix and their diffusional properties (e.g. see Chapman 1975), whilst some others have led directly to a potential tool for drug delivery systems (Gregoriadis 1971).

Investigations into the role of liposomes as transport vesicles for therapeutic agents have advanced considerably in recent years. Some areas that have attracted attention include the nature of interaction of solutes (proteins, drugs etc.) with liposomes; the factors governing the rate and site of uptake of liposomes after *in vivo* administration; the mechanism of liposome uptake by cells; and the immunological properties of liposomes. Variations of lipid composition and net charge of liposomal membrane as well as size of liposomes have been recognised as important physical characteristics that may determine the behaviour of the liposome *in vitro* and *in vivo*.

1.2. Physico-chemical Properties of Liposomes

1.2.1. Chemical Constituents

The word liposome has been adopted generally to describe mesomorphic lipid-water structures. Chemical components of liposomes are *lipids* and/or *phospholipids*. Lipids are fatty acid derivatives with various head group moieties. They are widely occurring in nature and have been the subject of various chemical manipulations, for instance to prepare liposomes with certain physico-chemical characteristics (e.g. fluidity, shelf-life, blood circulation time, rate of drug release, etc.). *In vivo*, they are subject to conversion by gastrointestinal lipases to their constituent fatty acids and head groups. Triglycerides are lipids made from three fatty acids and a glycerol molecule (a three-carbon alcohol with a hydroxyl [OH] group on each carbon atom). Mono- and diglycerides are glyceryl mono- and diesters of fatty acids. Phospholipids are similar to triglycerides except that the first hydroxyl of the glycerol molecule has a polar phosphate- containing group in place of the fatty acid. Phospholipids are *amphipathic*, being both hydrophylic and hydrophobic. The head group of a phospholipid is hydrophilic (water-loving) and its fatty acid tail is hydrophobic (water hating) (Figure 1.2.). The phosphate moiety of the head group is negatively charged. In the related galactolipids, the phosphate group is replaced by galactose. These molecules are non-ionic (Bowtle 2000).

Phospholipids are named according to the identity of the organic polar head group. For example, if the polar head group is choline, the phospholipid is called phosphatidylcholine. The term *phosphatidylcholine* actually refers to the family of phosphoglycerides (phospholipids) that contain choline as the polar head group. The fatty acid components may be specified in order to distinguish different phosphatidylcholines (also known as 'lecithin'). Lecithin can be derived from both natural and synthetic sources. Lecithin from natural sources is in fact a mixture of phosphatidylcholines, each with chains of different length and varying degrees of unsaturation (New 1990).

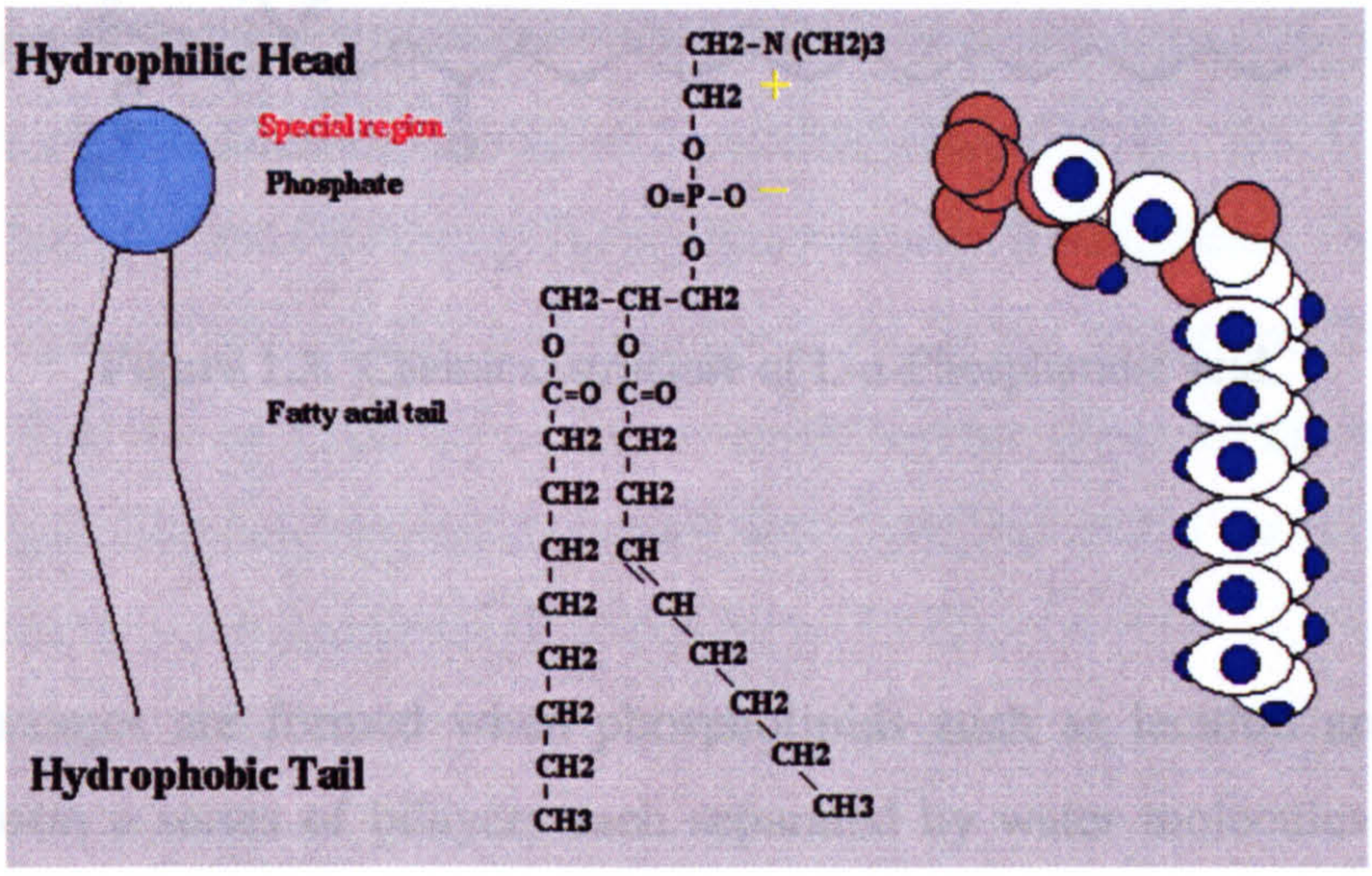


Figure 1.2. The hydrophilic and hydrophobic parts in the structure of a phospholipid molecule (from: http://www.cbc.umn.edu/~mwd/cell_www/chapter2/membrane.html).

Phosphatidic acid (Figure 1.3.) serves as general intermediate in the biosynthesis of phospholipids (Kennedy 1961). It is a structurally simple molecule and probably was the first phospholipid which appeared in the course of evolution (Epps et al 1978). Under conditions similar to that of the primitive Earth, in a series of experiments, Oro and his team have synthesised mono- and dipalmitoyl glycerophosphates (phosphatidic acids) and subsequently phosphatidylcholine and phosphatidylethanolamine, two of the most important amphiphilic lipids which are used in the structure of liposomes and membranes (for a review see Oro 1994). Liposomes, as initial biomembranes, might have had vital functions not only to protect the first informative molecules (probably RNA) but also, in our opinion, to direct replication through specific interactions with these nucleic acids (Kahveci et al 1994).

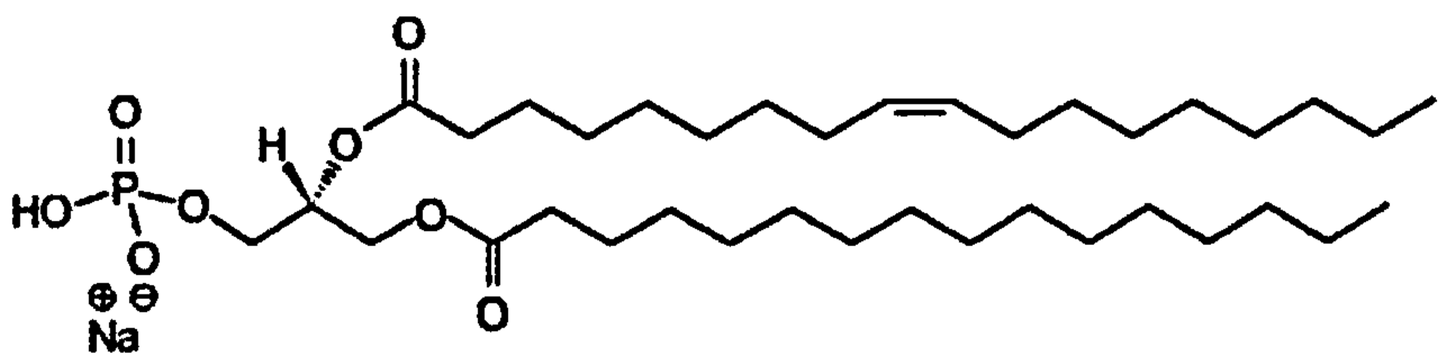


Figure 1.3. Chemical structure of L-α-Phosphatidic acid.

Liposomes are formed when phospholipids such as lecithin are present in water and form a series of bilayers each separated by water molecules by input of energy. The underlying mechanism for the formation of liposomes is basically the hydrophilic-hydrophobic interaction between phospholipids and water molecules. The amphipathic phospholipid molecules form a closed bilayer sphere in an attempt to shield their hydrophobic group from the aqueous environment while still maintaining contact with the aqueous phase via the hydrophilic head group. In more general terms hydration of phospholipids in aqueous media results in the arrangement of phospholipid molecules in bilayers to form closed spherules known as liposomes. During this process liposomes can entrap hydrophilic solutes (or drugs) that are present in the hydration media. Lipophilic (fat loving) molecules or drugs (e.g. vitamin E, cyclosporin A, phenytoin, ...) can also be incorporated into liposomal bilayers by dissolving these molecules together with the lipids. Alternatively lipid soluble molecules/drugs may be complexed with cyclodextrins and subsequently encapsulated within the liposome aqueous compartment (McCormack and Gregoriadis 1994).

1.2.1.1. Sterols

In addition to lipid and/or phospholipid molecules, liposomes may contain sterols in their structure. Sterols are important components of most natural membranes, and incorporation of sterols into liposome bilayers can bring about major changes in the properties of these membranes. The most widely utilised sterol

in the manufacture of the lipid vesicles is cholesterol. Cholesterol has an -OH group at position 3 of ring A (Figure 1.4.), a CH_3 at position 10 where rings A and B join, another CH_3 at position 13 where rings C and D join and an eight membered hydrocarbon chain extending from the five membered ring D. Inside membranes cholesterol has its free OH group facing the aqueous environment while its rings occupy the spaces between the hydrophobic parts of the phospholipid molecules. Cholesterol does not by itself form bilayer structures, but it can be incorporated into phospholipid membranes in very high concentrations, up to 1:1 or even 2:1 molar ratios of cholesterol to phosphatidylcholine (New 1990).

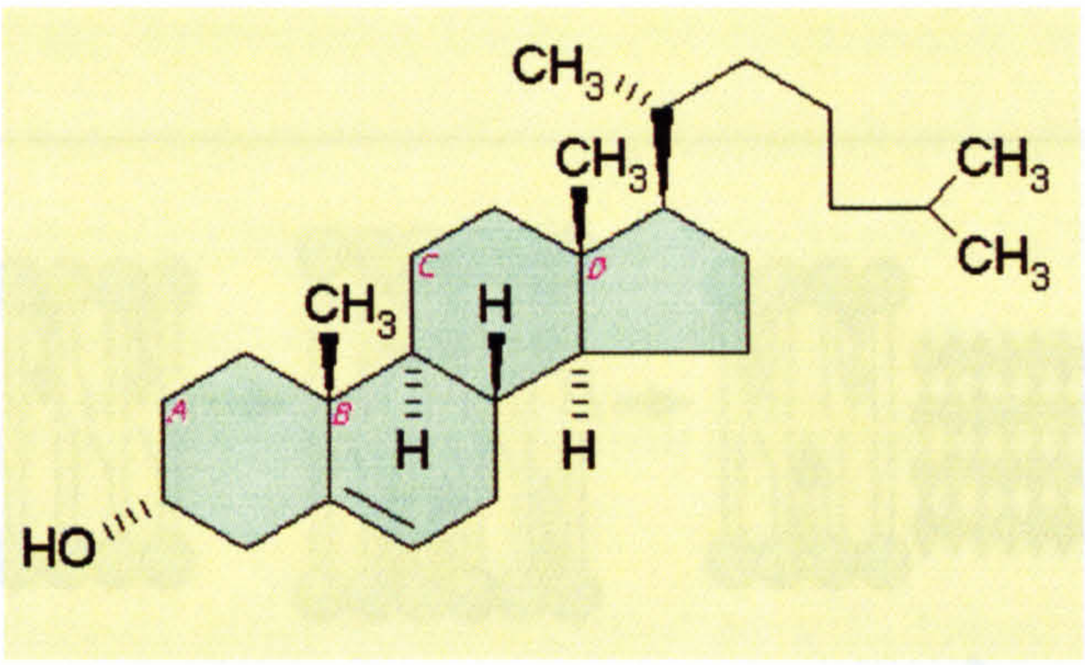


Figure 1.4. The structure of cholesterol.

Cholesterol is used, in liposomal formulations, to increase the stability of the lipid vesicles by modulating the fluidity of the lipid bilayer. In general, cholesterol modulates fluidity of phospholipid membranes by preventing crystallization of the acyl chains of phospholipids and providing steric hindrances to rapid movement. Stabilization of liposomes by cholesterol in general (New 1990), and in serum/blood in particular (Mayhew 1979; Hong 1997), is a well known phenomena. A schematic representation of the cholesterol effects on the structure of plasma membranes is demonstrated in Figure 1.5.

The amount of cholesterol to be used in the liposomal formulation depends mainly on the application area for which the liposomes are being manufactured. In previous work we have detected that both anionic and neutral liposomes can interact with model membrane systems (in the form of fusion/aggregation) when containing a 10% molar ratio of cholesterol (Mozafari and Hasirci 1998). Anionic vesicles containing 10% cholesterol were also able to incorporate DNA molecules in the presence of Ca^{2+}. Increasing the cholesterol content of the anionic vesicles from 10% to 40%, however, rendered them not only unable to undergo fusion/aggregation with model membranes but also unable to incorporate the DNA molecules. Consequently these liposomes containing 40% or more cholesterol can not be useful in gene and drug delivery applications.

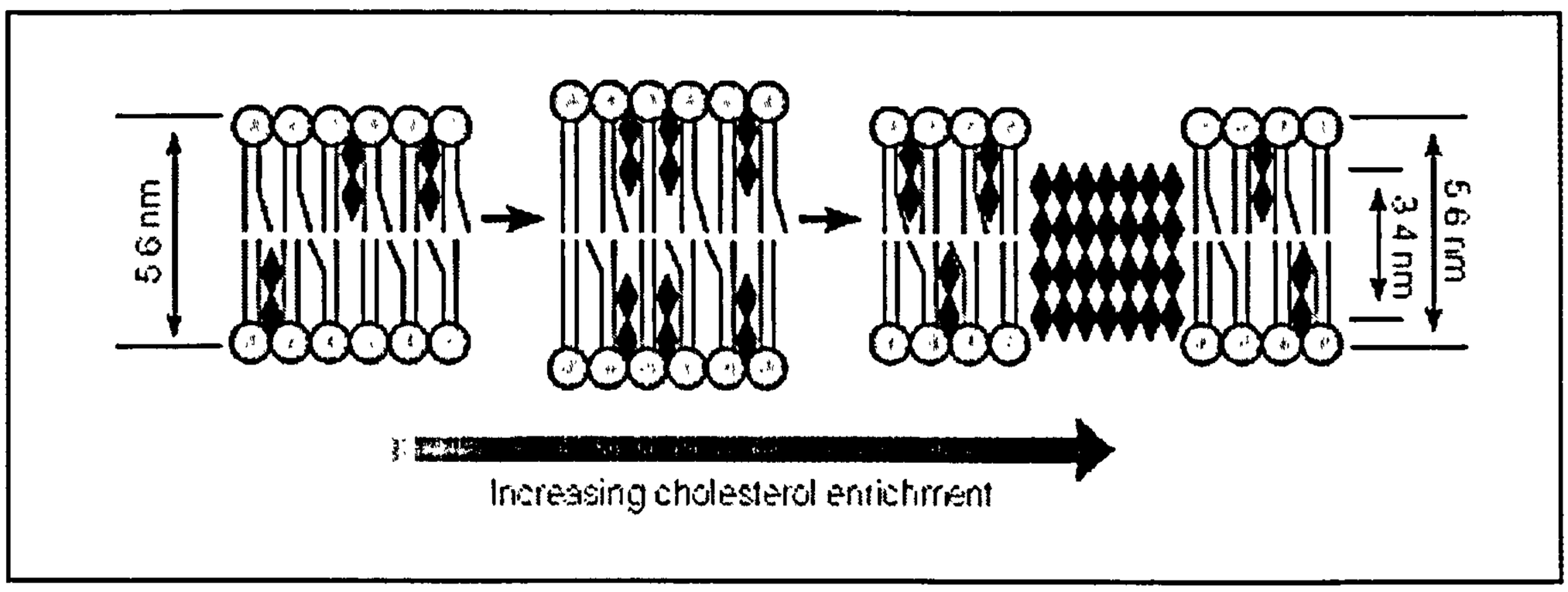


Figure 1.5. A schematic model of the effects of cholesterol (◆) on plasma membrane structure (adapted from Tulenko et al 1998).

1.2.2. Transition Temperature (T_C)

Liposomes can be prepared from a variety of lipids (usually phospholipids) and lipid mixtures. They can also be formed from whole lipid extracts of cell membranes, e.g. erythrocyte ghosts (Bangham 1968). Formation of liposomes depends on certain intrinsic and extrinsic factors including the length of the acyl chains, pH and temperature. Amphipathic lipid molecules such as soaps and

phospholipids have an important characteristic, i.e. they can undergo a thermotropic phase transition at temperatures much lower than their final melting point. The detailed molecular nature of the major thermotropic phase transition of these long-chain amphiphilic molecules was defined first by infra-red spectroscopic studies (Chapman 1966).

When water diffuses into the lipid bilayer it does so into the polar (ionic) region only when the temperature is reached at which the hydrocarbon chains "melt" (the T_C transition temperature). If the temperature is higher than this there is a simultaneous dissociation of the ionic structure by the penetration of water and melting of the hydrocarbon chain region. The temperature of transition (T_C) depends upon the nature of the hydrocarbon chains, the polar region of the molecule, the amount of water molecules present and on any solutes dissolved in the water. Once the water has penetrated and the sample is then cooled to below the T_C, the hydrocarbon chains rearrange themselves into an orderly crystalline lattice, but the water is not necessarily expelled from the system (Chapman and Arrondo 1981).

Each phospholipid series is characterised by a T_C below which its fatty acyl chains are in a quasi-crystalline array and above which the chains are in a more fluid state. Other changes accompanying chain melting are decreased bilayer thickness and increased area per molecule. These changes have been studied extensively by a variety of physical techniques including X-ray diffraction (Luzzati and Husson 1962), differential scanning calorimetry (DSC) and fluorescence depolarisation (Chapman et al 1967). In general T_C is lowered by decreased chain length, by unsaturation of the acyl chains, as well as presence of branched chains and bulky side groups (e.g. cyclopropane rings) (Szoka and Papahadjopoulos 1980). In phospholipids composed of the same acyl chain in both positions, T_C increases by about 14-17°C with every two-methylene unit addition to the chain. Most commonly used phospholipid molecules and their gel to liquid crystalline transition temperatures are presented in Table 1.1. Vesicles composed of phospholipids that are at temperatures below the transition temperatures of the phospholipids are known as "solid" or "gel" and those at temperatures above their T_C, as "fluid" liposomes (liquid crystalline vesicles).

In the study of model biomembranes, lecithin-water systems have received much attention because of the common occurrence of these lipids in animal cell membranes. The heat absorbed at T_C for lecithin-water systems at high water content is seen to be chain-length dependent. For the series of fully saturated lecithins a difference of 2 kcal/mole occurs for a chain length difference of two methylene groups.

An understanding of phase transitions and fluidity of phospholipid membranes is important both in the manufacture and exploitation of liposomes. This is due to the fact that the phase behaviour of a liposomal membrane determines such properties as permeability, fusion, aggregation and protein binding, all of which can markedly affect the stability of liposomes and their behaviour in biological systems (New 1990). Phase transition temperature of the lipid vesicles has also been reported to affect the pharmacokinetics of liposome-encapsulated drugs, such as doxorubicin (Gabizon et al 1993).

When developing a new liposomal formulation or method, controlling the transition temperature of the lipid components is very useful. Choosing a lipid possessing high T_C (e.g. DPPC, see Table 1.1.) where the lipid vesicle would be in the gel phase, at storage temperatures, would provide a non-leaky packaging system. Alternatively, a lipid with a transition temperature between the starting temperature and the ending temperature of the system would provide a means of releasing packaged material as the lipid passes through its phase transition temperature and the vesicle becomes leaky. These characteristics of liposomes can be utilised in drug delivery to tumour areas which, due to higher rate of metabolism, have higher temperatures compared with normal tissues. Also, one should consider how the transition temperature of the lipid could affect the processing steps. For instance, using a high transition lipid in liposome formulation, which causes the liposome to be rigid below its T_C, could present some technical problems when filtration is necessary during the manufacture or sterilisation steps.

Table 1.1. Most commonly used phospholipids in liposome preparations and their gel to liquid crystalline transition temperatures (adapted from Weiner 1990).

Phospholipid	Abbreviation	Transition temperature (^{o}C)
Egg phosphatidylcholine	PC	-15 to -7
Dilauryloyl phosphatidylcholine	DLPC	-1.8
Dimyristoyl phosphatidylcholine	DMPC	23
Dipalmitoyl phosphatidylcholine	DPPC	41
Distearoyl phosphatidylcholine	DSPC	55
Dioleoyl phosphatidylcholine	DOPC	-22
Dilauryloyl phosphatidylglycerol	DLPG	4
Dimyristoyl phosphatidylglycerol	DMPG	23
Dipalmitoyl phosphatidylglycerol	DPPG	41
Distearoyl phosphatidylglycerol	DSPG	55
Dioleoyl phosphatidylglycerol	DOPG	-18
Dimyristoyl phosphatidylethanolamine	DMPE	50
Dipalmitoyl phosphatidylethanolamine	DPPE	66
Phosphatidylserine	PS	7
Dimyristoyl phosphatidylserine	DMPS	38
Dipalmitoyl phosphatidylserine	DPPS	51
Dimyristoyl phosphatidic acid	DMPA	51 (pH 6.0)
Dipalmitoyl phosphatidic acid	DPPA	67 (pH 6.5)
Sphingomyelin	SPH	32
Dipalmitoyl sphingomyelin	DPSPH	41
Distearoyl sphingomyelin	DSSPH	57

1.2.3. Surface Charge

Besides the phase transition property of phospholipids, the surface charge of liposomes can also be varied. They could be neutral (with phospholipids such as phosphatidylcholine, or phosphatidylethanolamine), negative (with acidic phospholipids such as phosphatidylserine, phosphatidylglycerol, phosphatidic acid, or dicetylphosphate) or positive (by the use of lipids such as DOTAP (dioleoyl trimethylammonium propane), DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride), or stearylamine) in physiological pH ranges. Liposomal charge is an important characteristic that may determine the fate of liposomes both *in vitro* and *in vivo*. In addition, the net charge of the liposomal membrane is an important parameter in terms of liposome interaction with drug molecules. From a toxicity point of view, liposome charge has been shown to have crucial roles. There are considerable number of reports on the toxicity of cationic liposomes (e.g. Campbell 1983; Filion and Phillips 1997, 1998; Dokka et al 2000; Nagahiro et al 2000). On the other hand, anionic liposomes are reported to be less cytotoxic than cationic delivery systems (Chawla et al 1979; Campbell 1983; Welz et al 2000). It has been postulated that negatively charged liposomes, in general, associate more effectively and are taken up more readily by the cells compared with neutral liposomes (Heath et al 1985; Monkkonen et al 1994). Due to these reasons anionic liposomes were utilised in the studies reported in this thesis.

1.3. Liposome Preparation Techniques

There are several methods of liposome preparation giving rise to vesicles of different sizes ranging from 20nm to several microns in diameter and composed of one or more bilayers. Conventional liposome preparation techniques were discussed extensively by Gregoriadis (1984; 1993) and New (1990). More novel techniques are being introduced for liposome preparation, each with its own advantages and possible limitations. The key point to grasp in considering the manufacture of liposomes is that phospholipid membranes form as a result of unfavourable

interactions between phospholipids and water. Thus the emphasis in making liposomes is not towards assembling the membranes, but towards getting the membranes to form vesicles of the right size and structure, and to entrap materials with high efficiency and in such a way that these materials do not leak out of the liposome randomly. Other aspects of ideal liposome preparation procedures are discussed in chapter 2.

The detailed mechanism of liposome formation by different techniques is out of the scope of this thesis. Almost for each liposome preparation method a different liposome formation mechanism can be suggested. Lasic (1988) for example proposed that liposomes form when hydrated bilayered phospholipid flakes (BPF) are generated and subsequently vesiculate into larger liposomes. Gould-Fogerite and Mannino (1993) proposed the involvement of structures called *cochleate cylinders* as intermediates in the formation of large unilamellar vesicles (LUV) by *calcium-EDTA chelation*, *agarose plug diffusion* and *rotary dialysis* techniques. Talsma et al (1994), for their *bubble method*, postulated that the continuous generation of gas/water interfacial areas might produce lipid monolayers at these interfaces that could be the starting material for further vesicle formation.

The basic underlying principle for the formation of liposomes, regardless of the preparation methodology, is the hydrophilic/hydrophobic interactions between lipid-lipid and lipid-water molecules. Input of energy (e.g. in the form of sonication, homogenisation, shaking, heating, etc.) results in the arrangement of the lipid molecules, in the form of bilayered vesicles, to achieve a thermodynamic equilibrium in the aqueous phase. Lasic et al (2001) have proposed that symmetric membranes prefer to be flat (spontaneous curvature $\equiv C_O = 0$) and energy is required to curve them. Whether spherical lipid membranes form in one stage (with curvatures initially as they form) or in two stages, as suggested by Lasic and co-workers for the above-mentioned case, it seems that energy in one form or another is a requirement for the production of liposomes. In the heating method, explained in this thesis, the main form of energy for the formation of liposomes is heating while stirring is used to facilitate homogeneous distribution of the ingredients.

1.3.1. Conventional Methods

Conventional methods of making liposomes can be said to involve four basic stages: drying down of lipids from organic solvents, dispersion of the lipids in aqueous media, purification of the resultant liposomes, and analysis of the final product (New 1990). However, organic solvent residues, remaining in the lipid and/or aqueous phases of the liposomes during their preparation, could result in toxicity (Cortesi et al 1999). Recent developments in the field of liposome technology have made it possible to prepare liposomes without using any volatile organic solvent or detergent, examples of which are the polyol dilution method (Kikuchi et al 1994), the bubble method (Talsma et al 1994) and the heating method developed in our laboratory (Mozafari et al 2001, 2002a, 2002b, 2002c) which is explained in this thesis.

Table 1.2. shows the physical properties and entrapment efficiencies of some liposome formulations prepared by conventional methods. Only a few of the conventional liposome preparation procedures are capable of entrapping large quantities of water-soluble drugs (Vemuri and Rhodes 1995). Drug molecules can be entrapped in liposome vesicles by reverse-phase evaporation technique (Szoka and Papahadjopoulos 1978), ether injection technique (Deamer and Bangham 1976; Schieren et al 1978), and freeze-thaw method (Pick 1981), to name a few. These techniques yield large-unilamellar or multilamellar vesicles based on the selected method. Without an elaborate process scheme all the above methods produce heterogeneous mixture of liposomes. The original method of Bangham et al (1965) appears to be the simplest by comparison with the methods listed above. This method, however, has limited use because of its low encapsulation ability and impossibility to scale to larger batch sizes.

Table 1.2. Physical properties of conventional liposome formulations (adapted from Mayer et al 1986).

Vesicle Type	Preparation Method	Vesicle Diameter (μm)	Entrapped Agent	Entrapment Efiiciency (%)
SUV	Sonication	0.02-0.04	Cytosine, Arabinoside, Methotrexate, CF	1-5
SUV	French press	0.02-0.05	CF, Inulin, Trypsin, BSA	5-25
SUV	Detergent removal	0.036-0.05	CF, Inulin	12
LUV	Detergent removal	0.1-10.0	Inulin, Cytochrome C, CF	12-42
LUV	Reverse phase evaporation	0.2-1.0	CF, Cytosine, Arabinoside, 25s RNA, DNA, Insulin, Albumin	28-45
LUV	Solvent vaporization	0.1-0.5	Chromate, Glucose, DNA	2-45
LUV	Extrusion	0.056-0.2	^{22}Na, Inulin, Methotrexate, Cytosine, Arabinoside	15-60
MLV	Mechanical mixing	0.4-3.5	^{22}Na, CF, Glucose, Albumin, DNA	1-8.5
MLV	Sonication-freeze-thaw	0.17-0.26	Asparaginase	50-56
MLV	Freeze-thaw	0.5-5.0	^{22}Na, Inulin	35-88
MLV	Dehydration-rehydration	0.3-2.0	CF, Sucrose, Albumin, Factor VIII, ATP	27-54
MLV	Solvent-evaporation - sonication	0.3-2.0	Inulin, Streptomycin sulfate, Chloramphenicol oxytetracycline,	6.3-38

SUV: small unilamellar vesicle, **LUV**: large unilamellar vesicle, **MLV**: multilamellar vesicle
CF: carboxyfluorescein.

1.4. Classification of Liposomes

According to the number of lamellae, size, and preparation method, phospholipid vesicles can be classified into the following groups (Hope et al 1985; Mayer et al 1986; Lichtenberg and Barenholz 1988; New 1990; MacDonald 1991; Vemuri and Rhodes 1995; Chatterjee and Banerjee 2002; Mozafari et al 2002a, 2002c):

EIV: vesicles prepared by the ether injection method (ether injection vesicles; 20-200nm)

FATMLV: frozen and thawed multilamellar vesicles

FPV: vesicles prepared by the French press technique (French press vesicles; 50nm)

GUV: giant unilamellar vesicles (cell size vesicles with diameters > 1μm)

HM-liposomes: vesicles prepared by the heating method

LUV: large unilamellar vesicles (≥100nm)

LUVET: large unilamellar vesicles prepared by extrusion technique

MLV: multilamellar vesicles (0.1-10μm)

MLV-REV: multilamellar vesicles made by the reverse-phase method

MUL: medium-unilamellar vesicles (also called intermediate-sized unilamellar vesicles; > 100nm)

MVL: multivesicular liposomes (or multivesicular vesicles) consists of several small vesicles enclosed in one large phospho/lipid vesicle

OLV: oligolamellar vesicles

REV: single or oligolamellar vesicles made by reverse-phase evaporation

SPLV: stable plurilamellar vesicles

SUV: small unilamellar vesicles (20-50nm)

ULV: unilamellar vesicles (20-1000nm)

VET: vesicles prepared by extrusion technique.

When liposomes are described based on the number of bilayers they are termed unilamellar vesicles (ULV) or multilamellar vesicles (MLV). Descriptions based on the liposome size include small unilamellar vesicles (SUV), large

unilamellar vesicles (LUV), and giant unilamellar vesicles (GUV). Ether injection vesicles (EIV) and reverse-phase evaporation vesicles (REV) are examples of the descriptions based on method of preparation. The 'liquid crystalline vesicles' (liposomes) formed when dried lipids are suspended in excess of aqueous solution are multilamellar concentric vesicles, in which the lipid bilayers are separated by layers of aqueous medium. Small unilamellar vesicles are obtained generally from sonicated multilamellar vesicle suspensions. Electron micrographs of a freeze fractured multilamellar vesicle (MLV) and small unilamellar vesicles (SUV) are demonstrated in Figure 1.6. The periodicity of phospholipid bilayers in the structure of MLV, and the single bilayer nature of SUV are clearly evident in the photographs.

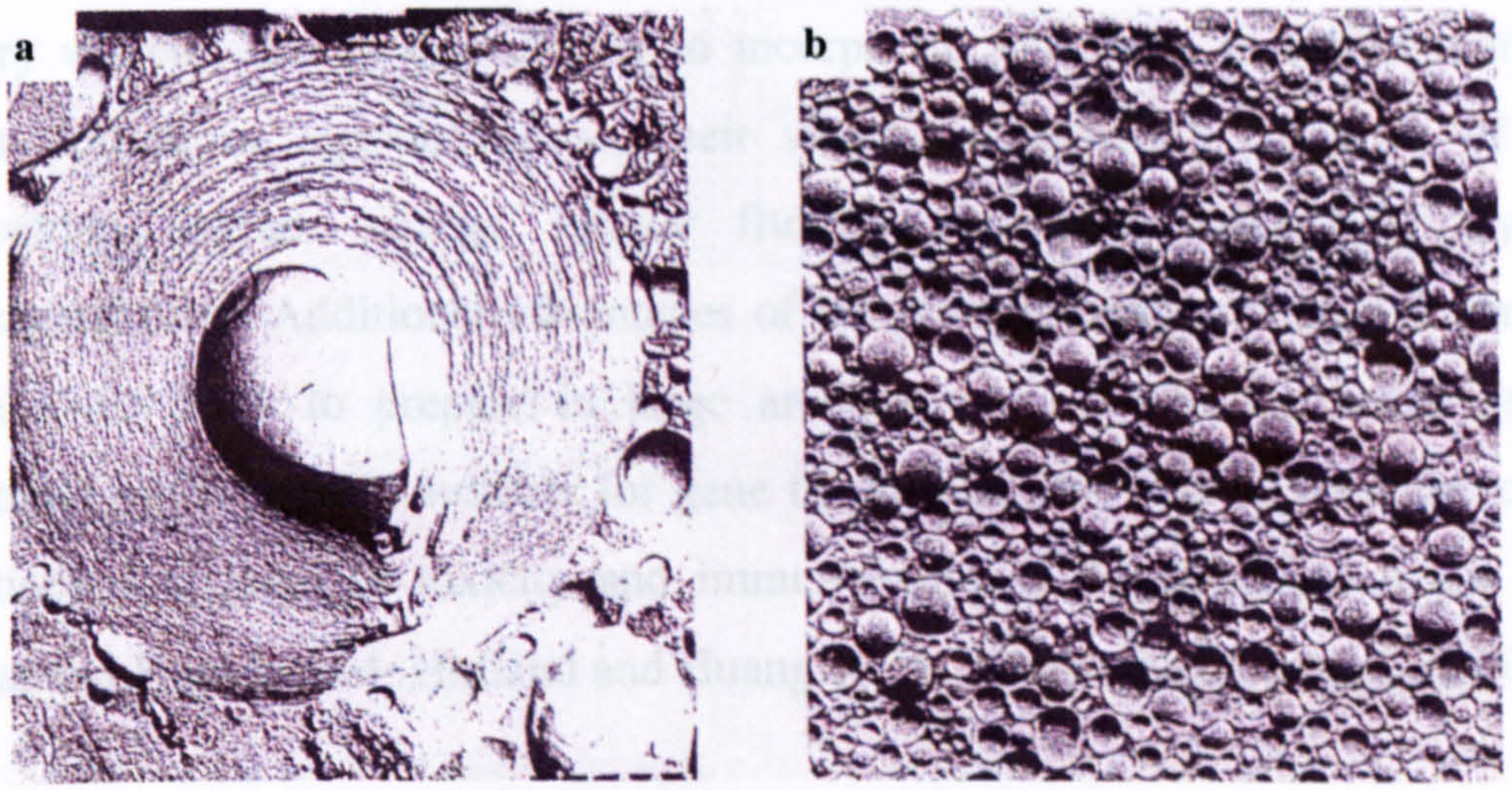


Figure 1.6. Electron micrographs of: a) MLV; b) SUV (original magnification ×140,000) (from Bangham 1992).

1.5. Applications of Lipid Vesicles

Liposomes are not a recent discovery, and there has been a spectacular progress in the field since Bangham's first publication on aqueous dispersions of liquid crystals of phospholipids which have permeability properties similar to those of biological membranes (Bangham et al 1965). Starting from the 1960's, liposomes became the most favourite model membrane system and provided the opportunity to

study the relation between structure and function of biological membranes such as permeability (Bangham et al 1965; Papahadjoupolos and Watkins 1967; Scarpa and De Gier 1971), transport phenomena via reconstituting membrane proteins into the bilayer (Bangham and Hill 1986; Anner et al 1988) and dynamics of membrane lipids (De Gier 1988). As a simplified model membrane system, liposomes are being used by many groups to study the formation and characteristics of the first living cells which emerged in the course of evolution (Kahveci et al 1994; Pozzi et al 1996; Oberholzer et al 1999; Monnard and Deamer 2001; Nomura et al 2001).

During the 1970's, considerable interest and excitement was caused by the proposal of the use of liposomes as a carrier to treat lysosomal storage disorders (Sessa and Weismann, 1970). It was soon realised that liposomes might be used as delivery vehicles due to their ability to incorporate molecules in either their lipid bilayer portion or aqueous phase, their structural versatility in terms of size, composition, surface charge, bilayer fluidity and their biodegradability and biocompatability. Additional advantages of the phospholipid vesicles are that they are relatively easy to prepare in large amounts and in pharmaceutical quality. Liposomes are especially suitable for gene transfer *in vitro* and *in vivo* due to their potentially low level of toxicity and immunogenicity (Sporlein and Koop 1991; Singhal and Huang 1994; Hofland and Huang 1995; Piperno-Neumann et al 2003).

Not only do liposomes serve as unique model membranes and nucleic acid delivery vehicles, but they have been also reported to be used as delivery systems of enzymes (Ohsawa et al 1985; Özden and Hasirci 1991), various drugs (Rahman et al 1980; Kumai et al 1985; Ostro and Cullis 1989; Shah and Misra 2004), hormones and blood factors (Weiner et al 1985; Kato et al 1993), antigens (Alving et al 1986; Gregoriadis et al 1987), and also in the fields of diagnosis (Gregoriadis et al 1974; Williams et al 1986), vaccine production (Gregoriadis 1990), cosmetics (Perrier and Redzianiak 1989; Muller et al 2002) and medical imaging (Torchilin 1996). In addition, liposomes can be effective carriers for nutritionally valuable ingredients (Keller 2001). Figure 1.7. shows the schematic representation of liposome application in delivery of antibiotics to a macrophage through endocytosis.

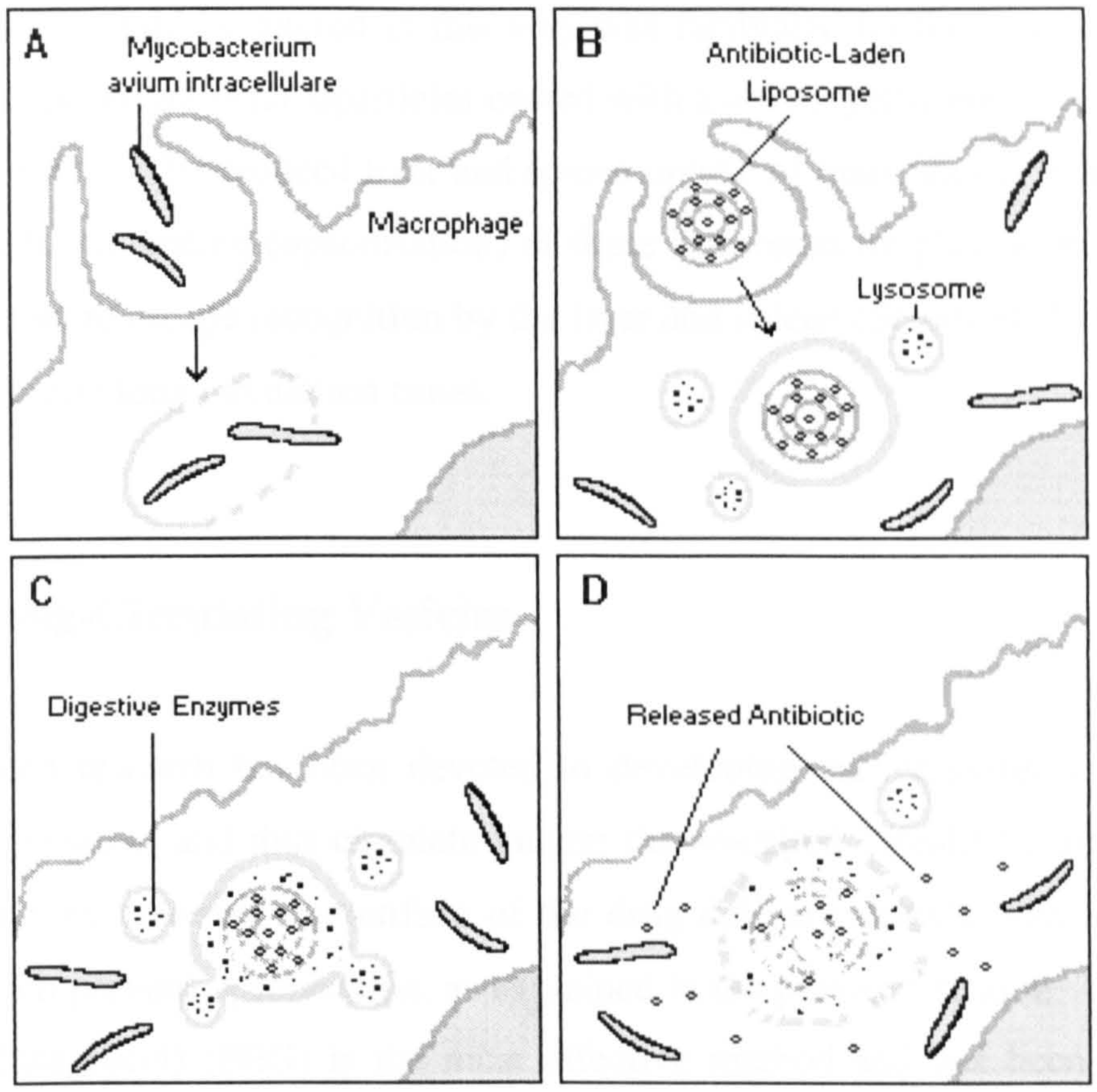


Figure 1.7. Uptake of antibiotic loaded multilamellar vesicle by a macrophage to enhance bacterial digestion (redrawn after Bangham 1992).

1.6. Fate of Liposomes *In Vivo*

The use of liposomes as drug delivery systems was originally hampered by the realisation that liposomes are rapidly cleared from the circulation and largely taken up by the liver macrophages (Gregoriadis and Ryman 1972). Macrophage uptake of liposomes by the liver and spleen (the reticuloendothelial system) affected their development as drug delivery systems for many years. However, some subsequent discoveries helped liposomes transfer from the bench to the clinic. One of them was the finding that the presence of liposome surface ligands, such as monosialoganglioside or polyoxyethylene, decreased liposome clearance by partially preventing liver and spleen uptake of intravenously injected liposomes (Klibanov et al 1990; Gabizon and Papahadjopoulos 1992). The realisation that liposomal

biodistribution could be altered in this way was facilitated by the results of similar studies with polystyrene nanoparticles coated with a polyoxyethylene polymer (Illum and Davis 1984). The reduced liver and spleen uptake of liposomes is believed to be due to a reduced coating (opsonisation) of these liposomes by plasma proteins, thus enabling them to escape recognition by the liver and spleen (Senior et al 1991; Allen 1994) and enjoy long circulation times.

1.6.1. Long-Circulating Vesicles

Much research has been devoted to developing carrier systems which can avoid phagocytosis and thus circulate longer: the so-called "Stealth" particles. This can be done by covering the surface of the drug delivery vehicle with hydrophilic chains which prevent opsonisation, as explained in the previous section. Grafting of poly(ethyleneglycol) (PEG) is the most effective method and has been applied to nanoparticles (Gref et al 1994) and liposomes (Woodle and Lasic 1992) to produce sterically stabilised vesicles. The sterically stabilised liposomes are involved in passive targeting (outlined below) of the drug they carry. When sterically stabilised liposomes are injected into an individual, who has either a solid tumour or an internal infection, the liposomes will migrate and aggregate in the tumorous or infected area. As the liposomes become degraded they will release their drugs into the surrounding area (Allen 1994). This is an example of passive targeting because the liposomes are left to their own devices and yet they migrate and treat the injured area. An important consideration when using sterically stabilized liposomes is the size of the coating polymer. If it is too large it may interfere with the ligand-receptor binding of the liposome and the target cell.

Other methods of extending liposome blood circulation times include the incorporation into liposomes of cholesterol (Kirby and Gregoriadis 1983), polyvinyl-pyrrolidone polyacrylamide lipids (Torchilin et al 1994), glucoronic acid lipids (Namba et al 1990) or phospholipids with high phase transition temperatures such as distearoyl phosphatidylcholine (Forssen et al 1992). Liposome size also affects biodistribution and a size of between 70 and 200nm is necessary to achieve

prolonged circulation times with stealth liposomes (Litzinger et al 1994; Harashima et al 1995, 1996).

1.7. Modes of Liposomal Drug Delivery

Liposomal drug delivery systems have been considered as 'magic bullets' for more than three decades. The engineered versions of liposomes offer potential for exquisite levels of specificity and drug targetability which are very important characteristics of the lipid vesicles. Liposomes can be employed for the delivery of drugs to selected organs and tissues by two main mechanisms known as 'passive' and 'active' targeting as described in the following sections.

1.7.1. Passive Targeting

Passive targeting uses the natural course followed by the liposomes upon injection into the circulatory system as the method of delivering the drug. Liposomes with a mean diameter of 100nm for example, can selectively extravasate in tissues characterised by leaky vasculature such as solid tumors (Hobbs et al 1998; Hashizume et al 2000; McDonald and Baluk 2002), thus exhibiting target specificity with negligible adverse effects to normal tissues (Figure 1.8.). Liposomes with larger diameters (generally ≥1μm) are taken up by the reticuloendothelial system (RES) in a passive manner. This is very useful in the targeting of various diseases associated with the RES such as candiasis, leishmaniasis and listeria. In these diseases the macrophages of the infected individual play a role in the disease, therefore if the macrophages are destroyed then will be the disease as well (Daemen et al 1995; Davis 1997). The liposome, once engulfed by the macrophage, will be degraded and the drug will be released within the macrophage. Therefore the drug will be delivered directly to the target area (see Figure 1.7.). In this scenario the liposome is allowed to follow its natural course yet at the same time it is able to deliver the drug passively without any interference to its target. In an *in vivo* experiment, using hamsters and passively targeted liposomes, it has been observed

that the treatment of leishmaniasis with the liposomal encapsulated drug is 1000 times more effective than with the free form of the drug (Lasic 1993).

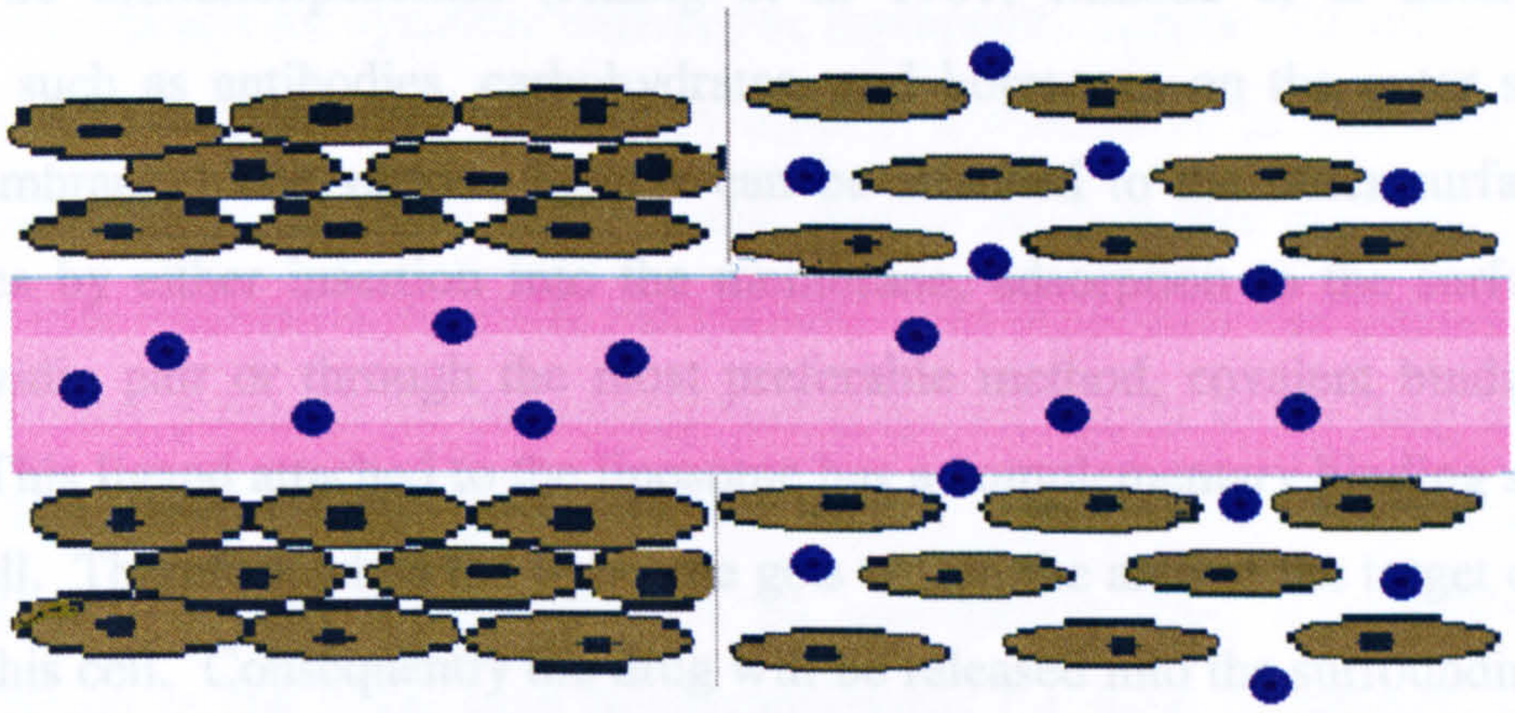


Figure 1.8. Accumulation of liposomes within solid tumours - (right) liposome extravasation from the disorganised tumour vasculature and (left) liposomes in normal tissue.

1.7.2. Active Targeting

Active transport, in contrast to passive transport, involves the directed movement of the lipid vesicle to the given organ, tissue or cell before release of the drug occurs. Active transport generally ensures that a greater amount of the drug is delivered to the target cells. This can be achieved via appropriately engineered modifications to the liposomal structure. For active targeting of liposomes, thermolabile (Tynell et al 1976, Weinstein et al 1979), pH sensitive (Connor et al 1984, Liu and Huang 1990), photo sensitive (Gürsel and Hasirci 1995), and antibody-coated vesicles (Sunamoto et al 1987) have been designed. Antibodies, lectins and sugar residues are examples of surface-coating groups which could be specifically recognised by cell-surface structures. It should be noted however that targeted carriers encounter the same biochemical and anatomical constraints as other carriers do (Allen and Moase 1996). There are two main types of liposomes used in active transport, namely immunoliposomes and virosomes.

1.7.2.1. Immunoliposomes

The immunoliposomes (Huang et al 1981; Mizoue et al 2002) possess moieties such as antibodies, carbohydrates, and hormones on the outer surface of their membrane. The various ligands can be attached to the outer surface of the liposomes by either insertion into the membrane, adsorption to the surface, via a biotin avidin pair or through the most preferable method, covalent binding (Lasic 1993). This ligand attached to the liposome has a complementary binding site on the target cell. Therefore when the liposome gets within the area of the target cell it will bind to this cell. Consequently the drug will be released into the surrounding region. In a recent study, immunoliposomes have been used for gene targeting to human brain cancer cells, which has resulted in a 70-80% inhibition in cancer cell growth (Zhang et al 2002).

1.7.2.2. Virosomes

Virosomes (Kara et al 1971; Almeida et al 1975), or artificial viruses, are another type of liposome used in active targeting and contain reconstituted viral proteins. Viruses have developed the ability to fuse with cells thus allowing for release of their contents directly into the cell. This is due to the presence of fusogenic proteins on the viral surface that facilitate this fusion. If these fusogenic viral proteins are reconstituted on the surface of a liposome then the liposome also acquires the ability to fuse with cells. This is an incredibly useful tool in active transport because it allows the direct release of the liposomal contents into the cell. As there is no diffusion of the drug involved it results in a more effective delivery. The most common viruses used in the construction of virosomes are the Sendai, Semliki Forest, influenza, herpes simplex, and vesicular stomatites viruses. The presence of a virus not only allows the liposome to target a particular cell but also allows it to fuse with the cell ensuring direct delivery of the drug (Lasic 1993).

1.8. Liposomal Drug Delivery to the Lungs

Growing attention is being given to the potential of the pulmonary route as a non-invasive means for systemic delivery of therapeutic agents (Yu and Chien 1997; Newman 2001). This route is a highly attractive alternative to injection for many drugs, including nucleic acid based drugs, due to the fact that the lung provides a huge but thin absorptive mucosal membrane. Not only does the pulmonary route offer enormous potential for delivering biotherapeutic agents to the lung itself but to other body organs as well. The lung is a unique organ which can be accessed for drug delivery by direct inhalation of an appropriate drug formulation. Drug administration to the lungs may be advantageous for local treatments of diseases such as asthma or other related respiratory distress syndromes, or for systemic delivery of bioactive materials such as peptides and nucleic acids that are unstable in the gastrointestinal tract. The advantage of topical drug administration to the lung is the potential of delivering an adequate drug dose to the target site with reduced undesirable extrapulmonary side effects.

A method that is commonly used in hospitals for delivery of drugs or surfactants to premature babies, is through a tube directly to the lower regions of the lungs (Figure 1.9.). These babies require administration of a surfactant and also treatment with oxygen and mechanical ventilation. About 20-60% of premature babies develop chronic lung disease (Ivey et al 1977). The development of this condition has been related to an acute inflammatory response to oxygen-derived free radical generation (Juliano and McCullough 1980). In addition, the premature babies, as well as cystic fibrotic patients (Wagner and Gardner 1997), are deficient in an endogenous antioxidant glutathione. Inhaled or directly administered glutathione is rapidly cleared from the lungs. However, incorporation of glutathione in a controlled drug delivery system, such as liposomes, offers a method of prolonging the glutathione levels in the lungs (Kellaway and Farr 1990). Therefore, it is vital to develop and evaluate liposomal delivery systems that are suitable for lung administration of glutathione to patients with low pulmonary glutathione levels.

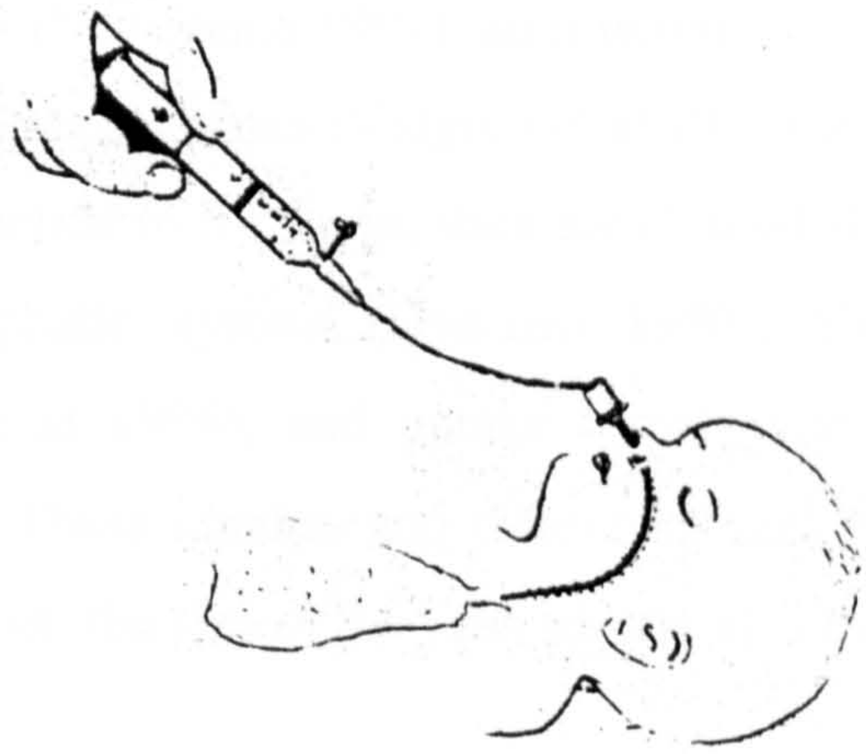


Figure 1.9. Delivery of lung surfactants (with or without liposomes) to premature babies.

There are many distinct advantages of liposomes including biocompatibility, targetability, ideal specific gravity and the possibility of producing them in different size ranges which makes them particularly useful for drug administration to the lung. The attractiveness of using liposomes as a pulmonary drug delivery system also stems from the fact that phospholipids are naturally occurring components of lung surfactant and, therefore, should not pose a toxicological risk to this organ. The earliest reports of liposomal administration to the respiratory tract concerned the potential replacement of pulmonary surfactant in the treatment of respiratory distress syndrome in new born babies (Ivey et al 1977). The advantages of liposomes for drug delivery to the lungs have also been related to their ability to contain the drug within the local regions of the lungs, thus reducing the extrapulmonary side-effects (Juliano and McCullough 1980), and also their ability to release the drug at a slower more controlled rate (Shek et al 1994). In addition, it is possible to manufacture liposomes to deliver drugs via the lungs for systemic activity (Boswell et al 1998). However, the main advantage of using liposomes is the fact that they are made of endogenous materials which are safe (Kellaway and Farr 1990) and allow flexibility in the formulation and design of the final product (New 1990).

Liposomal delivery systems have been used to deliver various types of drugs to the lungs. These include: peptides (Griffiths et al 1998), antiasthmatics (Saari

1998), antitumor agents (Maruyama 1997), antimicrobial agents (Gangadharam et al 1995), markers for imaging purposes (Vidgren et al 1995) and genes (Ma et al 2002). When liposomes are applied to the lungs, they are cleared slowly by the mucociliary process, via the lymphatic system (Oyarzun 1980), phagocytosis by alveolar macrophages (Stern et al 1986), and uptake by alveolar type II epithelial cells (Hallman et al 1981). These uptakes and clearances may be modified by the lipid composition of liposomes, the size of the vesicles and also their surface properties.

The region where the inhaled particles deposit generally depends on their size and density. Deposition in the lower regions of the lungs, bronchioles and alveoli, requires an aerodynamic particle (vesicle) diameter of 5-6μm (Stahlhofen 1980). Therefore formulation of liposome delivery system for lung delivery requires careful particle size characterisation. Methods of particle size determination of liposomes are explained in chapter 2 of this thesis.

1.8.1. Methods of Administration

A major factor to consider in the delivery of liposomal products to the lung is their method of administration. Drugs are delivered to the lungs via compressed metered dose inhalers (MDI), nebulizers or dry powder inhalers. The MDI is not suitable for liposome delivery because the solvents that are used as aerosol propellant would damage the liposomes. Moreover, when using an MDI, only a small amount of the carrier system can be delivered per actuation. Nebulization has been the simplest means to deliver liposomes to the respiratory tract (Saari 1998). Dry powder inhalers are becoming very popular and numerous workers have reported the development of the new improved devices and designs (Taylor and Farr 1993). However, not much attention has been given to dry powder inhalers for liposomal delivery. Before any liposomal formulation could be utilised for pulmonary drug delivery some critical points should be addressed: toxicity of the liposomal formulation, stability of the liposomes, drug entrapment efficiency and size variation of the formulation (Mozafari et al 2002b).

1.9. Aims of this research work

The primary aim of this thesis is to formulate and characterise a liposomal system appropriate for gene and drug delivery to the lung. A first task was to develop a methodology for manufacture of liposomes without employing toxic agents such as the organic solvents generally used to prepare liposomes, and without using any hazardous device such as probe sonication. Since one of the disadvantages of liposomal formulations is their production cost it was intended to minimise the number of steps and required chemicals and instruments in the developed methodology. Further objectives of this thesis have been:

- to prepare liposomes of different sizes utilising both conventional and the new technique developed in our laboratory;

- to study the morphology, size range, polydispersity, thermal behaviour and stability of liposomes prepared by the new technique;

- to assess the cytotoxicity of the new liposomal formulation with and without DNA using human bronchial epithelial cell line (16HBE14o) as a relevant pulmonary model in comparison with the cytotoxicity of liposomes prepared by the conventional methods;

- to measure drug entrapment efficiency and release characteristics of the produced lipid vesicles using the anticancer agent 5-fluorouracil (5FU) as a water-soluble model drug;

- to evaluate the cytotoxicity of 5FU as free (un-encapsulated) drug as well as encapsulated in the liposomes prepared by the new methodology *in vitro* using HBE cell culture;

- to measure drug entrapment efficiency and release characteristics of the new liposomal formulation using glutathione (GSH) as an antioxidant drug;

- to construct a gene transfer vector employing the new liposomal formulation and measure its DNA entrapment efficiency as well as gene transfer efficiency using HBE cells.

2: A NEW TECHNIQUE FOR PREPARATION OF NON-TOXIC LIPOSOMES: THE HEATING METHOD

2.1. Introduction

In recent years liposomes have attracted great interest as ideal models for biological membranes as well as carriers for drugs, diagnostics and vaccines (Lasic and Papahadjopoulos 1998). While the use of liposomes as models for biomembranes is confined to the research laboratory, their successful application in drug delivery and/or genetic engineering will depend not only on a demonstration of the superiority of the liposome carrier for the intended purpose, but also upon technical and economic feasibility of the system in practice. For drug delivery applications liposomal formulations should have high drug-trapping efficiencies, narrow size distribution and the potential to include in their structure a wide range of lipid molecules, especially lipids that promote liposome stability. In addition to the above properties, for nucleic acid delivery, liposomes should also be able to protect the DNA from degradation by nucleases (Chonn and Cullis 1998). Despite the enormous effort in research and development on liposomes as drug carriers, only a small number of liposomal products have arrived on the market so far. This may be due to various reasons including: toxicity of some liposomal formulations, poor loading of drug compounds into liposomes, instability of the liposomal drug carriers, and high cost of liposomal products.

There are numerous lab-scale and a few large-scale techniques for liposome preparation (e.g. see: Gregoriadis 1984, 1993; Lichtenberg and Barenholz 1988; New 1990; Basu and Basu 2002), some of which are mentioned in chapter one of this thesis. However, most of these techniques are not suitable for the encapsulation of sensitive substances because of their exposure either to mechanical stresses

(sonication, high-shear homogenisation, or high pressures) or potentially harmful chemicals (volatile organic solvents, detergents or low/high values of pH) during the preparation. The majority of liposome preparation techniques involve the application of volatile organic solvents (mainly chloroform, ether, or methanol), as a first step, to dissolve or solubilise the lipids. These solvents not only affect the chemical structure of the entrapped substance but will also remain in the final liposome formulation and contribute to toxicity and influence the stability of the vesicles (Deamer and Uster 1983; Vemuri and Rhodes 1995; Cortesi et al 1999). In general, residual solvents in pharmaceuticals, known as organic volatile impurities (OVIs), have no therapeutic benefits but may be hazardous to human health as well as the environment (Dwivedi 2002). In addition to the above mentioned disadvantages, application of volatile organic solvents or detergents necessitates performance of two additional steps in the liposome preparation: *i*) removal of these solvents/detergents, and *ii*) assessment of the level of residual organic solvents or detergents in the liposomal formulations (Barenholz and Amselem 1993; Vemuri and Rhodes 1995; Van Winden et al 1998b). Hence avoiding the utilisation of these solvents will potentially bring down the time and cost of liposome preparation.

Several techniques have been suggested for the removal of detergent and solvent traces from liposomes which include gel filtration, vacuum and dialysis. It has been reported that even after removal of ether residues, by gel filtration, from liposomes prepared by the *reverse phase evaporation method* trace amounts of ether still remained in the formulation and this was responsible for REVs (vesicles made by reverse-phase evaporation method) being more leaky to entrapped solutes when compared with liposomes prepared in the absence of ether (Allen 1984). Additionally, Weder and Zumbuehl (1984) have reported, for liposomes prepared by the *detergent dialysis method*, that after dialysis 3 to 4% residual detergent was still present in the final preparation. Therefore, a method which would produce lipid vesicles avoiding the above-mentioned shortcomings will be very useful in gene and drug delivery applications.

An important point in the development of a liposomal dosage form and evaluation of a suitable preparation process is whether it is possible to prepare, isolate and characterise the particular liposomal system on an industrial scale with clearly defined and reproducible properties. Another crucial point in this regard is the stability of the produced lipid vesicles (Brandl et al 1993). In order to prepare liposomes on a large scale for clinical applications, it is necessary to employ techniques which will meet the requirements of the pharmaceutical industry. One of the most important steps in the manufacturing of liposomes is sterilisation. It has been argued that liposomes can only be sterilised by filtration and that any other method involving chemical or physical treatment, especially heating, would destroy the liposomal structure and consequently release the encapsulated drug (Friese 1984). However, most viruses can not be removed by filtration, so that intensive microbiological control is necessary, and furthermore, the filtering process is so time-consuming that it is preferably avoided when dealing with large batches of the liposomal dispersions. A promising breakthrough regarding liposome sterilisation came with the work of Kikuchi and coworkers (1991). This group reported that it is possible to apply an ordinary heat sterilisation (121°C, 20min) and obtain liposomes which retain their structural integrity with high encapsulation efficiency after the heat treatment.

In this chapter a new method for the production of liposomes without the use of any hazardous chemical or process is described. This method involves the hydration of the liposome components in an aqueous medium followed by the heating of these components, in the presence of glycerol, up to *c.* 120°C. Glycerol is a water-soluble and physiologically acceptable chemical with the ability to increase the stability of the lipid vesicles (Kikuchi et al 1994) and does not need to be removed from the final liposomal product. Since heating is the main step in this methodology it is termed the `*Heating Method*` and the resultant liposomes are referred to as *HM-liposomes* (Mozafari et al 2001, 2002a, 2002b, 2002c). Employment of heat abolishes the need to carry out any further sterilisation procedure hence reducing the time and cost of liposome production by the heating method. Moreover, HM-liposomes possess the above-mentioned required

characteristics to be employed for both drug and nucleic acid delivery applications in addition to model membrane systems. A negatively charged, cholesterol containing, lipid composition was utilised throughout the present study. The rationale for employing this composition is provided in the next section.

2.1.1. Chemical Composition of the HM-liposomes

Physical and chemical stability as well as drug incorporation and leakage of liposomes are very much affected by their chemical composition (Lichtenberg and Barenholz 1988; New 1990). Chemical composition has also been postulated to be responsible for toxicity behaviour of some liposome formulations (Campbell 1983; Filion and Phillips 1997, 1998; Dokka et al 2000; Nagahiro et al 2000). In the preparation of HM-liposomes, no hazardous chemical or process was involved. The main component of the HM-liposomes is DPPC (see Table 1.1.), which naturally occurs as one of the main phospholipids in human lung surfactant (Banerjee and Bellare 2001). DPPC has also been detected to significantly reduce the toxicity of cationic liposomes towards macrophage cells *in vitro* (Filion and Phillips 1997). The other phospholipid constituent of the liposomes is DCP which was used to confer negative charges to the lipid vesicles. Due to several reports on the toxicity of cationic lipid vesicles (Campbell 1983; Filion and Phillips 1997, 1998; Dokka et al 2000; Nagahiro et al 2000) anionic liposomes were utilised in this study. It has been postulated that negatively charged liposomes, in general, associate more effectively and are taken up more readily by the cells compared with neutral liposomes (Heath et al 1985; Monkkonen et al 1994) and both neutral and cationic liposomes (Katragadda et al 2000). Application of DCP in the structure of liposomes has also been reported to stabilise the prepared vesicles possibly by interfering with aggregation/fusion of the vesicles (Talsma et al 1994). Cholesterol was used, in our formulation, to increase the stability of the liposomes by modulating the fluidity of the lipid bilayer. Stabilization of liposomes by cholesterol in general (New 1990; Mozafari and Hasirci 1998), and in serum/blood in particular (Mayhew et al 1979; Ropert et al 1996; Hong et al 1997), is a well known phenomena. Glycerol, which was used in the preparation

of HM-liposomes, is a water-soluble and physiologically acceptable chemical, and increases the stability of the lipid vesicles (Kikuchi et al 1994). The long term stability of the HM-liposome formulation in this study is therefore believed to be due to the stabilising characteristics of the ingredients.

Since a good amount of knowledge has been gathered in our previous studies regarding the liposomes with 7:2:1 molar ratio of PC (egg phosphatidylcholine):DCP:CHOL (Mozafari 1996; Zareie et al 1997; Mozafari and Hasirci 1998; Mozafari et al 1998a, 1998b) the same molar ratio was used during the works of this thesis. The only exception is that instead of PC a more homogeneous phospholipid (i.e. DPPC) was used in the HM-liposome formulation. Nevertheless, utilisation of DPPC, as a natural component of human lung, in the liposomal formulation would be more appropriate for pulmonary drug delivery applications. The other advantage of employing DPPC as a major liposome component is its transition temperature (T_C = 41°C) which is higher than that of egg phosphatidylcholine. This will cause the DPPC vesicles to be in gel phase, sufficiently stable and non-leaky (in respect to the entrapped material) at storage temperatures while PC vesicles will be in liquid crystalline phase. Liposomes, such as DPPC vesicles, with a high transition temperature can be utilised in targeted drug delivery to tumours as explained in chapter one (section 1.2.2.). Alternatively, localised drug delivery through this type of liposomes can be achieved by employing ultrasonic hyperthermia (Tacker and Anderson 1982; Pitt 2003).

2.1.2. Characterisation of the Liposomes

Following preparation of liposomes, especially when using a new technique, characterisation is required to ensure adequate quality of the product. Methods of characterisation have to be meaningful and preferably rapid. Of particular importance will be the size and size distribution of the formulated liposomes (Rao 1984; Goren et al 1990). Maintaining a constant size and/or size distribution for a prolonged period of time is an indication of liposome stability. Electron microscopic

methods are widely used in research work for establishing the morphology, size and stability of liposomes. With respect to a statistically meaningful analysis of size distribution of the lipid vesicles, methods such as light scattering, which measure the size of large number of vesicles in an aqueous medium, are more appropriate than microscopic techniques. Ideally these two techniques need to be employed along with other inexpensive and routine laboratory techniques, such as gel permeation chromatography, to provide a comprehensive and reliable characterisation of the liposomal formulations.

The characteristics and stability of liposomes prepared by the heating method, employing the aforementioned lipid components, are studied using various techniques and results are presented in this chapter.

2.2. Materials and Methods

2.2.1. Chemicals

Dipalmitoylphosphatidylcholine (DL-α-phosphatidylcholine, Dipalmitoyl, C=16:0, DPPC), dicetylphosphate (dihexadecylphosphate, DCP), Cholesterol (5-Cholesten-3β-ol, CHOL), glycerol and Sephadex G-50 were purchased from Sigma Chemical Co (Dorset, UK). Osmium tetraoxide (OsO_4) was purchased from Agar Scientific Ltd, Essex, UK. All solvents (chloroform, methanol, diethyl ether, cyclohexane, Analar grade) were obtained from Sigma Chemical Co (Dorset, UK). All other chemicals were of commercial analytical grade. Purity of the lipids (DPPC, DCP, and CHOL) was further confirmed by thin layer chromatography as explained in section 2.2.2.2.

Phosphate-buffered saline (PBS) was prepared by dissolving 4.3mM NaH_2PO_4, 1.47mM KH_2PO_4, 2.68mM KCl, and 137mM NaCl in 1l distilled water and adjusting the pH to 7.4.

2.2.2. Experimental Methods

2.2.2.1. Liposome Preparation

2.2.2.1.1. Preparation of conventional liposomes

Multilamellar vesicles (MLV) were prepared by the thin-film hydration method similar to the conventional technique of Bangham et al (1965). For this, a 3ml chloroform:methanol (2:1, v/v) solution of DPPC/DCP/CHOL (7:2:1 molar ratio), containing 10mM of total lipid, was evaporated to dryness in a round-bottomed flask using a rotary evaporator (EYELA, New Rotary Vacuum Evaporator NE-1, Birmingham, UK), in a water bath at 50°C. To remove traces of the solvents the flask was flushed with nitrogen gas for 1 hour and also placed under vacuum for a time period of at least 1 hour. The lipid film was then hydrated with 2ml sterile phosphate buffered saline (PBS, pH: 7.4), well above the phase transition temperature (T_C) of the main lipid component (i.e. DPPC, T_C = 41°C) at 50°C. Multilamellar vesicles (MLV) were formed either by hand-shaking the flask after adding glass beads of ~1-2mm diameter into the flask or by vortex agitation.

To obtain vesicles with smaller diameters the MLV suspension was extruded 11 times, with an extruder (LiposoFast™-Basic, Glen Creston Ltd, UK), above T_C through two-stacked polycarbonate filters of either 100 or 400nm pore size as described by MacDonald et al (1991). An odd number of passages through filters was performed to avoid contamination of the sample with the particles (e.g. large vesicles or unorganised lipid aggregates) which had not passed through the filter membranes. The liposomal suspension was left at room temperature for 1 hour under N_2 to allow the stabilisation of the lipid vesicles. The liposomes were either prepared immediately prior to use or stored under N_2 atmosphere at 2-8°C until use. Figure 2.1. demonstrates schematically the preparation of liposomes by the above mentioned conventional technique.

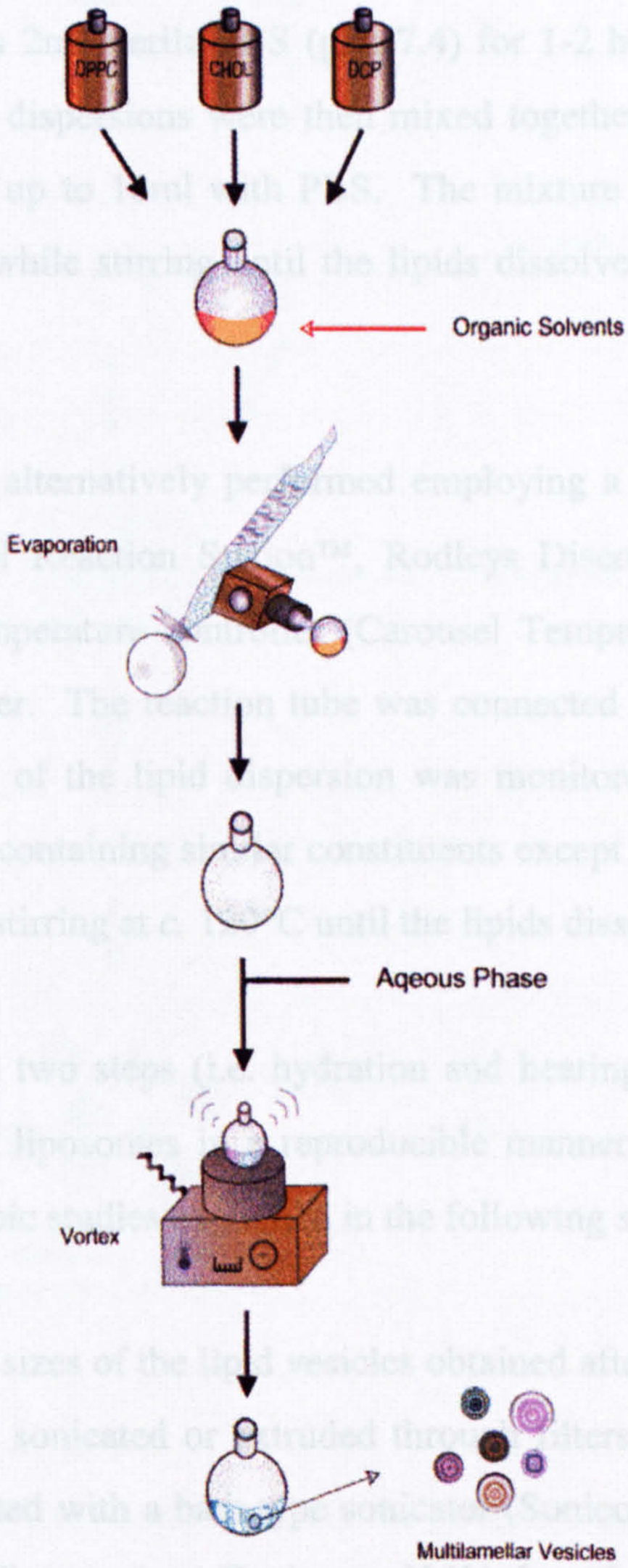


Figure 2.1. Preparation of liposomes by the conventional technique.

2.2.2.1.2. Preparation of liposomes by the heating method

HM-liposomes composed of DPPC/DCP/CHOL (7:2:1 molar ratio), containing 10mM of total lipid, were prepared as follows: the lipids DPPC, DCP, and CHOL, were hydrated each in 2ml sterile PBS (pH: 7.4) for 1-2 hours under N_2 at room temperature. The lipid dispersions were then mixed together, 0.3ml glycerol added, and the volume made up to 10ml with PBS. The mixture was heated to *c.* 120°C in a silicone oil bath while stirring until the lipids dissolved. Typically the heating step took up to 30min.

The heating step was alternatively performed employing a 12-well carousel reaction instrument (Carousel Reaction Station™, Rodleys Discovery Tech. Ltd., Essex, UK) fitted with a temperature controller (Carousel Temperature Controller RR98073) and magnetic stirrer. The reaction tube was connected to a nitrogen gas cylinder and the temperature of the lipid dispersion was monitored by inserting a thermometer into a next tube containing similar constituents except the lipids. Again the reaction proceeded while stirring at *c.* 120°C until the lipids dissolved.

The above mentioned two steps (i.e. hydration and heating) were sufficient for successful preparation of liposomes in a reproducible manner as confirmed by light scattering and microscopic studies explained in the following sections.

In order to reduce the sizes of the lipid vesicles obtained after the heating step the vesicles were either bath sonicated or extruded through filters as follows. The MLV suspension was sonicated with a bath type sonicator (Sonicor, Model Number SC-52, Sonicor Instrument Corporation, Copiague, N.Y.) for 10min, to provide a more homogeneous population of liposomes with smaller diameters. The liposomal suspension was alternatively extruded, as described in the previous section, through two-stacked polycarbonate filters of either 100 or 400nm pore size. The HM-liposomes were either prepared immediately or stored under N_2 atmosphere at 2-8°C until use. A schematic representation of liposome preparation by the heating method is provided in Figure 2.2.

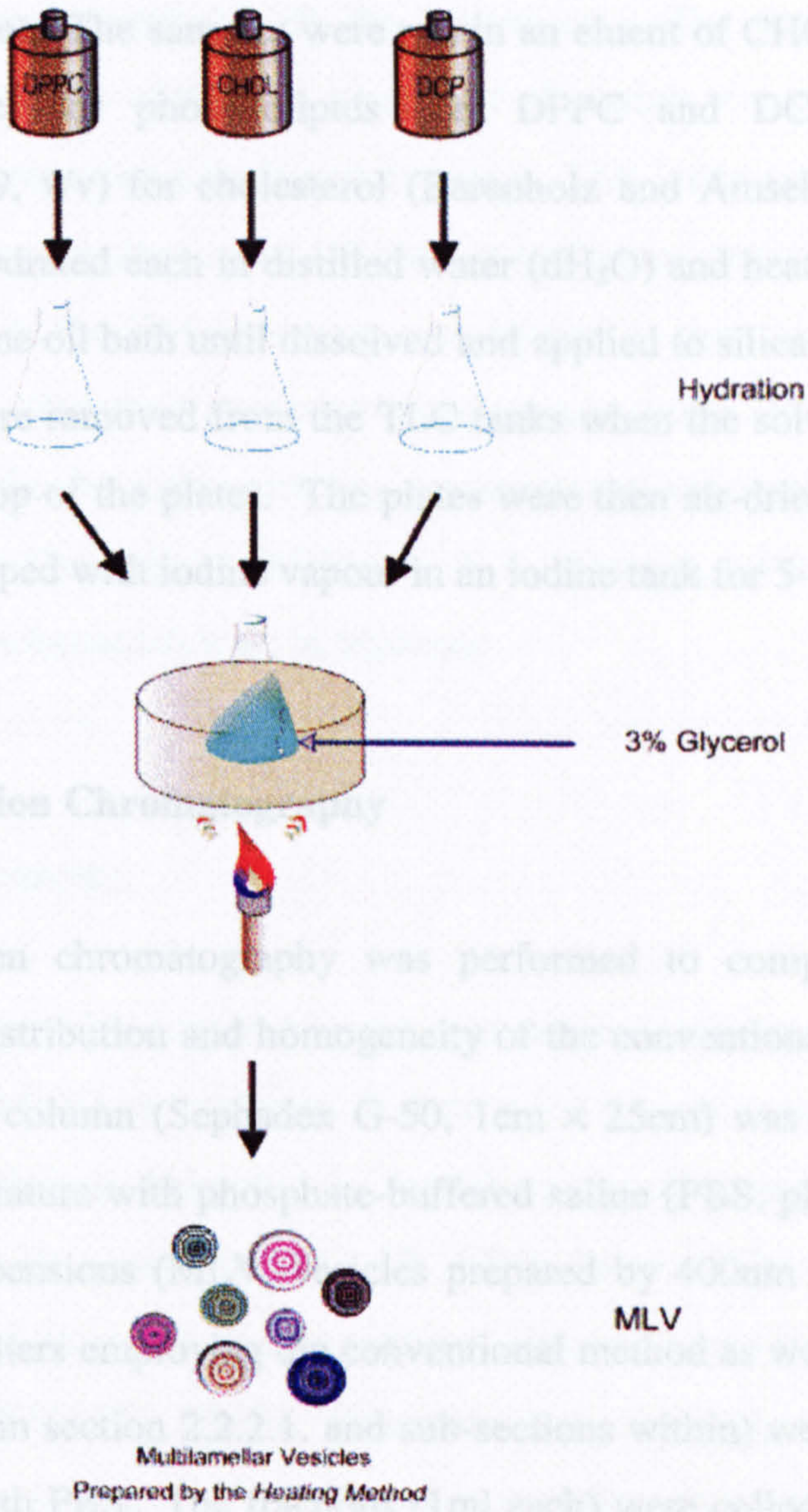


Figure 2.2. Representative liposome preparation by the heating method.

2.2.2.2. Thin Layer Chromatography

The effect of heating on the lipids was checked by thin layer chromatography (TLC) utilising un-heated lipids as a control for comparison. Samples of DPPC,

DCP, and CHOL (10µl each, 20mg/ml, dissolved in chloroform:methanol, 2:1 v/v) were spotted onto 0.25mm layers of silica gel on 20×20cm plates (2.5cm from the lower edge of the plate). The samples were run in an eluent of $CHCl_3$:CH_3OH:H_2O (65:25:4, by volume) for phospholipids (i.e. DPPC and DCP) and diethyl ether:cyclohexane (1:9, v/v) for cholesterol (Barenholz and Amselem 1993). The same samples were hydrated each in distilled water (dH_2O) and heated, individually, to *c.* 120°C in a silicone oil bath until dissolved and applied to silica gel as explained above. The plates were removed from the TLC tanks when the solvent ascended to within 3-4cm of the top of the plates. The plates were then air-dried for 15min in a fume hood and developed with iodine vapour in an iodine tank for 5-10min.

2.2.2.3. Gel Permeation Chromatography

Gel permeation chromatography was performed to compare the elution characteristics, size distribution and homogeneity of the conventional liposomes and HM-liposomes. The column (Sephadex G-50, 1cm × 25cm) was equilibrated and eluted at room temperature with phosphate-buffered saline (PBS, pH: 7.4). Two ml of the liposomal suspensions (MLV, vesicles prepared by 400nm filters and those prepared by 100nm filters employing the conventional method as well as the Heating Method as explained in section 2.2.2.1. and sub-sections within) were applied to the column and eluted with PBS. The fractions (1ml each) were collected by a fraction collector (Frac-100, Pharmacia, Sweden) with a flow rate of 20ml/hr. The absorbance of the eluents was detected at 410nm using a double-beam UV/VIS Spectrometer (Perkin Elmer, Lambda 40, USA).

2.2.2.4. Light Scattering

The mean particle size and polydispersity index of the conventional liposomes and HM-liposomes were determined by dynamic light scattering at *c.* 25°C with a Brookhaven ZetaPlus Particle Sizing instrument (BI-MAS Option,

Brookhaven Instruments Corporation, Holtsville, New York, USA), at the wavelength of 677nm, using a 15mW solid state laser as the light source and the MAS OPTION software supplied with the instrument. The scattered light was detected at a scattering angle of 90°. As a measure of particle size distribution of the dispersion, the system reports a polydispersity index. This index ranges from 0.0 for an entirely monodisperse system up to 1.0 for a polydisperse particle dispersion. In addition, the stability of the HM-liposomes was studied at time zero and also two, four and eight months after their preparation and storage under N_2 atmosphere at 2-8°C. Liposomal samples for light scattering measurements were prepared in filtered distilled H_2O using 0.22μm filters (Millipore, S.A., France). The light scattering measurements were performed at least in triplicate.

2.2.2.5. Optical Microscopy

Multilamellar vesicles prepared by the conventional technique (MLV) and the heating method (HM-MLV), as explained in section 2.2.2.1., were examined using an optical microscope (Nikon Labophot-2, Japan), with oil immersion at 1000× magnification fitted with a camera (JVC, TK 1270, Japan). The images from the camera were captured to a PC using a Ulead Videostudio 3.0 SEDV software package (USA) as BITMAPS (.BMP). Repeatability of the images was ascertained by taking three or more pictures from the same sample; representative figures are presented.

2.2.2.6. Nikon Eclipse Microscopy

Five μl of 1% (w/v) OsO_4 was added to 2ml of the liposomal suspension prepared by the heating method employing 400nm polycarbonate filters. The suspension was hand shaken and then centrifuged at 4°C, 100000g, for 60min in a Beckman L-80 refrigerated ultracentrifuge (USA). The supernatant was discarded and the pellet was suspended in 2ml PBS (pH: 7.4). Liposomes were visualised by a

Nikon Eclipse E600 microscope (Tokyo, Japan), with 1000× magnification and standard brightfield illumination, using a Spot RT Slider camera and Spot software (Diagnostic instruments, Michigan, USA) which enabled further magnification of the images.

2.2.2.7. Transmission Electron Microscopy

Liposomes prepared by using 100nm polycarbonate filters were studied under transmission electron microscope (TEM). For this the standard osmic acid method of Lewis and Knight (Lewis and Knight 1972) was employed. Liposomes were stained with 1% OsO_4, as explained in the previous section, and embedded in Taab TO28 epoxy resin (Taab Embedding Materials, UK). The resin was polymerised in an oven at 60°C for 24h and ultra-thin pale gold sections (50-90nm) were obtained on a Reichet-Jung ultramicrotome (Wien, Austria) with triangular knives prepared on an LKB knife maker using a 5×25mm plate glass knife.

The sections were examined using a Jeol JEM-1200 EX transmission electron microscope (Japan) operating at 80.0 kV. The TEM has its calibration checked every six months using a standard graticule of 2160 lines/mm. The diameter of liposomes was determined by a Digimatic caliper (Mitutoyo Ltd. England) using the scale bar on the electron micrographs. Reproducibility of the images was ascertained by taking three or more pictures from the same sample; representative TEM figures are presented.

2.2.2.8. Scanning Tunnelling Microscopy

The scanning tunnelling microscopy (STM) used in this study was constructed by Zareie (Zareie 1995) and used successfully for imaging of several biopolymers as well as liposomal preparations (Zareie et al 1996a, 1996b, 1997, 1998; Mozafari et al 1998a, 1998b, 2002c). Structural details of this STM is

provided in Appendix 1. A simplified structure of a scanning tunnelling microscopy is depicted in Figure 2.3.

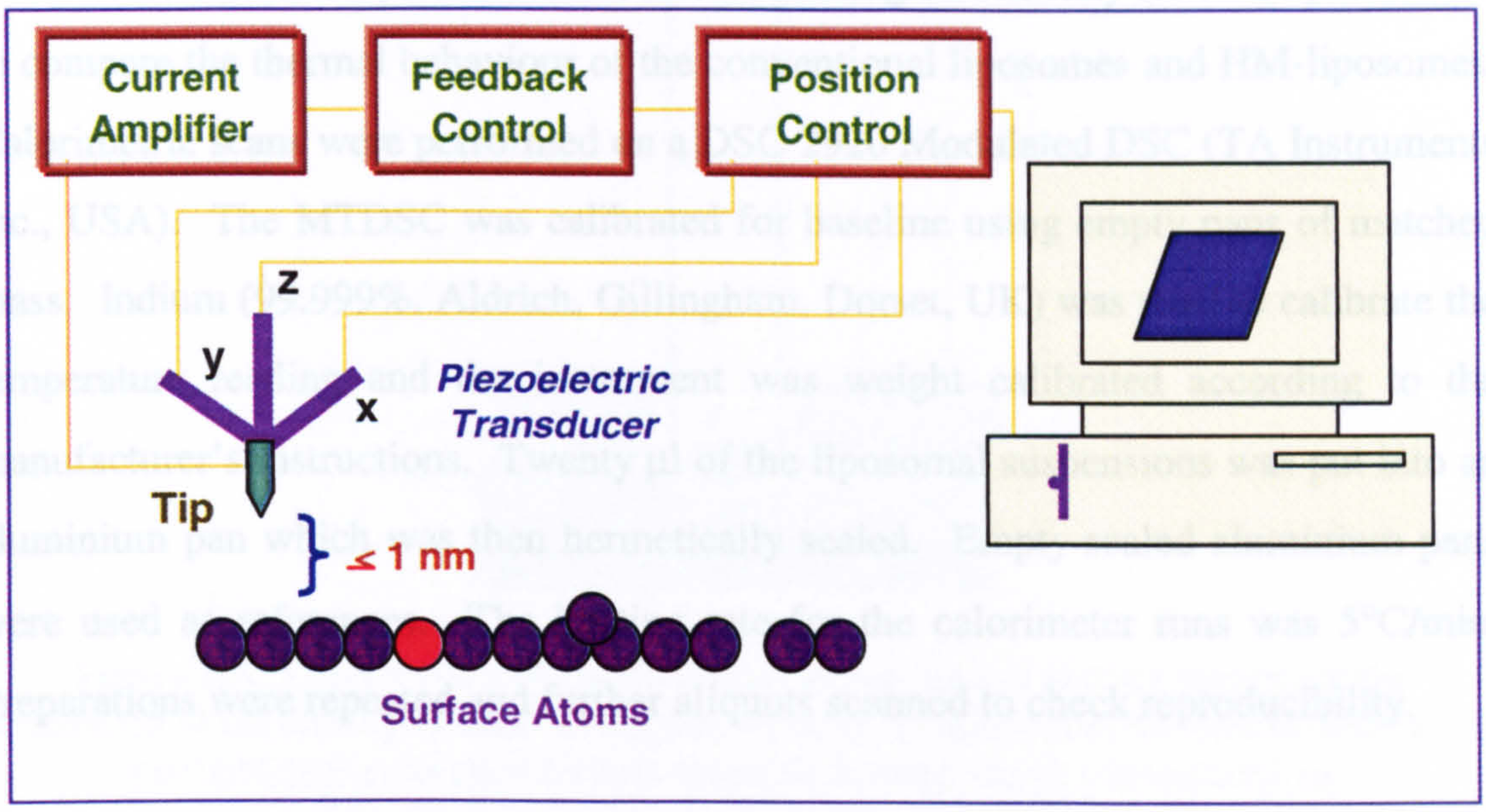


Figure 2.3. Schematic presentation of main components of a scanning tunnelling microscope.

For STM studies, unilamellar vesicles were prepared using 100nm pore-size filters by the conventional method and the heating method as explained in section 2.2.2.1. Typically a 20µl liposomal suspension was deposited on highly oriented pyrolytic graphite (HOPG) and was then dried at room temperature under atmospheric pressure in a clean room with a relatively slow drying rate. The STM was operated in air at atmospheric pressure with a tip-to-substrate bias of 800mV-1.5V (sample positive) and tunnelling current of 20-50 pA. Etched tips of Pt/Ir (80:20) wires (0.5mm in diameter, Digital Instruments, Santa Barbara, CA) were used in constant current mode. The diameter of liposomes was determined by a Digimatic caliper (Mitutoyo Ltd. England) using the dimensions of the STM images.

2.2.2.9. Thermal Analysis of Liposomes

Modulated temperature differential scanning calorimetry (MTDSC) was used to compare the thermal behaviour of the conventional liposomes and HM-liposomes. Calorimetric scans were performed on a DSC 2920 Modulated DSC (TA Instruments Inc., USA). The MTDSC was calibrated for baseline using empty pans of matched mass. Indium (99.999%, Aldrich, Gillingham, Dorset, UK) was used to calibrate the temperature reading and the instrument was weight calibrated according to the manufacturer's instructions. Twenty µl of the liposomal suspensions was put into an aluminium pan which was then hermetically sealed. Empty sealed aluminium pans were used as references. The heating rate for the calorimeter runs was 5°C/min. Preparations were repeated and further aliquots scanned to check reproducibility.

2.3. Results

2.3.1. Preparation of Liposomes by the Heating Method

In the context of introducing new liposome preparation techniques one of the fundamental questions will be whether it is possible to produce and characterise the liposomal formulation on a large scale with clearly defined and reproducible properties. In this chapter the physico-chemical characteristics of liposomes produced by a novel method, termed the heating method, are presented. Four different microscopies in addition to other analytical techniques confirmed successful formation of closed continuous lipid bilayers (i.e. liposomes) in a reproducible manner. The methodology has the potential to be scaled up for example by using a larger version of the simultaneous heating and stirring reactor simplified in Figure 2.4.

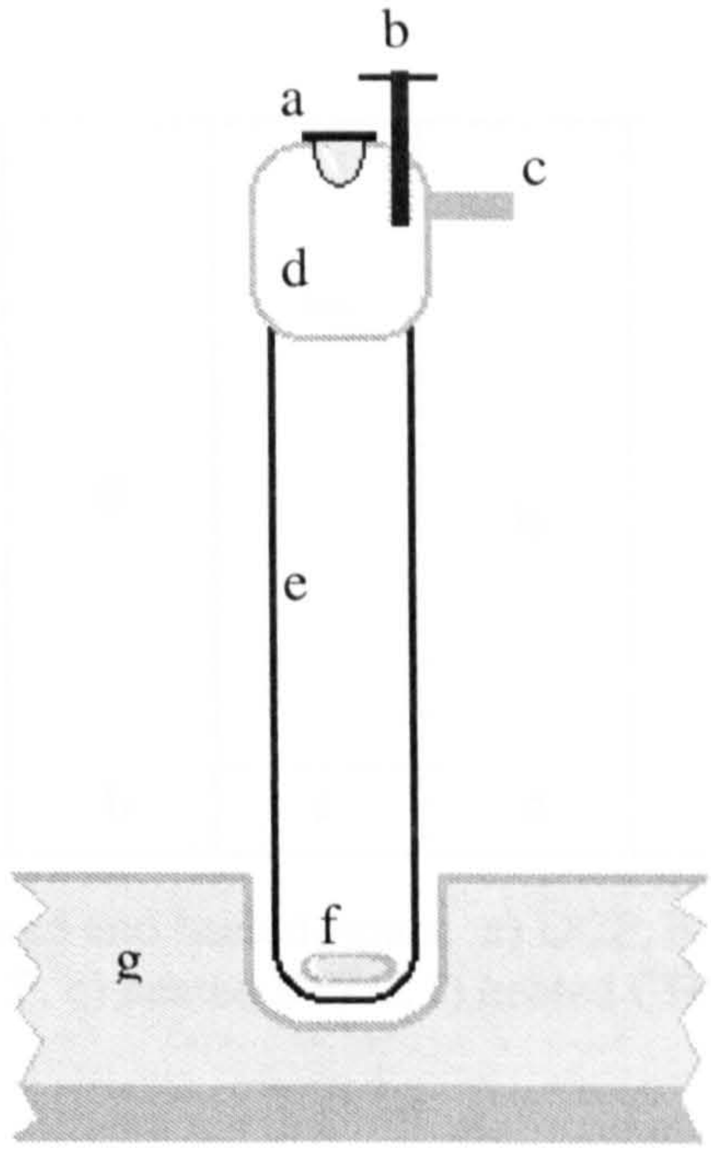


Figure 2.4. Cross section of the simultaneous heating and stirring reactor for the preparation of the HM-liposomes. a) stopper, b) valve, c) inlet/outlet for vacuum and gas, d) gas tight cap, e) reaction tube, f) magnetic stirring bar, g) stirring hotplate.

Another important question in the development of a liposomal formulation is the stability of the product. In this respect, the effect of aging on the morphology and size of the HM-liposomes was evaluated by direct microscopic observations and indirect light scattering measurements as explained in the following sections.

2.3.2. Thin Layer Chromatography

The effect of heating on the lipid molecules was checked by thin-layer chromatography (TLC). Figure 2.5. is a typical example of the chromatograms obtained for the heated and un-heated lipids. TLC confirmed that the lipids used in this study were pure and the high temperature employed during the preparation of the HM-liposomes did not cause degradation of these lipids. The average of three R_f values for unheated (control) and heated lipids respectively are as follows: DCP: 0.16 and 0.18; DPPC: 0.21 and 0.22; and CHOL: 0.36 and 0.39.

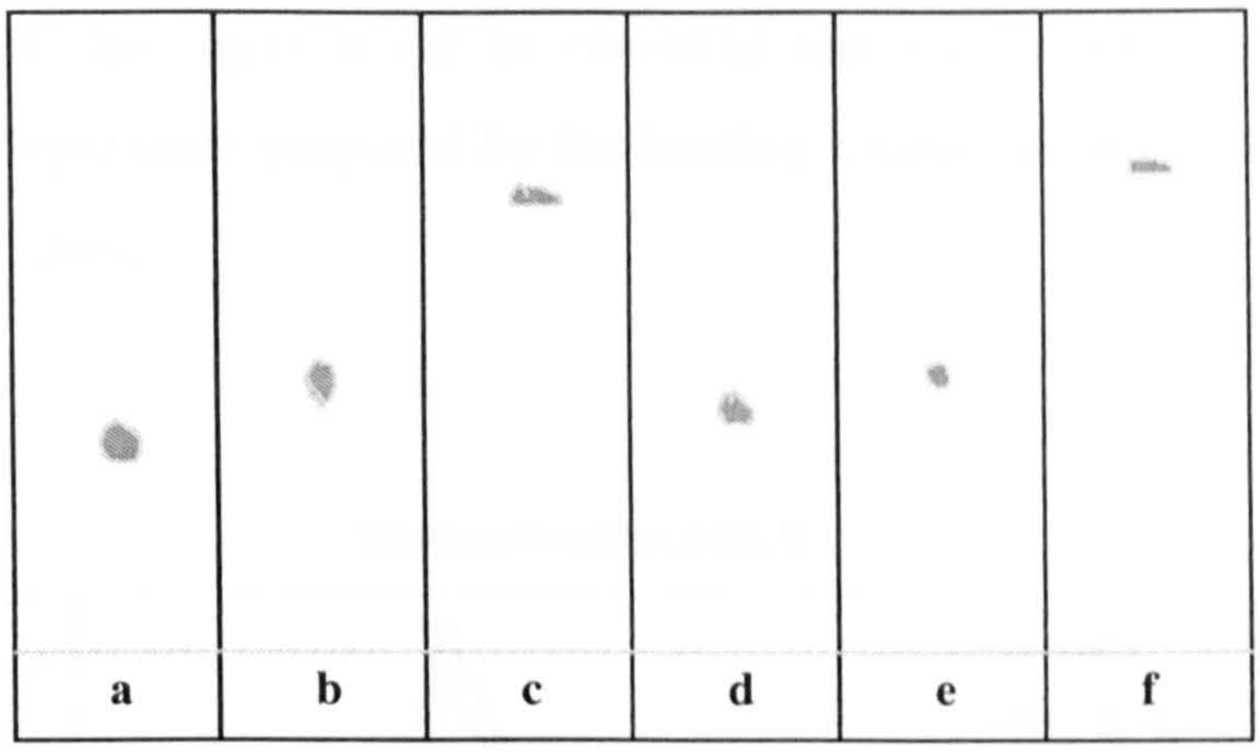


Figure 2.5. TLC of un-heated and heated lipids. a) DCP, b) DPPC, c) CHOL, d) heated DCP, e) heated DPPC, f) heated CHOL.

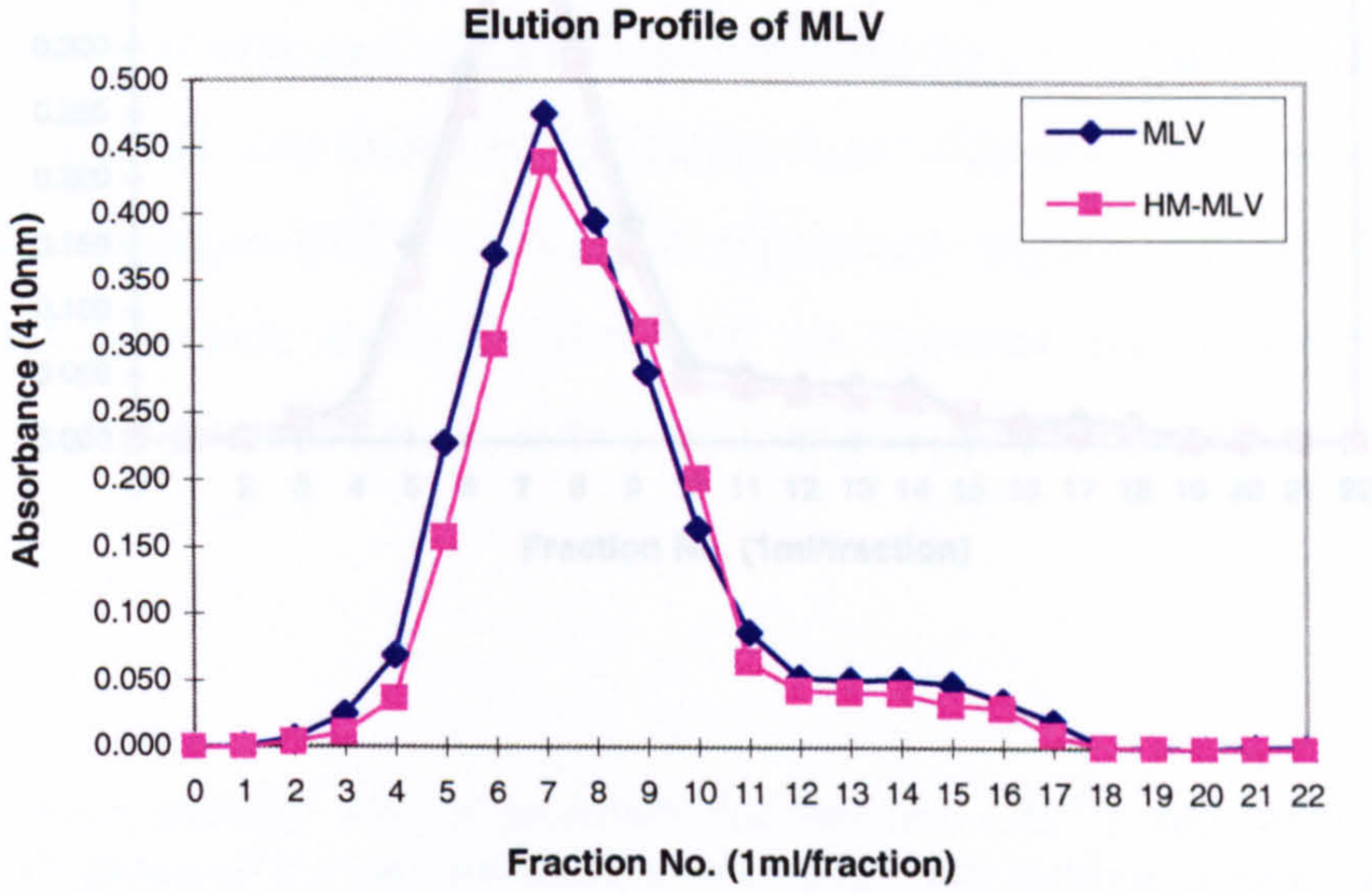
2.3.3. Gel Permeation Chromatography

Gel permeation chromatography (GPC), or gel filtration chromatography, is a very informative assay that does not involve expensive sophisticated instrumentation (Barenholz and Amselem 1993). GPC was performed in order to compare the elution characteristics, size distribution and homogeneity of the conventional liposomes and HM-liposomes. The absorbance of liposomal fractions was monitored at 410nm. It is known that the absorbance obtained at 410nm is due to turbidity caused by the lipoidal vesicles in their intact form (Sessa and Weissmann 1970).

The elution curves of the conventional liposomes and HM-liposomes are shown in Figure 2.6. It can be observed that multilamellar vesicles (both conventional MLV and HM-MLV) exhibit a wider size distribution (Figure 2.6.a) compared with vesicles extruded through 400nm filters (Figure 2.6.b) and 100nm filters (Figure 2.6.c). The small amount of particles (probably very small vesicles), coming after the liposomal fractions in the fraction numbers 13-16, was detected to be slightly less for the HM-liposomes compared to the conventional liposomes.

The overall elution profile of the six types of liposomes is demonstrated in Figure 2.6.d. From this figure it can be observed that the elution profile and size distribution of the liposomes prepared by the heating method is similar to that of the conventional liposomes.

a



b

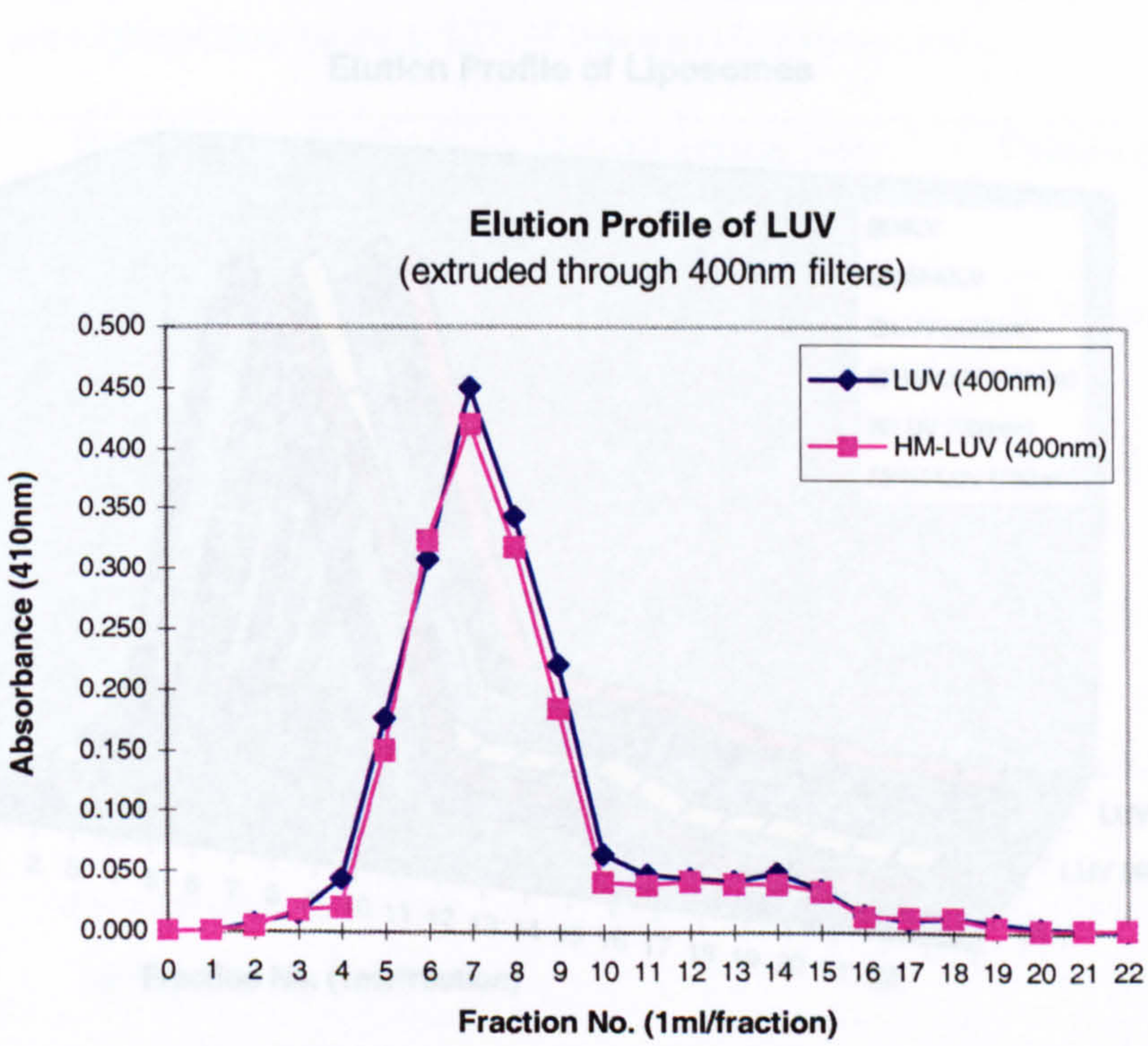
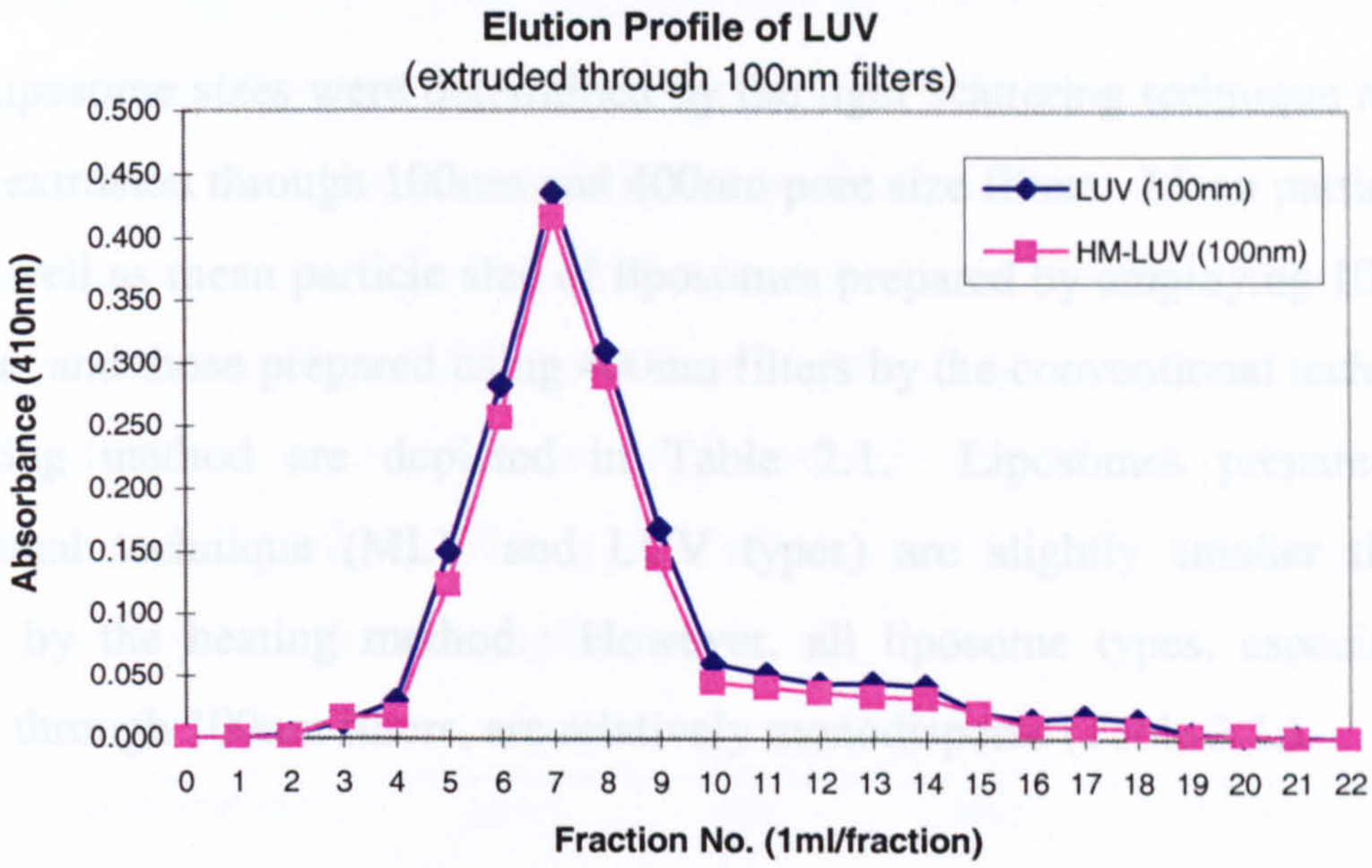


Figure 2.6. Gel permeation chromatography of conventional liposomes and HM-liposomes. a) multilamellar vesicles prepared by the conventional (MLV) and the heating method (HM-MLV); b) unilamellar vesicles prepared by the conventional (LUV) and the heating method (HM-LUV). Numbers in parenthesis are the filter sizes through which the vesicles were extruded.

c



d

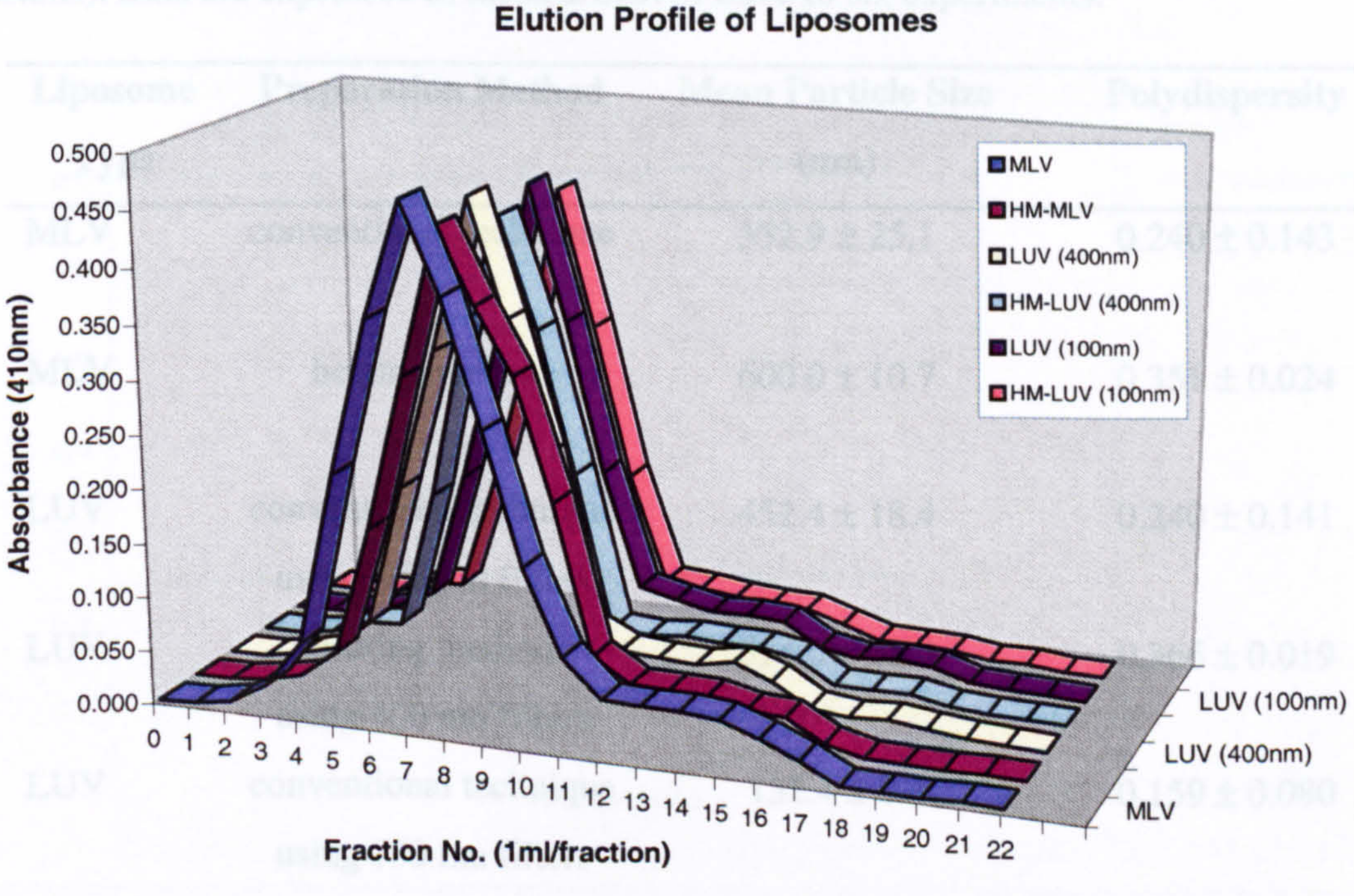


Figure 2.6. ***(continued)*** Gel permeation chromatography of liposomes and HM-liposomes. c) unilamellar vesicles prepared using 100nm filters by the conventional (LUV) and the heating method (HM-LUV); d) overall elution profile of the lipid vesicles. Numbers in parenthesis are the filter sizes through which the vesicles were extruded.

2.3.4. Light Scattering

Liposome sizes were determined by the light scattering technique before and after the extrusion through 100nm and 400nm pore size filters. Mean particle size of MLV as well as mean particle size of liposomes prepared by employing 100nm pore size filters and those prepared using 400nm filters by the conventional technique and the heating method are depicted in Table 2.1. Liposomes prepared by the conventional technique (MLV and LUV types) are slightly smaller than those prepared by the heating method. However, all liposome types, especially those extruded through 100nm filters, are relatively monodisperse (Table 2.1.).

Table 2.1. Mean particle size of multilamellar vesicles (MLV) and large unilamellar vesicles (LUV) prepared by the conventional technique and heating method (see text for details). Data are expressed as mean ± S.D. of three to six experiments.

Liposome Type	Preparation Method	Mean Particle Size (nm)	Polydispersity
MLV	conventional technique	552.9 ± 25.1	0.240 ± 0.143
MLV	heating method	600.0 ± 10.7	0.351 ± 0.024
LUV	conventional technique using 400 nm filters	452.4 ± 18.4	0.240 ± 0.141
LUV	heating method using 400 nm filters	514.6 ± 15.3	0.366 ± 0.019
LUV	conventional technique using 100 nm filters	152.4 ± 8.4	0.159 ± 0.080
LUV	heating method using 100 nm filters	156.8 ± 5.9	0.136 ± 0.040

2.3.5. The Aging Effect

Effect of aging on the size of HM-liposomes was also studied by the light scattering method (Figure 2.7.). The diameters of HM-liposomes were measured initially as well as after two, four and eight months storage under N_2 atmosphere at 2-8°C. The heating method resulted in the formation of stable liposomes for at least eight months at least. Figure 2.7. indicates a slight but insignificant size enlargement after eight months compared with the initial measurements (4.0% for MLV, 2.6% for 400nm filtered and 8.1% for 100nm filtered vesicles) for the HM-liposomes upon storage at the above mentioned conditions.

Time (month)	zero	two	four	eight
Liposome type				
MLV	600 ±7.1	600 ±13.3	601 ±10.7	624 ±11.5
LUV (400)	508 ±8.4	517 ±7.2	515 ±8.5	521 ±9.7
LUV (100)	148 ±5.4	153 ±6.0	160 ±6.6	160 ±7.6

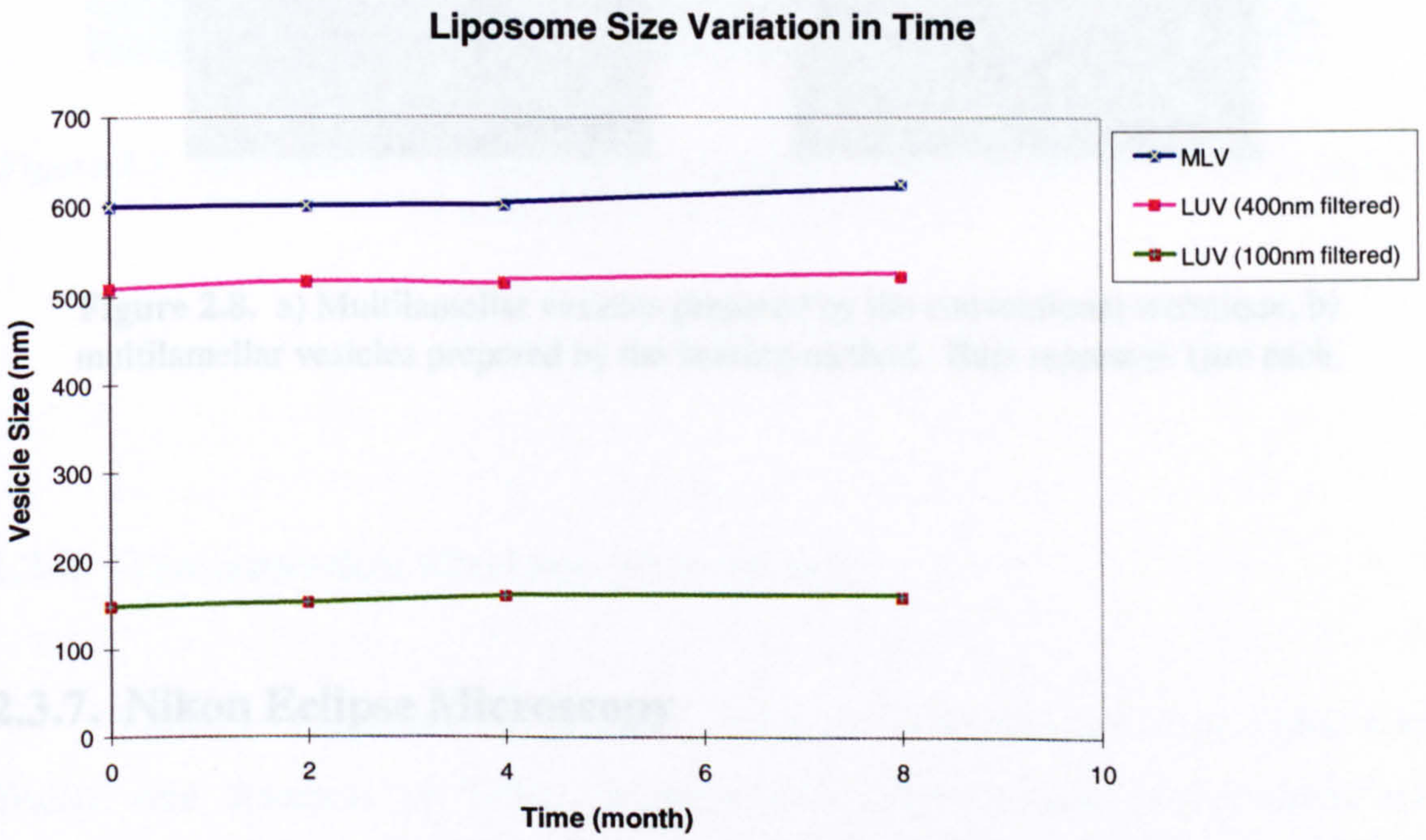


Figure 2.7. Time stability of HM-liposomes stored at 2-8°C under N_2 atmosphere. Average liposome sizes are provided above the graph. Data are expressed as mean ± S.D. of three to four experiments.

2.3.6. Optical Microscopy

Vesicles in micrometer size ranges (multilamellar vesicles produced before the extrusion step) were imaged by optical microscopy. Multilamellar vesicles prepared by the conventional method are shown in Figure 2.8.a and those prepared by the heating method are shown in Figure 2.8.b. Both figures suggest presence of liposomes with similar shape and size distributions. The closed spherical multilamellar vesicles (MLV) can be clearly seen in these photographs. No other lipidic structure (e.g. micelle, hexagonal (H_{II}) phase or lamellar L_α phase) was detected in the optical microscopy pictures.

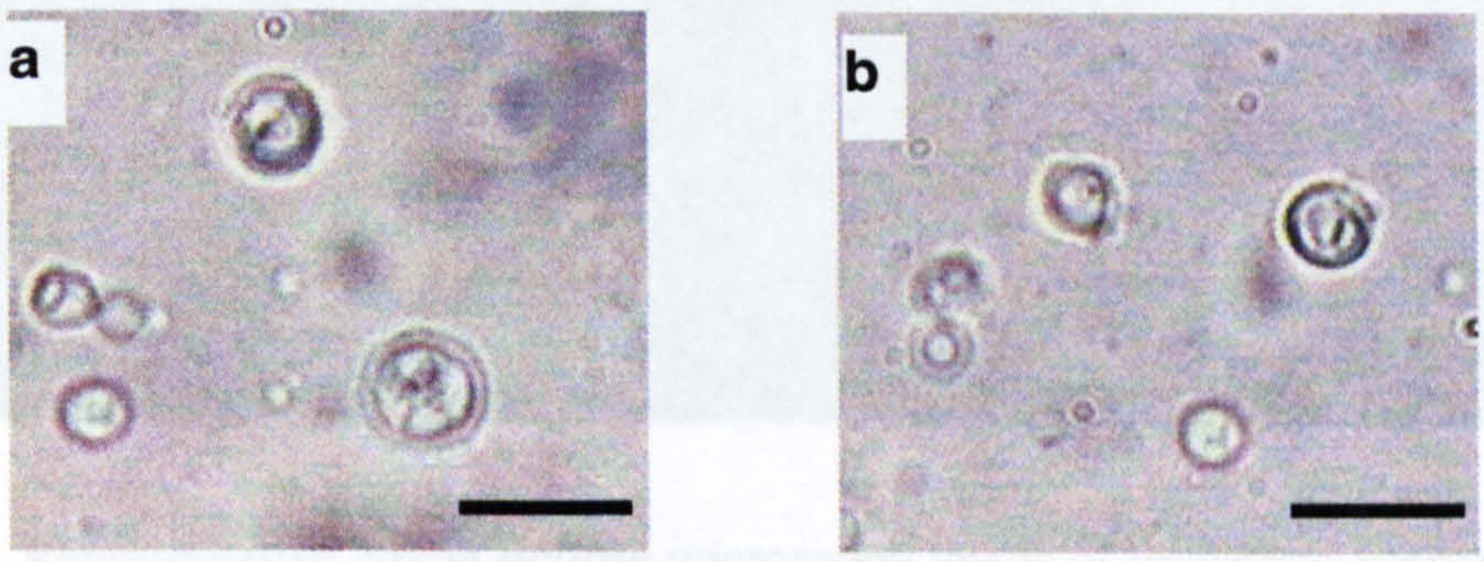


Figure 2.8. a) Multilamellar vesicles prepared by the conventional technique, b) multilamellar vesicles prepared by the heating method. Bars represent 1μm each.

2.3.7. Nikon Eclipse Microscopy

Figure 2.9. demonstrates HM-liposomes extruded through 400nm sized filters visualised by Nikon Eclipse microscopy. A total number of 28 vesicles can be observed in Figure 2.9. which have a mean diameter of 375.4nm (± 94.2). This figure shows the presence of spherical vesicles which possess homogenous size

distribution. Again, as with the MLV explained in the previous section, no other lipidic structure was detected through Nikon Eclipse microscopy.

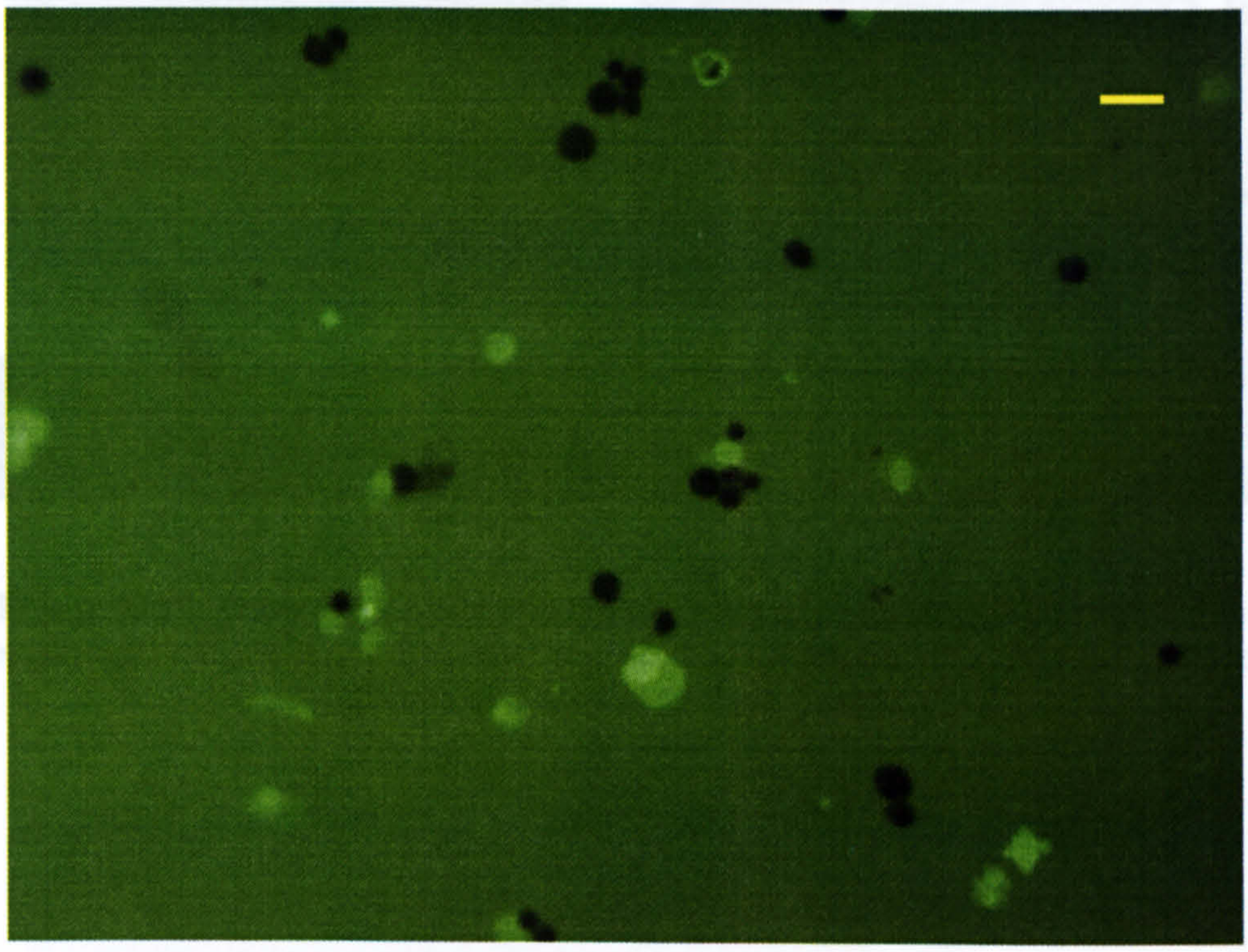


Figure 2.9. Representative Nikon Eclipse microscopy image of liposomes prepared by the heating method. Bar represents 1μm.

2.3.8. Transmission Electron Microscopy

Vesicles in nanometer size ranges (after extrusion through 100nm pore size filters) were observed by TEM. Representative TEM images of liposomes are demonstrated in Figure 2.10. Vesicles prepared by the conventional technique (Figure 2.10.a) and the heating method (Figure 2.10.b) have diameters of 97.4nm and 84.2nm respectively. TEM images reveal that the extruded vesicles, prepared by the conventional technique and the heating method, are in the form of large unilamellar vesicles (LUV).

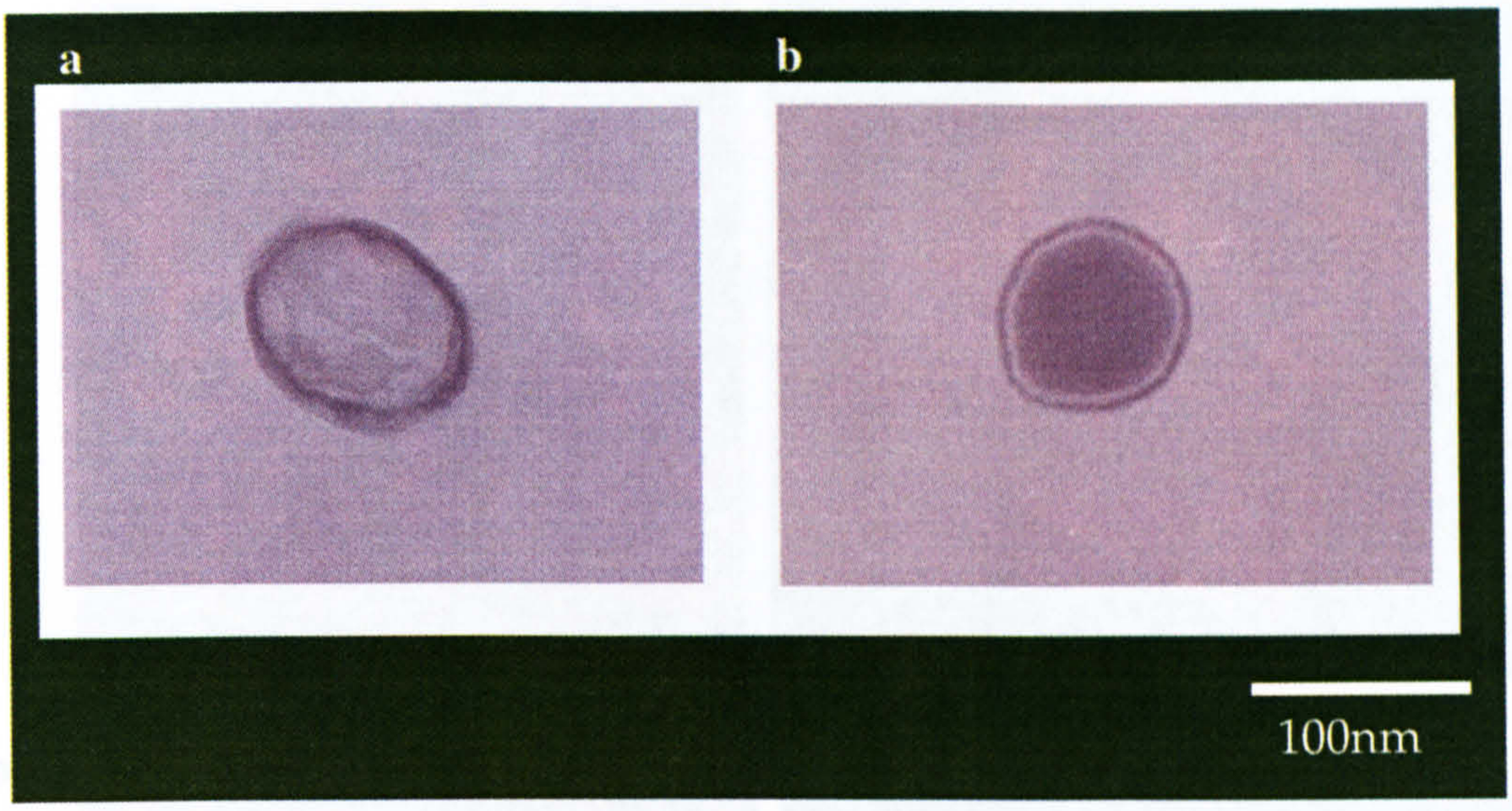


Figure 2.10. Transmission electron micrographs of a) liposomes prepared by the conventional technique; b) liposomes prepared by the heating method. Bar represents 100nm and is valid for both images.

2.3.9. Scanning Tunnelling Microscopy

The ultrastructure of the liposomes extruded through 100nm filters was studied by high-resolution scanning tunnelling microscopy (STM). Figure 2.11. shows the effect of storage on the morphology of the HM-liposomes. Figures 2.11.a, 2.11.b and 2.11.c demonstrate vesicles after two, six and eight months of their preparation and storage under N_2 at 2-8°C respectively. Two months old HM-liposomes in Figure 2.11.a have diameters of 38.0, 48.6 and 49.4nm. Diameters of six months old vesicles in Figure 2.11.b are 42.1, 83.5 and 92.3nm, and diameters of eight months old vesicles in Figure 2.11.c are 60.1 and 71.2nm. Liposomes possess similar mean bilayer thickness of around 10nm in average as calculated from the height-distance curves (line profiles) represented in Figure 2.12. The reason for the differences in size measurements performed using light scattering and microscopic techniques will be explained in section 2.4.3.

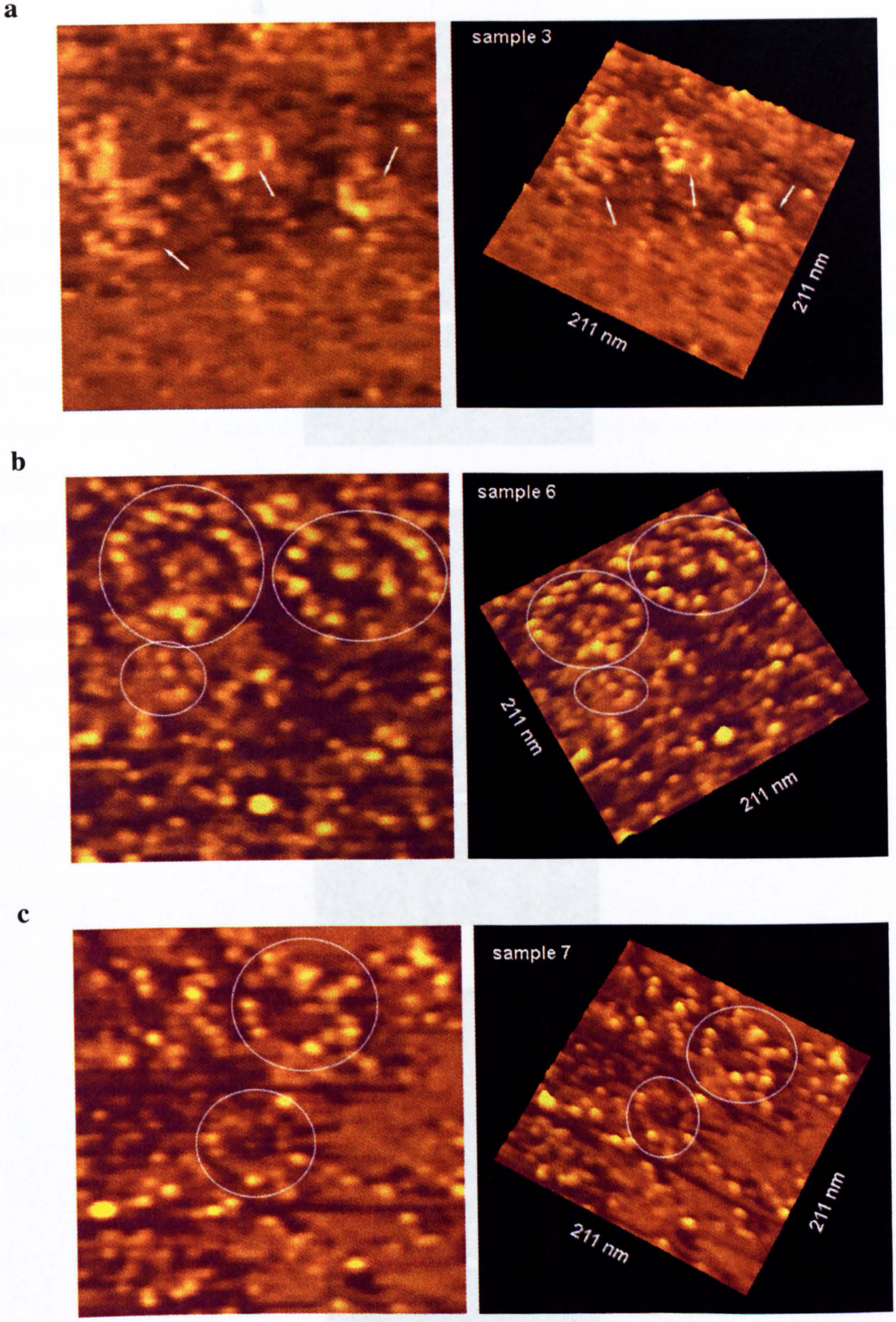
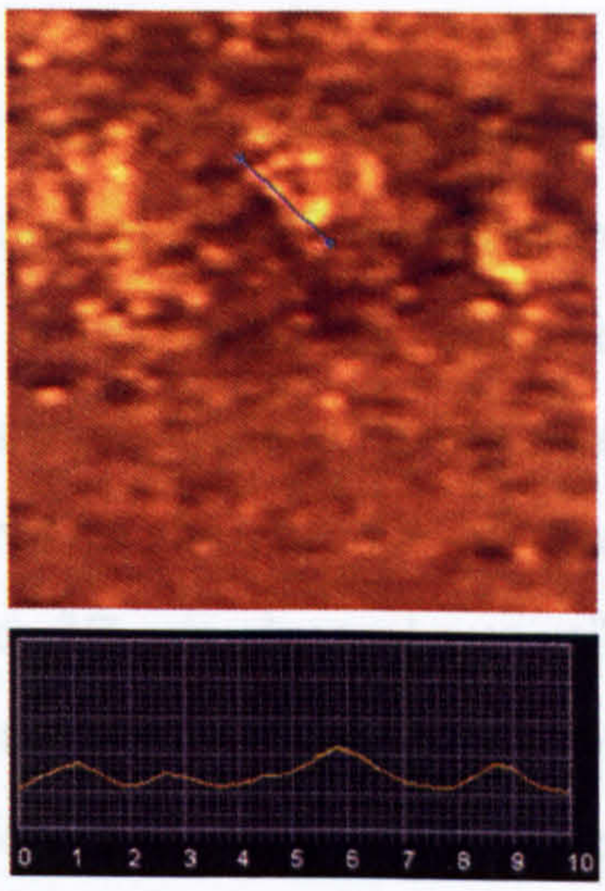
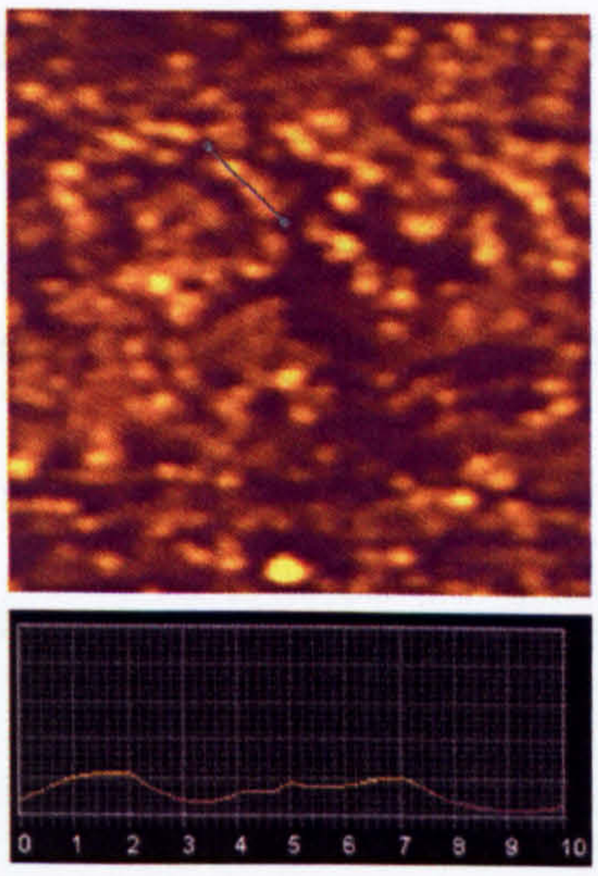


Figure 2.11. Two and three-dimensional scanning tunnelling micrographs of a) two months old HM-liposomes indicated by arrows; b) six months old HM-liposomes inside circles; c) eight months old HM-liposomes inside circles.

a



b



c

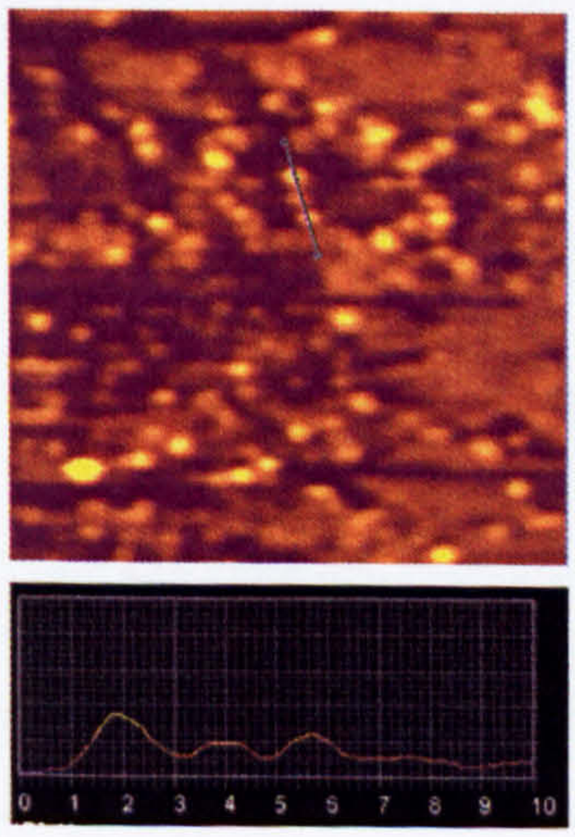


Figure 2.12. Representative profiles of the surface corrugations taken along lines on the bilayers of the vesicles shown in Figure 2.11. a to c respectively. The horizontal scales are arbitrary units proportional to the lines selected on each of the corresponding top diagrams.

2.3.10. Thermal Analysis of Liposomes

The thermal behaviours of conventional liposomes and HM-liposomes obtained from modulated temperature differential scanning calorimetry are presented in Figure 2.13. Both types of liposomes are composed of DPPC, DCP and CHOL. The conventional liposomes were prepared using chloroform and methanol and the HM-liposomes were prepared in the absence of these solvents. However, in the preparation of HM-liposomes 3% v/v glycerol was utilised. The inlet in Figure 2.13. is the thermal behaviour of the two liposome types with two varying parameters (i.e. presence/absence of chloroform and methanol and presence/absence of glycerol). In order to bring the number of these parameters down to one 3% v/v glycerol was added to the conventional liposomes after their preparation. It can be seen in Figure 2.13. that HM-liposomes exhibit slightly different thermal behaviour to the conventional liposomes. It seems that the presence of glycerol in both liposome types has reduced the difference in the thermal behaviour of the vesicles.

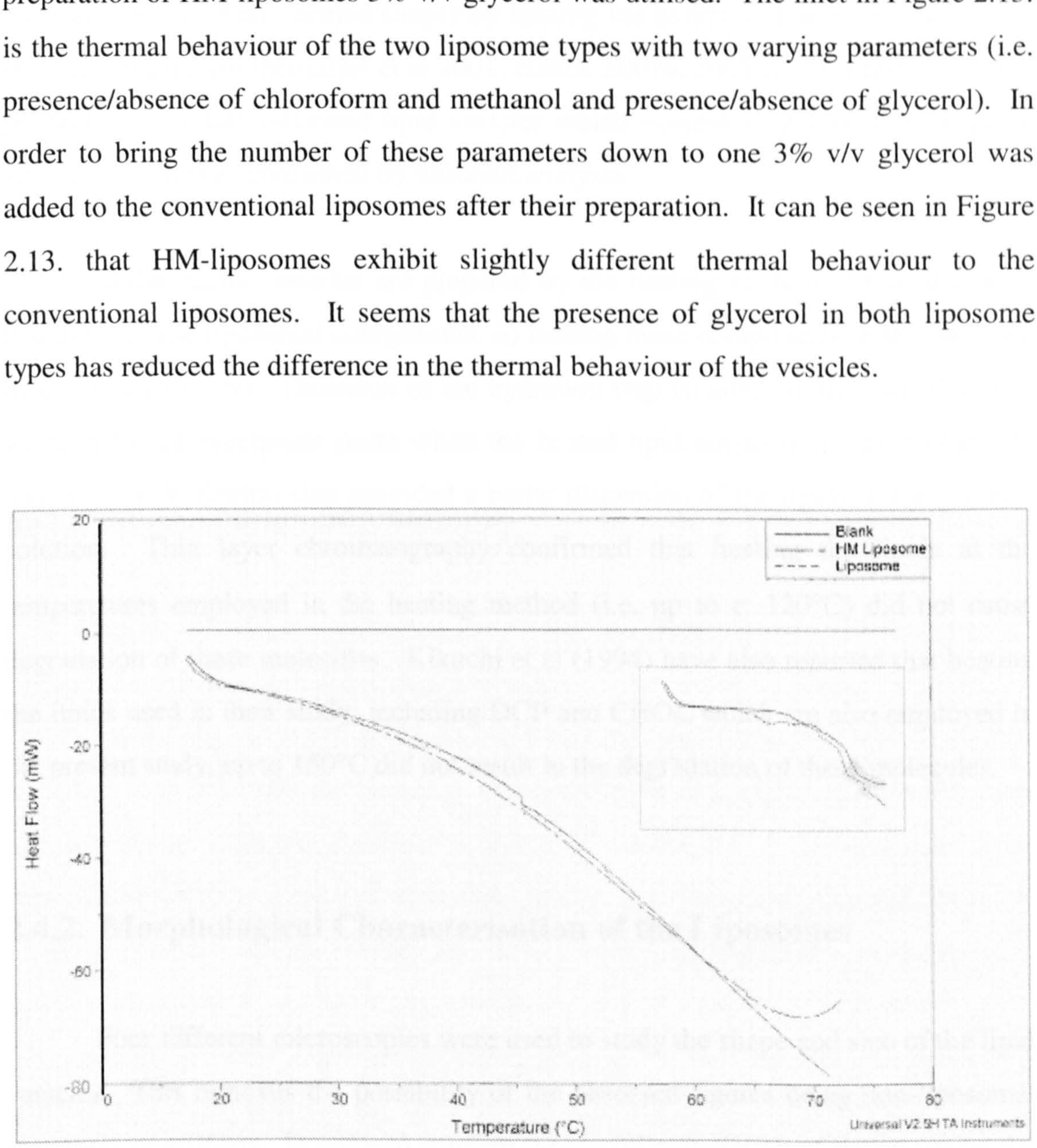


Figure 2.13. Modulated temperature differential scanning calorimetry of liposomes prepared by the heating method (HM) and conventional liposomes after addition of glycerol to the conventional liposomes (see text for details). Inlet shows the DSC scan of the vesicles before addition of glycerol to the conventional liposomes.

2.4. Discussion

2.4.1. Formation of Liposomes by the Heating Method

A novel technique for the preparation of liposomes without the use of volatile organic solvents, detergents, or high shear forces is described. In this heating method, liposomes are formed simply by heating the hydrated lipid molecules in the presence of glycerol (Mozafari et al 2001, 2002a, 2002b, 2002c). This resulted in the production of stable bilayered lipid vesicles which possess relatively monodisperse size distributions as confirmed by different analyses.

Multilamellar vesicles are prepared by the heating method in two stages: i) hydration of the liposomal components, ii) heating these components in the presence of glycerol (3% v/v). Omission of the hydration step resulted in the formation of a white coloured precipitate paste when the heated lipid suspension was cooled. In addition, the hydration step provided a better dispersion of the lipids in the glycerol solution. Thin layer chromatography confirmed that heating the lipids at the temperatures employed in the heating method (i.e. up to *c.* 120°C) did not cause degradation of these molecules. Kikuchi et al (1994) have also reported that heating the lipids used in their study, including DCP and CHOL which are also employed in the present study, up to 150°C did not result in the degradation of these molecules.

2.4.2. Morphological Characterisation of the Liposomes

Four different microscopies were used to study the shape and size of the lipid vesicles. This removes the possibility of the observed figures being non-liposomal structures or artifacts. In order to confirm successful preparation of liposomes by the heating method before the extrusion step, optical microscopy examinations were performed. Formation of spherical multilamellar vesicles and absence of other

structures, such as hexagonal (H_{II}) or lamellar L_α phase, was detected (Figure 2.8.). The multilamellar vesicles obtained by the heating method were similar in shape and size distribution to the conventional liposomes.

The multilamellar vesicles were extruded using 100nm and 400nm filters. The term 'liposome extrusion' has become synonymous with the process whereby multilamellar vesicles are forced through filters with defined pore size (Hope et al 1993). The extrusion step was performed in order to produce unilamellar vesicles with a homogeneous size distribution. Nikon Eclipse microscopy confirmed that the HM-liposomes extruded through 400nm filters are monodispersed spherical particles (Figure 2.9.). Liposomes extruded through 100nm filters were examined by TEM and STM. Transmission electron micrographs revealed that the heating method is capable of producing spherical unilamellar liposomes similar to those obtained by the conventional technique (Figure 2.10.). STM investigations are discussed in the next section.

2.4.2.1. Scanning Tunnelling Microscopy

Scanning tunnelling microscopy (STM) is a relatively new but fast growing surface analysis and imaging technique. Since its invention by Binnig et al (1982a, 1982b), which led to a Nobel prize award later in 1986 because of this invention, STM has been gradually increasing in popularity in the imaging of micro- and mostly nano-structures. Compared with other forms of microscopy, STM has unparalleled capabilities which include: (1) ultra-high resolution down to atomic dimensions; (2) three-dimensional images, especially with a very high sensitivity in the vertical direction; (3) a variety of operating conditions, such as vacuum, air and even liquids; (4) observation range from 10^{-6} to 10^{-10} m; and (5) the ability to do tunnelling spectroscopy (Feng et al 1989).

Sample preparation for STM, explained in section 2.2.2.8. for an in-air-operated STM, is very mild and easy and does not involve treatments such as

vacuum, freeze fracture, fixation, embedding and staining. The first report of STM images of a biological substance, DNA, appeared in 1983 (Binnig and Rohrer 1983), introducing the possible application of STM in biology. This was followed by further imagings of DNA and many other biological samples and molecules (Baro et al 1985, 1986; Feng et al 1988, 1989; Leggett et al 1993; Mozafari 1996; Zareie et al 1996b, 1997, 1998; Mozafari et al 1998a, 1998b; Wang et al 2001) including lipid films (Horber et al 1988), lipid bilayers (Smith et al 1987) and liposomes (Fowler et al 1992; Zareie et al 1996a; Mozafari 1996; Mozafari et al 1998a, 1998b, 2002c). Similar to many (if not all) other newly introduced instruments there arose some concerns regarding the STM analysis (Lindsay 1993). The vast number of publications on biological and other applications of STM, however, has substantially reduced the amount of doubts concerning the validity of STM images. Besides, in our previous studies (Mozafari 1996; Mozafari et al 1998b) and the present one (partially published in Mozafari et al 2002c), the data obtained through different types of microscopies were all in accordance with the morphological data obtained through STM.

It is believed that regularly packed structures (such as liposomes) are much easier to image and distinguish and the resolution is higher compared to individual or randomly distributed molecules (Feng et al 1989). We were the very first group who imaged a liposome/DNA system, as a gene delivery vector, by STM (Zareie et al 1997) introducing a new application area (i.e. gene therapy) for this device. A representative STM image of liposome/DNA complexes from that study is shown in Figure 2.14. In addition to enabling liposome diameter measurements, STM is very useful in determining the bilayer thickness of the liposomes by its analytical ability in the vertical axis. This and the three-dimensional image analysis in general, are not feasible by the other microscopies described in the present chapter. The calculated average bilayer thickness of the HM-LUV (large unilamellar vesicles obtained by the heating method) is approximately 10nm which is less than bilayer thickness of the small unilamellar vesicles (i.e. around 13nm) which were produced by a conventional technique (Zareie et al 1997). Among the factors which could be responsible for this bilayer thickness variation are difference in liposomal components (i.e. replacement

of PC with DPPC), liposome type (LUV and SUV), phase (gel or liquid-crystalline state) or preparation method. However, STM images obtained in the present study attest that the HM-liposomes are similar in shape to the conventional liposomes and are stable for at least eight months.

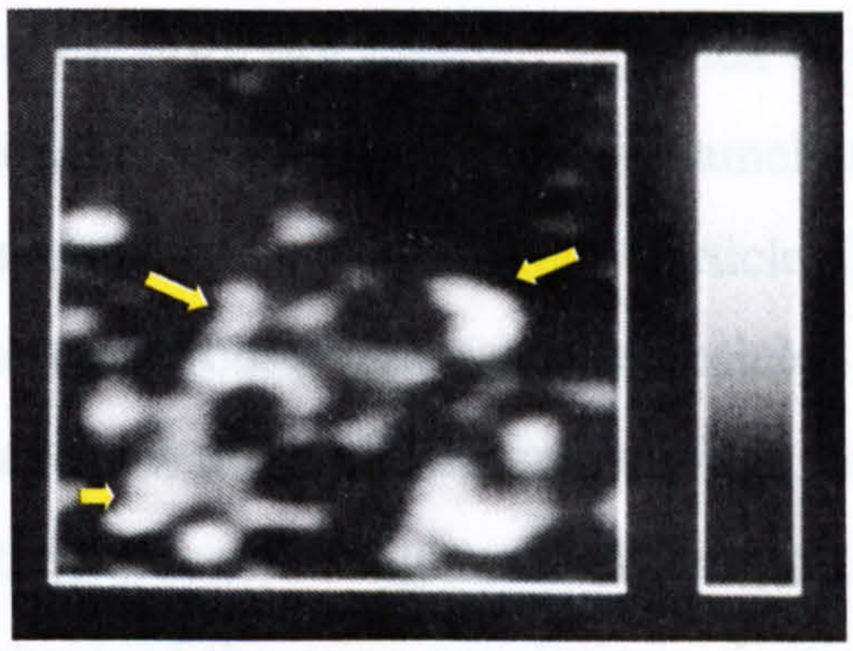


Figure 2.14. Scanning tunnelling micrograph of a liposome/Ca^{2+}/DNA complex (From: Zareie et al 1997). Arrows indicate possible locations of DNA molecules.

2.4.3. Size Distribution of the Liposomes

Determination of liposome size distribution is an obligatory quality control assay due to the following reasons: (1) The size distribution of liposomal formulations is an important parameter with respect to the physical properties and stability (Lichtenberg and Barenholz 1988; Goren et al 1990); (2) size distribution, when combined with composition, defines plasma pharmacokinetics, organ biodistribution and stability of liposomes and their associated/entrapped substances in plasma and other organs (Blume and Cevc 1990; Goren et al 1990; Gabizon et al 1993); (3) for injectables, there is a regulatory requirement to quantify the concentration of large particles (Barenholz and Amselem 1993); (4) liposome size is a major factor in their permeation through tumor microvessels and their local

residence in tumor tissue (Nagayasu et al 1999); (5) in pulmonary applications the deposition region of the liposomes depends mainly on density, shape and size of the lipid vesicles.

Each of the currently used liposome size determination techniques has its own advantages and limitations. The advantage of light scattering, for example, is that it provides cumulative average information of the size of a large number of vesicles at the same time. However, it does not provide any idea of the shape of the lipidic system (e.g. oval, spherical, hexagonal (H_{II}) or lamellar) and it assumes any aggregation of more than one vesicle as one single particle. Microscopic techniques, on the other hand, make direct observation of the vesicles possible, hence provide information on the shape of the vesicles as well as presence/absence of any aggregation, provided they have the right resolution for the particle size under investigation. The drawback of the microscopic investigations is that the number of particles which can be studied at any particular time is limited. The general approach for the determination of size distribution of liposomal formulations should hence be to use as many different techniques as possible (also recommended by Barenholz and Amselem 1993). Based on this, four different microscopies as well as gel permeation chromatography and light scattering techniques were employed to determine liposome sizes/size distributions in this study. For all liposome formulations, the first stage in the size analysis should be light microscopy. This method does not require any pretreatment or fixation of the liposomes. In addition, most of the liposome preparation techniques initially result in the formation of micrometer sized vesicles which can be observed by optical microscopies.

The monodispersity of the extruded vesicles was confirmed through GPC elution curves and light scattering investigations. In terms of monodispersity the data obtained through light scattering are in agreement with those obtained through the microscopic studies. However, the mean particle size values obtained through light scattering for the extruded vesicles are bigger than the sizes obtained by the microscopic studies. It should be noted, as explained above, that light scattering technique provides data concerning a larger number of particles when compared with

microscopic studies. Variations between particle sizes obtained by light scattering and microscopic techniques have been encountered in our previous works as well (Zareie et al 1997; Mozafari and Hasirci 1998; Mozafari et al 1998b). Nevertheless, these two methodologies have different sample preparation requirements and sensitivities. No signs of liposome aggregation was detected for both liposome types as confirmed by the microscopic examinations. Lack of aggregation (and/or fusion), which is expected for similarly charged vesicles due to electrostatic repulsion and also detected before for anionic vesicles (Mozafari and Hasirci 1998), indicates that the big vesicle size values obtained through light scattering were not due to aggregation of the vesicles. Moreover, observation that DPPC-containing vesicles are larger than the filter pores through which they were extruded has also been reported by other groups (Nayar et al 1989; MacDonald et al 1991). The explanation of how vesicles which are solid at ambient temperatures can pass through pore sizes smaller than their diameters relies on the fact that the extrusion process was carried out at temperatures above the T_C of the lipid constituents, when liposomes are flexible, while liposome sizes were measured at room temperature.

2.4.4. Storage Stability of the HM-liposomes

In order to have a useful liposomal formulation, the physical stability issue has to be addressed during the developmental stages of the formulation. This issue is even more important for the commercial development and utilisation of a liposomal product as a drug delivery system. Therefore, any liposomal formulation designed for clinical use will need to be tested initially for adequate storage stability with regard to changes in size, vesicle aggregation and vesicle fusion.

The light scattering method revealed that HM-liposomes, in the form of multilamellar vesicles or unilamellar vesicles obtained by extrusion through 400 and 100nm filters, retain their physical stability for at least eight months. STM observations also confirmed the same result for HM-liposomes prepared by using 100nm filters. A slight size enlargement (up to 8.1%) observed for the vesicles after

eight months storage under N_2 at 2-8°C may be due to the swelling of the vesicles. This can not be due to vesicle aggregation or fusion as these should result in liposome size enlargements of bigger magnitudes. In addition, STM studies of the two, six and eight months old vesicles also show absence of liposomal aggregates. As explained in the previous section the electrostatic repulsion forces between the anionic HM-liposomes is the main factor in preventing their aggregation. This is a desirable characteristic for liposomes in gene and drug delivery particularly in aerosol formulations.

2.4.5. Thermal Analysis of the Liposomes

The thermal behaviour of HM-liposomes was compared with that of liposomes prepared by the conventional technique. For this purpose a modulated temperature differential scanning calorimetry (MTDSC) was employed. With conventional DSC, the sample is subjected to a constant heating rate. The registered heat flow to increase the sample temperature may be divided into two components, one depending on the heat capacity of the sample and the other depending on thermally activated or kinetically driven process occurring in the sample. Only the first component which is related to the heat capacity depends proportionally on the heating rate. This difference is exploited in MTDSC (Van Winden et al 1998a).

Modulated temperature DSC employs a periodic heating rate oscillation in place of a conventional linear temperature program in order to gain additional information about the thermal properties of materials (Reading et al 1994). The signal from the instrument is separated into two components - a thermally *reversing* heat flow that is a function of temperature and heating rate, and a *non-reversing* heat flow that is a function of temperature and time. The former parameter is most readily identified with the heat capacity of the sample whereas the non-reversing heat flow includes contributions from irreversible processes such as crystallisation, chemical reactions and loss of volatile materials. The important advantages of MTDSC over conventional DSC include higher resolution without loss of sensitivity, improved

signal to noise ratio, reduced baseline curvature, the possibility of measuring heat capacities in a single run and the separation of overlapping thermal events.

Both liposome types are composed of similar lipid components with same molar ratio. HM-liposomes contained 3% v/v glycerol while conventional liposomes did not contain glycerol. The main component of the liposomes is DPPC which typically possesses a pretransition at *c.* 32°C and main transition at *c.* 41°C (Papahadjopoulos et al 1973; Kikuchi et al 1994). It is well known that the thermal behaviour of DPPC is affected by the co-existence of cholesterol and charged lipids in the membrane according to the added ratio or the alkyl-chain length (Kikuchi et al 1994). It was confirmed from MTDSC studies that, once glycerol is present in equal amounts in both liposome types, HM-liposomes demonstrate similar thermal behaviour to the conventional liposomes. Hence the methodology, including application of heating, did not change the thermal properties of the HM-liposomes. The slight difference between the thermal behaviour of conventional liposomes and HM-liposomes in Figure 2.13. is probably due to the presence of trace amounts of chloroform and methanol as this is the only variable between the two liposome types in this figure.

2.5. Conclusions

A new liposome preparation method is introduced in this chapter in which no hazardous chemical (e.g. volatile organic solvents or detergents) or methodology (e.g. sonication or high pressures) is required. Liposomes prepared by this heating method (HM-liposomes) exhibited long term stability, narrow size distribution and spherical bilayered morphologies. Another important point is that due to the employment of heat (and filtration when smaller sized vesicles required), in the manufacture of HM-liposomes, there is no need to carry out further sterilisation steps which consequently reduces the time and cost of liposome production by the heating method. From the liposomologist point of view the heating method is ideal because she/he is not subject to any harmful chemical or process during preparation of the HM-liposomes.

In general, the heating method is an easy methodology, capable of producing sterile and stable lipid vesicles in only two steps. The heating method also possesses the potential for mass production of non-toxic liposomes to be utilised in gene and drug delivery applications.

3: *IN VITRO* CYTOTOXICITY EVALUATION OF THE HM-LIPOSOMES TOWARDS HUMAN RESPIRATORY EPITHELIAL CELLS

3.1. Introduction

Liposome science and technology is one of the fastest growing scientific fields contributing to areas such as drug delivery, cosmetics, structure and function of biological membranes and investigations of the origin of life to name a few. This is due to several advantageous characteristics of liposomes such as ability to incorporate not only water soluble but also lipid soluble agents, specific targeting to the required site in the body and versatility in terms of fluidity, size, charge and number of lamellae. Despite the enormous research and development works on liposomes, only a small number of liposomal products have been approved for human use so far. A major drawback in the clinical utilisation of liposomes is the toxicity associated with some of these lipid vesicles. For instance there are many reports on the toxicity of cationic liposomes (Panzner and Jansons 1979; Chawla et al 1979; Campbell 1983; Filion and Phillips 1997, 1998; Dokka et al 2000; Nagahiro et al 2000) with the exact mechanism of toxicity remaining unclear. One reason for the cationic liposome toxicity is believed to be the interaction of the cationic lipids with cell organelle membranes, specifically the anionic lipids making up these membranes (Xu and Szoka 1996). For example, in mitochondrial membranes, cardiolipin is the major anionic lipid, and interaction of this lipid with cationic species would be detrimental to the basic energy reactions of the cell (for a review see Dass et al 1997). Another postulated mechanism, for cationic lipid-mediated toxicity in the lung, is the involvement of reactive oxygen intermediates (Dokka et al 2000). On the other hand, volatile organic solvents employed in the manufacture of liposomes, including chloroform and methanol, may remain in the final liposome preparation and contribute to potential toxicity and influence the stability of the vesicles (Vemuri and

Rhodes 1995; Cortesi et al 1999). Residual solvents in pharmaceuticals, generally known as organic volatile impurities (OVIs), not only have no therapeutic benefits but may also be hazardous to the environment and human health (Dwivedi 2002). It has been suggested that organic solvents can exert toxicity towards cells via two types of mechanism: a) at the molecular level, or b) at the phase level. Molecular toxicity represents the effects caused by organic solvents that are dissolved within the aqueous phase and include enzyme inhibition, protein denaturation and membrane modifications such as membrane expansion, structure disorders and permeability changes. Phase toxicity effects include the extraction of nutrients, disruption of the cell wall (extraction of outer cellular components), and the limited access to nutrients caused by cell attraction to interfaces, the formation of emulsions and the coating of cells (Bar 1987; Hocknull and Lilly 1990).

Although there are measures to remove the OVIs from liposome formulations (see chapter two), these are practically very difficult and time consuming procedures. In addition, the level of OVIs in the final liposomal products must be assessed to ensure the clinical suitability of the liposomes. Consequently it would be much preferable to avoid utilisation of these solvents in the manufacture of the lipid vesicles. There are a number of liposome preparation techniques, such as the polyol dilution method (Kikuchi et al 1994) and the bubble method (Talsma et al 1994), in which no volatile organic solvent or detergent is being used. However, in terms of toxicity, the vesicles produced by these methods have not been characterised.

In the previous chapter a new method for the preparation of liposomes without employment of volatile organic solvents, detergents, sonication or homogenisers was introduced. Following the physico-chemical characterisation of the liposomes produced by this heating method, the possibility of toxicity of these vesicles needed to be addressed. Here we report a comparison of the cytotoxicity of two liposome preparations, one prepared using chloroform and methanol by a conventional method, and the other prepared by the heating method. A human bronchial epithelial cell line (16HBE14o) was utilised in the cytotoxicity studies as a relevant *in vitro* model for pulmonary applications of liposomes as explained in

section 3.1.3. below. Cell viability was evaluated using two well known cytotoxicity tests, the neutral red uptake (NRU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, in the presence of serum. The NRU assay is based on the uptake of neutral red dye which accumulates in the lysosomes of uninjured cells and the MTT assay is an indicator of mitochondrial metabolic activity. The effect of incorporation of plasmid DNA, with the aid of Ca^{2+}, on the toxicity of the liposomes was also evaluated. This had the aim of evaluating the cytotoxicty of HM-liposome/Ca^{2+}/DNA complex (vesicles of which were prepared by the heating method) when used as a gene transfer vector, in comparison with the cytotoxicity of the same complex prepared by the conventional technique. In order to understand the liposomal cytotoxicity issue it would be helpful to review the *in vitro* fate of liposomes which is provided in the next section.

3.1.1. Liposome-cell interactions *in vitro*

Various mechanisms have been postulated for the interaction of liposomes with cells, some of which are illustrated in Figure 3.1. The interaction of the lipid vesicles with cultured cells could involve the following non-exclusive mechanisms:

1) Incorporation of intact vesicles by endocytosis;

2) Fusion of vesicles with the plasma membrane;

3) Exchange of phospholipids (and probably proteins) between the liposomal and the plasma membranes;

4) Adsorption of liposomes or fragments of liposomal membrane to the cell surface without true incorporation;

5) Contact-mediated release of the material incorporated in the lipidic and/or the aqueous phases of the liposome without any need for internalisation of the liposome; and

6) Any combination of the above mechanisms (Campbell 1980; New et al 1990; Lasic 1998).

Investigations into the liposome interaction with cells have started some 35 years ago shortly after liposomes were first proposed and tested by Gregoriadis (1976) as a drug delivery system. The mode of liposome interaction with the target cell membrane seems to be partly determined by the liposome physical characteristics (membrane fluidity, charge and size), and the nature of the target cell. Juliano and stamp (1975) observed a difference in rate of serum clearance for large multilamellar liposomes and small unilamellar ones. They concluded that large multilamellar liposomes were more rapidly cleared from the circulation than small unilamellar vesicles. Serum has been reported to affect the stability of liposomes and their ability to interact with cells (Bonte and Juliano 1986) in addition to suppressing the transfection efficiency of cationic liposomes (reviewed by Oku et al 2001). Deol and Khuller (1997) reported that serum proteins have more affinity to positively charged liposomes and observed higher leakage in cationic liposomes compared with the anionic liposomes. In order to obtain a more realistic understanding of liposome behaviour towards cells, therefore, *in vitro* experiments should be conducted in the presence of serum.

3.1.1.1. Phagocytosis/endocytosis

Cells with phagocytic activity take liposomes up into endosomes. These endosomes fuse with lysosomes where cellular digestion takes place. Lysosomal enzymes disintegrate liposomes and the liposomal phospholipids become hydrolysed to fatty acids, which can then be incorporated into host phospholipids (New et al 1990). During the process of liposome breakdown in lysosomes, the contents of the liposomal aqueous and/or lipidic compartment(s) are released, after which they will either remain sequestered in the lysosomes until exocytosis (particularly if they are highly charged at low pH), or they will slowly leak out of the lysosome and gain access to the rest of the cell (Campbell 1980). It should be noted that, the low pH and enzymes of the lysosomes are able to inactivate the liposomal encapsulated agent(s) (Lasic 1998).

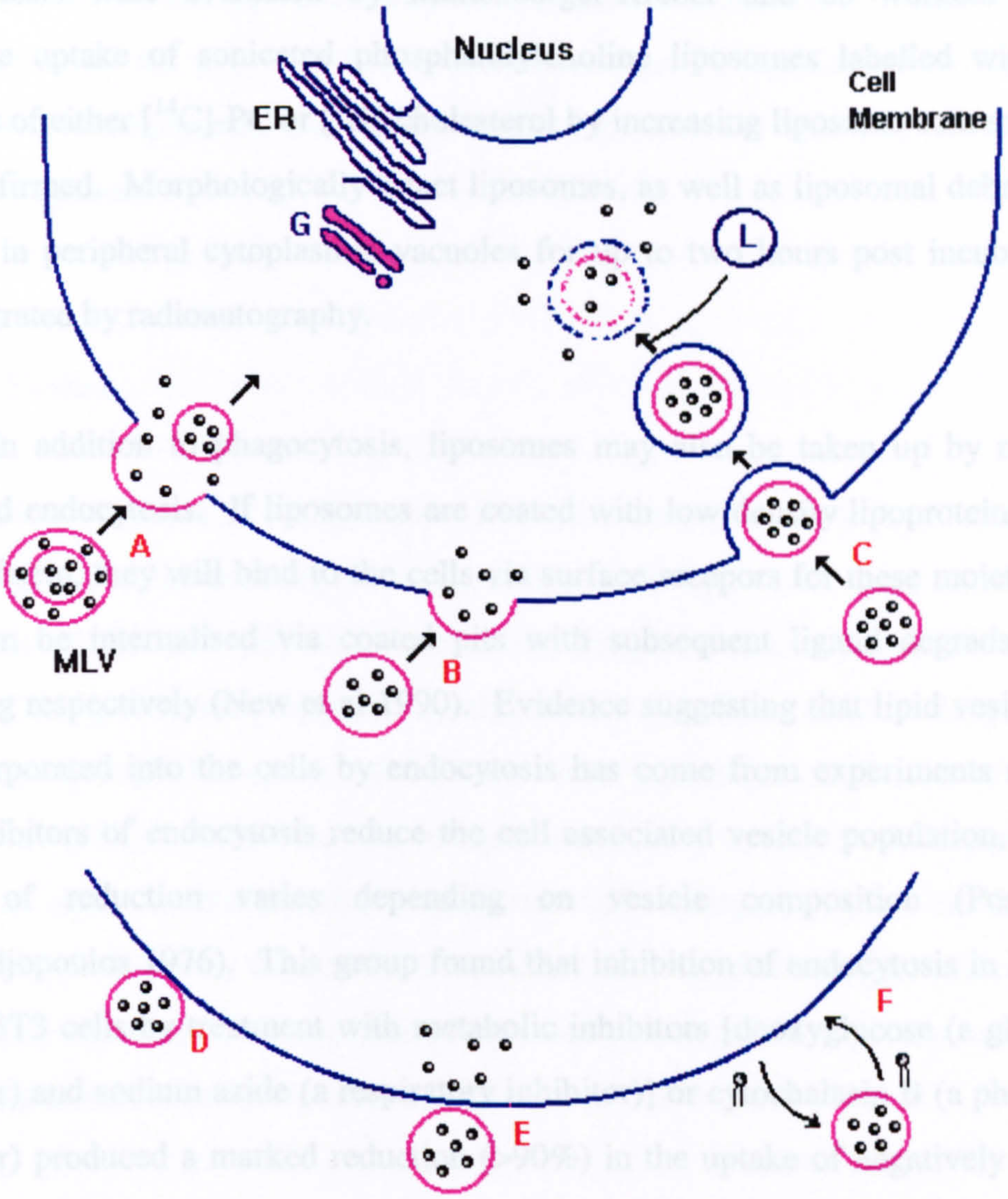


Figure 3.1. Schematic representation of the main modes of liposome interaction with cells. A: Fusion of the outermost bilayer of a multilamellar vesicle (MLV) - or a multivesicular liposome (MVL) - with the cell membrane and release of the inner vesicle(s) as well as the entrapped drug into the cytoplasm. B: Fusion of a unilamellar vesicle and concomitant release of the contents. C: Endocytosis/phagocytosis of liposomes followed by lysosomal (L) digestion and possible release of the entrapped material. D: Adsorption of liposomes on the cell membrane without uptake and with complete retention of aqueous and lipid contents within the liposome, separate from the cell. E: Contact-release of the material incorporated in the liposomal lipidic or aqueous phases without liposome internalisation. F: Lipid transfer mechanism through which lipid molecules are exchanged between the liposomal and cell membranes. Secondary intracellular processes may concentrate the liposomal materials within cell organelles such as golgi bodies (G), lysosomes (L) or endoplasmic reticulum (ER).

The kinetics of liposome uptake by mouse peritoneal macrophages via phagocytosis were evaluated by Mattenberger-Kreber and co workers (1976). Saturable uptake of sonicated phosphatidylcholine liposomes labelled with trace amounts of either [^{14}C]-PC or [^{3}H]-cholesterol by increasing liposome concentrations was confirmed. Morphologically intact liposomes, as well as liposomal debris, were evident in peripheral cytoplasmic vacuoles for up to two hours post incubation as demonstrated by radioautography.

In addition to phagocytosis, liposomes may also be taken up by receptor-mediated endocytosis. If liposomes are coated with low density lipoproteins (LDL) or transferrin, they will bind to the cells via surface recepors for these moieties, and will then be internalised via coated pits with subsequent ligand degradation, or recycling respectively (New et al 1990). Evidence suggesting that lipid vesicles can be incorporated into the cells by endocytosis has come from experiments showing that inhibitors of endocytosis reduce the cell associated vesicle population, and the extent of reduction varies depending on vesicle composition (Poste and Papahadjopoulos 1976). This group found that inhibition of endocytosis in BALB/c mouse 3T3 cells by treatment with metabolic inhibitors [deoxyglucose (a glycolysis inhibitor) and sodium azide (a respiratory inhibitor)] or cytochalasin B (a phagocytic inhibitor) produced a marked reduction (>90%) in the uptake of negatively charged small unilamellar vesicles and large multilamellar vesicles that were solid at 37°C (PS:DPPC:DSPC). The same treatments were much less effective in reducing the cell associated vesicle population (<35%) for vesicles of similar charge (anionic) and size composed of fluid phospholipids (PS:PC), indicating that fluid liposomes may interact with cells via non-endocytic mechanism(s). Similar findings showing that endocytosis is not a major pathway in the interaction of charged fluid vesicles with cells have also been reported in studies with anionic fluid vesicles in which the negative charge is conferred by dicetylphosphate (Weissman et al 1977).

Endocytosis has also been postulated as a main mechanism of the interaction of liposome-DNA complexes with cells. After the lipid-DNA complex gains entry into cells by endocytosis (Zabner et al 1995), it destabilises the endosomal lipidic

layer components. In the case of cationic liposome systems, it has been proposed that endosomal anionic lipids laterally diffuse into the lipid-DNA complex and form a charge neutral ion pair with the liposomal cationic lipids (Xu and Szoka 1996). This results in displacement of the DNA from the cationic lipid and release of the DNA into cytosol.

3.1.1.2. Fusion

Close approach of the liposomal and cell membranes can lead to fusion of the two (Bangham 1992) resulting in mixing of liposomal lipids with those of the plasma membrane of the cell. By this mechanism, drugs entrapped within the aqueous milieu of the liposome are injected directly into the cytoplasm, whereas drugs trapped within the hydrophobic space are delivered into the bilayer membrane of the cell (Dass et al 1997). Fusion may be brought about quite readily by incorporation of fusogens, such as lysolecithin, surfactants and detergents, into the liposome composition. However, these materials possess the risk of being quite toxic to cells, presumably because they continue to manifest their non-specific membrane perturbing effects after incorporation within the cell (New et al 1990).

In addition to composition, the preparation method of liposomes is an important parameter influencing their interaction with cells via fusion and/or other mechanisms. This is because the preparation method, and more precisely the procedures and instruments used during liposome preparation, determine, among other characteristics, the stability and release properties of the lipid vesicles. It has been suggested that unilamellar vesicles of DPPC produced by sonication are leaky, indirect evidence for which is provided by the work of Grant and McConnell (1973). They documented the loss of solute into the surrounding medium from liposomes during the process of fusion. Other groups have reported that some unilamellar liposomes fuse their membranes with those of the target cell and introduce their contents directly into the cytosol (Pagano and Huang 1975; Poste and

Papahadjopoulos 1976). Pagano and Huang (1975) determined that the kinetics of uptake of unilamellar liposomes were independent of the liposome charge.

A combination of both fusion and endocytosis for multilamellar vesicles (MLV) has been reported by Batzri and Korn (1975). It has been suggested that the fusion with the membrane of the target cell, leads to introduction of a multilamellar liposome without its outermost bilayer (Weissmann et al 1977). Internalisation of MLV in mouse spinal cells has been directly observed through electron microscopy (Azzazy et al 1995). Sessa and Weissmann (1968) reported that the outermost aqueous compartment, which accounted for about 10% of the total trapped volume of the MLV in their studies, merged its contents immediately with the cytosol, while the remaining aqeous compartment remained lipid bound. The intracytoplasmic liposome minus its outer bilayer may either be generally degraded in situ or may undergo a secondary fusion event with membrane organelles such as the lysosomes. Once liposomes are inside the cell cytoplasm (e.g. following fusion of a MLV with the plasma membrane) there is the possibility of the interaction of proteins with the lipid vesicles. Utsumi et al (1981) have found that cytoskeletal proteins such as actin, α-actin and tubulin have a strong tendency to associate with liposomes.

In experiments with the phagocytic soil bacterium, *Acanthamoeba castellanii*, Batzri and Korn (1975) have observed that the phospholipid composition of the liposome determines whether uptake by phagocytosis or fusion predominates. At 28°C, egg phosphatidylcholine liposomes and DMPC (dimyristoylphosphatidylcholine) liposomes favour uptake via phagocytosis while DPPC (dipalmitoylphosphatidylcholine) and DSPC (distearoylphosphatidylcholine) liposomes favour uptake by fusion. Positively charged egg phosphatidylcholine liposomes were more avidly taken up than neutral or negatively charged liposomes, but positively charged DPPC vesicles displayed only slightly preferential stimulus for uptake as compared to neutral or negatively charged DPPC liposomes. Phagocytic uptake was inhibited by either 4°C incubation or 2,4-dinitrophenol. The uptake by *A. castellanii* of unilamellar DPPC liposomes, presumably by fusion, resulted in the loss of 60% of the aqeous contents (D-[^{3}H]-glucose) of the vesicles. This observation

agrees with the findings of Grant et al (1973) who observed a 96% loss of entrapped solute upon fusion of unilamellar DPPC liposomes with *Acholeplasma Laidlawii*. The finding that the prokaryotic cells take up cationic vesicles more readily is contrary to the results obtained for the eukaryotic cells which were observed to take up anionic vesicles more avidly (Heath et al 1985; Monkkonen et al 1994; Katragadda et al 2000). These works emphasis the importance of the type and nature of the cell on the mechanism of liposome-cell interactions.

3.1.1.3. Lipid exchange

In addition to fusion and endocytosis, another possibility - i.e. lipid exchange - has been proposed as a mechanism for liposome-cell interaction. Lipid exchange is a long-range interaction that involves the exchange of liposomal lipids for the lipids of various cell membranes. It depends on the mechanical stability of the liposomal bilayer and can be reduced by 'alloying' the liposomal membrane with cholesterol (which gives rise to greatly improved mechanical properties, such as an increased stretching elastic modulus, resulting in stronger membranes and reduced permeability) (Lasic 1998).

Scherphof et al (1978) provided evidence for egg phosphatidylcholine exchange between liposomes (irrespective of size) and high density lipoproteins and consequent leakage of an aqueous compartment marker into surrounding medium. Black and Gregoriadis (1976) observed cholesterol exchange between liposomes and serum components. Hellings et al (1974) reported the purification of a protein from beef heart that catalyses phospholipid exchange between liposomes. Similar proteins, which transfer lipid molecules from one membrane to another, have been found in other organs including liver and lung (Zilversmit 1984) as well. It has been demonstrated that phospholipids transfer between two vesicle populations, not by collisional processes, but, rather, as monomers (or multimers) diffusing through aqueous solution (Roseman and Thompson 1980; Nichols and Pagano 1981). However, although the process occurs via diffusion, the physical characteristics of

both the donor and the acceptor vesicles determine the net rate of transfer. This is due to the fact that the vesicle physical properties determine both the rate of association and the rate of dissociation of the monomer from the bilayer membrane.

Pagano and co workers (1974, 1975) carried out *in vitro* experiments with cells that do not normally engage in phagocytosis (Chinese hamster lung cells). They have shown that uni- and multilamellar vesicles exchange membrane lipids and trapped solutes with cells via vesicle-cell fusion and vesicle-cell lipid exchange. At 37°C, the vesicle-cell fusion interaction predominates, but at 2°C, or when recipient cells are depleted of energy stores, vesicle-cell lipid exchange becomes significant. The fusion event was depicted by the cellular association of both the aqueous compartment, containing [^{3}H]-inulin, and the phospholipid component labelled with [^{14}C]-PC, in exactly the same proportions as exists within liposomes. In contrast, under conditions favouring lipid exchange, the phospholipid marker became cell associated while the aqueous compartment marker did not.

3.1.1.4. Adsorption

Another major liposome-cell interaction mechanism is adsorption of liposomes to the cell membrane. It can often occur with little or no internalisation of either aqueous or lipid components of liposomes (New et al 1990). In adsorption, liposomes attach to cell surfaces when the attractive forces (e.g. electrostatic, electrodynamic, van der Waals, hydrophobic insertion, hydrogen bonding, specific 'lock-and-key', etc.) exceed the repulsive forces (e.g. electrostatic, steric, hydration, protrusion, ondulation, etc.) (Lasic 1998).

Magee and co workers (1974) suggested that positively charged liposomes initially interact with negatively charged cells via adsorption and are subsequently internalised by fusion or endocytosis. The interaction of charged liposomes with the cell membrane, presumably by electrostatic attraction (Magee et al 1974) appears to be altered by α-globulin (a serum component) possibly by alteration of the liposome

charge. Black and Gregoriadis (1976) have shown that some serum components interact with liposomes in such a way as to alter the charge on the liposomes.

Adsorption may also take place as a result of binding by specific receptors to ligands on the vesicle membrane. It is thought that physical adsorption of liposomes may occur through binding to a specific cell surface protein (Pagano and Takeichi 1977). Adsorption is a prerequisite for ingestion of the liposome by cells, but it is not fully understood what factors determine whether or not a liposome is consumed thereafter by pinocytosis or phagocytosis. Leserman et al (1981) have shown that attachment of liposomes to cell membranes via certain surface proteins, but not others, can result in rapid uptake into the cell. In some cases, the liposomes may remain passively adsorbed on cell surface indefinitely (New et al 1990).

3.1.1.5. Contact release

Contact-release of aqueous contents of liposomes occurs by a poorly understood mechanism in which contact with the cell causes an increase in permeability of the liposome membrane. This leads to release of water-soluble solutes in high concentration in the closed vicinity of the cell membrane, through which these solutes may, under certain circumstances, then pass. Cell-induced leakage of solutes has been observed to be greater in membranes with cholesterol concentrations above 30 mol % (Van Renswoude and Hoekstra 1981). This phenomenon can provide a very effective means for introducing materials into specific cells without the need for ingestion of the whole liposome, and would be of particular value for cells which are not effectively phagocytic. The method will work best under conditions where flow and turbulence of the medium surrounding the cells is reduced, and where physical interactions between liposomes and cells are strengthened by means of receptor-ligand binding between the two membranes. Whether this process takes place to a significant extent may depend upon membrane composition as well as the nature of the compounds themselves (New et al 1990).

3.1.2. Liposome toxicity

Liposomes, generally being composed of ingredients from natural sources, are accepted as being biocompatible and relatively non-toxic (Van Rooijen and Nieuwmegen 1983; Stewart et al 1992; Storm et al 1993; Mercadal et al 1995; Cheng and Scheule 1998). Due to this reason, probably, the number of reports on the toxicity evaluation of liposomes (without drug) is limited and works are mainly devoted to compare the toxicity of free drug with that of liposome incorporated drug. Some examples of the drugs the toxicity of which have been studied in comparison with the liposome incorporated form of the same drugs are valinomycin (Daoud and Juliano 1986), amphotericin B (Juliano et al 1987), doxorubicin (Mayer et al 1989; Gabizon 1992), anamycin (Zou et al 1995), isoniazid and rifampicin (Deol and Khuller 1997) and paclitaxel (taxol) (Ceruti et al 2000). It was reported that the liposome encapsulated forms of the above mentioned drugs were less toxic than the free forms of the drugs.

However, there is also a need to evaluate the toxicity of the liposomes themselves to ensure the clinical suitability of these leading nanocarrier systems. Towards this end, the cytotoxicity of two types of lipid vesicles, namely conventional liposomes and HM-liposomes, each of two different sizes, with and without DNA and Ca^{2+}, as well as the cytotoxicity of their ingredients was evaluated in the human bronchial epithelial cell line 16HBE14o-. The reasons for using this cell line are outlined in the next section.

3.1.3. Rationale for the use of 16HBE14o- cell line

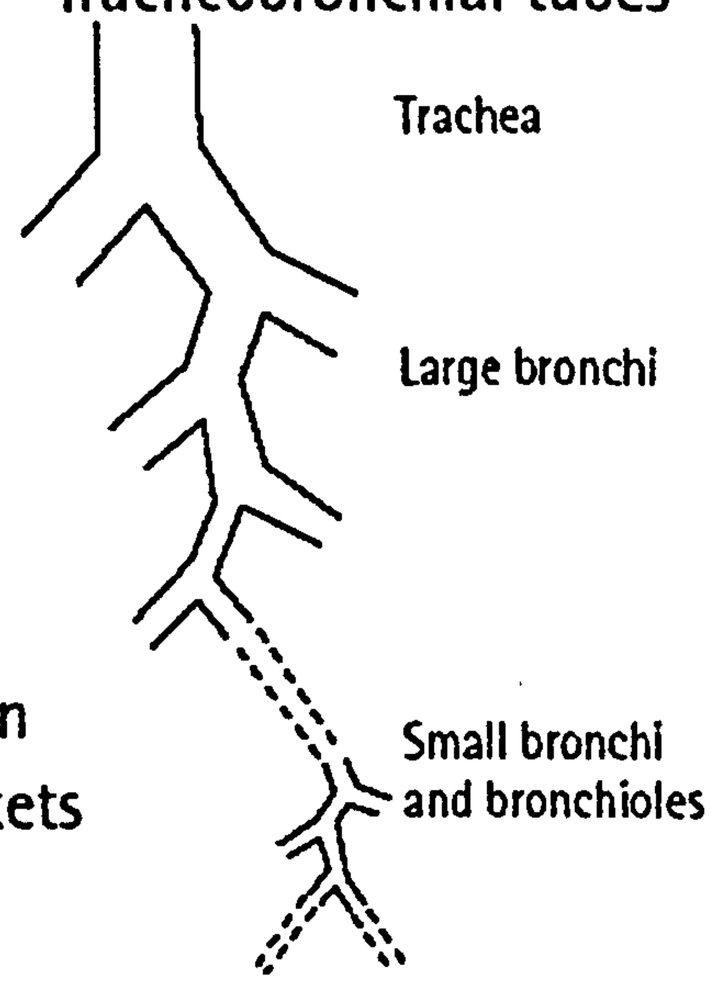
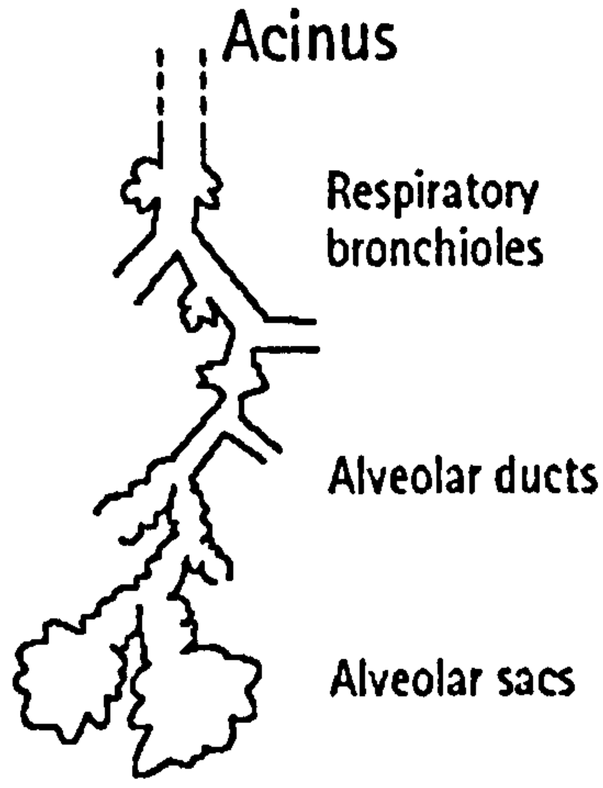
Inhalation to the lung is an increasingly important route for drug delivery. In 1998, more than 20% of the drug delivery products sold worldwide were aimed at the central regions of the lung, for the treatment of asthma, chronic obstructive pulmonary disease and other bronchial-related disease (Forbes 2000). In addition, there are pharmaceuticals in development for delivery to the peripheral lung

primarily for systemic absorption rather than local delivery. Direct targeting of the lung results in an immediate onset of drug action and reduced side effects. The permeability of the lung to a variety of drugs, including peptides and proteins, has also been recognised for some time (Enna and Schanker 1972; Patton and Platz 1992). Gene therapy is another rapidly emerging area, and several inherited and acquired lung diseases are potentially treatable through the delivery of therapeutic genes to the respiratory epithelium (Curiel et al 1996). An overview of human lung structure, given below, will help us in the choice of an appropriate *in vitro* model for pulmonary drug application studies.

3.1.3.1. Human respiratory tract

The respiratory tract is usually considered as two distinct regions; the central conducting airways and peripheral alveolar regions (Table 3.1.). Deposition of pharmaceutical aerosols occurs primarily via impaction at airway bifurcations, gravitational sedimentation within the airway tubules and diffusion within the alveoli. The alveolar epithelium is extremely thin and has an extensive surface area which is patrolled by alveolar macrophages. The airway epithelium is pseudostratified, features leakier tight junctions and a smaller surface area than the alveolar epithelium, and is protected by a mucociliary clearance mechanism. The human airway epithelium is composed of at least six distinct epithelial cell types: ciliated cells, mucous goblet cells, Clara cells, serous cells, basal cells and dense core-granulated cells (Harkema et al 1991; Gruenert et al 1995). The cellular composition of the epithelium varies substantially between species and at different levels of the lung between the trachea and the terminal bronchioles (Harkema et al 1991). Ciliated cells, mucous goblet cells and Clara cells make up almost the entire surface of the epithelium. Basal cells and dense core-granulated cells do not form part of the epithelial surface under normal conditions, and the other cell types, including serous cells, represent <5% of the epithelial cells (Gruenert et al 1995).

Table 3.1. The airways and alveolar region of the pulmonary tract (adapted from Forbes 2000).

Structure of the lung	Airways	Alveolar region
	Tracheobronchial tubes	Acinus
Airway generation is shown in brackets		
Primary function	Transport of air to the gas-exchange regions	Gas exchange
Mechanisms of aerosol deposition	Impaction and sedimentation (particles 3–10 μm)	Sedimentation and diffusion (particles <3 μm)
Epithelium	Pseudostratified, ciliated, mucus-secreting epithelium	Squamous epithelium

The human airway epithelial surface principally consists of ciliated cells, which are the most abundant cell at all levels of the airways (Sturgess 1989; Gruenert et al 1995). In the higher airways, the ciliated cells are interspersed by secretory

cells, mainly mucus-secreting goblet cells. At lower levels the ciliated cells are interspersed mainly by Clara cells. Considering this distribution of epithelial cells, the ciliated cell is clearly the most appropriate single cell-type for modelling of the airway epithelium. Diagrammatic representations of the typical epithelium in the higher airways, at the bronchiolar level and in the alveolar region are illustrated in Figure 3.2.

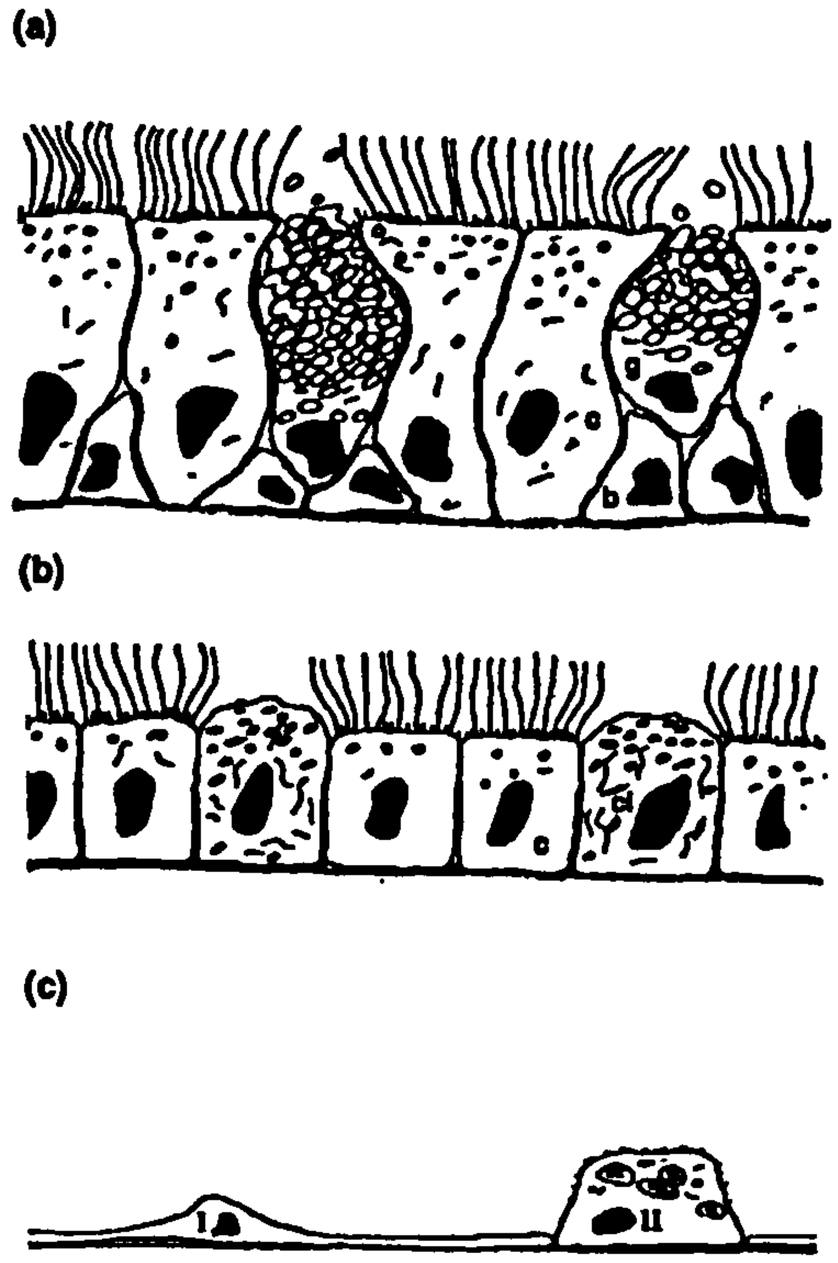


Figure 3.2. Typical lung epithelia showing the main cell types: a) the bronchial epithelium showing the pseudostratified nature of the columnar epithelium, principally consisted of ciliated cells, interspersed with goblet cells and the basal cells (designated as c, g and b respectively); b) the bronchial epithelium representing the cuboidal nature of the epithelium mainly composed of ciliated cells and interspersed with Clara cells (c and cl respectively); c) the alveolar epithelium showing the squamous nature of the epithelium, comprised of the extremely thin type I cells (I), which accounts for approximately 95% of the epithelial surface and the cuboidal type II cells (II) (from Forbes 2000).

3.1.3.2. Importance of airway epithelial cell lines

For the treatment of lung diseases, pharmaceutical aerosols are a well established means of localised drug delivery. The most frequently used aerosols

include small amounts of bronchodilator or steroid delivered directly to the lung to treat obstructive airway diseases. The major site of deposition in the lung from current pharmaceutical aerosol devices is the airways, where the epithelium is both the principal barrier to drug absorption and the first cellular metabolic barrier encountered by inhaled compounds. Recent improvements in delivery device technology have created much optimism over using inhalation to the deep lung for the delivery of drugs with poor oral absorption to the systemic circulation (Service 1997; Patton 1998). Despite the popularity of inhalation as a drug delivery route, assessing the fate of inhaled compounds is difficult because of the inaccessibility, delicate nature and complex structure of the lung. The interpretation of results obtained in animal and tissue models is complicated by inter-species variation, imprecise delivery of drug to the lung and concerns over preparation viability. Airway cell lines are more convenient models in which to assess absorption mechanisms and cytotoxicity issues, compared with intact lung models which are not usually capable of discerning such properties. In general, the pharmaceutical industry relies on appropriate *in vitro* models for the evaluation of absorption, metabolism and toxicity of drugs. The advantages of using cell culture models include:

- Small amounts of compounds are required for experiments,
- Easier and more economical than *in vivo* experiments and reduces animal usage,
- Rapid, with a high throughput capacity,
- Provides mechanistic information about epithelial transport,
- Environmental conditions, such as temperature and pH, can be controlled,
- Drug analysis is simplified by the use of aqueous buffer solutions (Forbes 2000).

The value of epithelial cell culture is best illustrated by reference to the well characterised Caco-2 cell line, the foremost cell culture model of intestinal drug absorption (Artursson and Borchardt 1997; Mainprize and Grady 1998). Caco-2 cell monolayers are used to study drug transport mechanisms, assess absorption enhancement strategies, and predict oral bioavailability (Brayden 1997; Polli and Ginski 1998). Caco-2 cell monolayers are also used in high throughput screening programmes and to generate data for regulatory purposes. Compared with the

development of the intestinal cell culture models, interest in the use of cell lines to model the respiratory epithelium is comparatively recent. At present there is no lung equivalent of the Caco-2 cell line to serve as a well-established *in vitro* model of the respiratory epithelium (Forbes 2000; Forbes et al 2003). A summary of the general applications of epithelial cell culture models, outlined in Box 3.1., illustrates their potential value in the development of dosage forms.

Box 3.1. Summary of the applications of epithelial cell culture models in the design and development of drugs (Forbes 2000).

- Estimation of permeability characteristics of drug candidates,
- Deduction of drug transport pathways,
- Determination of structure-activity relationships for active transport and efflux processes,
- Determination of optimal physicochemical characteristics for passive absorption,
- Assessment of chemical strategies for absorption enhancement,
- Elucidation of drug metabolism,
- Rapid assessment of potential toxic effects of drug candidates or formulations.

To usefully predict the fate of compounds delivered to the lung, a model of the airway epithelium should reflect the drug transport characteristics and metabolic activity encountered *in vivo*. Primary cultures of mixed populations of human airway epithelial cells provide the closest *in vitro* representation of the airway epithelium. However, the lack of availability of normal human airway tissue, the limited amount of cells generated by primary cell culture, and donor variation are major limitations. It has been detected that cultured human surface airway epithelial cells have a limited life span and senesce or terminally differentiate (Lechner and Laveck 1985; Gruenert et al 1990). Despite the optimisation of culture conditions, the mucociliary

differentiation of primary cultures becomes significantly impaired after two to three passages (Gray et al 1996). Major factors that promote the differentiation of airway epithelial cells in culture are the extracellular matrix (such as collagen gel), the composition of the growth medium (such as inclusion of retinoic acid) and culture at an air-liquid interface (Gruenert et al 1995).

Cell lines have the advantage of providing a much more convenient and reproducible model than primary cell cultures. Airway cell lines derived from human airway epithelium are beginning to find use as drug transport models (Mathias et al 1996; Cavet 1997; Forbes and Lansley 1998; Rao et al 1998; Forbes et al 2003). Three airway-derived cell lines in particular have shown promise as *in vitro* models of the airway epithelium; BEAS-2B, 16HBE14o- and Calu-3. The 16HBE14o- cell line, used in this study, was developed by transformation of cultured bronchial-surface epithelial cells from a one-year-old male heart-lung patient (Cozens et al 1994). This cell line was developed to study the chloride channel activity of the cystic fibrosis transmembrane conductance regulator (CFTR) in normal airway epithelial cells (Cozens et al 1994). Early studies concentrated on the ability of airway-derived cell lines to model the physiological processes of the airway epithelium particularly ion transport, and characterisation of their cell biology (Van-Scott et al 1991). There has also been considerable interest in their use for pathophysiological studies, such as the response of the epithelium to injury and inflammatory mediators.

16HBE14o- and Calu-3 cell lines both form polarised cell layers and have been identified as two of the better differentiated airway-derived cell lines (Gruenert et al 1995) with potential as drug absorption models (Mathhias et al 1996). Both 16HBE14o- and Calu-3 cells were shown to express the proteins of the major intracellular junctions: functional tight junctions, desmosomes and zonulae adherentes (Forbes 2000). The Calu-3 cell line has tight junctions; the 16HBE14o- cell line has a similar morphology to native airway epithelia, including cilia and tight junctions (Mobley and Hochhaus 2001). The 16HBE14o- cells maintain, in addition to many differentiated epithelial morphological features, functional characteristics

including ability to generate transepithelial resistance across a confluent cell monolayer. Freeze-fracture electron microscopy of cultured 16HBE14o- cells has revealed extensive and well-formed tight junctional belts (Godfrey and Jeffrey 1998). Recently, polarised 16HBE14o- cell layers have been used to study drug transport (Forbes and Lansley 1998; Rao et al 1998; Forbes et al 2003) and gene delivery (Stern et al 1998). Furthermore, 16HBE14o- cells have been found to be a suitable cell line for *in vitro* respiratory toxicity evaluations (Westmoreland et al 1999) due to their aforementioned characteristics.

3.2. Materials and Methods

3.2.1. Chemicals

Dipalmitoylphosphatidylcholine (DPPC), dicetylphosphate (DCP), Cholesterol (CHOL), glycerol, 0.25% trypsin-EDTA solution, neutral red solution (NR, 3-amino-7-dimethylamino-2-methyl phenazine hydrochloride (3.3g/l)), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Co. (Dorset, UK). Minimum essential medium (EAGLE) containing Glutamax-1, fetal calf serum, and penicillin/streptomycin (10000 U/mL, 10000 µg/mL) were obtained from GibcoBRL® Life Technologies Ltd (Paisley, UK). The plasmid (pcDNA3.1/His B/*lac*Z, 8578 nucleotides) was supplied by Invitrogen (Nederlands). All solvents (chloroform, methanol, Analar grade) were obtained from Sigma Chemical Co. (Dorset, UK). All other chemicals were of commercial analytical grade.

Phosphate-buffered saline (PBS) was prepared by dissolving 4.3mM NaH_2PO_4, 1.47mM KH_2PO_4, 2.68mM KCl, and 137mM NaCl in distilled water and adjusting the pH to 7.4.

3.2.2. Experimental methods

3.2.2.1. Liposome preparation

3.2.2.1.1. Preparation of conventional liposomes

Conventional liposomes were prepared by the thin-film hydration method as follows: a chloroform/methanol (2:1, v/v) solution of DPPC/DCP/CHOL (7:2:1 molar ratio), containing 10mM total lipid, was evaporated to dryness in a round bottomed flask connected to a rotary evaporator (EYELA, New Rotary Vacuum Evaporator NE-1, Birmingham, UK), in a water bath at 50°C. To remove traces of the solvents the flask was flushed with nitrogen gas for 1 hour and also placed under vacuum for a time period of at least 1 hour. The lipid film was then hydrated, above the phase transition temperature (T_c) of the lipids, with 2ml sterile (autoclaved) phosphate buffered saline (PBS, pH: 7.4), and multilamellar vesicles (MLV) formed by vortex agitation or by hand-shaking the flask after adding glass beads of ~1-2mm diameter into the flask. The liposome suspension was extruded 11 times, with an extruder (LiposoFast™-Basic, Glen Creston Ltd, UK), above T_C through two-stacked polycarbonate filters of either 100 or 400nm pore size. The liposomes were either utilised immediately after preparation or stored under N_2 at 2-8°C until use.

3.2.2.1.2. Preparation of liposomes by the heating method

HM-liposomes with the same composition as the conventional liposomes were prepared as follows: DPPC, DCP and CHOL were hydrated in PBS and then heated up to 120°C in the presence of glycerol (3% v/v) in a silicone oil bath. Alternatively the heating step was performed employing a 12-well carousel reaction instrument (Carousel Reaction Station™, Rodleys Discovery Tech. Ltd., Essex, UK). The carousel instrument was fitted with a temperature controller (Carousel Temperature Controller RR98073) and magnetic stirrer. The carousel tube was connected to a nitrogen gas cylinder during the process. Formation of MLV at the

end of the heating stage was confirmed by light scattering and microscopic examinations and results are presented in chapter two. The effect of heating on the lipids has been checked by thin-layer chromatography and no degradation was detected as explained in chapter two (Mozafari et al 2002c).

In order to provide a more homogeneous population of liposomes with smaller diameters the liposomal suspension was extruded, as described in the previous section, through two-stacked polycarbonate filters of either 100 or 400nm pore size. The HM-liposomes were utilised shortly after preparation or stored under N_2 at 2-8°C until use.

3.2.2.2. Plasmid DNA

The plasmid pcDNA3.1/His B/*lac*Z (8578 nucleotides) (Invitrogen) grown in competent Escherichia coli DH5α cells, prepared by alkaline lysis, and purified by CsCl-EtBr density gradient ultracentrifugation (Sambrook et al 1989) was kindly provided by Mr A. R. Evans (Liverpool John Moores University, England, UK). The purity of plasmid preparations was determined by agarose gel electrophoresis and optical density (the $OD_{260/280}$ ratio was between 1.8 and 1.9). Plasmid concentration was measured by UV absorption at 260nm, by multiplying the absorbance of the sample by a factor of 50 (Muller et al 1993). The plasmid samples were stored at –20°C until use.

3.2.2.3. Incorporation of DNA to liposomes

The ternary complexes of liposome/Ca^{2+}/DNA (vesicles of which were prepared by the conventional technique) or HM-liposome/Ca^{2+}/DNA (vesicles of which were prepared by the heating method) were constructed by introducing plasmid (15µg / 285µg liposome) and then calcium (50mM) to the liposomal suspensions followed by incubation of the mixture for 30min under N_2 at room

temperature. Formation of similar complexes has been confirmed and well documented in our previous studies by light scattering, UV spectrophotometry, spectrofluorometry and different microscopic techniques (Mozafari 1996; Zareie et al 1997; Mozafari and Hasirci 1998; Mozafari et al 1998a, 1998b).

3.2.2.4. Cells

Immortal human respiratory epithelial cells (16HBE14o-), kindly donated by Dr A. R. Evans (Liverpool John Moores University, England, UK), were maintained at 37°C, 5% CO_2 and 95% humidity in T75 tissue culture flasks (Starstedt, Leicester, UK). Complete growth media (cMEM) consisted of 89% (v/v) minimum essential medium (MEM, EAGLE) with Glutamax-1 supplemented with fetal calf serum (10% v/v) and 1% (v/v) penicillin/streptomycin (10000 U/ml, 10000 µg/ml). One T75 flask of cells was routinely sub-cultured (passaged) into two new flasks when the cells were almost 90% confluent. In order to passage the cells, the cMEM was removed from the flasks and the cells were washed twice with 20ml PBS. The adherent cells were removed from the flasks by trypsinisation. For this trypsin-EDTA solution (0.25%, 5ml) was added and the cells incubated for 10min (at 37°C, 5% CO_2 and 95% humidity), by which time the cells could be loosened by gentle tapping of the flask. Cell dissociation from the flask surface was confirmed by observation through light microscopy. The activity of trypsin was neutralised by addition of 10ml cMEM. The clusters of aggregated cells were broken up by pipetting several times using a 10ml disposable pipette to obtain an homogenous cell suspension. Two new T75 flasks were then seeded by the addition of cell suspension (5ml) and cMEM (7.5ml) to each flask. After day one and day four the culture medium was removed and replaced with 12.5ml cMEM per flask. These cells were usually almost 90% confluent after 6 days by which time they were either passaged into new flasks or seeded into 96-well plates (BDH, Leicester, UK). The cells were sub-cultured (passaged) no more than 50 times. When the cells were required to be seeded in 96-well plates (for toxicity tests) a flask grown to almost 90% confluence was trypsinised and neutralised and then diluted with cMEM to a total volume of

45ml. This volume of cells (approximately 50000 cells/ml) was enough to seed two 96-well plates containing 200µl medium/sample solution per well.

3.2.2.5. Toxicity assays

HBE cells were plated in 96-well plates (BDH, Leicester, UK) and used for toxicity tests at 50-60% confluency (approximately 50000 cells/ml). The cells were treated with the reagents, diluted with cMEM to provide the required concentration, and incubated for 24 hour prior to toxicity assessment. *In vitro* cytotoxicity was evaluated using the Neutral Red Uptake (NRU) method of Borenfreund and Puerner (1985) and the MTT method of Mosmann (1983) except that both methods were performed simultaneously in the same microplate and the incubation time with NR and MTT was 2 hours. This 'parallel NRU/MTT assay' was developed recently by Evans (2003) in our laboratory. Six of the 96-well plate columns were allocated to the NRU and the other six to the MTT assay as explained in the following sections. Cytotoxicity of all the ingredients of the liposomes individually (i.e. DPPC, DCP, and CHOL each dissolved in corn oil by vortex mixing), liposomes prepared by conventional method and HM-liposomes (each of two sizes of 100 and 400nm) with and without calcium and plasmid, as well as the cytotoxicity of Ca^{2+} was evaluated as explained above. The concentration range used for each reagent except calcium was from zero to 87.5µg/ml which covers liposome concentrations used in most of the gene transfer studies. The concentration range for calcium was from zero to 250mM, well above the 50mM concentration used in the gene transfer formulation. Triton X-100 (78 ppm) was used as positive control as recommended by Evans (2003). Viability was expressed as a percentage of the control untreated cells. All toxicity experiments were repeated on a minimum of three independent occasions.

3.2.2.5.1. Neutral Red Uptake (NRU) assay

Neutral Red (NR) is a weak, cationic, water-soluble dye that is taken up by viable, uninjured cells and accumulates in the lysosomes. It can be detected and quantified spectrophotometrically upon lysis of the cells (Borenfreund and Puerner 1985). NR solution was diluted in cMEM to give a working solution of 50µg/ml and then incubated for 24 hours at 37°C. Any NR crystals that may have formed during this incubation period were filtered out using a 0.2µm syringe filter prior to the assay. After removal of medium from those wells of the 96-well plate allocated to the NRU assay, 100µl of the NR-cMEM mix was added to each well and the plate incubated (at 37°C, 5% CO_2 and 95% humidity) for two hours to allow the NR to be taken up by the cells. After this incubation period, the NR-cMEM mix was removed from each well and the cells were washed with PBS (200µl/well) to remove any excess NR. After adding NR destain (1% glacial acetic acid in 50% ethanol, 100µl/well) the 96-well plate was shaken on an orbital shaker for 15min to achieve a homogenous colour in each well. Absorbance of each well of the plate was determined at 540nm using the microplate reader (Titertek Multiskan® MCC/340 MKII Labsystems, Finland). Absorbance readings from wells treated with the agents were compared to the readings of control wells (untreated cells). Results are expressed as percentages of control wells.

3.2.2.5.2. MTT assay

This procedure is basically that of Mosmann (1983) and utilises the fact that MTT, a yellow soluble tetrazolium salt, is taken up into cells and reduced by mitochondrial succinate dehydrogenase to an insoluble blue formazan product. This product accumulates in the cell, as it can not pass through the plasma membrane and can be detected and quantified spectrophotometrically upon lysis of the cells.

A stock solution of 5mg/ml MTT in PBS was prepared and stored at 4°C protected from light. This solution was diluted ten times in cMEM before each

application. After removal of medium from the wells allocated to the MTT assay, 100µl of MTT-cMEM mix was added to each well and the cells were incubated for two hours (at 37°C, 5% CO_2 and 95% humidity) to allow for reduction of the MTT. The MTT-cMEM mix was removed from each well and then isopropanol (100µl/well) was added as a destain. The 96-well plate was shaken on an orbital shaker for 15min to achieve a homogenous colour in each well. Absorbance reading from each well of the plate was determined at 540nm using the microplate reader (Titertek Multiskan® MCC/340 MKII Labsystems, Finland). Absorbance readings from wells treated with the agents were compared to the readings of control wells (untreated cells). Results are expressed as percentages of control wells.

3.2.2.6. Statistical analysis

Data are expressed as mean ± standard deviation of the mean from three or more experiments (performed on separate occasions and each time six wells were devoted to each sample). A two way analysis of variance of the viability data, taking account of liposome concentration and method of preparation was performed. A two sample t-test taking account only of the method of liposome preparation was also performed. All analyses were carried out using the Minitab statistical package (v 13.1, MINITAB Inc. PA, USA). A P value of less than 0.05 was considered to indicate a significant difference.

3.3. Results

Cytotoxicities of two liposomal formulations as well as two liposomal gene transfer vectors and their constituents were evaluated using a cell culture model of the human bronchial epithelial cells. Figure 3.3. is a microscopic image of a monolayer of the 16HBE14o- cells used in this study. The "cobblestone" appearance of these cells, as described by the originators of this cell line (Cozens et al 1994), can be seen in this Figure.

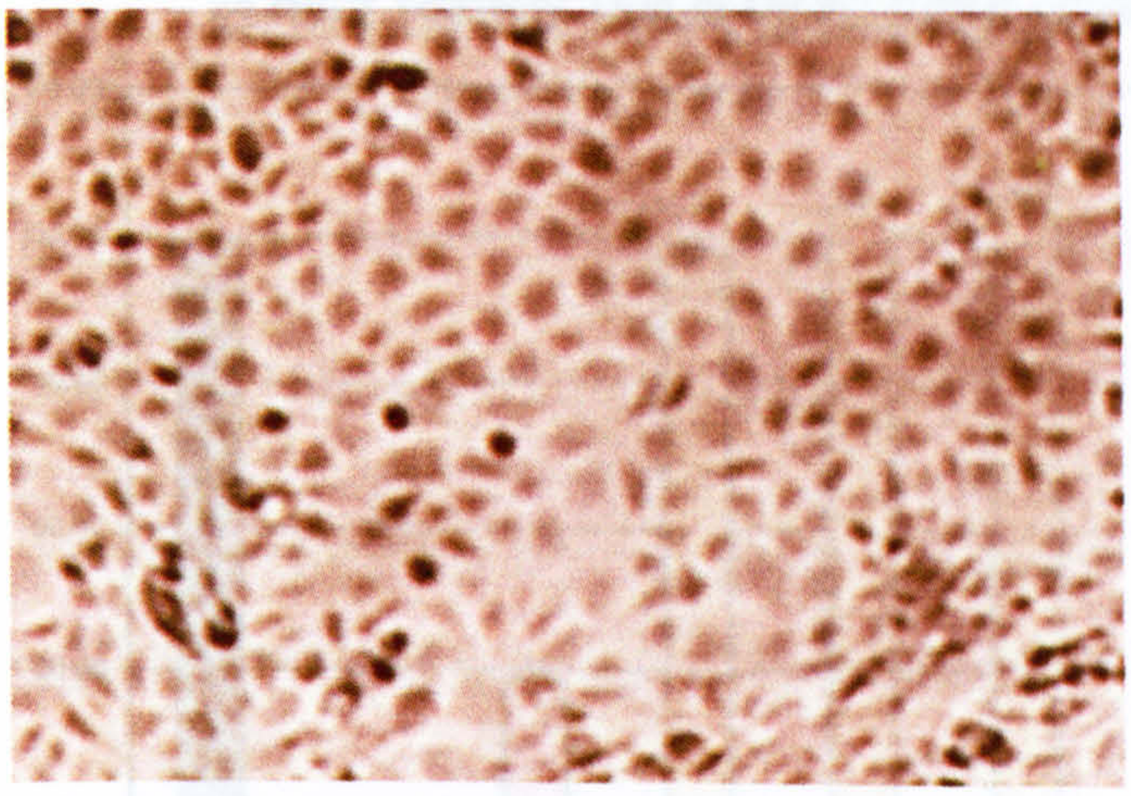
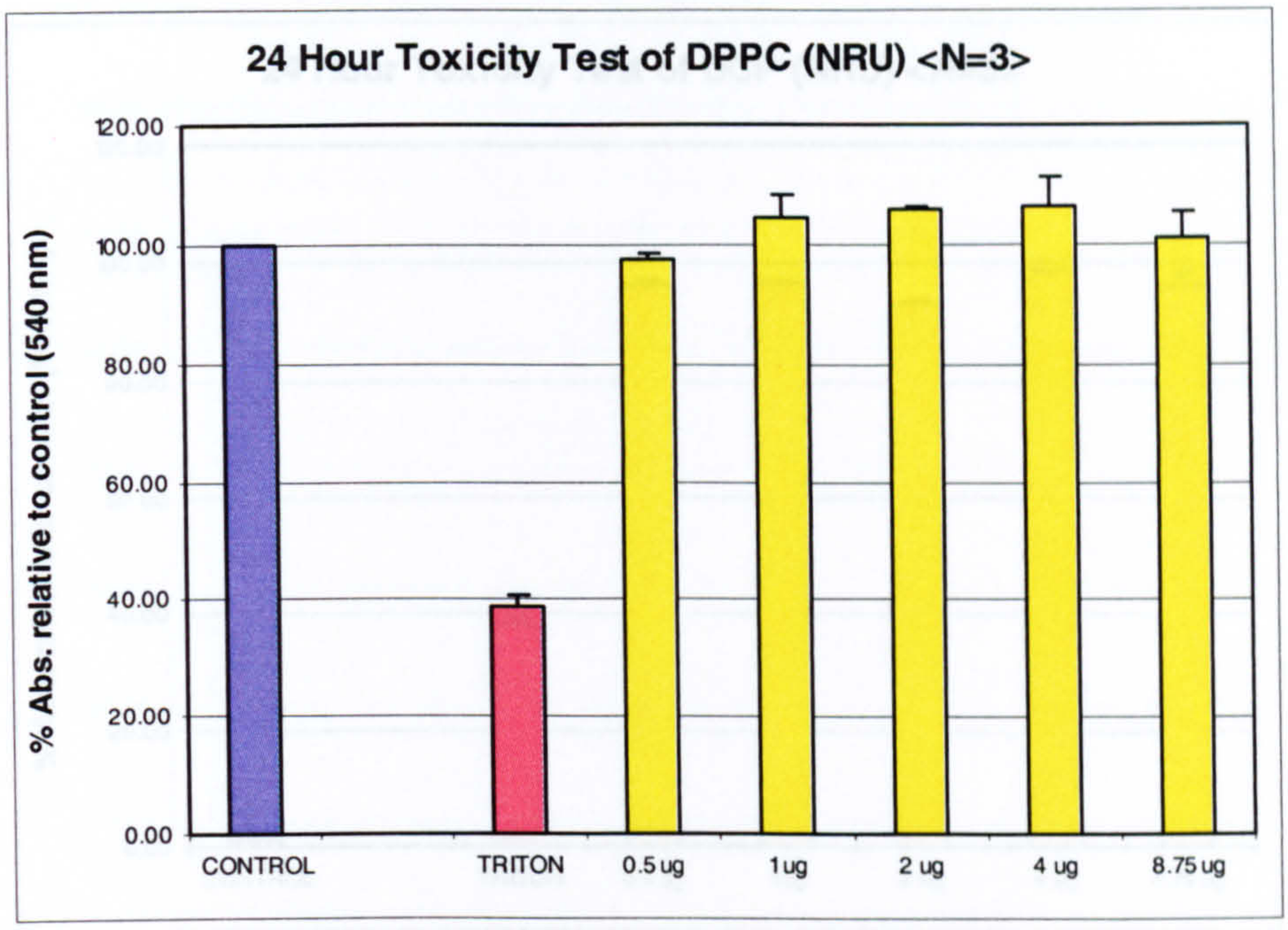


Figure 3.3. Microscopic image of the human lung epithelial cells (16HBE14o-) in T75 tissue culture flasks.

The cytotoxicity evaluation of the liposomal constituents DPPC, DCP and CHOL are depicted in Figures 3.4., 3.5. and 3.6. respectively. The overall data indicate that the liposomal constituents are well tolerated by the HBE cells as attested by both NRU (part a in each Figure) and MTT (part b in each Figure) assays. DPPC was found to cause a very slight loss in cell viability - i.e. ~2% according to NRU and ~4% according to MTT assay - at 0.5µg/ml while no cell death was detected at other concentrations (Figure 3.4.).

The negatively charged phospholipid DCP caused only 1-7%, as evaluated by the NRU, and 1-5%, as evaluated by the MTT assay, decrease in cell viability (Figure 3.5.). Cholesterol, on the other hand, was found to cause a slightly larger loss in cell viability - i.e. 0-10% according to NRU and 4-12% according to MTT assay - in the concentration range tested (Figure 3.6.).

a



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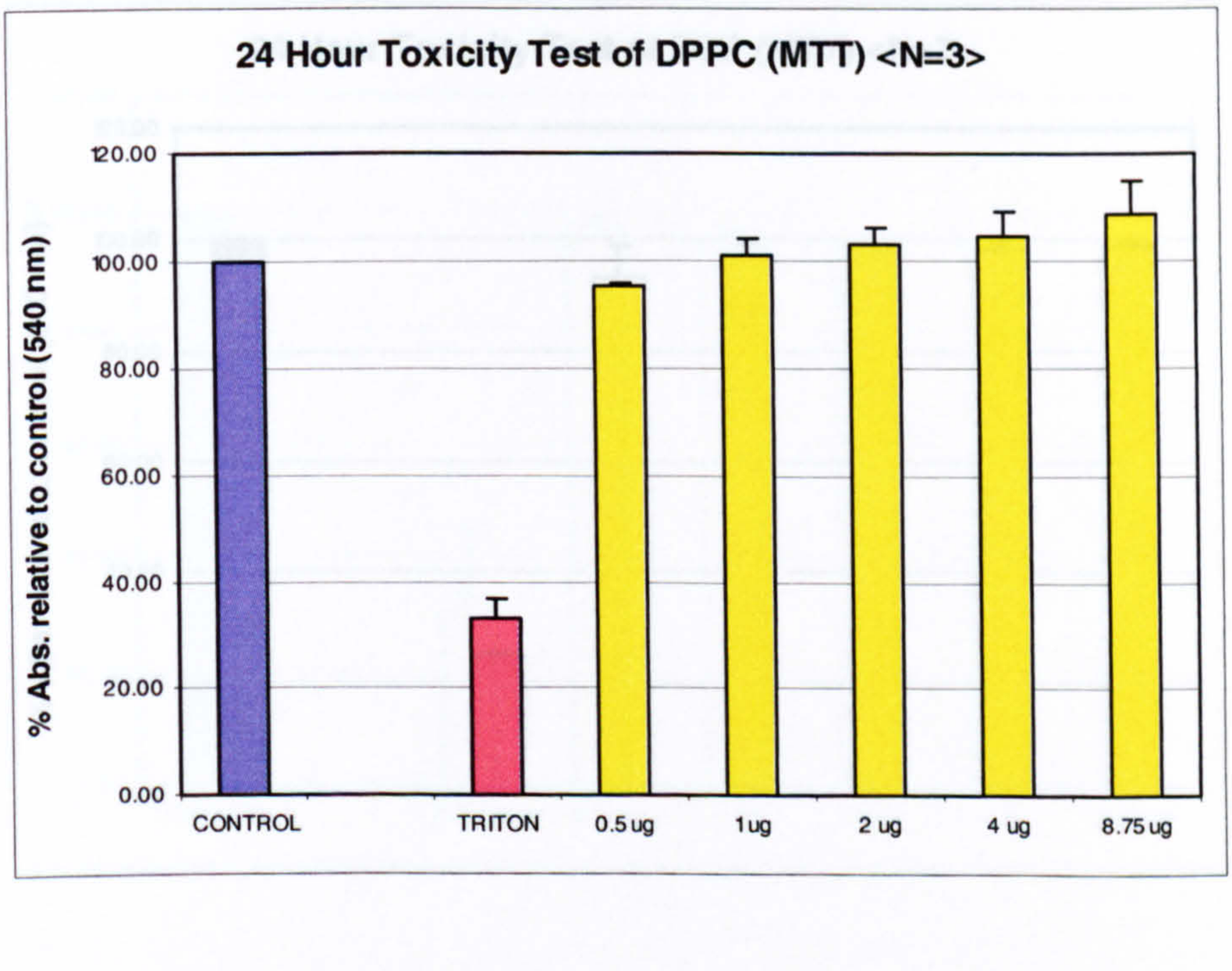
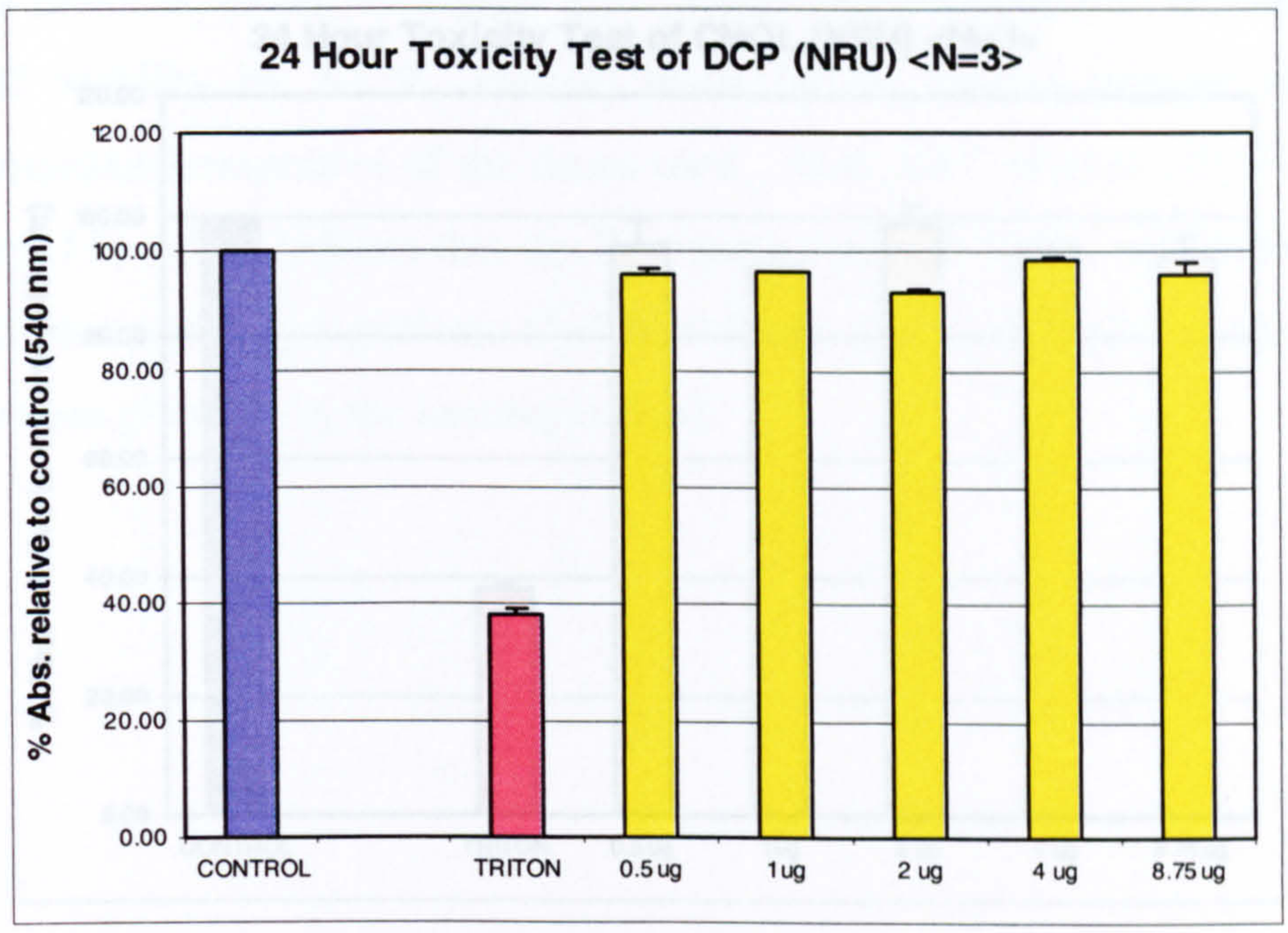


Figure 3.4. Cytotoxicity evaluation of DPPC in the HBE cells. Cell viability was determined after 24 hr by (a) neutral red, and (b) MTT assays. Triton X-100 (78 ppm) was used as positive control. Results represent means + S.D. of data obtained from at least three independent experiments.

a



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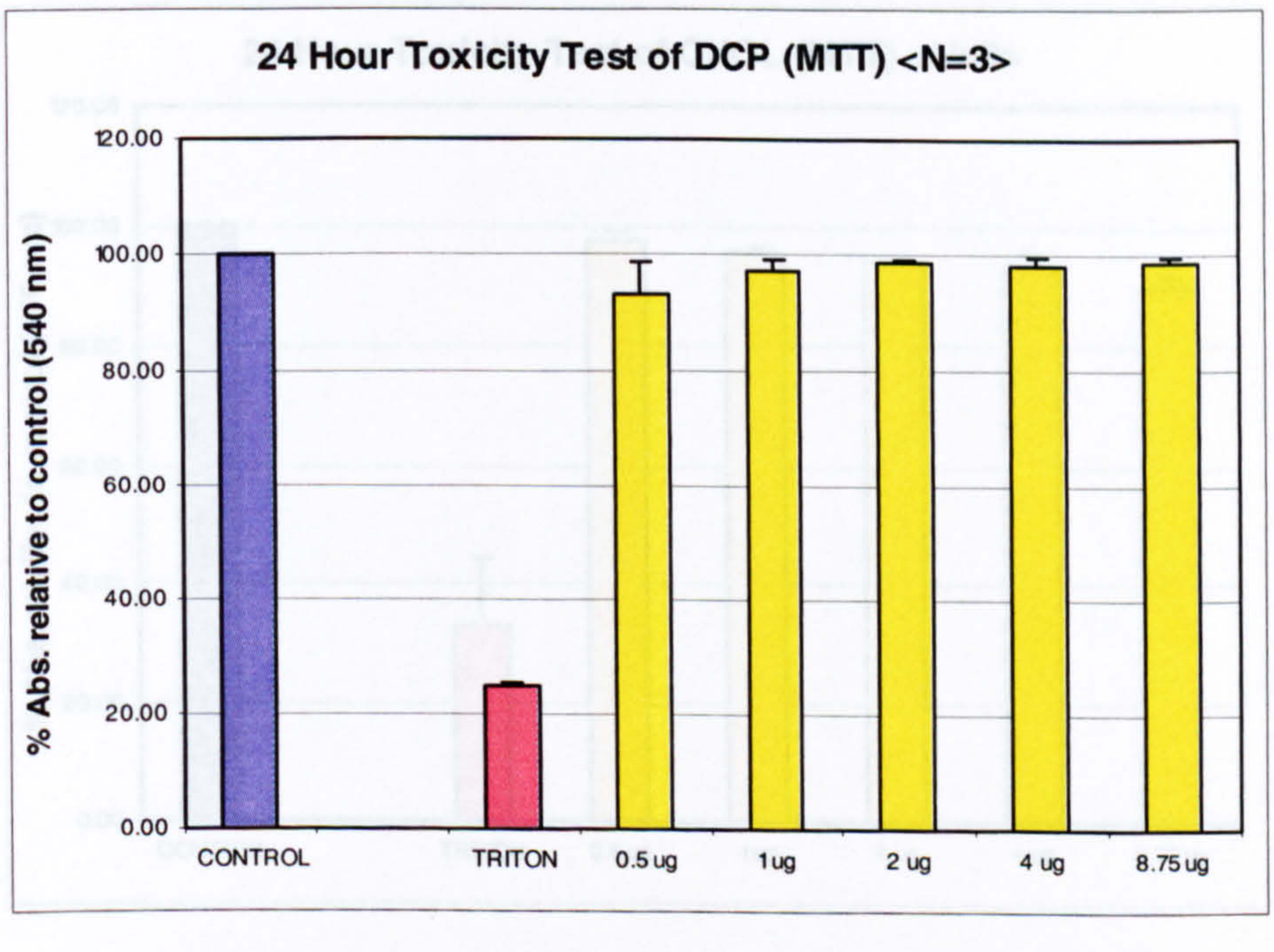
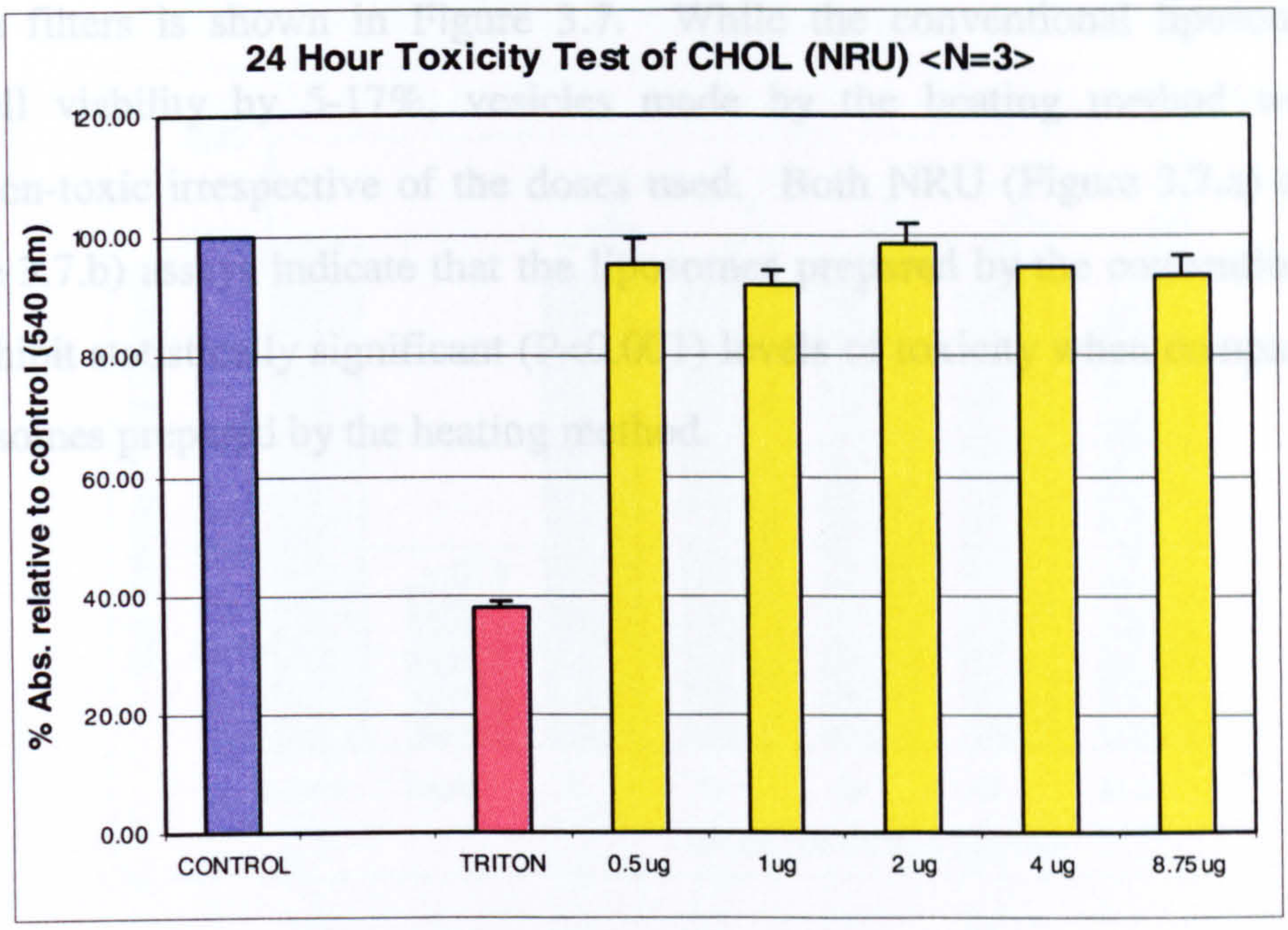


Figure 3.5. Cytotoxicity evaluation of DCP in the HBE cells. Cell viability was determined after 24 hr by (a) neutral red, and (b) MTT assays. Triton X-100 (78 ppm) was used as positive control. Results represent means + S.D. of data obtained from at least three independent experiments.

a



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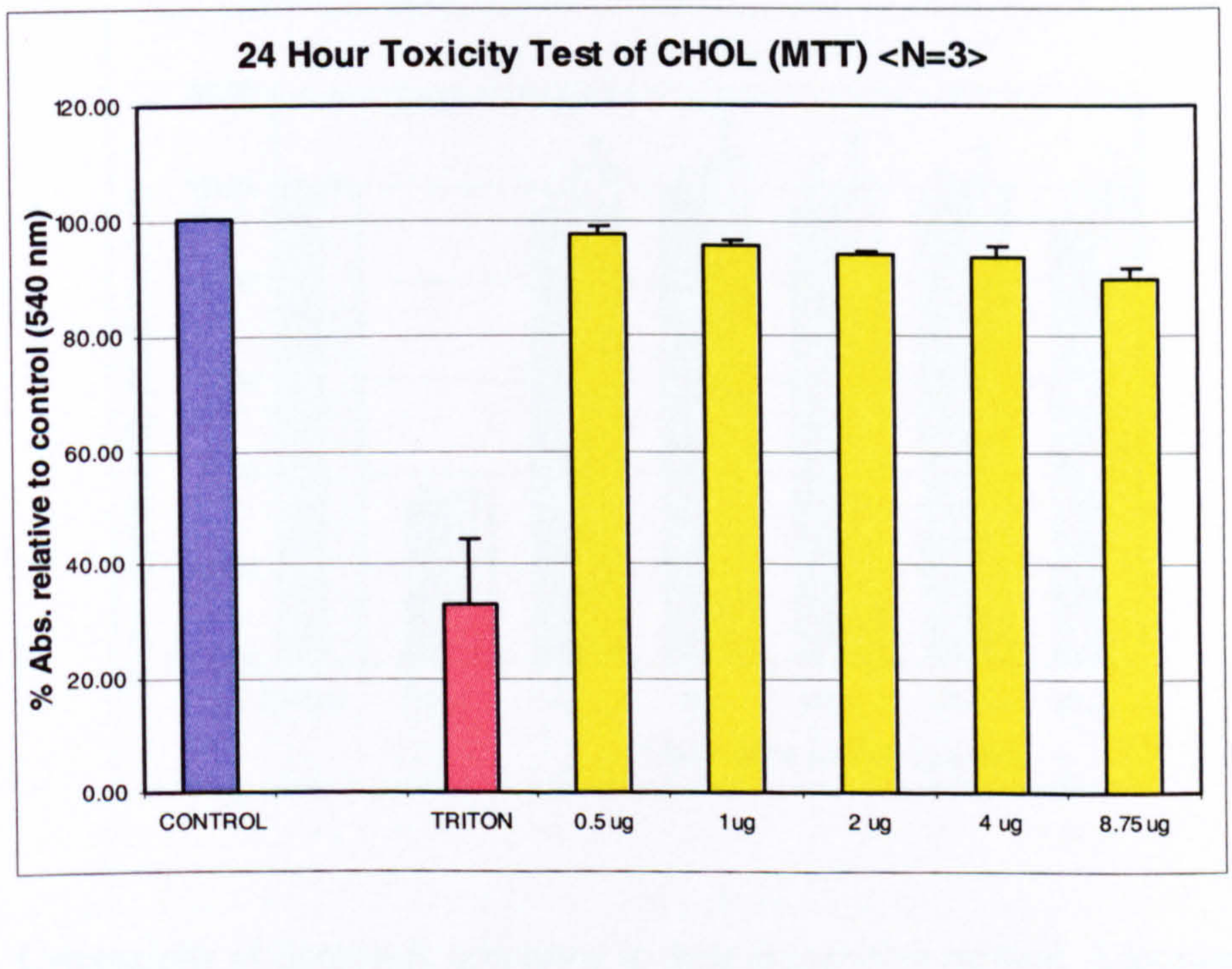
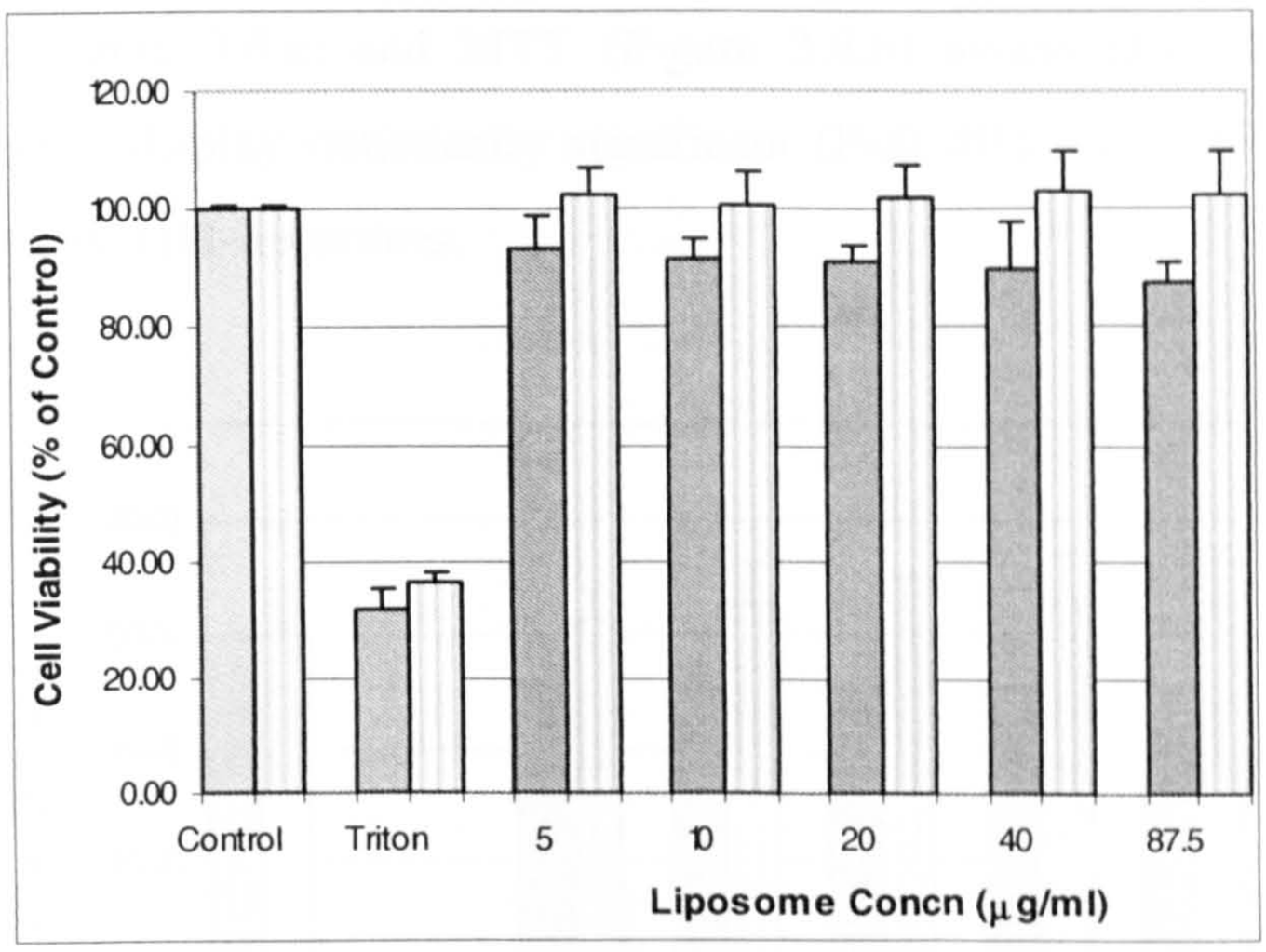


Figure 3.6. Cytotoxicity evaluation of CHOL in the HBE cells. Cell viability was determined after 24 hr by (a) neutral red, and (b) MTT assays. Triton X-100 (78 ppm) was used as positive control. Results represent means + S.D. of data obtained from at least three independent experiments.

The cytotoxicity evaluation of the two populations of liposomes prepared using 100nm filters is shown in Figure 3.7. While the conventional liposomes decreased cell viability by 5-17%, vesicles made by the heating method were completely non-toxic irrespective of the doses used. Both NRU (Figure 3.7.a) and MTT (Figure 3.7.b) assays indicate that the liposomes prepared by the conventional technique exhibit statistically significant ($P<0.001$) levels of toxicity when compared with the liposomes prepared by the heating method.

a



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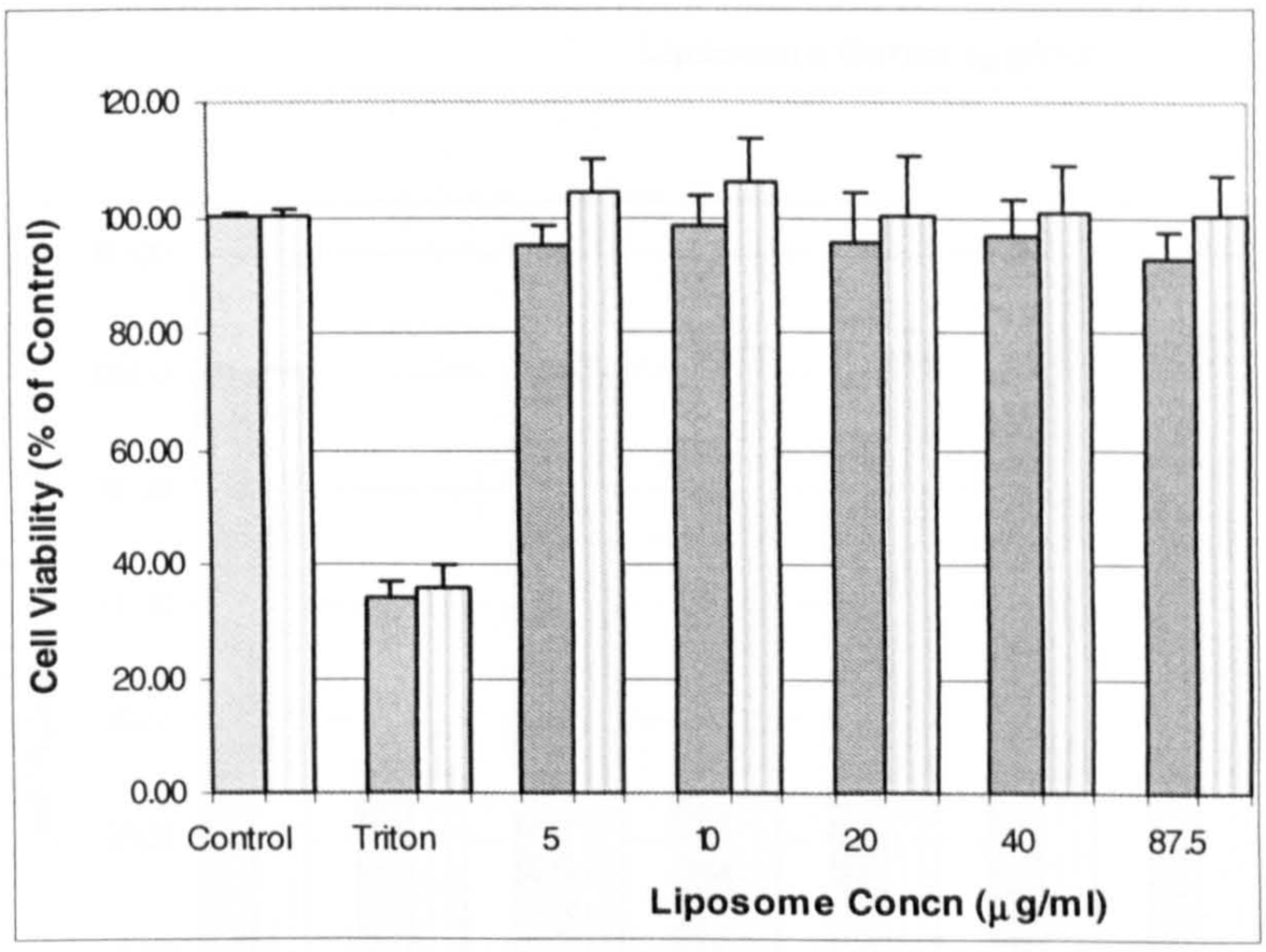
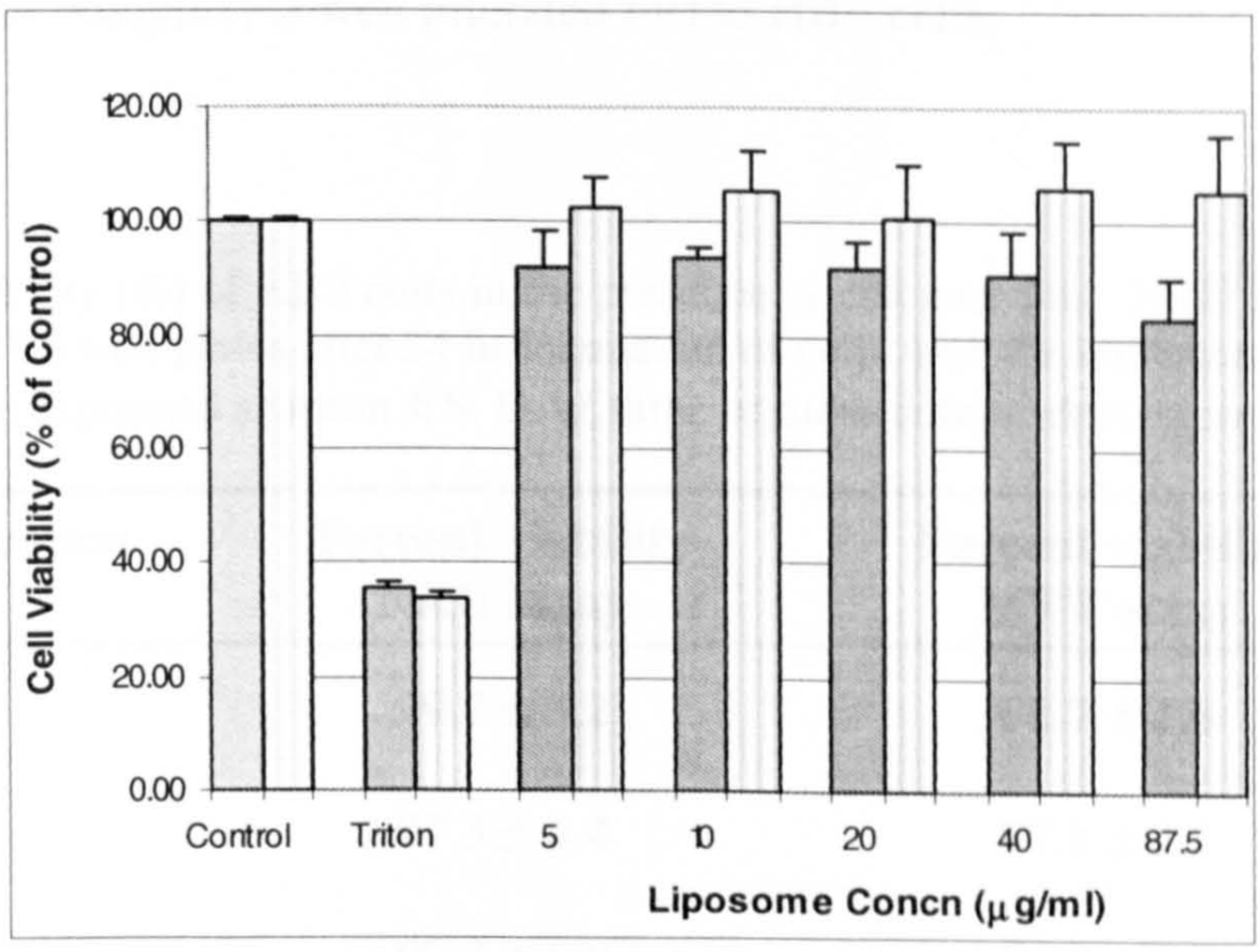


Figure 3.7. Cytotoxicity of liposomes according to their preparation method. Liposomes were prepared by extrusion through 100nm filters. Cell viability was determined after 24 hr by (a) neutral red, and (b) MTT assays. Normal bars: liposomes prepared by the conventional method; slashed bars: liposomes prepared by the heating method. Triton X-100 (78 ppm) was used as positive control. Results represent means + S.D. of data obtained from at least three independent experiments and the two liposome types differed significantly ($P<0.001$).

Figure 3.8. demonstrates cell viability studies of the cultured cells incubated with the conventional liposomes and HM-liposomes prepared using 400nm filters. Again both NRU (Figure 3.8.a) and MTT (Figure 3.8.b) assays attest that the conventional liposomes display statistically significant ($P<0.001$) levels of toxicity when compared with the HM-liposomes.

a



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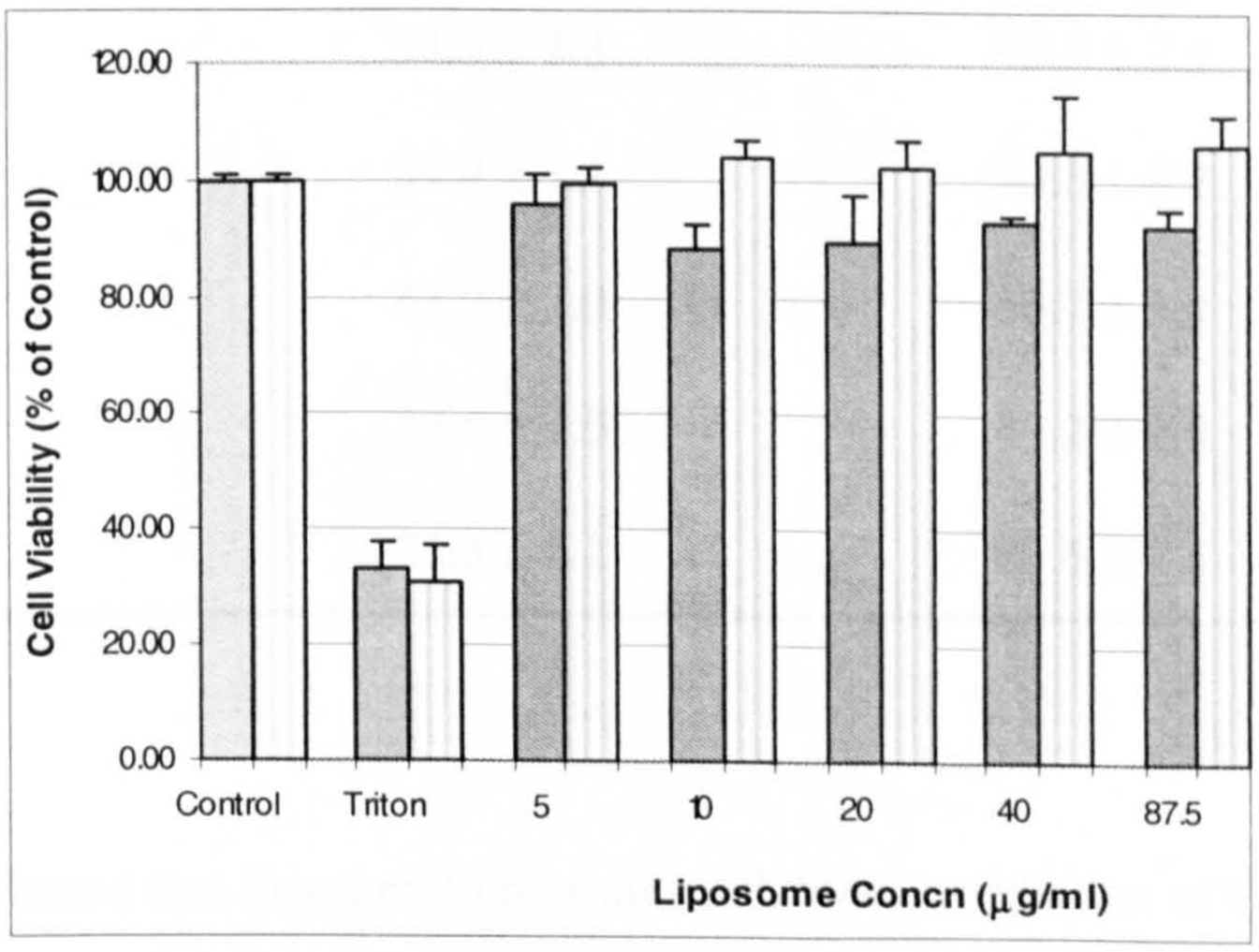


Figure 3.8. Cytotoxicity of liposomes according to their preparation method. Liposomes were prepared by extrusion through 400nm filters. Cell viability was determined after 24 hr by (a) neutral red, and (b) MTT assays. Normal bars: liposomes prepared by the conventional method; slashed bars: liposomes prepared by the heating method. Triton X-100 (78 ppm) was used as positive control. Results represent means + S.D. of data obtained from at least three independent experiments and the two liposome types differed significantly ($P<0.001$).

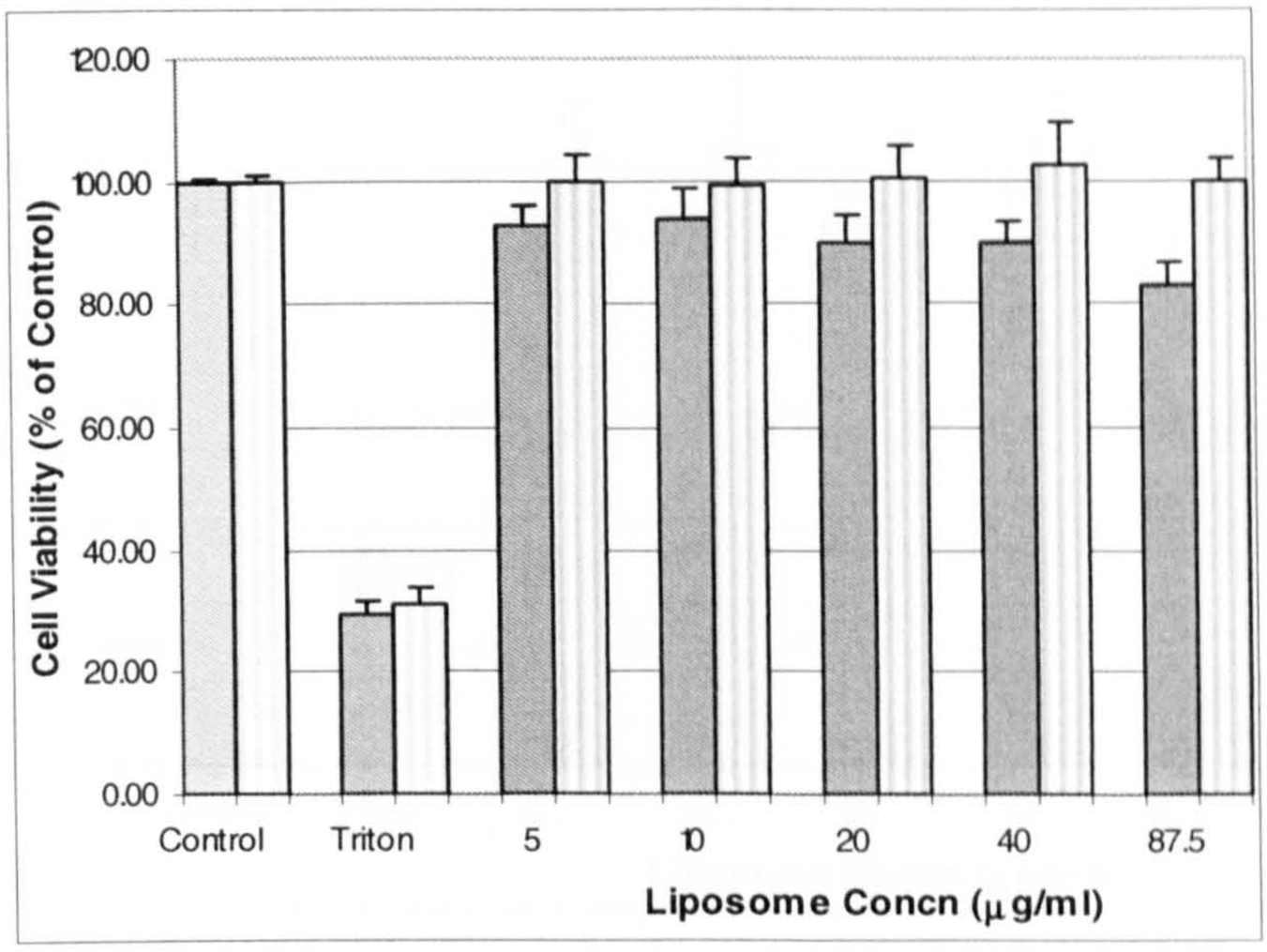
The effect of the incorporation of plasmid DNA into the anionic liposomes on their toxicity towards the HBE cells was also evaluated. For the incorporation of DNA molecules into the anionic liposomes calcium ions were employed. Table 3.2. shows the cytotoxicity of different concentrations of Ca^{2+} ions determined by NRU and MTT assays. Both assays confirm that 50mM calcium (the amount used in the liposome/Ca^{2+}/DNA complex) is well tolerated by the HBE cells.

Table 3.2. Cell viability (%) of HBE cells in the presence of calcium ions. NRU and MTT assays performed in 96-well plates after 24 hr incubation of cells with the indicated amounts of calcium. Data are expressed as mean ± S. D. of three or more independent experiments.

Ca^{2+} concentration (mM)	Percent viability NRU assay	Percent viability MTT assay
5	99.7 ± 3.2	98.7 ± 2.6
10	97.3 ± 4.4	97.1 ± 2.7
25	97.2 ± 0.8	94.8 ± 2.4
50	94.4 ± 2.8	93.3 ± 2.4
100	89.9 ± 7.1	90.6 ± 8.2
150	43.2 ± 2.0	56.7 ± 3.9
200	23.6 ± 2.5	43.2 ± 2.8
250	21.3 ± 2.1	23.6 ± 2.6

Results indicated that incorporation of plasmid, by the mediation of Ca^{2+}, into the two populations of liposomes used in this study did not significantly change their cytotoxicity. This was the same for 100nm (Figure 3.9.) and 400nm (Figure 3.10.) lipid vesicles as confirmed by both NRU (Figure 3.9.a and 3.10.a) and MTT (Figure 3.9.b and 3.10.b).

a



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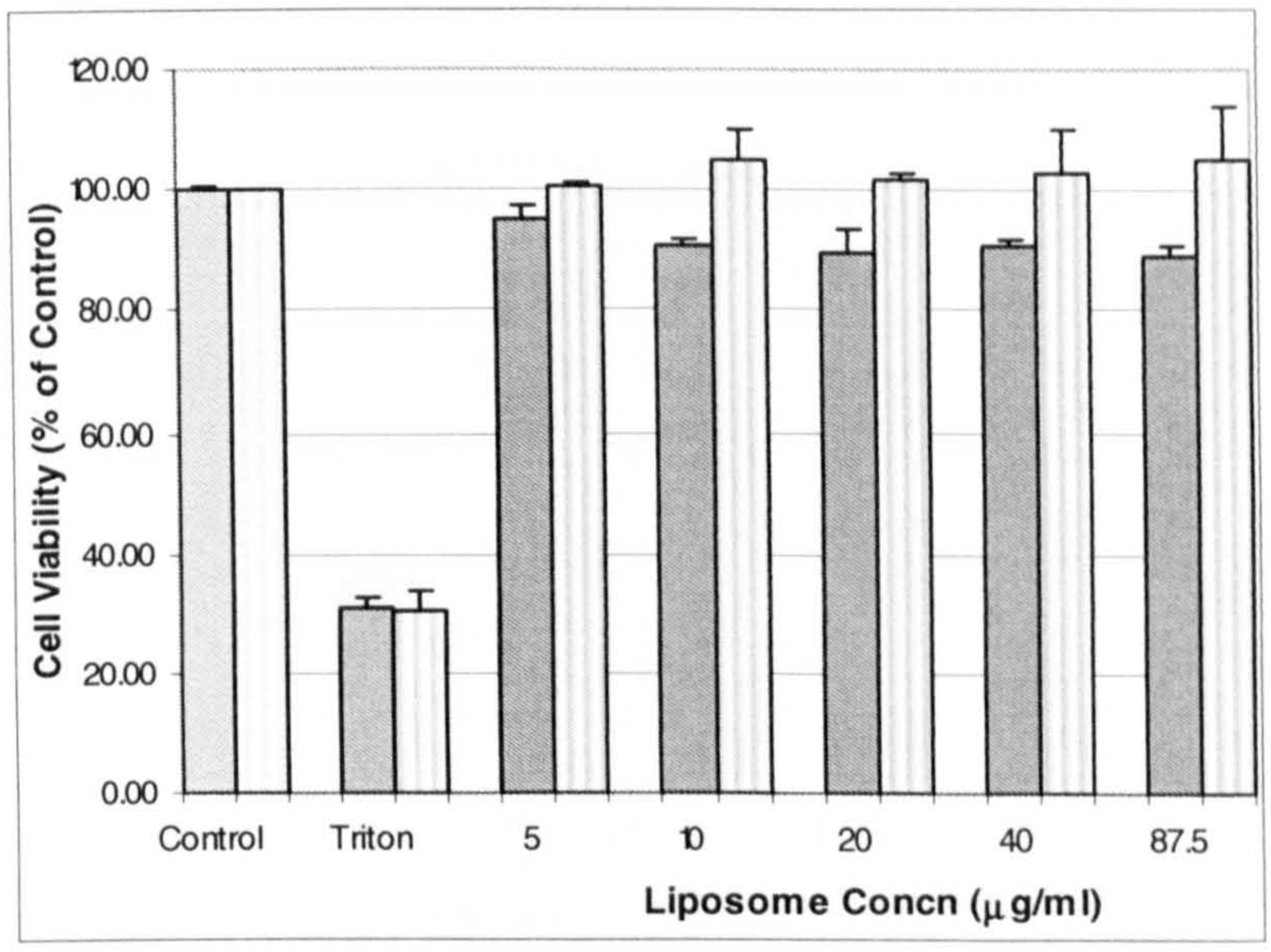
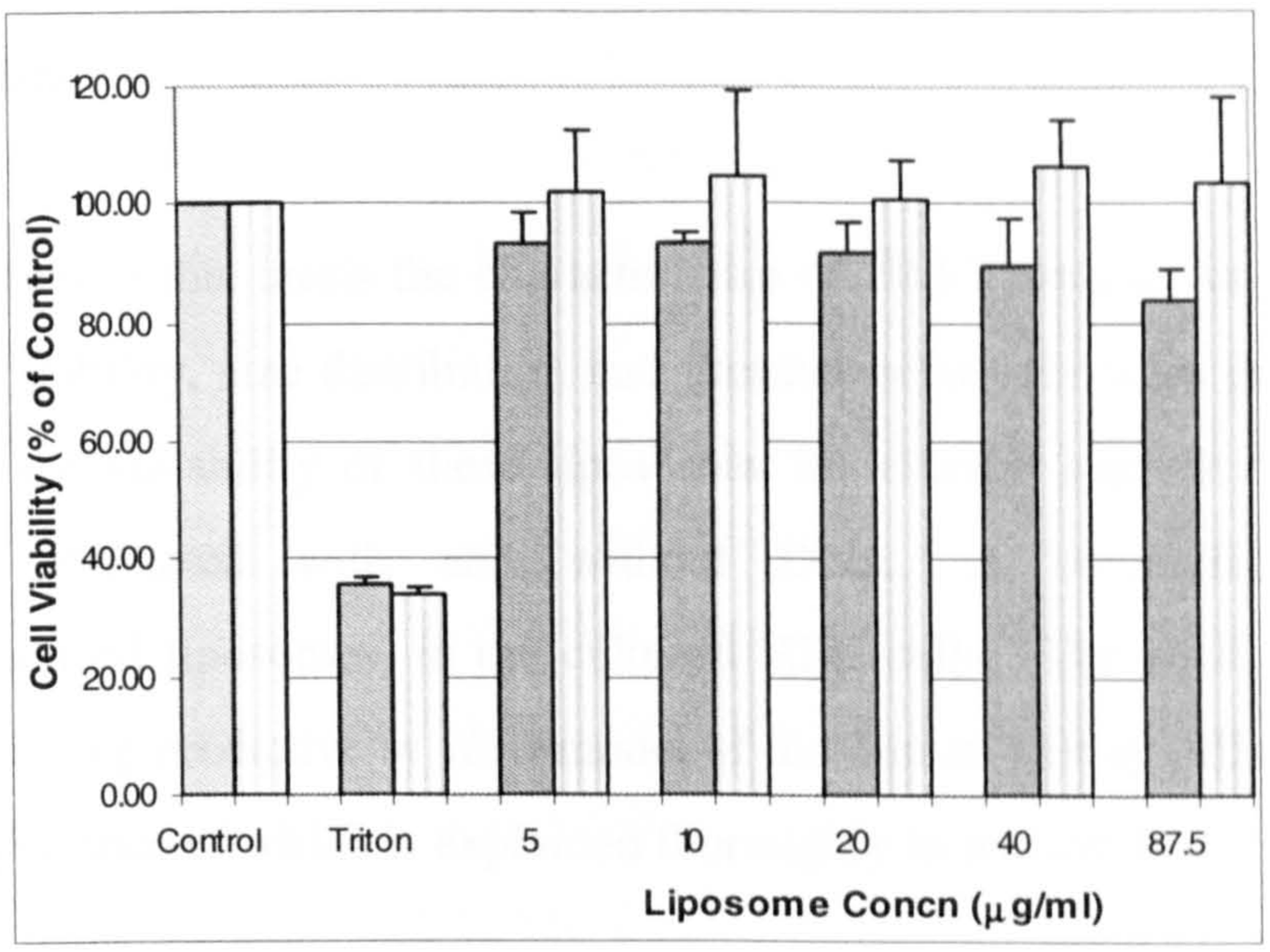


Figure 3.9. Cytotoxicity of liposome/Ca^{2+}/DNA complexes according to their preparation method. Liposomes were prepared by extrusion through 100nm filters. Cell viability was determined after 24 hr by (a) neutral red, and (b) MTT assays. Normal bars: liposomes prepared by the conventional method; slashed bars: liposomes prepared by the heating method. Triton X-100 (78 ppm) was used as positive control. Results represent means + S.D. of data obtained from at least three independent experiments and the two liposome types differed significantly ($P<0.001$).

a



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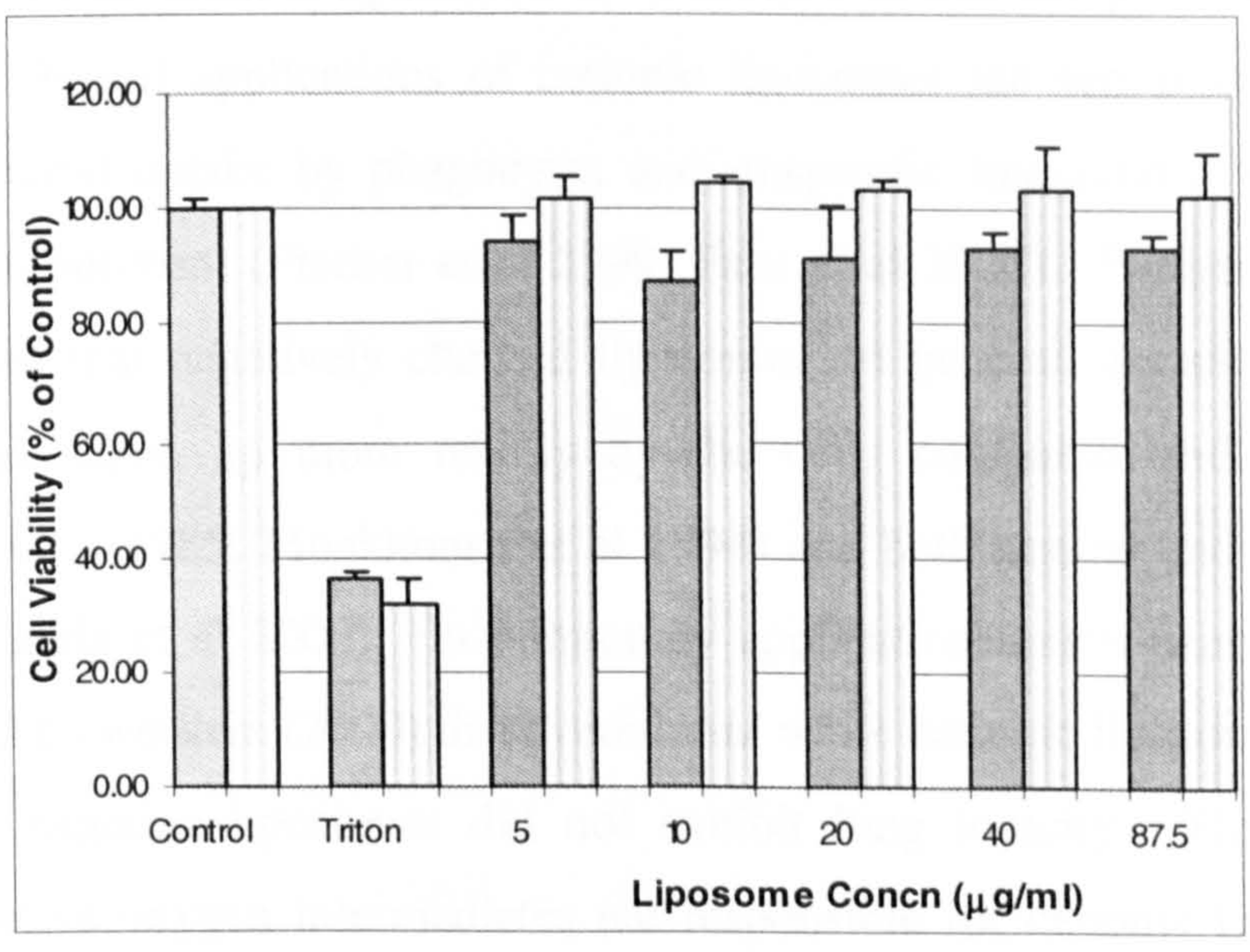


Figure 3.10. Cytotoxicity of liposome/Ca^{2+}/DNA complexes according to their preparation method. Liposomes were prepared by extrusion through 400nm filters. Cell viability was determined after 24 hr by (a) neutral red, and (b) MTT assays. Normal bars: liposomes prepared by the conventional method; slashed bars: liposomes prepared by the heating method. Triton X-100 (78 ppm) was used as positive control. Results represent means + S.D. of data obtained from at least three independent experiments and the two liposome types differed significantly ($P<0.001$).

3.4. Discussion

In chapter two of this thesis the characteristics of HM-liposomes in terms of their morphology, stability, size distribution and thermal behaviour were presented. To further assess the suitability of these liposomes for clinical applications their cytotoxicity was evaluated with and without DNA, in comparison with conventionally produced liposomes, in the cultured HBE cells. The HBE cell line was used as a promising predictive *in vitro* model of the human airway cells (Forbes et al 2003) the importance of which is explained thoroughly in section 3.1.3.

Due to several reports on the toxicity (Panzner and Jansons 1979; Chawla et al 1979; Campbell 1983; Filion and Phillips 1997, 1998; Dokka et al 2000; Nagahiro et al 2000) and other limitations (Litzinger 1997; Fischer et al 1999; Fahr et al 2002) of cationic lipid vesicles, anionic liposomes were utilised in this study. Among the limitations in the clinical applications of cationic liposomes are serum instability (Litzinger 1997), rapid uptake by phagocytes and unspecific interactions with the biological microenvironment (Fischer et al 1999; Fahr et al 2002). Furthermore, it has been postulated that negatively charged liposomes, in general, associate more effectively and are taken up more readily by the cells compared with neutral liposomes (Heath et al 1985; Monkkonen et al 1994) and both neutral and cationic liposomes (Katragadda et al 2000). In pulmonary application assays using mouse models Dokka and co workers (2000) discovered that while cationic liposomes were toxic neutral and negative liposomes did not exhibit lung toxicity. This group proposed that reactive oxygen intermediates are responsible for cationic liposome-mediated toxicity in the lung. There are other suggested mechanisms for the toxicity of cationic lipids/liposomes which have not yet been completely proven. In addition to alterations of the net charge of cell membranes, adverse effects on the activity of ion channels, membrane receptors and enzymes have been postulated as possible mechanisms of cationic lipid toxicity (Felgner et al 1994).

The effect of liposomes on the HBE cells was evaluated using two cytotoxicity assays instead of just one. This is because certain chemicals have been reported to give divergent results in different toxicity tests including the NRU and MTT assays (Olivier et al 1995; Chiba et al 1998). Besides, Evans et al (2001) have recently found that in some cases one of the NRU or MTT assays can be more sensitive in detecting the toxicity of non-viral transfection reagents. Although not much difference in the sensitivity of these two assays was observed in this study, using two different viability tests bestows the results with more certainty. Both cytotoxicity assays showed that HM-liposomes, extruded through 100nm or 400nm filters, were completely non-toxic in the HBE cell line. However, liposomes prepared by the conventional technique, using chloroform and methanol, decreased cell viability by 5-17% (vesicles extruded through 100nm filters) and 4-17% (vesicles extruded through 400nm filters). This is despite the fact that not only one but two common measures (i.e. N_2 flushing and vacuum) were employed to remove the OVIs (organic volatile impurities) from the conventional liposomes. In most of the liposome manufacture methods, in which volatile organic solvents are used, only one of these procedures is carried out to remove the OVIs.

The chemical composition of the conventional liposomes and HM-liposomes in this study is same and the difference between them, apart from the presence/absence of the organic solvents, is the employment of glycerol in the manufacture of HM-liposomes. Glycerol is a water-soluble and physiologically acceptable chemical with the ability to increase the stability of the lipid vesicles (Kikuchi et al 1994). On the other hand, chloroform and methanol are well known toxic agents (Timbrell 2000; Dwivedi 2002) which exert cytotoxicity through different mechanisms including destabilisation of membrane proteins (Ivanov 2001). The toxicity observed for the liposomes prepared by the conventional technique is possibly caused by the presence of chloroform and/or methanol employed in their manufacture. While it is arguable that the OVIs in the liposomal products may eventually be brought down to an acceptable level in terms of affecting such properties as stability, permeability and toxicity, the situation is less obscure when such agents are not present at all. It has been shown that trace amounts of these

potentially toxic agents remain in the liposomal formulation, no matter how much effort is undertaken to remove them (Deamer & Uster 1983; Cortesi et al 1999). Due to this reason, mainly, application of organic solvents such as chloroform, methanol or methylene chloride to solubilise and mix lipids is not recommended (US regulations allow 50 ppm of chloroform and 500 ppm of methylene chloride in the formulation) (Lasic 1998). We propose that as a result of liposome interaction with cells, small amounts of the volatile organic solvents, entrapped in the lipid vesicles, can be released into the cells and cause cytotoxicity. It is possible that even if these liposomes are not taken up by the cells (through endocytosis or fusion) the organic solvents present in their structure, and initially those present in their lipid phase, can interact with the cells after being released from the liposomes for example by the mechanism of lipid exchange (Figure 3.11.).

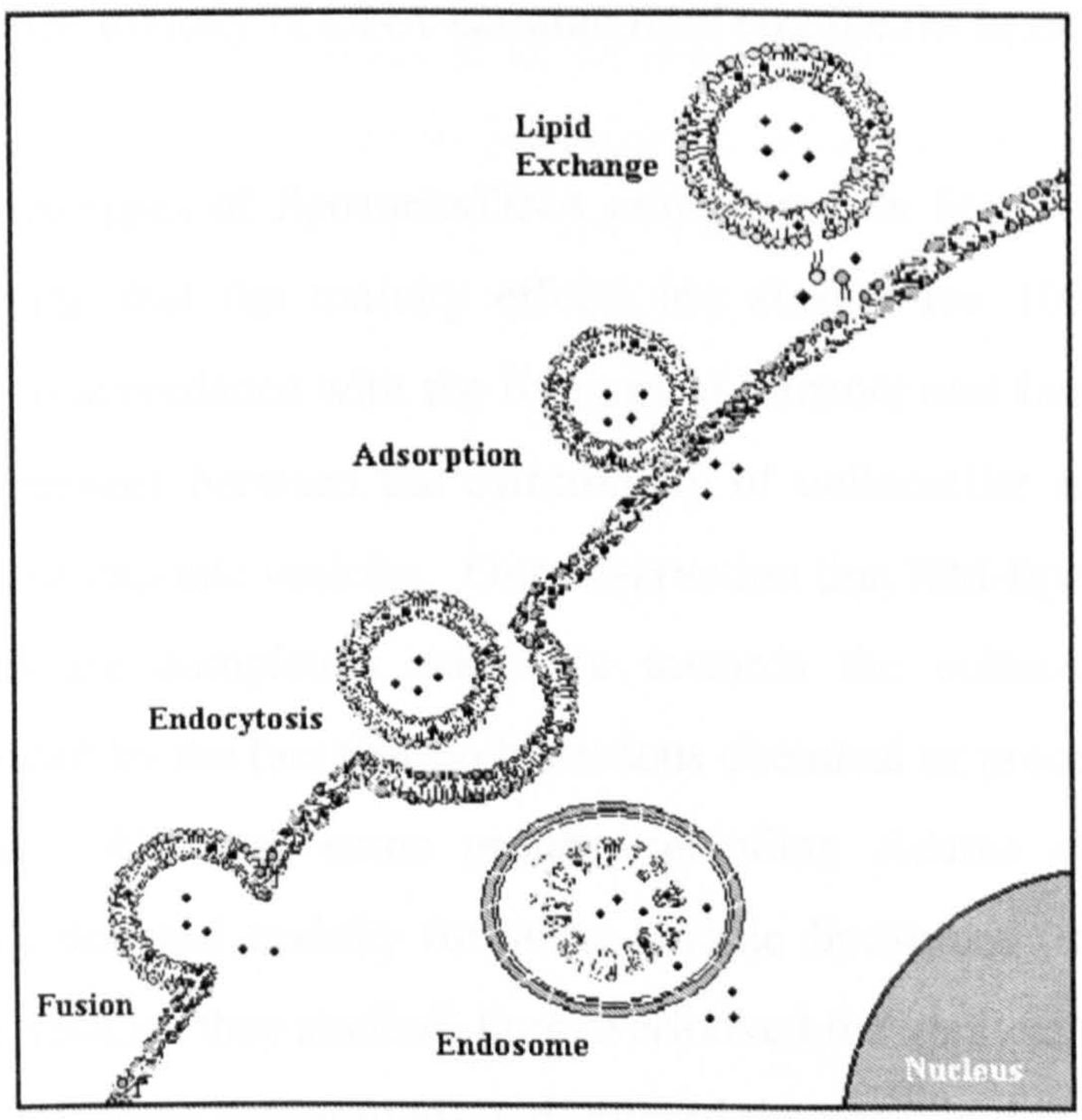


Figure 3.11. Different modes of liposome interaction with cell through which trace amounts of the organic solvents (♦) present in the lipid and/or aqueous phases of the liposome can be released to the cell and cause cytotoxicity.

Another finding was that the presence of DNA as a plasmid vector does not affect the toxicity of the anionic liposomes although Filion and Phillips (1997) reported that incorporation of DNA marginally reduced the toxicity of cationic liposomes in their study. It seems that binding a poly-anionic agent (e.g. DNA) to cationic vesicles has the potential to reduce the toxicity, possibly by decreasing the amount of positive charges on the surface of these vesicles. Since the amount of DNA used in the cationic liposome vectors is not to an extent to completely neutralise the positive charges, these vectors remain predominantly cationic hence potentially toxic. In agreement with this, there are reports of *in vitro* and *in vivo* toxicity of several cationic liposome-DNA complexes. For instance, some DNA-cationic lipid complexes have been found to cause toxicity and cell injury in dog airway epithelial cells (Fasbender et al 1995) as well as high level of toxicity towards mouse macrophages (Filion and Phillips 1998). In addition Nagahiro and co workers (2000) have reported toxicity of DNA-cationic lipid complexes in rat lung *in vivo*.

For the two types of liposome/DNA complexes, as found for the unloaded liposomes, it seems that the toxicity effects are similar for 100nm and 400nm vesicles. This is in accordance with the findings of Panzner and Jansons (1979) who observed no differences between the cytotoxicity of unilamellar and multilamellar anionic, neutral and cationic vesicles. Our observation that HM-liposomes (with and without plasmid) are completely non-toxic towards the cultured HBE cells is potentially explained by the fact that no hazardous chemical or process is involved in their preparation. Although some groups, including Adams et al (1977) and Campbell (1983), detected toxicity for some anionic liposomes (to a lesser degree than the cationic vesicles they studied) they overlooked the application of the organic solvents in their liposome preparations and ignored the contribution of these solvents to the toxicities they observed. This should be noted in the future toxicity evaluations of liposomal formulations.

At this stage it is clear that HM-liposomes possess long-term stability and significant advantages over conventional liposomes including absence of the residual

organic solvents and detergents and lack of toxicity. As gene transfer vehicles HM-liposomes also proved to be non-toxic vectors and further characterisation of the HM-liposome/DNA complexes, including their DNA incorporation by the mediation of Ca^{2+} ions and transfection efficiencies, are reported in chapter six.

3.5. Conclusions

Results of this study indicate that liposomes prepared by the heating method, complexed or not complexed with plasmid DNA, are completely non-toxic to HBE cells, while liposomes prepared by the conventional method using volatile organic solvents are significantly toxic towards the same cells and hence should be utilised with caution. These results were verified by not one but two different cytotoxicity assays. The two sizes of vesicles tested exhibited similar toxicity profiles. In general terms, the heating method is proposed as a rapid liposome preparation technique, with the potential for mass production of non-toxic liposomes to be utilised in gene and drug delivery applications.

4: ENCAPSULATION OF 5-FLUOROURACIL IN HM-LIPOSOMES: CHARACTERISATION AND *IN VITRO* TOXICITY USING HUMAN AIRWAY EPITHELIAL CELLS

4.1. Introduction

The major aim in drug therapy of tumors is the selective killing of tumor cells while avoiding any harm to the normal cells. One potential approach to improve therapeutic efficacy in chronic and difficult to treat disorders including cancer is to increase the targeted dose by regional therapy (Sharma et al 2001). In addition to enhancing the drug exposure to the tumor, the regional chemotherapy approach also minimises the systemic side effects of the cytotoxic agents. Regional chemotherapy has been used in selected situations for treating colon cancer, carcinomatous meningitis, melanoma, superficial bladder cancer and intraperitoneal ovarian cancer (Ghussen and Kruger 1989; Markman 1996; Kemeny et al 1999; Markman 1999). Using this regional therapy approach, Wattenberg and co workers demonstrated that lower doses by nasal aerosol administration of the drug budesonide was as potent as a high dose (which was more than four times higher than the low dose) by oral administration in preventing pulmonary tumor formation in mice (Wattenberg et al 1997, 2000).

In the treatment of chronic diseases such as lung tumors, besides local drug delivery, repeated dosing (usually at high frequencies) is commonly required. However, such dosing is associated with peaks at which the drug could be toxic and troughs at which the drug could be ineffective. A sustained release system can prolong the release of drugs at the site of action and reduce the fluctuations in drug levels, thereby rendering chronically administered drugs more safe, effective, and reliable. Colloidal drug delivery systems, such as liposomes and polymeric particles,

have been used to sustain drug delivery at various sites in the body including the respiratory tract (Lai et al 1993; Suarez et al 2001; Konduri et al 2003). An attractive attribute of liposomes for regional cancer therapy applications is that vesicles with a mean diameter around 100nm seem to be effective in blood-to-tumor drug transfer and possess longer retention times in tumor tissue (reviewed by Nagayasu et al 1999). These characteristics are due to the tumor vessel leakiness (McDonald and Baluk 2002) and do not require employment of any targeting strategy in the structure of liposomes.

The antineoplastic agent 5-fluorouracil (5-FU) was used in this study as a water-soluble model anticancer drug. This drug has been employed in the therapy of different solid tumors such as breast cancer and cancer of the gastointestinal tract (stomach, colon, rectum) for more than four decades. Although 5-FU is one of the oldest anticancer drugs, it remains the standard therapy of advanced colorectal cancer and is also one of the major drugs in the treatment of head and neck cancer and breast cancer (Curreri at al 1985; Chang et al 1989; Dine et al 1999). In addition, this drug is under investigation for the chemotherapy of non-small cell lung cancer (Muller 2002; Focan et al 2003), the leading cause of cancer deaths in human.

The chemical structure of 5-FU is depicted in Figure 4.1. It is a pyrimidine-base analogue that acts as an antimetabolite to block the synthesis of deoxythymidylic acid and to disrupt normal RNA function (Heidelberger et al 1957; Bosch et al 1958). Its mode of action involves incorporation of the 5-FU anabolite 5-fluorouridine-5'-triphosphate into RNA which effects both the processing and function of RNA and also interferes with DNA synthesis by inhibition of the enzyme thymidylate synthetase via the 5-FU anabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate (Heidelberger 1981). It has been suggested that the relative contribution of RNA-directed and DNA-directed mechanisms of 5-FU cytotoxicity depends on both the concentration of 5-FU and the duration of exposure (van Kuilenburg 2004). Sobrero and co workers (1997) have reported that short-term exposure to high concentrations of 5-FU induce RNA-directed 5-FU toxicity, whereas longer exposures to lower concentrations induce DNA-directed effects.

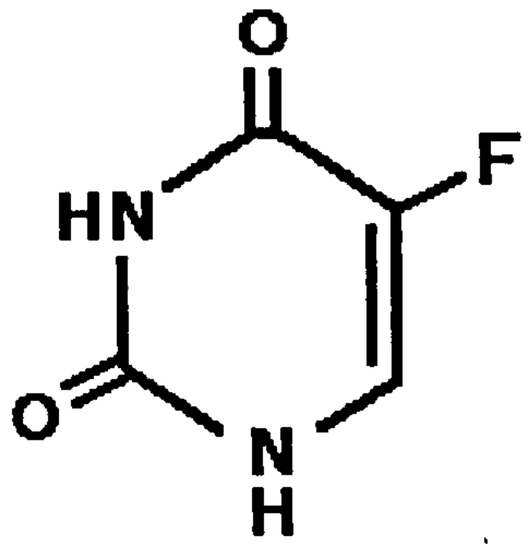


Figure 4.1. Chemical structure of 5-fluorouracil (5-FU).

Because 5-FU has a short plasma half-life of 10-20min, high doses (e.g. 400-600mg/m^2 weekly) have to be administered to reach therapeutic drug levels (Peters et al 1993; Holland et al 1997). The oral absorption of this drug is incomplete and unpredictable and when administered parenterally it manifests a short biological half-life due to fast metabolism (Namdeo and Jain 1999). Moreover, the clinical use of 5-FU is limited by its toxic effects not only on the tumor but on the healthy tissues as well. It has been reported that due to non-specific distribution in tumor and healthy tissues 5-FU was responsible for toxic side effects on bone marrow and the gastrointestinal tract (Chabner 1982; Hardman and Limbird 1996). Similar toxic effects of the drug have been encountered by other groups as well. Fraile et al (1980) detected gastrointestinal toxicity (stomatitis) and myelotoxicity while Au and Gunnarsson (1989) have reported high levels of 5-FU toxicity in the intestinal mucosa. In addition, Tuchman and co workers (1985) have detected a severe neurotoxicity reaction in the brain caused by this drug. Therefore it is essential to find means for localisation of 5-FU in the required site to enhance bioavailability, decrease the required dose and protect healthy tissues from toxic effects.

Several efforts have been made to increase the efficacy of 5-FU and reduce its side effects by encapsulation in liposomes (Mazumder 1981; Ozer 1992; Elorza et al 1993; Fresta et al 1993; El Maghraby et al 2001; Kaiser et al 2003). However, when using conventional liposome preparation methods the trapping efficiency of 5-FU is low (Kaiser et al 2003). This is because 5-FU has low water solubility at neutral pH values and does not interact with the lipid bilayers nor adsorb to them (Tsukada et al 1984; Fresta et al 1993). This effect particularly applies to small liposomes due to the small ratio of the internal to external aqueous volume (Kaiser et al 2003). The reported 5-FU trapping efficiencies for liposomal carriers is 2-10%, depending on the lipid composition (Ozer 1992; Fresta et al 1993; El Maghraby 2001). Kaiser and colleagues (2003) reported higher entrapment efficiencies for the drug, in vesicular phospholipid gels, but at non-physiological pH (e.g. pH: 8.0 and 8.6). Hence for efficient anticancer treatments there is a requirement for carriers which in the first place are not toxic themselves and possess high entrapment efficiencies as well as appropriate controlled release properties.

In this chapter, the main *in vitro* characteristics, i.e. entrapment efficiency, release kinetics and cytotoxicity, of 5-FU encapsulated in the conventional liposomes as well as the HM-liposomes are presented. For *in vitro* toxicity evaluations the human epithelial cell line, described in chapter 3, was employed as an ideal model for pulmonary drug delivery applications. Cell viability was evaluated using two widely used cytotoxicity tests namely the neutral red uptake (NRU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

4.2. Materials and Methods

4.2.1. Chemicals

Dipalmitoylphosphatidylcholine (DPPC), dicetylphosphate (DCP), Cholesterol (CHOL), glycerol, Sephadex G-50, 0.25% trypsin-EDTA solution, neutral red solution (NR, 4-amino-7-dimethylamino-2-methyl phenazine

hydrochloride (4.4g/l)), and MTT (4-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Co. (Dorset, UK). Minimum essential medium (EAGLE) containing Glutamax-1, fetal calf serum, and penicillin/streptomycin (10000 U/mL, 10000 µg/mL) were obtained from GibcoBRL® Life Technologies Ltd (Paisley, UK). All solvents (chloroform, methanol, Analar grade) were obtained from Sigma Chemical Co. (Dorset, UK). All other chemicals were of commercial analytical grade.

5-fluorouracil [5-fluoropyrimidine-2,4(1*H*,3*H*)-dione] was a product of Sigma Chemical Co. (Dorset, UK) with the following characteristics:

Molecular weight:	130.08
Empirical formula:	$C_4H_3FN_2O_2$
Solubility:	10mg/ml in H_2O
Melting point:	280-282°C
pKa:	8.15, 13.0

Phosphate-buffered saline (PBS) was prepared by dissolving 4.4mM NaH_2PO_4, 1.47mM KH_2PO_4, 2.68mM KCl, and 147mM NaCl in 1l distilled water and adjusting to pH 7.4.

4.2.2. Experimental methods

4.2.2.1. Liposome preparation

4.2.2.1.1. Preparation of conventional liposomes

Conventional liposomes were prepared by the thin-film hydration method as follows: a chloroform/methanol (2:1, v/v) solution of DPPC/DCP/CHOL (7:2:1 molar ratio), containing 10mM total lipid, was evaporated to dryness in a round

bottomed flask connected to a rotary evaporator (EYELA, New Rotary Vacuum Evaporator NE-1, Birmingham, UK), in a water bath at 50°C. To remove traces of the solvents the flask was flushed with nitrogen gas for 1 hour and also placed under vacuum for a time period of at least 1 hour. The lipid film was then hydrated, above the phase transition temperature (T_C) of the lipids at 50°C, with 2ml sterile PBS (autoclaved) containing 10-500mM 5-FU and multilamellar vesicles (MLV) formed by vortex agitation. The liposome suspension was extruded above T_C through two-stacked polycarbonate filters of 100nm pore size mounted in an extruder (LiposoFast™-Basic, Glen Creston Ltd, UK) fitted with two 0.1ml glass syringes. An odd number of passages (i.e. 11 times) was performed to avoid contamination of the sample by large vesicles or aggregates which might not have passed through the filter. The liposome suspension was left undisturbed at room temperature under N_2 for at least 30min to equilibrate.

4.2.2.1.2. Preparation of liposomes by the heating method

HM-liposomes with the same composition as the conventional liposomes were prepared as follows: DPPC, DCP and CHOL were hydrated each in PBS (pH: 7.4) and then mixed together and heated up to 120°C in the presence of glycerol (3% v/v) in a silicone oil bath. Loading of 5-FU into the HM-liposomes was achieved at two different temperatures of 120°C and 60°C. For loading at 120°C 5-FU (10-500mM in PBS, pH: 7.4) was added to the lipid suspension before the heating step. For loading at 60°C the drug (10-500mM in PBS, pH: 7.4) was introduced to the lipid suspension when the temperature dropped to 60°C.

In order to provide a more homogeneous population of liposomes with smaller diameters the liposomal suspension was extruded, as described in the previous section, through two-stacked polycarbonate filters of 100nm pore size. The HM-liposome suspension was left undisturbed at room temperature under N_2 for at least 30min for equilibration.

4.2.2.2. Thin layer chromatography

The effect of heating on 5-FU was checked by thin layer chromatography (TLC) in comparison with un-heated drug as a control. A 20µl sample of 5-FU (5mg in 50ml PBS, pH: 7.4) heated to 120°C in a silicone oil bath and a 20µl sample of control unheated 5-FU (5mg dissolved in 50ml methanol) were spotted on 0.25mm layers of silica gel on 20×20cm plates (2.5cm from the lower edge of the plate). The samples were run in an eluent of ethyl acetate, methanol and ammonium hydroxide (75:25:1) (Hawrylshyn et al 1964). The plates were removed from the TLC tanks when the solvent ascended to within 3-4cm of the top of the plates. The plates were then air-dried for approximately 5min in a fume hood and viewed under shortwave ultraviolet light (254nm). The TLC assay was performed in triplicate.

4.2.2.3. Separation of liposomes from unentrapped drug

Separation of the unentrapped (free) drug from liposomal 5-FU was performed using the mini-column centrifugation method (Fry et al 1978; New 1990; El Maghraby et al 2001) as follows. Sephadex G-50 (10g) was swollen in 120ml sterile PBS (pH: 7.4) at room temperature for at least 5h and stored at 4°C until use. To prepare the mini-columns, Whatman GF/B filter pads were inserted in the bottom of the barrels of 2.5ml disposable plastic syringes which were then filled with the hydrated gel. Excess buffer was centrifuged at 3000rpm for 3min using a Labofuge 400R centrifuge (Heraeus Instruments, Germany) and discarded. Then 500µl of the liposome suspension was added drop-wise to the centre of the top of the gel column, followed by centrifugation as above. To the mini-column, 625µl PBS (pH: 7.4) was added and centrifugation repeated. Liposomes (depending on their type and size) can be recovered from the first or the first and second stages of the centrifugation (New 1990). Both liposome types utilised in the present study required two centrifugation stages for complete recovery. This method was able to separate all the free drug as evidenced by the absence of any drug in the centrifugate in the initial two stages when performing the separation process after application of drug solution (without

liposomes). The eluants collected after third to tenth centrifugation rounds were pooled together as unentrapped drug sample. A major advantage of this technique is that the liposomes can be recovered with no dilution (Fry et al 1978; New 1990; Dipali et al 1996), since the excess void fluid has already been drained off in the previous spin before sample collection. The recovered liposomes were pooled together and applied to fresh mini-columns again, as above, to ensure complete removal of free drug. However, no unentrapped drug was detected after this step. Absence of 5-FU attached to the outer surface of liposomes was further checked by an interaction assay explained below.

4.2.2.4. Liposome 5-FU interaction assay

Before evaluation of the liposome interaction with 5-FU the λ_{max} of 5-FU was determined using a double-beam UV/VIS Spectrometer (Perkin Elmer, Lambda 40, USA) to be 266nm at three independent measurements. Liposomes were prepared by the conventional technique and the heating method as explained in section 4.2.2.1. with no drug. To these empty liposomes 400mM 5-FU was added and the mixtures were incubated at room temperature under N_2 for time periods of 1, 2, 3 and 4h after a brief hand-shaking. At the end of these time periods samples were applied to the mini-columns as explained in the previous section. The absorbance of the collected drug (eluants of third to tenth centrifugation rounds pooled together) were measured at 266nm using a double-beam UV/VIS Spectrometer (Perkin Elmer, Lambda 40, USA). A sample of 5-FU (400mM) without the liposomes was employed as control. The concentration of 5-FU was calculated from the calibration curves constructed at 266nm for each assay. An example of such calibration curves are represented in Appendix 2.

4.2.2.5. Determination of entrapment efficiency

Entrapment efficiency (EE) of 5-fluorouracil into conventional liposomes and HM-liposomes was calculated at the evaluated λ_{max} (i.e. 266nm, section 4.2.2.4.) using PBS (pH: 7.4) as blank. The concentration of 5-FU was calculated from the calibration curves constructed at 266nm for each assay. EE was calculated as a percentage of the initial drug added, after correction for the dilution factor, by using the following equation:

$$EE\ (\%) = 100 \times (c_{init} - c_{free}) / c_{init}$$

where c_{init} is the concentration of the initial 5-FU added, and c_{free} is the concentration of free (unentrapped) 5-FU.

4.2.2.6. Release of 5-FU from liposomes

Release kinetics of the liposomes prepared with 400mM initial concentration of 5-FU was investigated in phosphate buffered saline (pH: 7.4) at room temperature. Two millilitres of the liposomal suspension was placed in a dialysis tube (Size 10, Inf Dia 1 1/4", 31.7mm, Mol. Wt cut-off 12000-14000, Medicell International Ltd, London, England) which was closed with plastic clips and suspended in 100ml of PBS which was constantly stirred on a magnetic stirrer. At certain time intervals (15, 30 and 45min; 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 14h) 0.5ml aliquots were withdrawn. The release medium was replenished after each sample removal in order to have sink conditions. The amount of drug released was measured spectrophotometrically at 266nm using a double-beam UV/VIS Spectrometer (Perkin Elmer, Lambda 40, USA) and PBS as blank. The release assays were performed in triplicate.

4.2.2.7. Cells

Immortal human respiratory epithelial cells (16HBE14o-), kindly donated by Dr A. R. Evans (Liverpool John Moores University, England, UK), were grown and maintained at standard cell culture conditions as described previously in Chapter three (section 3.2.2.4.).

4.2.2.8. Toxicity assays

The cytotoxicity of non-encapsulated (free) 5-FU as well as 5-FU encapsulated in the HM-liposomes was evaluated by the NRU and MTT assays as explained in Chapter three (section 3.2.2.5. and subsections within). Both non-encapsulated and encapsulated drug forms were assessed in the concentration range of 10-500mM.

4.2.2.9. Statistical analysis

Data are expressed as mean ± standard deviation of the mean from three or more experiments. A two way analysis of variance of the data was performed. All analyses were carried out using the Minitab statistical package (v 14.1, MINITAB Inc. PA, USA). A P value of less than 0.05 was considered to indicate a significant difference.

4.3. Results

4.3.1. Thin layer chromatography

The effect of heating (120°C) on the chemical structure of 5-FU was studied in comparison with un-heated drug sample using thin-layer chromatography (TLC,

Figure 4.2.). Both samples produced single spots indicating the purity of the 5-FU sample utilised in this study as well as stability of the drug under the conditions of the preparation of the liposomal formulation. The average R_f values (n=3) for unheated and heated 5-FU samples were 0.48 and 0.46 respectively.

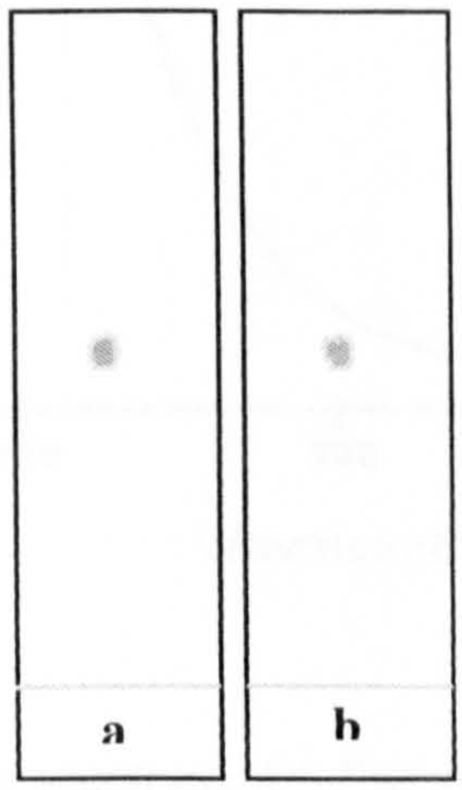


Figure 4.2. Thin layer chromatography of 5-fluorouracil (5-FU) on silica gel. (a) unheated 5-FU; and (b) 5-FU heated at 120°C.

4.3.2. Interaction assay

To find out the λ_{max} for 5-FU the absorbance of the drug was scanned at three different occasions one of which is indicated in Figure 4.3. It was detected that 5-FU has its highest absorbance at the wavelength of 266nm. This wavelength was used for quantifications of 5-FU throughout the present study.

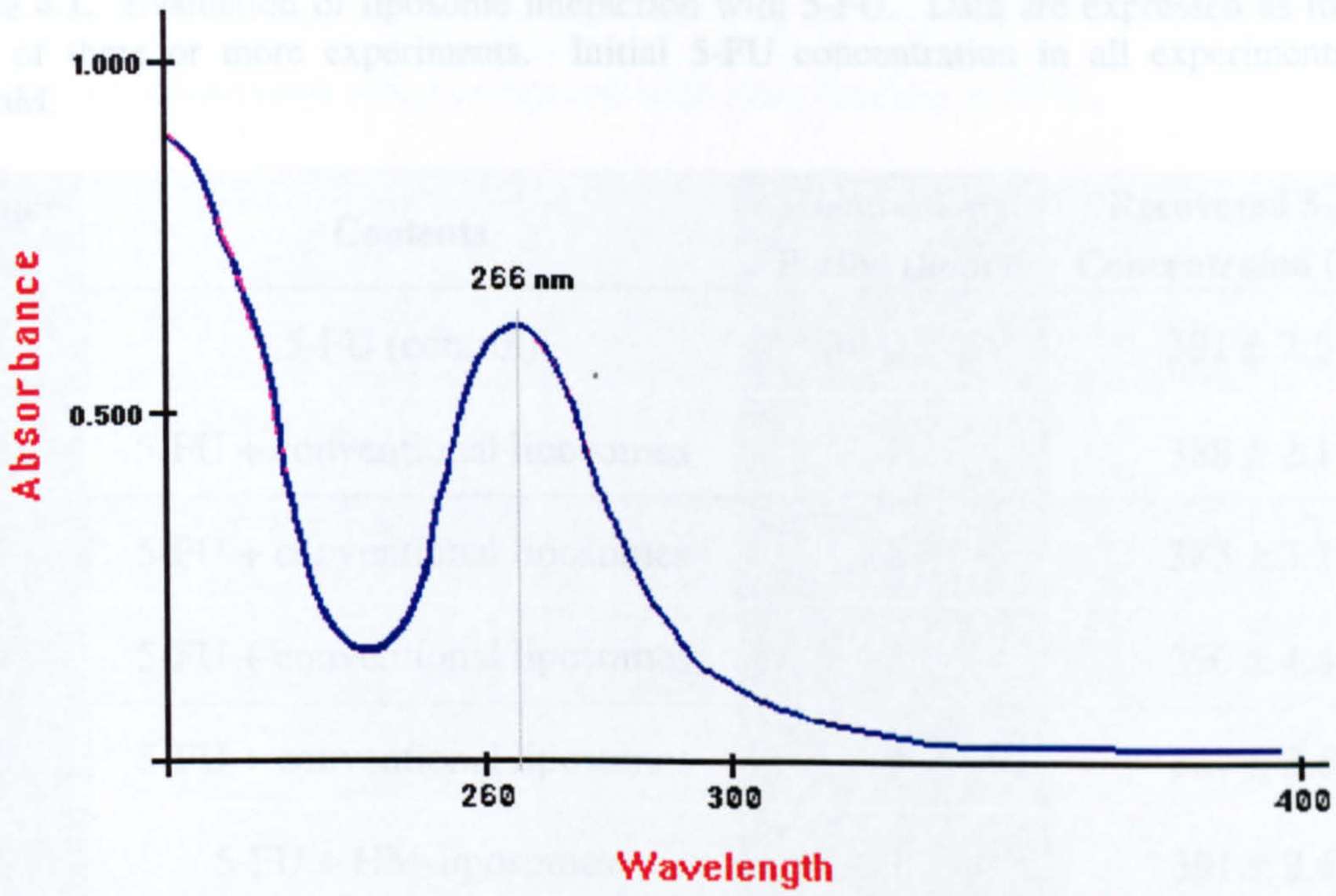


Figure 4.3. Spectrophotometric spectrum of 5-fluorouracil (0.04 mM) in PBS (pH: 7.4).

In order to find out if 5-FU interacts with the external surface of the liposomes an interaction experiment was carried out, results of which are presented in Table 4.1. This table clearly indicates that there is no difference between the amount of control sample (i.e. 5-FU without liposomes) recovered after mini-column centrifugation compared with 5-FU samples brought into interaction with liposomes from one to four hour time periods. Both liposome types (i.e. those prepared by the conventional technique and those prepared by the heating method) exhibited same results. Hence it seems that 5-FU does not interact with the preformed lipid vesicles.

Table 4.1. Evaluation of liposome interaction with 5-FU. Data are expressed as mean ± S.D. of three or more experiments. Initial 5-FU concentration in all experiments was 400mM.

Sample No.	Contents	Incubation Period (hour)	Recovered 5-FU Concentraion (mM)
1	5-FU (control)	0	391 ± 2.5
2	5-FU + conventional liposomes	1	388 ± 2.1
3	5-FU + conventional liposomes	2	385 ± 3.1
4	5-FU + conventional liposomes	3	390 ± 4.4
5	5-FU + conventional liposomes	4	387 ± 2.6
6	5-FU + HM-liposomes	1	391 ± 2.6
7	5-FU + HM-liposomes	2	387 ± 3.2
8	5-FU + HM-liposomes	3	387 ± 2.6
9	5-FU + HM-liposomes	4	386 ± 3.5

4.3.3. Entrapment efficiency

The entrapment efficiency of liposomes was determined spectrophotometrically and calculated from the calibration curves constructed at 266nm each time separately for each assay. Only curves possessing trend lines with R^2 higher than 0.99 were utilised in these calculations. Some examples of the calibration curves used for 5-FU quantifications are represented in Appendix 2. Results of entrapment efficiency assays are given in Table 4.2. It appears that liposomes prepared by the heating method (HM-liposomes) have higher entrapment efficiencies compared to the conventionally made liposomes at both drug loading

temperatures of 60 and 120°C. In addition drug loading at 120°C resulted in higher entrapment efficiencies when compared with drug loading at 60°C.

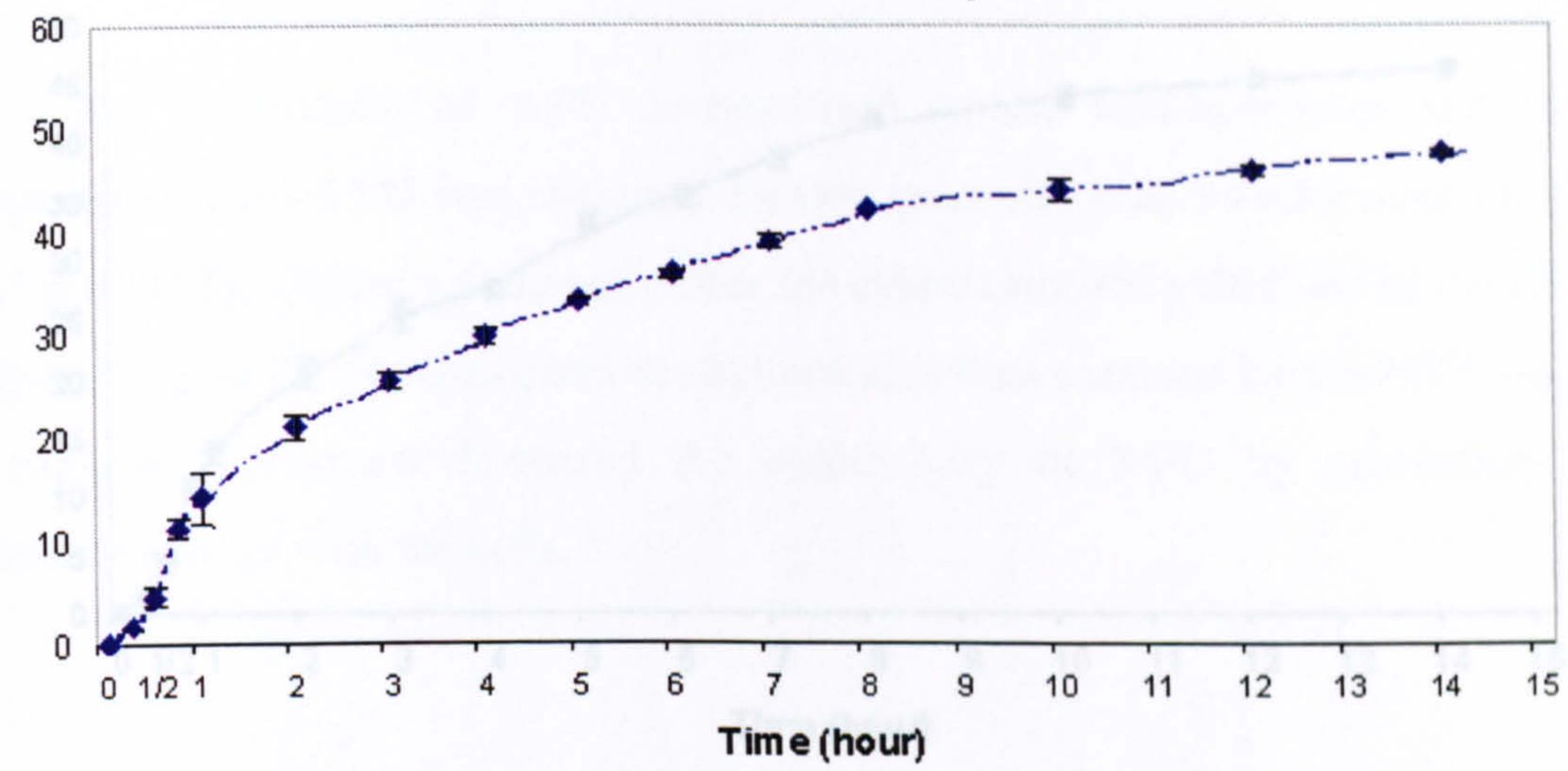
Table 4.2. Entrapment efficiency of 5-FU in liposomes prepared by the conventional technique and the heating methd (at 60 or 120°C). Data are expressed as mean ± S.D. of three or more experiments.

Liposome Type	Drug Loading Temperature (°C)	Entrapment Efficiency (%)
Conventional	50	19.7 ± 2.6
HM-liposomes	60	29.2 ± 3.5
HM-liposomes	120	38.4 ± 1.8

4.3.4. Release of 5-FU from liposomes

The release behaviour of the 5-FU loaded HM-liposomes is depicted in Figure 4.4.a. Almost 47.5% of the drug on average was released within the first 14 hours. The Higuchi plot (cumulative release vs. square root of time) of the release data is approximately linear indicating that 5-FU is entrapped within the liposomes (Figure 4.4.b.). Figure 4.5.a. and 4.5.b. demonstrate the release characteristics and the Higuchi plot of the 5-FU loaded conventional liposomes, respectively. Almost 46.1% of the drug on average was released within the first 14 hours.

a



b

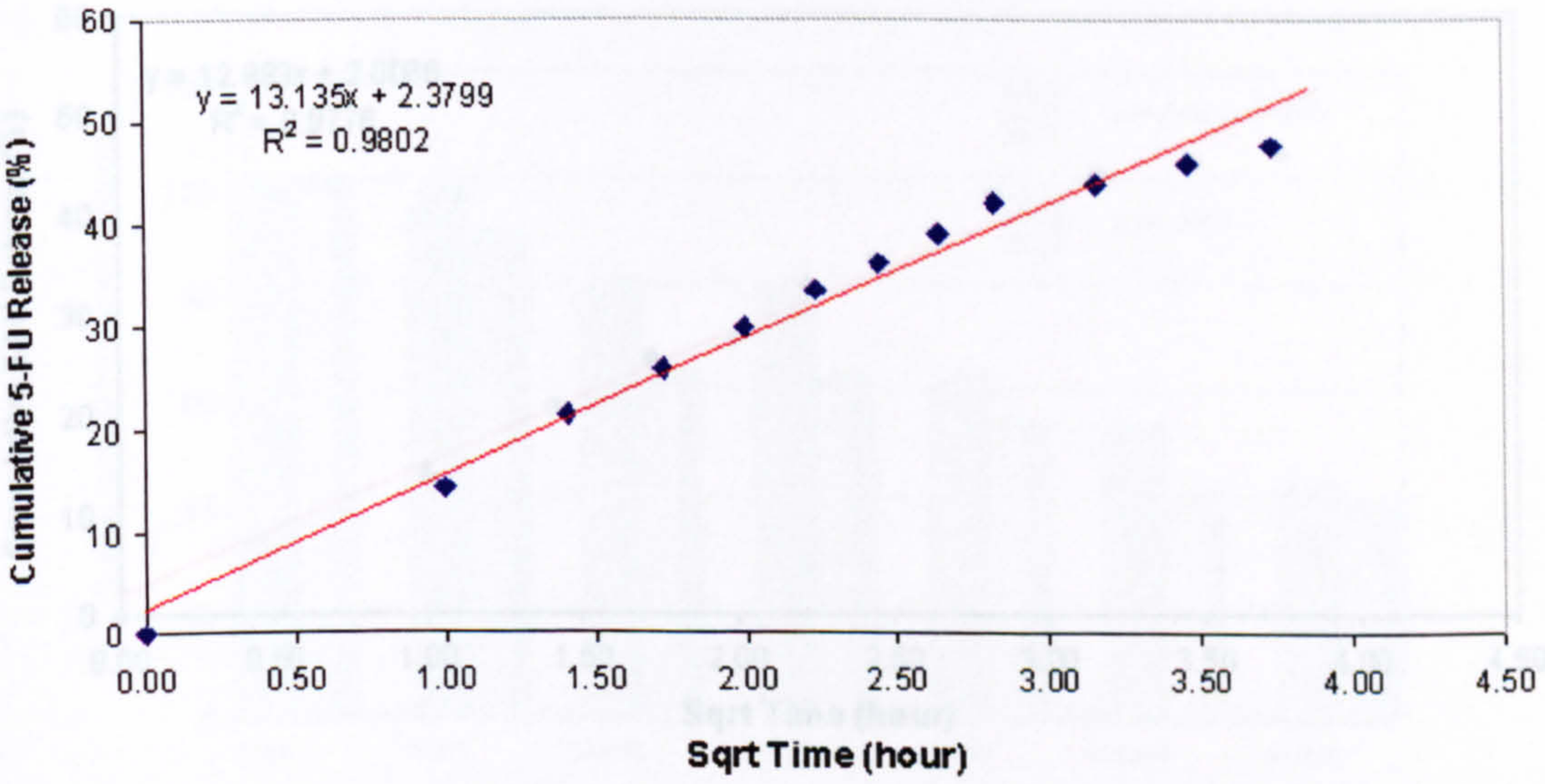
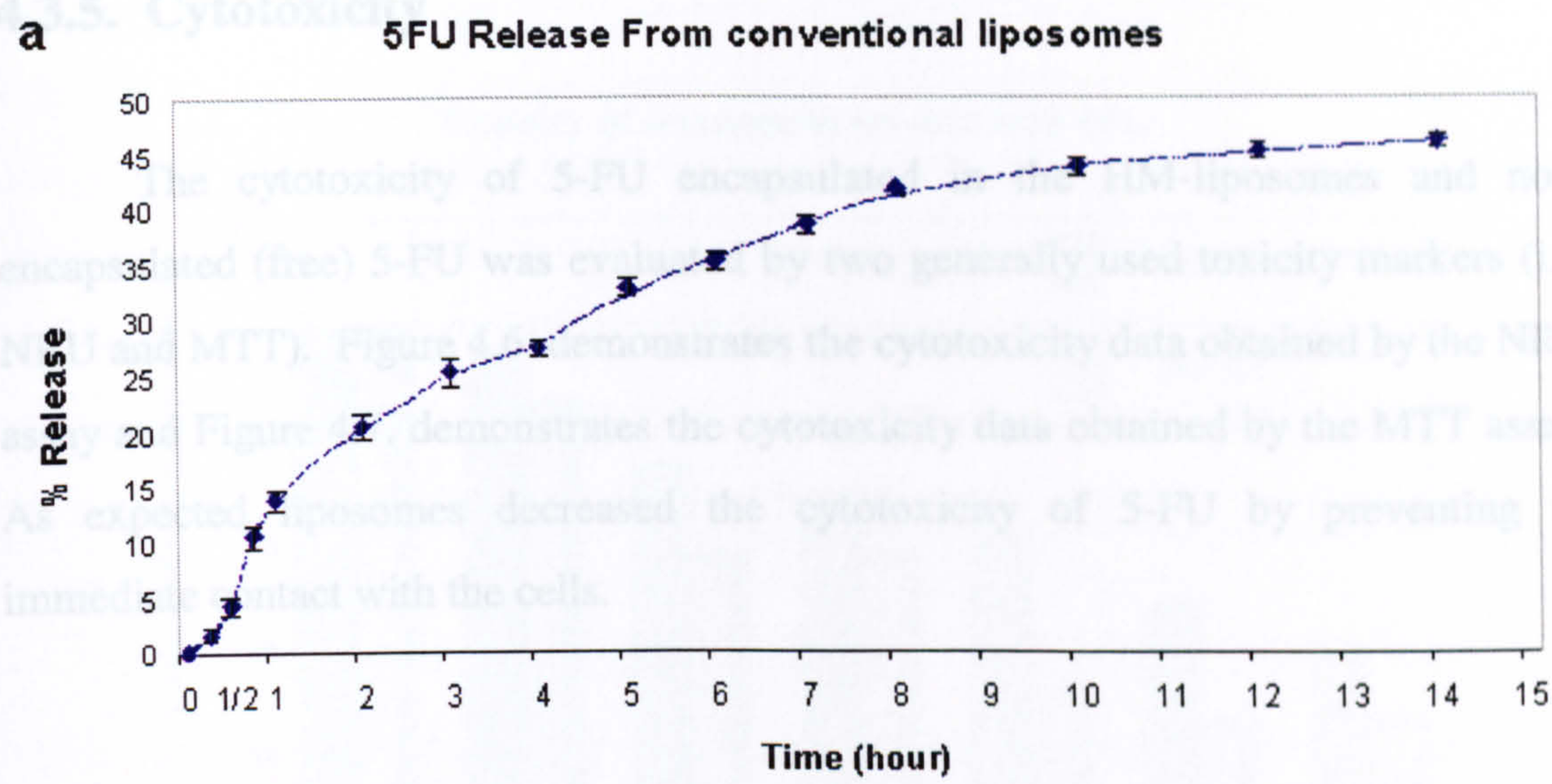


Figure 4.4. a) Release characteristics of 5-fluorouracil (400 mM) from the HM-liposomes in PBS (pH: 7.4). b) Higuchi plot for 5-FU release from the HM-liposomes.

a



b

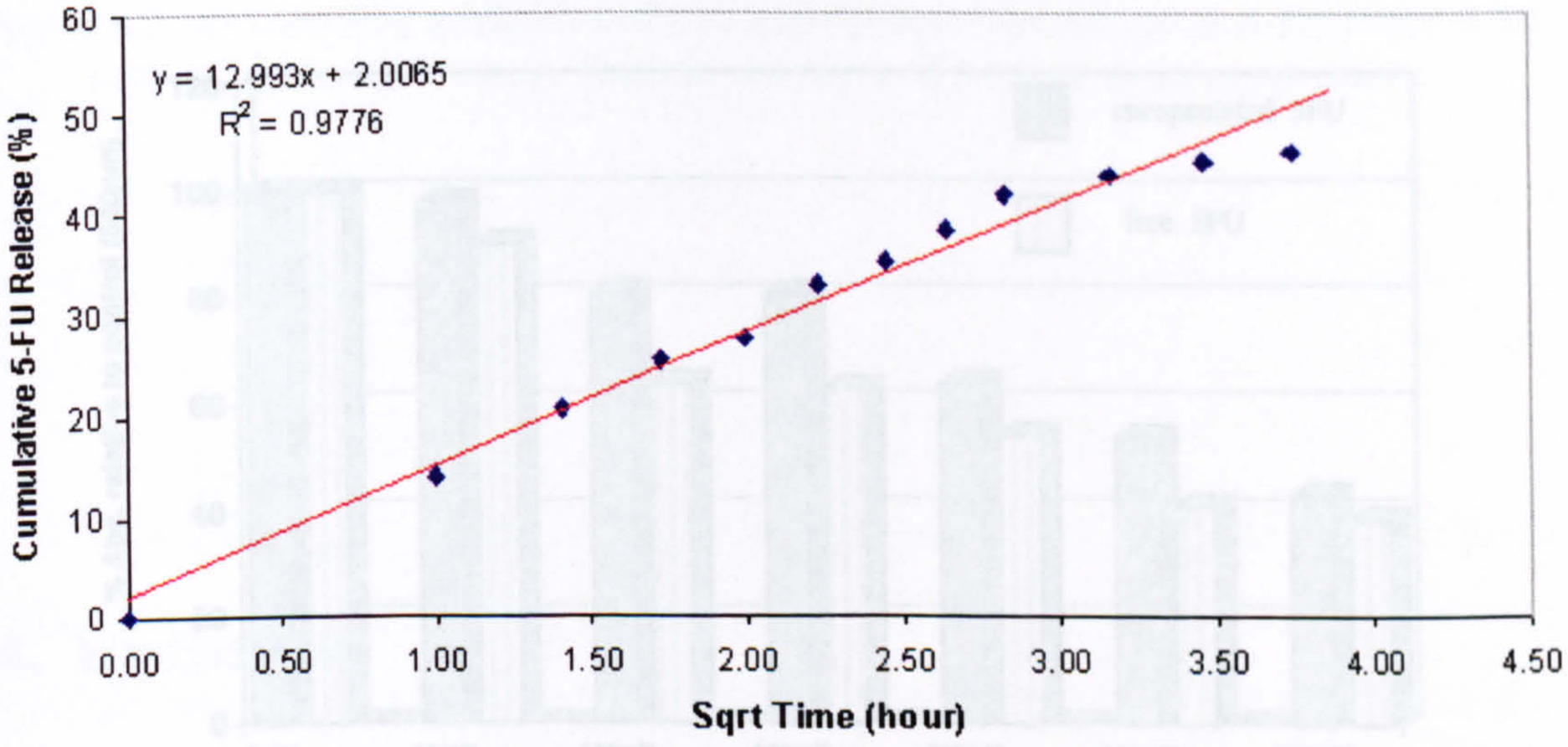


Figure 4.5. a) Release characteristics of 5-fluorouracil (400 mM) from the conventional liposomes in PBS (pH: 7.4). b) Higuchi plot for 5-FU release from the conventional liposomes.

4.3.5. Cytotoxicity

The cytotoxicity of 5-FU encapsulated in the HM-liposomes and non-encapsulated (free) 5-FU was evaluated by two generally used toxicity markers (i.e. NRU and MTT). Figure 4.6. demonstrates the cytotoxicity data obtained by the NRU assay and Figure 4.7. demonstrates the cytotoxicity data obtained by the MTT assay. As expected liposomes decreased the cytotoxicity of 5-FU by preventing its immediate contact with the cells.

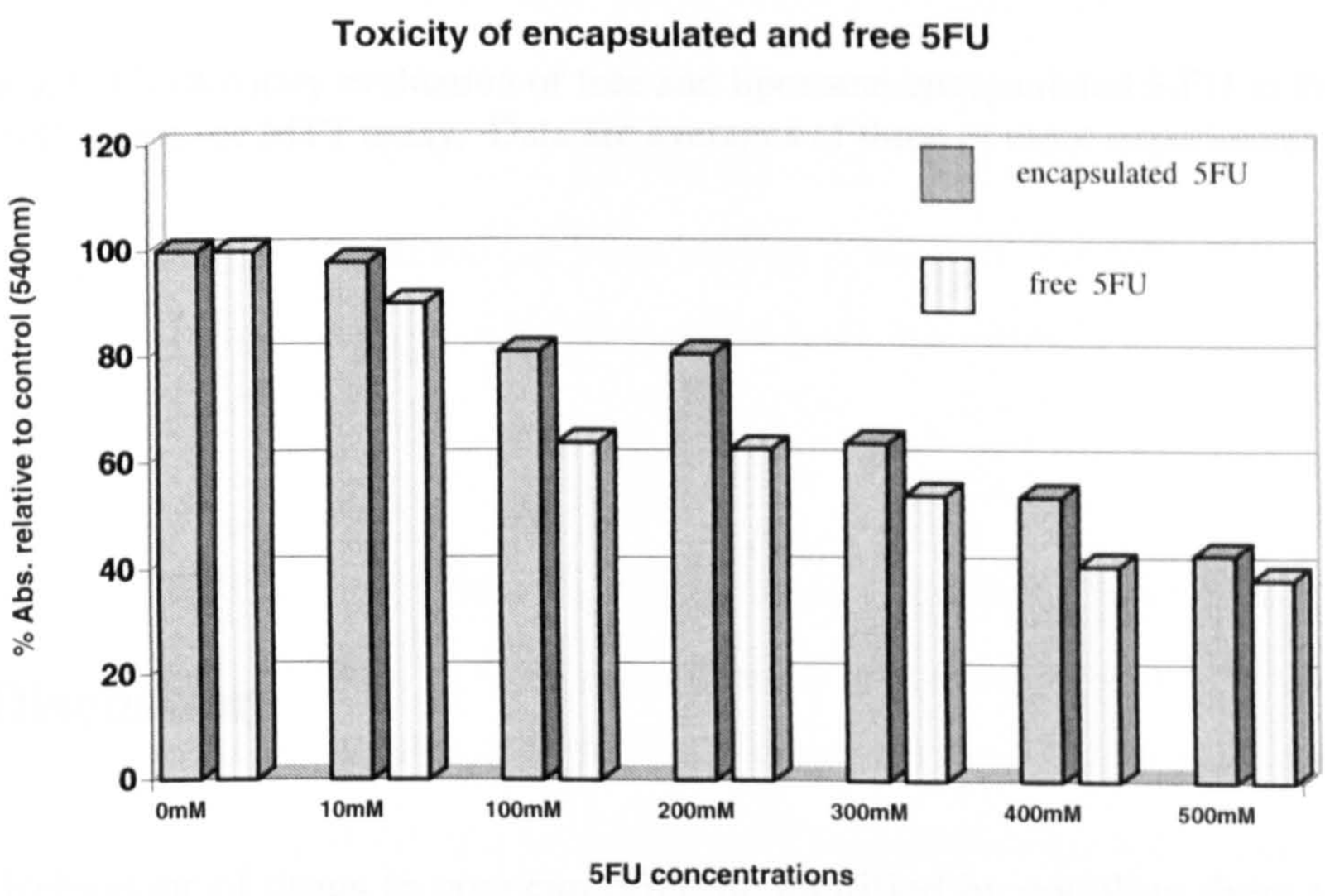


Figure 4.6. Cytotoxicity evaluation of free and liposome-encapsulated 5-FU in the HBE cells using the NRU assay. Data are averages of three or more experiments.

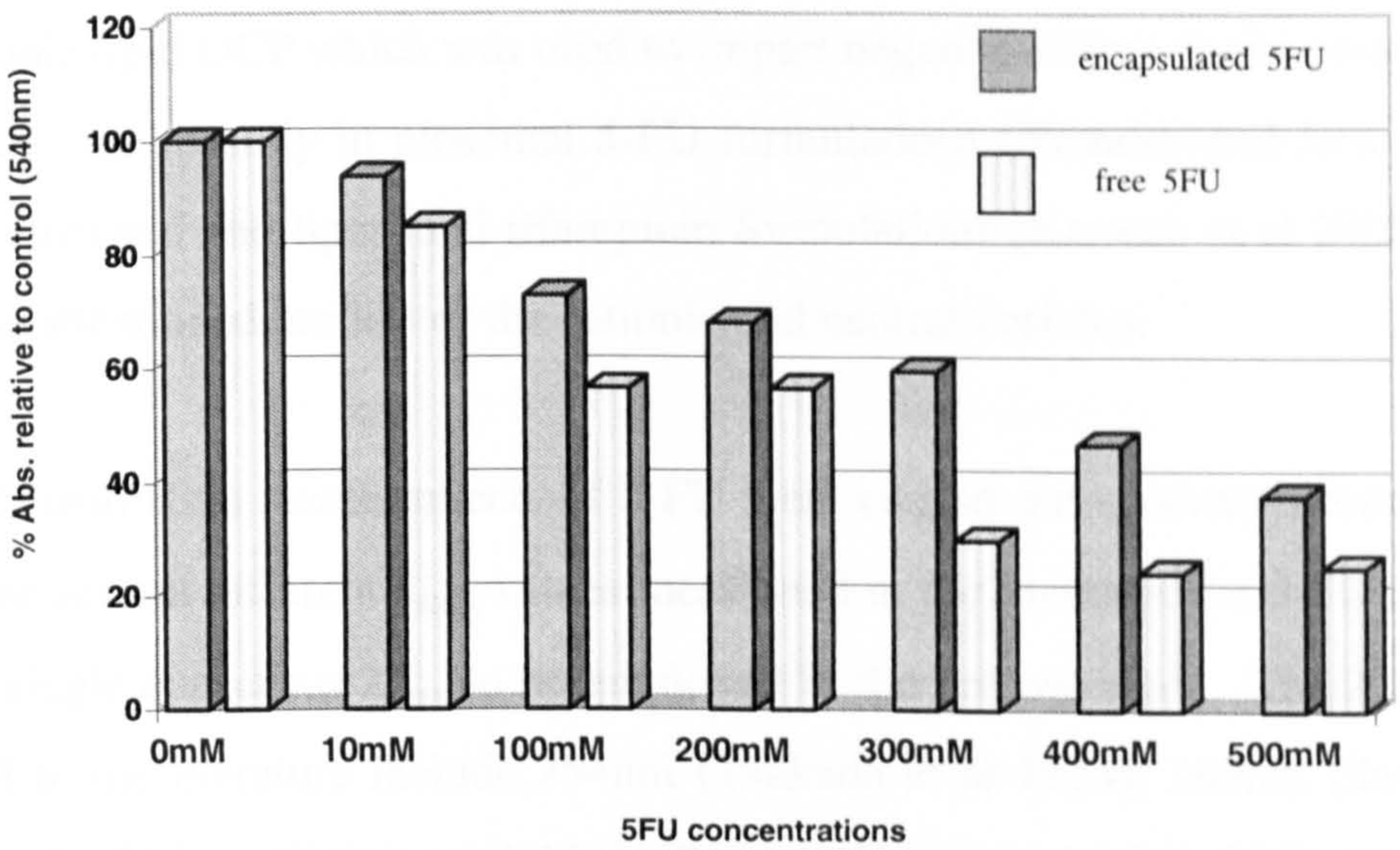


Figure 4.7. Cytotoxicity evaluation of free and liposome-encapsulated 5-FU in the HBE cells using the MTT assay. Data are averages of three or more experiments.

4.4. Discussion

Behaviour of drugs *in vivo* can often be modified by coupling them to carrier moieties. Plasma clearance kinetics, tissue distribution, metabolism and cellular interaction of drugs will be influenced by the behaviour of the carrier systems. In some cases manipulation of these pharmacodynamic attributes can lead to a higher therapeutic index of the drug. There is now considerable data available on the benefits of liposomal encapsulation for a variety of anticancer drugs (Abra et al 2002; Cullis 2003). In this study we have utilised negatively charged liposomes as carrier systems, since positively charged or neutral liposomes are less efficient for drug

delivery as well as having lower *in vivo* antitumor activity (Straubinger et al 1983; Crommelin 1984; Heath et al 1985; Fresta et al 1993). 5-FU was chosen for its remarkable antitumor activity and the need to reduce the side effects of this agent. The anionic lipid DCP which was used to impart negative charge to the vesicles have also been used recently in niosomal 5-FU formulations (Namdeo and Jain 1999) as well as niosomal and liposomal rifampicin formulations (Kamath et al 2000) due to the above mentioned hurdles of the cationic and neutral vesicles.

Quantitative measurements of 5-FU were carried out spectrophotometrically. There are several different λ_{max} values mentioned in the literature for 5-FU and there was no single consensus λ_{max} to be employed in the present work. The λ_{max} values reported in the literature include 254nm (Tsukada et al 1984), 260nm (Sawai et al 1997, 1998), 265nm (Kaiser et al 2003), 266nm (Namdeo and Jain 1999; Denkbas et al 1999, 2000), 266.2nm (Ciftci et al 1994, 1996), 270nm (Dine et al 1999) and 275nm (House et al 1998). Hence it was decided to determine the λ_{max} of the drug under the experimental conditions of the present study. This was found to be 266nm which is in agreement with three previous reports (Namdeo and Jain 1999; Denkbas et al 1999, 2000) and is very close to the 266.2nm used by Ciftci and co workers (1994, 1996).

In order to find out if there is any interaction between 5-FU and preformed liposomes and whether the drug will be associated with the surface of the vesicles an interaction assay was carried out. The tendency of a drug to interact by various means, such as polar and non-polar forces and/or electrostatic interactions with the bilayer, determines whether it would be incorporated into the aqueous compartments or into the bilayer structures or whether it would be firmly associated with the polar head groups of the bilayers via electrostatic interactions (Kulkarni et al 1995). It has been reported that 5-FU does not associate with the surface of bilayers (Tsukada et al 1984; Fresta et al 1993; Namdeo and Jain 1999; Kaiser et al 2003). This was also confirmed by the results of the interaction assay (see section 4.3.2.). Consequently 5-FU will mostly be entrapped within the aqueous compartments and this depends on the encapsulated volume and the solubility of the drug.

The liposomes prepared by both the conventional technique and the heating method were in the form of large unilamellar vesicles (LUV) which have a large internal aqueous volume surrounded by one lipid bilayer. LUV liposomes are suitable for incorporation of drugs such as 5-FU which can mainly be entrapped within their aqueous phase. For incorporation of materials to liposomes, in general, the material should either be water soluble (for incorporation into the liposomal aqueous spaces) or lipid soluble (for incorporation into liposomal lipid regions). In conventional liposome preparation methods practically there are no means of increasing the solubility of the drug material except employing high or low pH which will decrease the stability of the vesicles and render the final formulation not suitable for clinical use. In the heating method, however, heating serves, in addition to sterilisation of the formulation, to increase the solubility and entrapment within liposomes of the drugs with low/limited solubilities.

Liposomes prepared by the heating method exhibited much higher 5-FU entrapment efficiencies (up to almost two-fold) than the vesicles made by the conventional technique. The difference may presumably be due to the higher temperatures employed in the heating method which will increase the solubility of 5-FU in the aqueous medium. Preparation of the conventional liposomes and drug incorporation into them was carried out at 50°C which resulted in a 5-FU entrapment efficiency of 19.7%. Drug loading to the HM-liposomes was performed at two temperatures of 60°C and 120°C and resulted in 5-FU entrapment efficiencies of 29.2% and 38.4% respectively. It is evident that employing higher temperatures results in higher 5-FU entrapment efficiencies. Results of the TLC assays confirmed the heat stability of 5-FU at the above-mentioned temperatures in agreement with the recent findings of Kaiser and co-workers (2003).

The reported liposomal 5-FU entrapment efficiencies are 2-6% (Ozer 1992), up to 10% (Fresta et al 1993; El Maghraby et al 2001), 18.5% (Kaiser et al 2003) and 21% (Tsukada et al 1984). Namdeo and Jain (1999) have reported up to 22.7% 5-FU encapsulation efficiencies in 12 different niosomal formulations. All these values are

less than the encapsulation efficiencies obtained for the HM-liposomes in the present study. In a recent work, Kaiser and colleagues (2003) have reported up to 39.6% 5-FU entrapment efficiency in vesicular phospholipid gels (i.e. concentrated liposomes) which was achieved at pH: 8.6. However, at pH: 8.0 the 5-FU entrapment efficiency of their formulation was 33.3% and at pH: 7.4 it was only 18.5%. Hence at physiological pH values, HM-liposomes are superior to the vesicles reported in the literature in terms of 5-FU entrapment efficiency. The interesting finding is that, even at 60°C, high drug entrapment efficiency was obtained for the HM-liposomes in comparison with the works of other groups. This is very promising in entrapment of drugs which are sensitive to higher temperatures to the HM-liposomes.

The release of 5-FU was sustained following encapsulation in HM-liposomes and after 14 hours the vesicles showed release of 47.5% at pH: 7.4 (Figure 4.4.). Efflux of 5-FU from HM-liposomes was biphasic, with an initial faster release for 2-3 hours, followed by a period of slow release. This biphasic release pattern of water soluble drugs seems to be a characteristic of bilayered vesicles and have been reported for liposomes (Betageri and Parsons 1992) as well as niosomes (Baillie et al 1985; Namdeo and Jain 1999). The initial faster release of 5-FU could be partially due to the release of the drug present in the lipid phase of the vesicles. Tsukada and co-workers (1984) have reported that up to about 15% of the liposome-entrapped 5-FU could be encapsulated in the membrane matrix. Indeed results of the present work show that approximately 14.3% (Figure 4.4.) of the drug was released in the first hour. In general the release of 5-FU, at pH: 7.4, could be described as matrix-controlled diffusion with kinetics of up to 8 hours according to the square-root of time law (Higuchi 1960).

The sustained release characteristics of the HM-liposomes and absence of any burst effect indicate that the anionic HM-liposome formulation used in this study is stable. The high level of stability could be due to the presence of cholesterol in the structure of the HM-liposomes. This is in accordance with the microscopic studies which revealed that HM-liposomes are able to retain their structural stabilities for at least 8 months (see Chapter 2 of this thesis, also Mozafari et al 2002c).

The cytotoxicity of 5-FU encapsulated in the HM-liposomes and non-encapsulated (free) 5-FU was evaluated in the HBE cells by two generally used toxicity markers - namely NRU and MTT (Mozafari et al 2003). Two toxicity assays rather than one were used to increase the validity of the data. Both toxicity tests revealed a dose-dependent cytotoxicity for free and encapsulated 5-FU (Figures 4.7. and 4.8.). This verifies the fact that 5-FU is a potent cytotoxic agent. HM-liposomes decreased the cytotoxicity of 5-FU by reducing the release of drug when they come into contact with the cells, as was also reported for a liposomal paclitaxel formulation (Crosasso et al 2000). Arndt and colleagues (2001) have also observed a decrease in pulmonary toxicity of bleomycin when encapsulated in liposomes *in vivo*. The decrease in the cytotoxicity of 5-FU when entrapped into HM-liposomes is in accordance with the results of the release experiments in that HM-liposomes were successful in sustaining the release of the drug and also indicates their stability in the cultured cell medium for a 24 hour period. In chapter three of this thesis it was found that HM-liposomes were completely non-toxic towards the same cell line used in this chapter. Consequently the dose-dependent cytotoxicity observed for the HM-liposome encapsulated 5-FU formulation can be said to be caused by the drug only. It can be perceived from the cytotoxicity results that the HM-liposomes are able to deliver drugs to cells in an efficient and controlled fashion.

Once an appropriate targeting ligand is employed in the structure of the HM-liposomes they can deliver their therapeutic load to the target cell/tissue while avoiding release (and damage) to other cells/tissues. HM-liposomes are still applicable without employing any targeting strategy for local drug administrations - e.g. to the lungs in the treatment of small cell lung cancer (Moreira et al 2001) and non-small cell lung cancer which is the leading cause of cancer deaths in humans (Muller 2002; Focan et al 2003). Local administration of liposome encapsulated drug formulations have many advantages including reducing the required drug doses, less frequent dosing intervals (e.g. when compared with un-encapsulated 5-FU therapy), sustained release and increasing the bioavailability of the therapeutic agents.

4.5. Conclusions

A liposomal 5-FU formulation is introduced in this chapter which possesses high drug entrapment efficiency and sustained release properties. Results also indicated the stability of the drug loaded HM-liposomes in buffer at pH: 7.4 as well as when in contact with cultured cells. The formulation was detected to be able to release the anticancer drug to the cultured human lung epithelial cells in a controlled manner efficiently. This formulation has the potential to improve the pharmacological and pharmacokinetic profiles of antineoplastic agents, particularly in the lung.

5: HM-LIPOSOMES AS GLUTATHIONE DELIVERY SYSTEMS

5.1. Introduction

Glutathione (GSH) is among the most important low-molecular weight compounds functioning in cells to protect against chemically induced toxicity. It is the most abundant non-protein thiol in living organisms and plays a crucial role in intracellular protection against radiation and toxic compounds, such as heavy metals. GSH also provides protection against reactive oxygen species and other free radicals (Sies and Wendel 1978; Suntres 2002). Glutathione is involved in protein and nucleic acid synthesis, DNA repair, protection of enzymes, immune function, cellular differentiation and aging. It is a tripeptide composed of glutamate, cysteine and glycine in which the γ-carboxyl of glutamate is linked to the α-amino group of cysteine (Figure 5.1.). This substitution of the α-amino group of cysteine decreases the reactivity of the thiol group while the iso-peptide linkage renders the compound resistant to proteases and peptidases. Thus, GSH turnover can be controlled separately from protein metabolism, and the reactivity of the relatively stable thiol of GSH can be controlled by enzymes (Jones et al 1995).

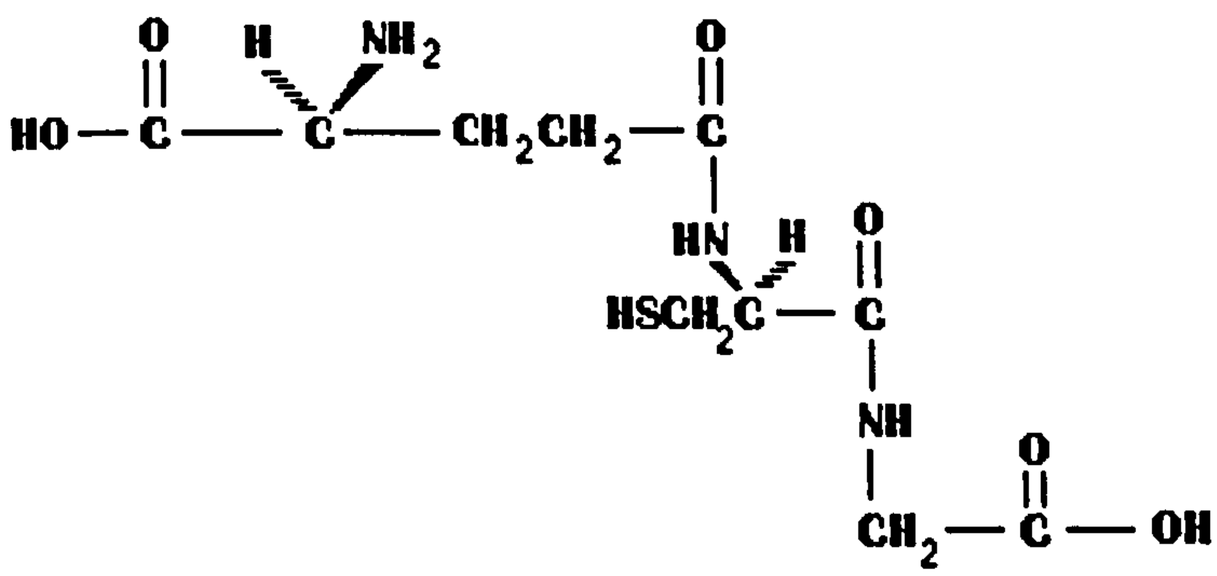


Figure 5.1. Chemical structure of glutathione (GSH).

GSH reacts non-enzymatically with reactive electrophilic compounds and also directly reduces some biomolecules (such as dehydroascorbate). Such reactions may be of great importance in extracellular compartments even if GSH-dependent enzymes are not present. Intracellularly, most GSH-dependent reactions are enzyme catalysed. GSH is used for rather specific detoxification reactions, such as formaldehyde removal by the formaldehyde dehydrogenase system (Koivusalo et al 1989) and also for removal of classes of compounds such as organic peroxides and electrophiles by enzymes with rather broad substrate specificities (DeLeve and Kaplowitz 1991). Each of these systems is ultimately dependent upon the concentration of GSH. The factors that affect this concentration, such as availability of amino acid precursors, activity of the rate-limiting enzyme for synthesis (γ-glutamylcysteine synthetase), rate of NADPH supply for glutathione disulfide (GSSG) reduction, and rates of removal of GSH from the cell, can determine the rate of detoxication of many reactive chemicals. A typical example of enzymatic detoxication reactions is the removal of hydrogen peroxide (H_2O_2) by the enzyme glutathione peroxidase (GSH-Px) while catalysing oxidation of GSH to GSSG (Figure 5.2.), providing the major line of defence against endogenous peroxides (Vani and Rawal 1996; Hurst et al 2001).

$$2\,GSH + H_2O_2 \xrightarrow{GSH\text{-}Px} GSSG + 2\,H_2O$$

Figure 5.2. Involvement of GSH (reduced form of glutathione) in the breakdown of hydrogen peroxides (H_2O_2) by the enzymatic action of glutathione peroxidase (GSH-Px). GSSG is the oxidised form of glutathione.

Studies have suggested that GSH serves additional antioxidant functions by reducing oxidised forms of ascorbate (semidehydroascorbate and dehydroascorbate)

back to the reduced and functional form (Brown 1994; Brown and Jones 1995). Because ascorbate reduces the α-chromanoxyl radical (1-*e* oxidised form of α-tocopherol) back to α-tocopherol, GSH functions to maintain tissue concentrations of the free radical scavengers, ascorbate and α-tocopherol (Buettner 1993).

A large number of xenobiotics either generate reactive oxygen species or are activated to electrophiles that can deplete cellular GSH, inactivate cell proteins and/or introduce mutations into DNA (Dahm and Jones 1995). In principle, simultaneous exposure to combinations of such chemicals can result in more severe GSH depletion than would occur from a single exposure and thus render tissues more likely to achieve a concentration of GSH which is insufficient to protect against injury (Jones et al 1995). Nagai and co-workers (2002) have suggested that GSH depletion by some toxic agents is responsible for apoptosis and cell susceptibility to tumor necrosis factor-induced cell death. Deficiency in endogenous glutathione has been reported for premature babies and patients with cystic fibrosis (Wagner and Gardner 1997; Hudson 2001) as well as idiopathic pulmonary fibrosis patients (Borok et al 1991).

Attempts to utilise the protective or therapeutic potential of glutathione have been hindered by its short circulating half-life (1.5h, Wendel and Cikryt 1980) and its inability to cross cell membranes (Puri and Meister 1983). Exogenously supplied glutathione is hydrolysed rapidly in the kidneys into its constituent amino acids which are then redistributed and resynthesised to the tripeptide in the liver (Hahn et al 1978; Griffith and Meister 1979). Thus, in order for glutathione to retain its efficacy at a specific target site, the rapid hydrolysis must be prevented or at least delayed. Liposome encapsulation may provide a means of achieving this and, indeed, had been shown to significantly improve the hepatoprotective effect of intravenously administered glutathione against acetaminophen-induced liver toxicity (Wendel and Jaeschke 1982).

Liposomes have also been employed for GSH delivery to the lungs (Jurima-Romet et al 1990; Jurima-Romet and Shek 1991; Jurima-Romet et al 1992; Suntres

and Shek 1994, 1995, 1996; Suntres 2002) because lung tissues are highly susceptible to toxic, oxidative environmental insults (Bend et al 1985; Menzel and Amdur 1986). Since pulmonary glutathione serves as an endogenous protective agent against lung injury (Boyd et al 1982), its augmentation by exogenously administered glutathione may provide additional protection. Inhaled or directly applied GSH is rapidly cleared from the lungs. However, incorporation of GSH in a carrier system, such as liposome, offers a method of protection for GSH and prolonging its levels in the lungs (Kellaway and Farr 1990). Furthermore, it is known that liposomes aid in the transfer of macromolecules, normally impermeable to cell membranes, to the cell interior (Jurima-Romet et al 1990; Suntres and Shek 1995, 1996). While *in vitro* and *in vivo* studies on liposomal delivery of glutathione have a history of more than two decades (e.g. see Wendel et al 1982) there is no liposomal glutathione formulation approved for human use on the market yet. The main reasons for this include concerns for the safety of the liposomal formulations for human use, relative high cost of preparation of these formulations and low drug entrapment efficiencies. Therefore, it is vital to develop and evaluate liposomal delivery systems that are suitable for pulmonary administration of glutathione to patients with low pulmonary glutathione levels.

A major advantage of liposomes for pulmonary applications is that their main constituents, i.e. phospholipids, are endogenous to the lung (Baxter et al 1969). Hence liposomes composed of naturally occuring phospholipids at an appropriate dose, should not pose a toxicological risk to the lungs. However, application of potentially toxic chemicals, including volatile organic solvents or some of the cationic lipids, in the preparation of liposomes, will affect their safety and stability and hence pose hazards in their clinical utilisations. Also employment of procedures, such as sonication or high shear-force homogenisation, which can harm the structure of the drug to be incorporated to the liposomes, are among the draw-backs to be taken into account when formulating liposomal drug delivery systems as explained in chapter 2 of this thesis. In order to overcome the above-mentioned disadvantages a liposomal GSH formulation was developed without using any harmful chemical or process, by the heating method (HM). DPPC was used as the main ingredient of the

liposomes since it is the major phospholipid component of the lung (Frosolono et al 1970; Jurima-Romet and Shek 1991). The physicochemical characteristics of this new formulation are presented in this chapter.

5.2. Materials and Methods

5.2.1. Chemicals

Dipalmitoylphosphatidylcholine (DPPC), dicetylphosphate (DCP), Cholesterol (CHOL), EDTA (ethylenediaminetetraacetic acid), DTNB [5′, 5′-dithiobis-(2-nitorbenzoic acid)], and glycerol were purchased from Sigma Chemical Co. (Dorset, UK). Tri-sodium citrate was purchased from BDH (VWR International, Lutterworth, Leicester, UK). All solvents (chloroform, methanol, Analar grade) were obtained from Sigma Chemical Co. (Dorset, UK). All other chemicals were of commercial analytical grade.

Glutathione (GSH) tripeptide (γ-Glu-Cys-Gly) was a product of Sigma Chemical Co. (Dorset, UK) with the following characteristics:

Molecular weight:	307.3
Empirical formula:	$C_{10}H_{17}N_3O_6S$
Solubility:	0.1 M in H_2O
Melting point:	192-195°C

Phosphate-buffered saline (PBS) was prepared by dissolving 4.4mM NaH_2PO_4, 1.47mM KH_2PO_4, 2.68mM KCl, and 147mM NaCl in 1l distilled water and adjusted to pH 7.4.

5.2.2. Experimental methods

5.2.2.1. Liposome preparation

5.2.2.1.1. Preparation of conventional liposomes

Liposomes were prepared by the conventional thin-film hydration method as follows: a chloroform/methanol (2:1, v/v) solution of DPPC/DCP/CHOL (7:2:1 molar ratio), containing 10mM total lipid, was prepared. The lipid solution was evaporated to dryness in a round bottomed flask employing a rotary evaporator (EYELA, New Rotary Vacuum Evaporator NE-1, Birmingham, UK) and a water bath adjusted at 50°C. To remove traces of the solvents the flask was flushed with nitrogen gas for 1 hour and also placed under vacuum for a time period of at least 1 hour. The lipid film was then hydrated, above the phase transition temperature (T_c) of the lipids (i.e. at 50°C), with 2ml PBS containing 400mM GSH and multilamellar vesicles (MLV) formed by vortex agitation. The liposome suspension was extruded 11 times through two-stacked polycarbonate filters of 100nm pore size mounted in an extruder (LiposoFast™-Basic, Glen Creston Ltd, UK) fitted with two 0.1ml glass syringes above T_c. The liposomal GSH suspension was left undisturbed at room temperature under N_2 for at least 30min to equilibrate.

5.2.2.1.2. Preparation of liposomes by the heating method

HM-liposomes with the same composition as the conventional liposomes were prepared as follows: DPPC, DCP and CHOL were hydrated each in PBS (pH: 7.4), under N_2 atmosphere to prevent oxidation of the lipids, and then mixed together and heated up to 120°C in the presence of glycerol (3% v/v) in a silicone oil bath. Loading of GSH (400mM) into the HM-liposomes was achieved by adding it to the lipid suspension before the heating step.

In order to provide a more homogeneous population of liposomes with smaller diameters the liposomal suspension was extruded, as described in the previous section, through two-stacked polycarbonate filters of 100nm pore size. The HM-liposome-GSH suspension was left undisturbed at room temperature under N_2 for at least 30min for equilibration.

5.2.2.2. Thin layer chromatography

The effect of heating on GSH was checked by thin layer chromatography (TLC) in comparison with control un-heated GSH. A 20µl sample of GSH (5mg in 50ml PBS, pH: 7.4) heated to 120°C in a silicone oil bath and a 20µl sample of control unheated GSH (5mg dissolved in 50ml PBS) were spotted on 0.25mm layers of silica gel on 20×20cm plates (2.5cm from the lower edge of the plate). The samples were run in an eluent of butyl alcohol, acetic acid and distilled water (80:20:20). The plates were removed from the TLC tanks when the solvent ascended to within 3-4cm of the top of the plates. The plates were then air-dried for 15min in a fume hood and developed with iodine vapour in an iodine tank for 5-10min.

5.2.2.3. Separation of liposomes from unentrapped GSH

Separation of unentrapped (free) GSH from liposomal GSH was carried out in a manner similar to the procedure reported in the literature for separation of GSH from 400nm filtered LUV liposomes (Jurima-Romet et al 1990; Jurima-Romet and Shek 1991; Jurima-Romet et al 1992; Suntres and Shek 1994). For this, liposomes were washed twice in PBS buffer (pH: 7.4) and ultra-centrifuged at 110×10^3 g for 1 hour at 4°C using a BECKMAN L-80 refrigerated ultracentrifuge (Beckman Instruments, Palo Alto, CA, USA).

5.2.2.4. GSH determination

GSH was measured with 5′, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent) as described by Beutler and co workers (1963). This is a simple spectrophotometric assay in which DTNB gives a coloured product (measured at 412nm) when bound to non-protein sulphydryls (Figure 5.3.).

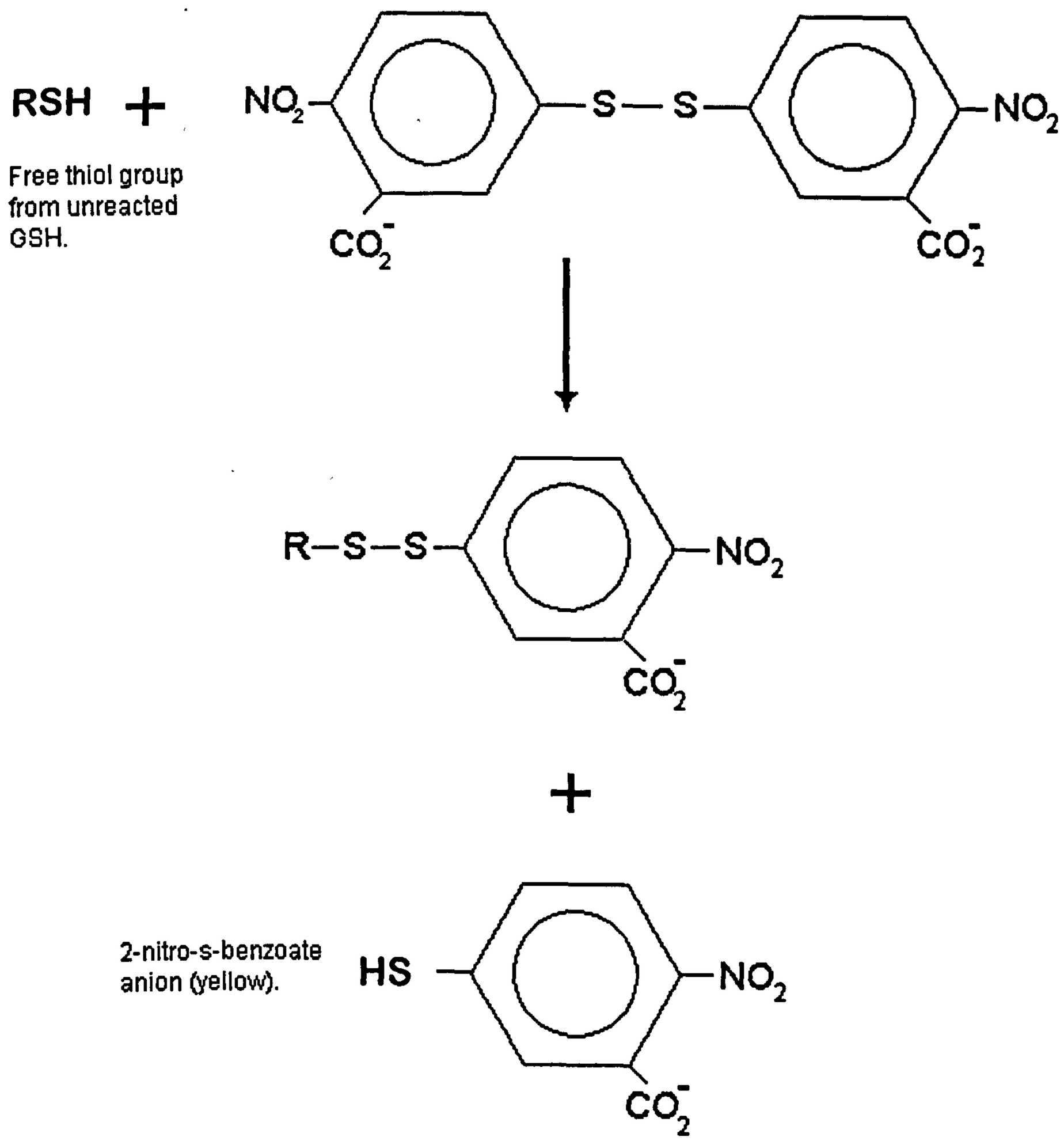


Figure 5.3. The principle reactions involved in the spectrophotometric determination of glutathione (GSH).

For quantification of GSH, 0.5 ml samples of GSH separated from the liposomes (see section 5.2.2.3.) were mixed each with a solution of DTNB (0.5 ml of 0.04% (w/v) DTNB in 1% (w/v) trisodium citrate). After 5 minutes the absorbance of the mixture was measured at 412nm using a double-beam UV/VIS Spectrometer (Perkin Elmer, Lambda 40, USA). GSH amounts were calculated by reference to calibration curves constructed for each assay separately. Appendix 3. represents one of such calibration curves.

5.2.2.5. Liposome GSH interaction assay

Liposomes were prepared by the conventional technique and the heating method as explained in section 5.2.2.1. but with no GSH. To these empty liposomes 400mM GSH was added and the mixtures were incubated at room temperature under N_2 for time periods of 1, 2, 3 and 4h after a brief hand-shaking. At the end of these time periods samples were centrifuged as explained in section 5.2.2.3. The absorbance of the collected GSH in the supernatants were measured as explained in the previous section. A sample of GSH (400mM) without the liposomes was employed as control.

5.2.2.6. Determination of entrapment efficiency

Entrapment efficiency (EE) of GSH in the conventional liposomes and HM-liposomes was determined spectrophotometrically as explained in section 5.2.2.4. The concentration of GSH was calculated from the calibration curves constructed for each assay. EE was calculated as a percentage of the initial drug added, after correction for the dilution factor, by using the following equation:

$$EE\ (\%) = 100 \times (c_{init} - c_{free}) / c_{init}$$

where c_{init} is the concentration of the initial GSH added, and c_{free} is the concentration of free (unentrapped) GSH.

5.2.2.7. Release of GSH from liposomes

Release kinetics of the liposomes prepared with 400mM initial concentration of GSH was investigated in phosphate buffered saline at pH value of 7.4 at room temperature. Two millilitres of the liposomal suspension was placed in a dialysis tube (Size 10, Inf Dia 1 1/4", 31.7mm, Mol. Wt cut-off 12000-14000, Medicell International Ltd, London, England) which was closed with plastic clips and suspended in 100ml of PBS which was constantly stirred on a magnetic stirrer. At certain time intervals (15, 30 and 45min; 1, 2, 3, 4, 5, 6, 7, 8, 10, 12 and 14h) 0.5ml aliquots were withdrawn. The release medium was replenished after each sample removal in order to have sink conditions. The amount of drug released was measured spectrophotometrically at 412nm, as explained in section 5.2.2.4., using a double-beam UV/VIS Spectrometer (Perkin Elmer, Lambda 40, USA).

5.3. Results

5.3.1. Thin layer chromatography

The effect of heating (120°C) on the chemical structure of GSH was studied in comparison with un-heated GSH sample using thin-layer chromatography (TLC, Figure 5.4.). Both samples produced single spots indicating the purity of the GSH sample utilised in this study as well as stability of the drug under the conditions of the preparation of the liposomal formulation. The average R_f values (n=4) for unheated and heated GSH samples were 0.39 and 0.40 respectively.

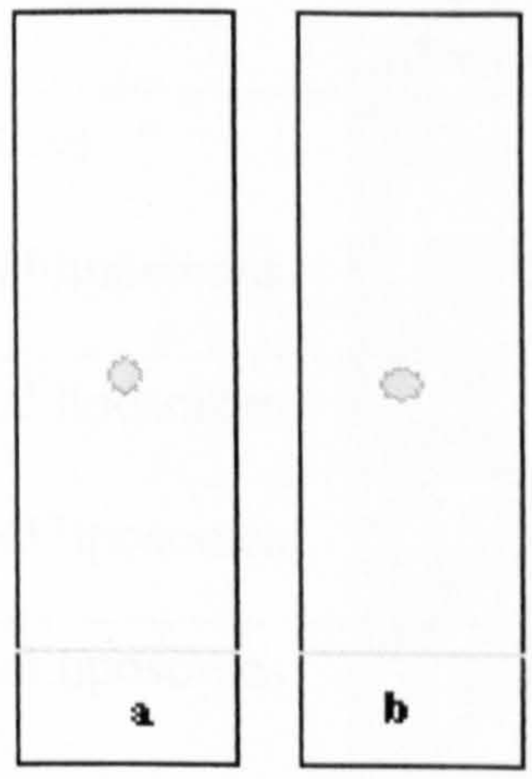


Figure 5.4. Thin layer chromatography of unheated (a) and heated (b) glutathione (GSH).

5.3.2. Interaction assay

In order to find out if GSH interacts with the external surface of the liposomes an interaction experiment was carried out, results of which are presented in Table 5.1. This table clearly indicates that there is no difference between the amount of control sample (i.e. GSH without liposomes) recovered after centrifugation compared with GSH samples brought into interaction with liposomes from one to four hour time periods. Both liposome types (i.e. those prepared by the conventional technique and those prepared by the heating method) exhibited the same results. Hence it seems that GSH does not interact with the preformed lipid vesicles.

Table 5.1. Evaluation of liposome interaction with GSH. Data are expressed as mean ± S.D. of three or more experiments. Initial GSH concentration in all experiments was 400mM.

Sample No.	Contents	Incubation Period (hour)	Recovered GSH Concentraion (mM)
1	GSH (control)	0	393 ± 3.3
2	GSH + conventional liposomes	1	392 ± 2.6
3	GSH + conventional liposomes	2	388 ± 3.3
4	GSH + conventional liposomes	3	389 ± 2.7
5	GSH + conventional liposomes	4	389 ± 3.6
6	GSH + HM-liposomes	1	394 ± 3.4
7	GSH + HM-liposomes	2	390 ± 3.7
8	GSH + HM-liposomes	3	389 ± 2.1
9	GSH + HM-liposomes	4	391 ± 3.1

5.3.3. Entrapment efficiency

The entrapment efficiency of liposomes was determined spectrophotometrically and calculated from the calibration curves constructed at 412nm each time separately for each assay. Only curves possessing trend lines with R^2 higher than 0.99 were utilised in these calculations. Some examples of the calibration curves used for GSH quantifications are represented in Appendix 3. Results of entrapment efficiency assays are given in Table 5.2. It appears that liposomes prepared by the heating method (HM-liposomes) have slightly, but not

significantly, less GSH entrapment efficiencies compared to the conventionally made liposomes.

Table 5.2. Entrapment efficiency of GSH in liposomes prepared by the conventional technique and the heating method. Data are expressed as mean ± S.D. of three or more experiments.

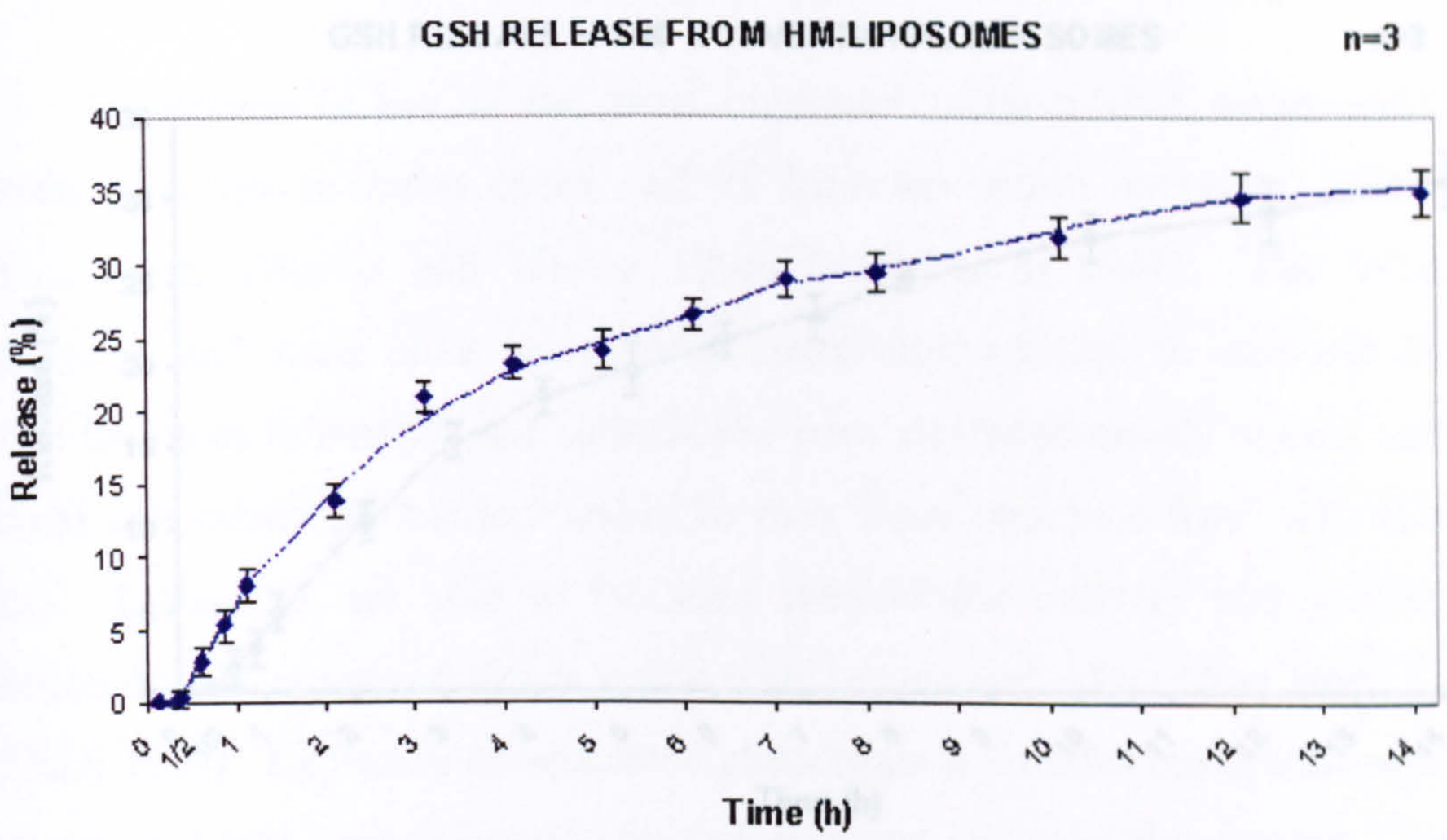
Liposome Type	Entrapment Efficiency (%)
Conventional	36.4 ± 2.4
HM-liposomes	33.8 ± 2.4

5.3.4. Release of GSH from liposomes

The release behaviour of the GSH loaded HM-liposomes is depicted in Figure 5.5.a. Almost 34.7% of the drug on average was released within the first 14 hours. The Higuchi plot (cumulative release vs. square root of time) of the release data is shown in Figure 5.5.b. Figure 5.6. a and b demonstrate the release characteristics and the Higuchi plot of the GSH loaded conventional liposomes respectively. Almost 31.1% of the drug on average was released within the first 14 hours.

It is evident that both types of liposome have similar release characteristics. The Higuchi plots of the release data for both liposome types are approximately linear indicating that GSH is entrapped within the liposomes.

a



b

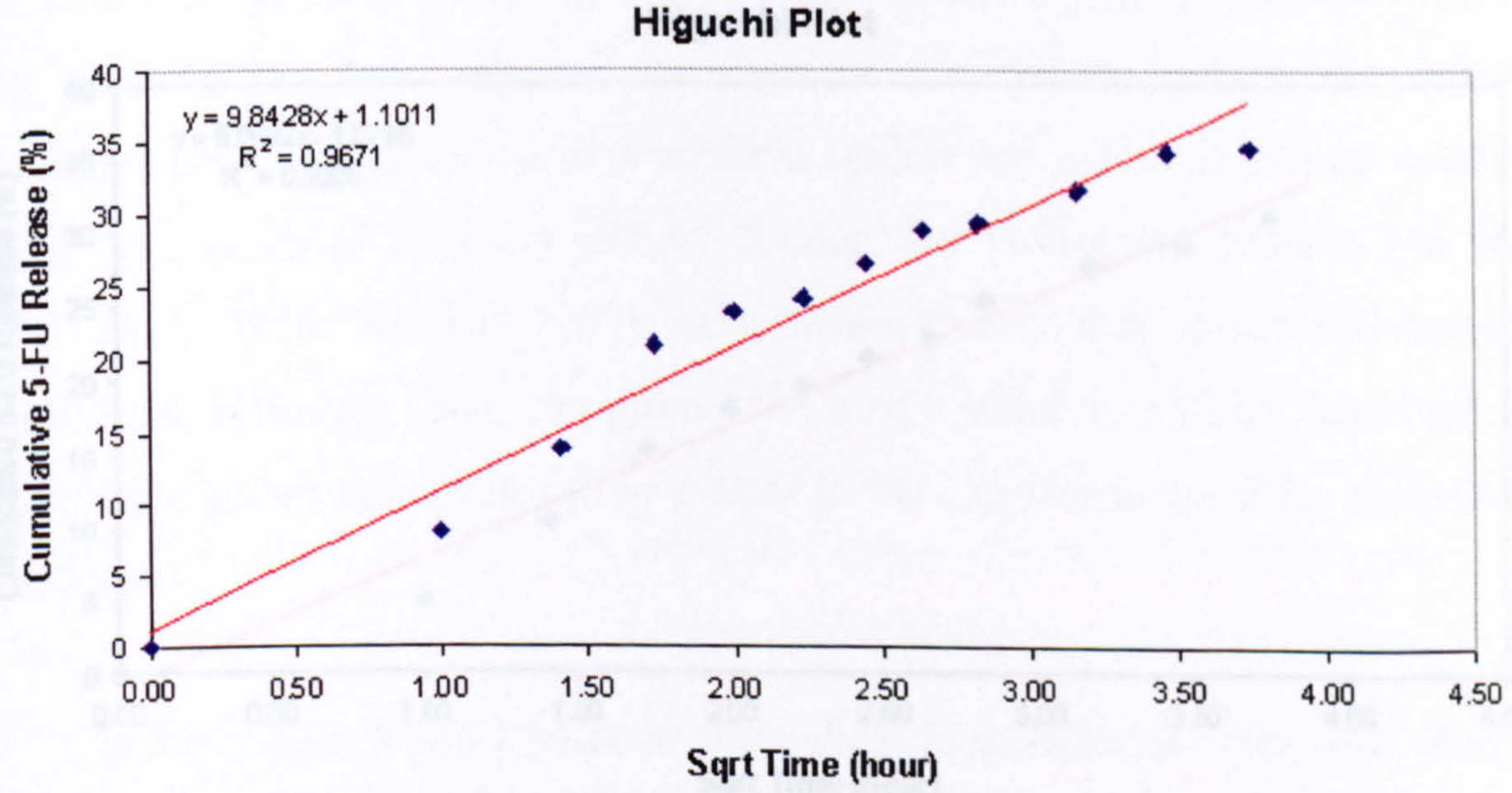


Figure 5.5. a) Release characteristics of GSH (400 mM) from the HM-liposomes in PBS (pH: 7.4). b) Higuchi plot for GSH release from the HM-liposomes.

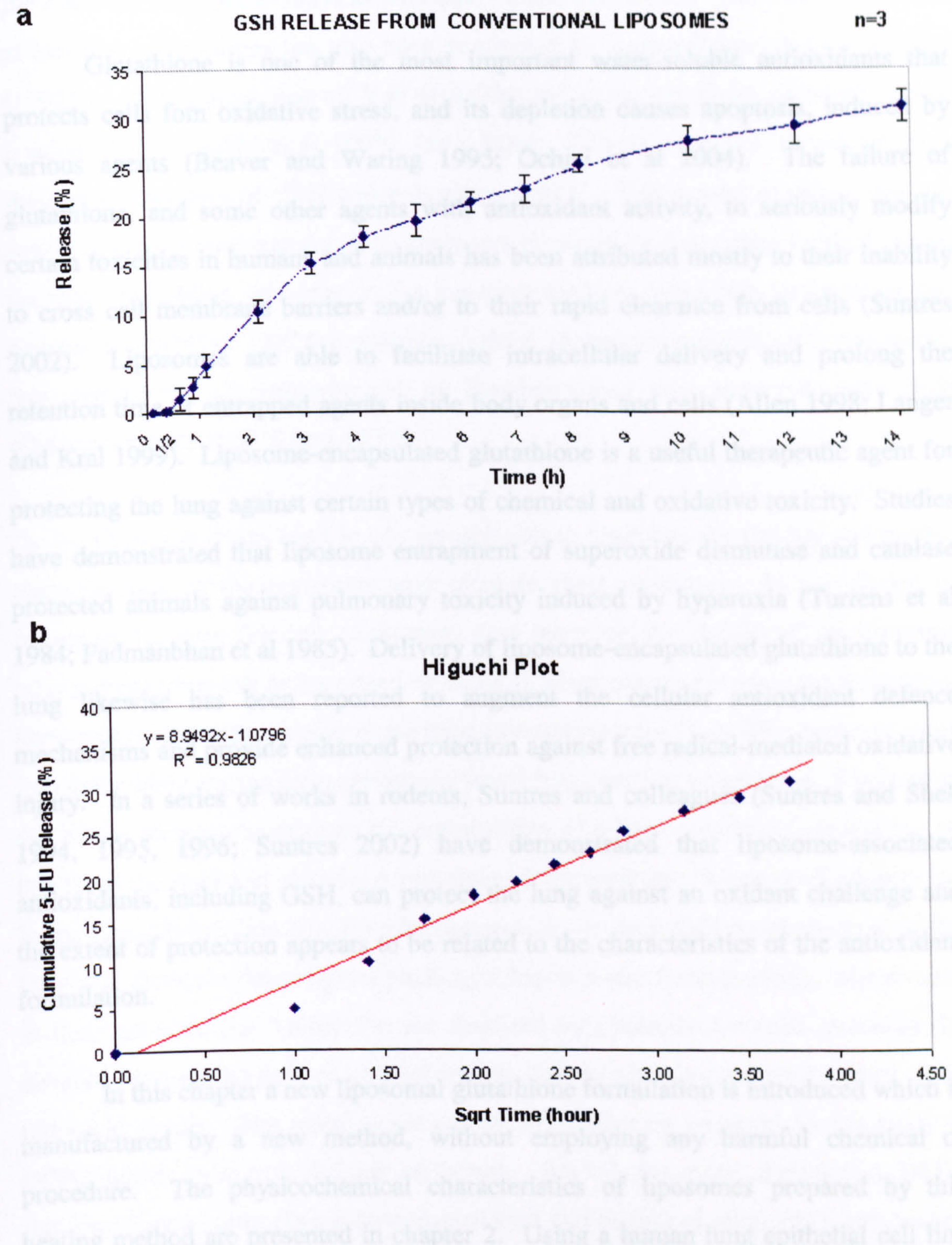


Figure 5.6. a) Release characteristics of GSH (400 mM) from the conventional liposomes in PBS (pH: 7.4). b) Higuchi plot for GSH release from the conventional liposomes.

5.4. Discussion

Glutathione is one of the most important water-soluble antioxidants that protects cells fom oxidative stress, and its depletion causes apoptosis, induced by various agents (Beaver and Waring 1995; Ochiai et al 2004). The failure of glutathione, and some other agents with antioxidant activity, to seriously modify certain toxicities in humans and animals has been attributed mostly to their inability to cross cell membrane barriers and/or to their rapid clearance from cells (Suntres 2002). Liposomes are able to facilitate intracellular delivery and prolong the retention time of entrapped agents inside body organs and cells (Allen 1998; Langer and Kral 1999). Liposome-encapsulated glutathione is a useful therapeutic agent for protecting the lung against certain types of chemical and oxidative toxicity. Studies have demonstrated that liposome entrapment of superoxide dismutase and catalase protected animals against pulmonary toxicity induced by hyperoxia (Turrens et al 1984; Padmanbhan et al 1985). Delivery of liposome-encapsulated glutathione to the lung likewise has been reported to augment the cellular antioxidant defence mechanisms and provide enhanced protection against free radical-mediated oxidative injury. In a series of works in rodents, Suntres and colleagues (Suntres and Shek 1994, 1995, 1996; Suntres 2002) have demonstrated that liposome-associated antioxidants, including GSH, can protect the lung against an oxidant challenge and the extent of protection appears to be related to the characteristics of the antioxidant formulation.

In this chapter a new liposomal glutathione formulation is introduced which is manufactured by a new method, without employing any harmful chemical or procedure. The physicochemical characteristics of liposomes prepared by this heating method are presented in chapter 2. Using a human lung epithelial cell line and two cytotoxicity markers it has been detected that HM-liposomes are completely non-toxic while conventional liposomes exhibited some degree of toxicity (chapter 3). Preparation of HM-liposomes involves heating the ingredients in order to dissolve the lipid molecules in the absence of the volatile organic solvents. Because of the presence of cholesterol in the liposomal formulation the temperature needed to

be as high as 120°C in order to solubilise cholesterol. This temperature did not harm the chemical structure of GSH as indicated by the TLC assay. The stability of GSH at elevated temperatures has also been confirmed by Jurima-Romet and co workers (1992) by determining that no loss of sulfhydryl group reactivity occurred during heating of GSH solution at 100°C for 1 hour. This is expected due to the high melting temperature of GSH which is above 190°C. Entrapment of drugs, that are sensitive to high temperatures, into liposomes prepared by the heating method can be achieved by adding the drug to the reaction medium when the temperature has decreased to 60°C or 70°C for example. Indeed, in the previous chapter it was shown that entrapment of 5-fluorouracil to HM-liposomes was achieved, with high efficiencies, both at 60°C and 120°C.

Cholesterol was used in our formulation to modulate the fluidity and release characteristics of the liposomes and increase their stabilities. In the design of liposomes for drug delivery, to increase drug retention, the rigidity of the liposome bilayer membrane can be increased by using lipids containing longer, more saturated fatty-acid chains with a higher phase transition temperature (T_C). This becomes more important when encapsulating relatively small drugs such as glutathione. Cholesterol can either decrease or increase the permeability of a lipid membrane, depending on the Tc of the lipid and also the environmental temperature. When lipid bilayers are in the liquid-crystalline (fluid) state, permeability is decreased by cholesterol as the fatty-acid chains undergo tighter packing (Chapman and Penkett 1966). Membranes in their gel-state (i.e. below Tc) are fluidised by cholesterol, which increases the distance between the lipid polar head groups, thereby allowing greater mobility of the fatty-acid chains. The main ingredient of the liposomes prepared in this study is DPPC which is a natural lung surfactant. Also it has been shown that DPPC liposomes are a better choice for encapsulation and release of relatively small, water-soluble compounds including GSH (Jurima-Romet et al 1992). The phase transition temperature of DPPC is 41°C and hence under the conditions of experiments and storage at or below room temperature, or even at normal body temperature, DPPC vesicles are in a gel state. Without cholesterol, these structures will be rigid and as a result lacking the required fluidity characteristics for efficient drug release and

interaction with cells. Consequently there was a need for employment of cholesterol in the liposomal formulation of the present study.

For the determination of GSH there are several procedures (e.g. see Griffith 1980; Anderson 1985; Farris and Reed 1987) in addition to commercially available kits such as Calbiochem®'s Glutathione Assay Kit (Calbiochem-Novabiochem Ltd, Nottingham, UK). This kit was used for GSH quantification at the initial stages of the present work. However, due to some problems such as obtaining non-reproducible and unusual results (i.e. higher amount of GSH was being quantified at the end of the assay compared to the initial amount of GSH used), application of this kit was abandoned. A spectrophotometric assay, developed by Beutler and co workers (1963), was utilised later on for GSH quantification. This assay which was found to be simple and reproducible has also been used by other groups for glutathione determination (Chamberlain 1998; Chamberlain et al 1998).

Results of the interaction assay indicated that GSH does not bind to liposomes externally. Consequently, the entrapment efficiency values obtained are for the GSH entrapped inside the aqueous phase of the lipid vesicles. Both conventional liposomes and HM-liposomes exhibited higher GSH entrapment efficiencies compared with the values reported in the literature. The reported liposomal GSH entrapment efficiencies are up to 21.0% (Jurima-Romet and Shek 1991) and 10.3-21.0% (Jurima-Romet et al 1992). These values are less than the encapsulation efficiencies obtained for the liposomes in the present study (i.e. 33.8% for HM-liposomes and 36.4% for conventional liposomes). A reason for high GSH entrapment efficiencies obtained for both liposomes in the present study, regardless of their preparation techniques, might be the composition and the ratio of the lipids used in their manufacture. Employing a lipid with high Tc (i.e. DPPC) as the main constituent of the liposomes (with 70% molar ratio) and cholesterol (with 10% molar ratio) as a membrane stabiliser seems to be a good proportion of the correct lipids for stable and efficient entrapment of GSH. Furthermore, presence of the negatively charged lipid DCP (with 20% molar ratio) prevents the aggregation and sedimentation of the liposomes by providing electrostatic repulsion between the

vesicles. In addition, liposomes prepared in the present study both by the conventional technique and heating method are in the form of large unilamellar vesicles (LUV) which are suitable for high entrapment of water-soluble agents, as discussed in chapter 4.

The importance of the effect of liposomal chemical composition on drug entrapment efficiency can be illustrated by findings of Jurima-Romet and colleagues (1992). This group have found that liposomes prepared from DMPC (dimyristoyl phosphatidylcholine) have a GSH entrapment efficiency of only about 3%. However, when they incorporated cholesterol at 30 mol% to these liposomes, entrapment efficiency of GSH increased to about 16% (i.e. more than five fold). Furthermore, Vyas and co-workers (2004) have recently reported that among four different phosphatidylcholine-based liposomes employed in their study, the formulation containing cholesterol and DCP had the highest entrapment efficiency for the drug rifampicin. These and similar studies clearly indicate the importance of liposomal chemical structure in different characteristics of the vesicles including stability and drug incorporation efficiency. The liposome composition selected in the present study was based, in addition to the fact that DPPC (the main ingredient of the HM-liposomes) is a natural lung component, on the previous data published in the literature (Papahadjopoulos et al 1977; Jurima-Romet et al 1992; Kahveci et al 1994; Borucu et al 1995; Zareie et al 1996a; Mozafari 1996; Filion and Phillips 1997; Zareie et al 1997; Mozafari and Hasirci 1998; Mozafari et al 1998a; Mozafari et al 1998b; Banerjee and Bellare 2001).

The release of GSH was sustained upon encapsulation in conventional liposomes and HM-liposomes and after 14 hours vesicles prepared by the conventional technique and heating method showed release of 31.1% (Figure 5.5.) and 34.7% (Figure 5.6.) respectively. The release of GSH from liposomes is very similar to the release observed for 5-FU (chapter 4). Efflux of GSH from HM-liposomes was biphasic, with an initial faster release for 2-3 hours, followed by a period of slow release. This biphasic release pattern of water soluble drugs seems to be a characteristic of bilayered vesicles and have been reported for liposomes

(Betageri and Parsons 1992) as well as niosomes (Baillie et al 1985; Namdeo and Jain 1999). In general the release of GSH could be described as matrix-controlled diffusion with kinetics of up to 8 hours according to the square-root of time law (Higuchi 1960).

5.5. Conclusions

Overall, the results of this investigation suggest that HM-liposomes may be of potential benefit for pulmonary delivery of GSH by prolonging retention of the encapsulated agent in the lung and facilitating its intracellular delivery. HM-liposomes were found out to be superior to previous liposomal GSH formulations in respect to drug entrapment efficiency. It is conceivable that HM-liposome encapsulation may enhance the therapeutic efficacy of pulmonary drugs by producing a local pharmacologic response within the target organ and minimising extrapulmonary adverse effects.

6: CONSTRUCTION OF A GENE DELIVERY VECTOR USING HM-LIPOSOMES AND TRANSFECTION OF HUMAN RESPIRATORY EPITHELIAL CELLS

6.1. Introduction

6.1.1. Nucleic acids as therapeutics

Inadequacy of conventional dosage forms in the treatment of existing health problems and the new challenges including AIDS and severe acute respiratory syndrome (SARS) makes the requirement for efficient formulations a matter of urgency. A new class of bioactive therapeutic agents are based on nucleic acid molecules. These nucleic acid drugs have the potential to offer healing of human (and animal) diseases at their cause rather than merely treating their symptoms. Based on their target site and mechanism of action, nucleic acid drugs can be subdivided into five classes: *i*) aptamers, *ii*) antigene compounds, *iii*) catalytic or ribozyme nucleic acids, *iv*) antisense compounds, and *v*) functional genes (Table 6.1.), explained in the following paragraphs.

i) Aptamers (from the latin *aptus*: to fit) are single-stranded or double-stranded nucleic acids which are capable of binding proteins or other small molecules (Ellington and Szostak 1990; Ess et al 1994). In another words aptamers are double-stranded DNA or single-stranded RNA molecules that bind specific molecular targets (Bock et al 1992). Aptamers, as therapeutic agents, would most likely bind proteins involved in the regulation and expression of genes (i.e. transcription factors). They have potential application in neoplastic and viral diseases. An aptamer approach to prevent HIV-1 replication by sequestering the tat protein of the virus has been

reported by Sullenger and co-workers (1990). Recently RNA aptamers have been used successfully to enhance tumor immunity in mice (Sandra-Marotto et al 2003).

Table 6.1. Nucleic acid drug classification (partially adapted from Stull and Szoka 1995).

Nucleic Acid	Target	Drug Type	Mechanism	Site of Action
Aptamer	Protein	DNA/ RNA	binding: interferes with biological function	intra- and/or extracellular
Antigene	Duplex DNA	DNA/ RNA	triplex formation: blocks transcription	nucleus
Ribozyme (Catalytic)	mRNA	RNA	hybrid assembly: cleavage and destruction of target	nucleus and/or cytoplasm
Antisense	mRNA	DNA/ RNA	hybrid assembly: translation arrest and/or RNase H activation	nucleus and/or cytoplasm
Functional Gene	Chromosome (ds* DNA)	DNA	repair of a defective gene or replacing a missing one	nucleus

*ds DNA: double stranded DNA

ii) Nucleic acids targeted to genomic DNA are called antigene nucleic acids (Helene and Toulme 1990; Orson et al 1991; Torigoe et al 2003). Antigene nucleic acids are designed to bind to single-stranded or double-stranded DNA and thereby interfere with replication or transcription. McShan and co-workers (1992) reported HIV-1 inhibition by two different triplex forming (antigene) oligonucleotides. In addition there are several reports of inhibition of cancer cell proliferation by antigene compounds (for a review see Stull and Szoka 1995).

iii) Ribozymes are RNA molecules which catalytically cleave covalent bonds in a target RNA (Chang et al 1990; Cech 1992). They possess an enzymatic, self-cleaving activity and catalytically cleave phosphodiester bonds in the target RNA strand. Other groups define ribozymes as a class of RNA molecules that can perform phosphodiester cleavage of nucleic acids in the absence of protein (Symons 1992). They can hybridise to and cleave target RNA molecules independent of cellular enzymes. These site-specific RNase properties of ribozymes have been applied to the treatment of HIV infection and cancer (Sarver et al 1990; Poeschla et al 1994). Ribozymes targeted *in vivo* for therapeutic applications are chemically synthesised or can be transcribed from a DNA template. Since the catalytic RNA is not consumed during the cleavage reaction, a large number of substrate molecules can be processed. These ribozymes have a turnover greater than one and are not modified during the reaction (Symons 1992). Several groups have shown that ribozymes are able to suppress foci formation, tumor cell growth and the tumorigenicity of the cells when injected into mice (Koizumi et al 1992, 1993; Kashani-Sabet et al 1994).

iv) Another group of nucleic acid drugs are antisense compounds (Uhlmann and Peyman 1990; Akhtar et al 1991). These nucleic acids have the ability to inhibit individual gene expression in the potential treatment of cancer and viral diseases (Wickstrom 1991). Antisense drugs utilise the ability of single-stranded oligonucleotides to hybridise with the target sequence in mRNA. In principle, this provides selective drug action only on the target, if the oligodeoxynucleotide (ODN) consists of about 15 or more nucleotides (Jaaskelainen and Urtti 2002). Hybridisation results in the arrest of translation due to steric blocking or by

recruitment of the enzyme RNase H to the binding site. Currently, a number of clinical trials are ongoing for treatment of various diseases (e.g. Crohn's disease, non-Hodgkin's lymphoma, HIV and cytomegalovirus infections) with antisense ODN's and the FDA has approved Vitravene (fomivirsen sodium) for the treatment of cytomegalovirus retinitis in AIDS patients (Jaaskelainen and Urtti 2002). Single stranded oligodeoxynucleotides are sensitive to extracellular and intracellular nucleases and rapid degradation *in vivo* restricts their use in most cases. Their stability can be increased by some modifications such as replacement of one of the non-bridging oxygen atoms in the phosphate with sulphur which leads to phosphorothioate oligonucleotides. Phosphorothioates (PS) shown in Figure 6.1. are one of the oligonucleotide chemical analogues now being studied most actively for use as therapeutic agents (Woodle and Leserman 1998). Other chemical analogues include phosphorodithioates (PDS) and methylphosphonates (MP) both shown in Figure 6.1. as well.

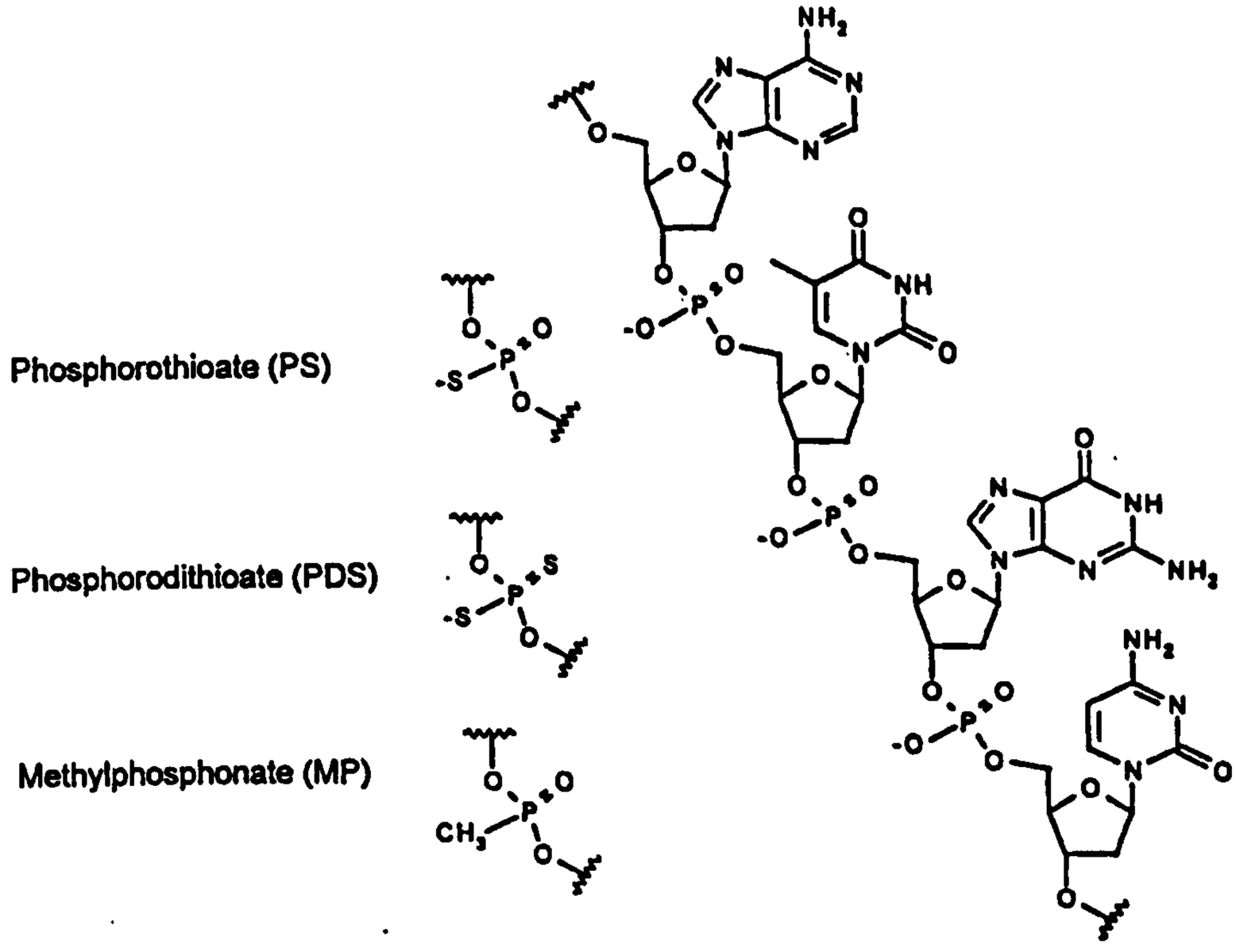


Figure 6.1. The chemical structure of common chemical analogues of oligodeoxynucleotides with each of the four common bases found in the DNA (From: Woodle and Leserman 1998).

v) Treatment of human genetic disorders requires intact functional genes to repair the defective genes in situ or to be added elsewhere in the genome (Hug and sleight 1991). Major causes of death and disease today are of genetic origin or have major genetic components. More than 4900 genetic disorders of man result from a defect in a single gene (McKusick 1990), and many more are probably caused by multiple defects (Hug and Sleight 1991). Gene replacement therapy can be used to overcome diseases such as cystic fibrosis (CF), α_1-antitrypsin deficiency, Huntington's disease, severe combined immunodeficiency (SCID), sickle cell disease, severe thalasemias, Gaucher's disease, and Lesch-Nyhan syndrome (Thompson and Thompson 1986; Hug and Sleight 1991; Cheng and Scheule 1998; Davies et al 2001).

In addition to the above-mentioned nucleic acids another class of polynucleotide therapeutic agents is DNA vaccines which, for instance, can be in the form of antigen-encoding plasmid DNA (Greogoriadis et al 1997; Perrie et al 2002) or synthetic oligodeoxynucleotides containing immunostimulatory sequences (Joseph et al 2002).

Two major obstacles to the creation of effective nucleic acid drugs are their relatively high molecular weight (>3300 dalton) and their sensitivity to degradation. These obstacles have created a significant delivery problem which needs to be solved if nucleic acid drugs are to become effective therapeutics (Stull and Szoka 1995).

6.1.2. Nucleic acid transfer methods

In general, a polynucleotide can not be inserted into a person's cell *in vivo*. It must be delivered to the cell using a carrier known as a `vector`. There are currently several methods of DNA/RNA transfer under investigation which utilise physical, chemical, colloidal or biological means for nucleic acid transfer. In the case of gene therapy one approach is to introduce genes into the somatic cells of subjects to correct inherited or acquired diseases through synthesis of a missing or defective

gene product. Several criteria must be met to provide effective gene therapy. The defective gene must be identified and its contribution to the pathophysiology of the disorder characterised. An animal model of the disease should be available and measurable parameters for an adequate therapeutic effect defined. The gene must also be delivered to either the affected tissue or provide a systemic effect from another tissue to alleviate the disease. Finally, the process of gene transfer or subsequent expression of the introduced gene should cause no adverse effects and ideally should be regulatable.

A variety of techniques have been used to introduce polynucleotides into cells both *in vitro* and *in vivo*. Generally nucleic acid transfer techniques are classified in two main groups: viral and non-viral vectors. Viral vectors include adenovirus (the most widely used viral vector), adeno-associated virus (AAV), retrovirus and sendai virus (Davies et al 2001). Viral vectors, in general, are believed to provoke mutagenesis and carcinogenesis (Bhattacharya and Huang 1998; Liu and Huang 2002). Zuckermann et al (1999), for instance, observed influenza-like symptoms and a cell-mediated immune response against the adenovirus vector administered into lung segments of cystic fibrosis subjects. In addition some (if not all) viral vectors (e.g. AAV) have another drawback which is their small size (Davies et al 2001). This makes insertion of large pieces of DNA, such as the cystic fibrosis transmembrane conductance regulator (CFTR) gene, problematic.

Non-viral vectors include calcium phosphate precipitation (Graham and Van der Erb 1973; Chen and Okayama 1987), microinjection (Anderson et al 1980), electroporation (Neumann et al 1982; Potter et al 1984), DEAE-dextran (Sussman and Milman 1984), particle bombardment by microprojectiles (Klein et al 1987), polymers such as branched (Boussif et al 1995; Fischer et al 1999) and linear (Chemin et al 1998) polyethylenimine (PEI), microspheres (Capan et al 1999) and liposomes (Hoffman et al 1978; Turner et al 2002) which are discussed in the next section.

6.1.2.1. Liposomes as polynucleotide carriers

In a paper published in 1978 Hoffman and colleagues showed that liposomes made of the zwitterionic lipid egg lecithin can efficiently incorporate DNA molecules (Hoffman et al 1978). Since then the number of works using liposomes as a gene transfer method has been increasing extensively. At present liposomes are accepted as one of the main standard gene transfer vectors and many companies compare the efficiency of their new gene delivery products with that of liposomes. Among the polynucleotide transfer methods liposomes are one of the most attractive vectors due to their biocompatibility, non-immunogenicity (unless needed to be immunogenic), ease of preparation, capability to accommodate large pieces of nucleic acids, targetability and ability to mediate cytoplasmic/nucleus delivery (e.g. see Tanner et al 1997; Bhattacharya and Huang 1998; Barron et al 1999; Bailey and Sullivan 2000; Oku et al 2001; Hofland et al 2002; Piperno-Neumann et al 2003 and references therein). Furthermore, unlike some other polynucleotide transfer methods (e.g. microprojectiles), their administration to the body is mild and non-invasive.

On some occasions it is required to complex DNA molecules with proteins or polycations to enhance the transfection efficiency. Importantly, the use of liposomes affords the flexibility to undertake these approaches, whereas some other polynucleotide delivery methods (e.g. calcium phosphate and polycations) do not (Fraley et al 1980). Liposomes are also being considered for delivery of nucleic acids to the human airways. Successful formulations of dry liposome-DNA powder aerosols as well as nebulised cationic liposome-DNA complexes have been reported (Schreier 1998). In aerosol formulations liposomes are not only required for protecting the polynucleotides from enzymatic degradation and targeting them to the site of action, they are also needed to protect the fragile polynucleotide structures from shearing caused by the aerosolisation process. Eastman et al (1997) found that naked DNA was rapidly degraded when aerosolised while coating with lipids effectively protected DNA from shearing.

The majority of liposomes employed in nucleic acid delivery are cationic in nature and have been used for delivery of different polynucleotides, including plasmid DNA and antisense nucleotides, into various cells (Felgner et al 1987; Felgner and Ringold 1989; Gao and Huang 1991; Farhood et al 1992; Farhood 1995; Takeuchi et al 1996; Lesage et al 2002). However, the net positive charge of these vectors promotes unspecific interactions with the biological milieu, opsonisation, destabilisation and rapid uptake by phagocytes (Fahr et al 2002). A further drawback of cationic liposomes is their toxicity (Panzner and Jansons 1979; Chawla et al 1979; Campbell 1983; Filion and Phillips 1997, 1998; Dokka et al 2000; Nagahiro et al 2000; Tousignant et al 2000) which, along with the other limitations, leads to low transfection efficiencies. Neutral liposomes also suffer from insufficient transfection efficiencies mainly due to low DNA/RNA entrapment, especially in small vesicles. Bailey and Sullivan (2000) have recently reported high plasmid trapping efficiencies (up to %80.0) for neutral liposomes of 200nm average size, however, their methodology is very lengthy (more than 24h) and involves application of ethanol, which is a cytotoxic agent (Triglia et al 1991a, 1991b), for plasmid entrapment to the liposomes.

Hence, the only possible liposome type which remains to be considered for polynucleotide delivery is the anionic type, unless the above-mentioned problems with the cationic and neutral vesicles could be rectified. Initially there seems to be two main problems with considering anionic vesicles as a vector:

i) cellular uptake of anionic entities is generally believed to be low (if not impossible),

ii) incorporation of negatively charged nucleic acids to similarly charged vesicles seems to be infeasible.

Perhaps due to these reasons the interest in employing anionic lipids/liposomes in nucleic acid delivery has been very low compared to cationic ones. However, works of some groups have been encouraging for the reconsideration of anionic liposomes. Regarding cell uptake of these liposomes, results obtained by different groups including Heath et al (1985), Monkkonen et al (1994) and Katragadda and co

workers (2000) indicated that anionic liposomes associate more effectively and are taken up more readily by the cells compared with neutral and even cationic liposomes.

In respect of the incorporation of nucleic acids into anionic liposomes one possible solution came from the works of Zhdanov and colleagues (Kahveci et al 1994; Zhdanov et al 1994), showing that it is possible to incorporate polynucleotides to anionic (and zwitterionic) vesicles by the mediation of divalent metal cations such as Mg^{2+} and Ca^{2+}. The idea, which probably stemmed from earlier works such as a study of DNA interaction with mitochondrial and model membranes in the presence of divalent cations (Bichenkov et al 1978), was successively utilised in the formulation and construction of anionic liposomal vectors (Mozafari 1996; Zareie et al 1997; Mozafari and Hasirci 1998; Mozafari et al 1998a, 1998b, 2001, 2002a, 2002b, 2002c). The polynucleotide carrier formulation is composed of triple complex of liposome-Ca^{2+}-DNA (or other nucleic acids), as shown in Figure 6.2.

One of the advantages of this liposomal vector is the mild approach used in the incorporation of DNA molecules into the liposomes, in contrast to harsh methods of DNA incorporation into liposomes, such as sonication, reported in the literature (Fraley et al 1980). Evidence for the presence of DNA and other polynucleotides in these anionic complexes came from studies using different techniques including spectrofluorimetry, turbidity measurements, light scattering and direct visualisation by fluorescence microscopy and scanning tunnelling microscopy (Mozafari 1996; Zareie et al 1997; Mozafari and Hasirci 1998; Mozafari et al 1998a, 1998b). Based on these investigations a mechanism for polynucleotide interaction with anionic and zwitterionic lipid vesicles was proposed (Mozafari and Hasirci 1998). These formulations have subsequently been adopted and/or adapted by other research groups employing neutral (Kharakoz et al 1999; Bailey and Sullivan 2000) and anionic (Fillion et al 2001) vesicles.

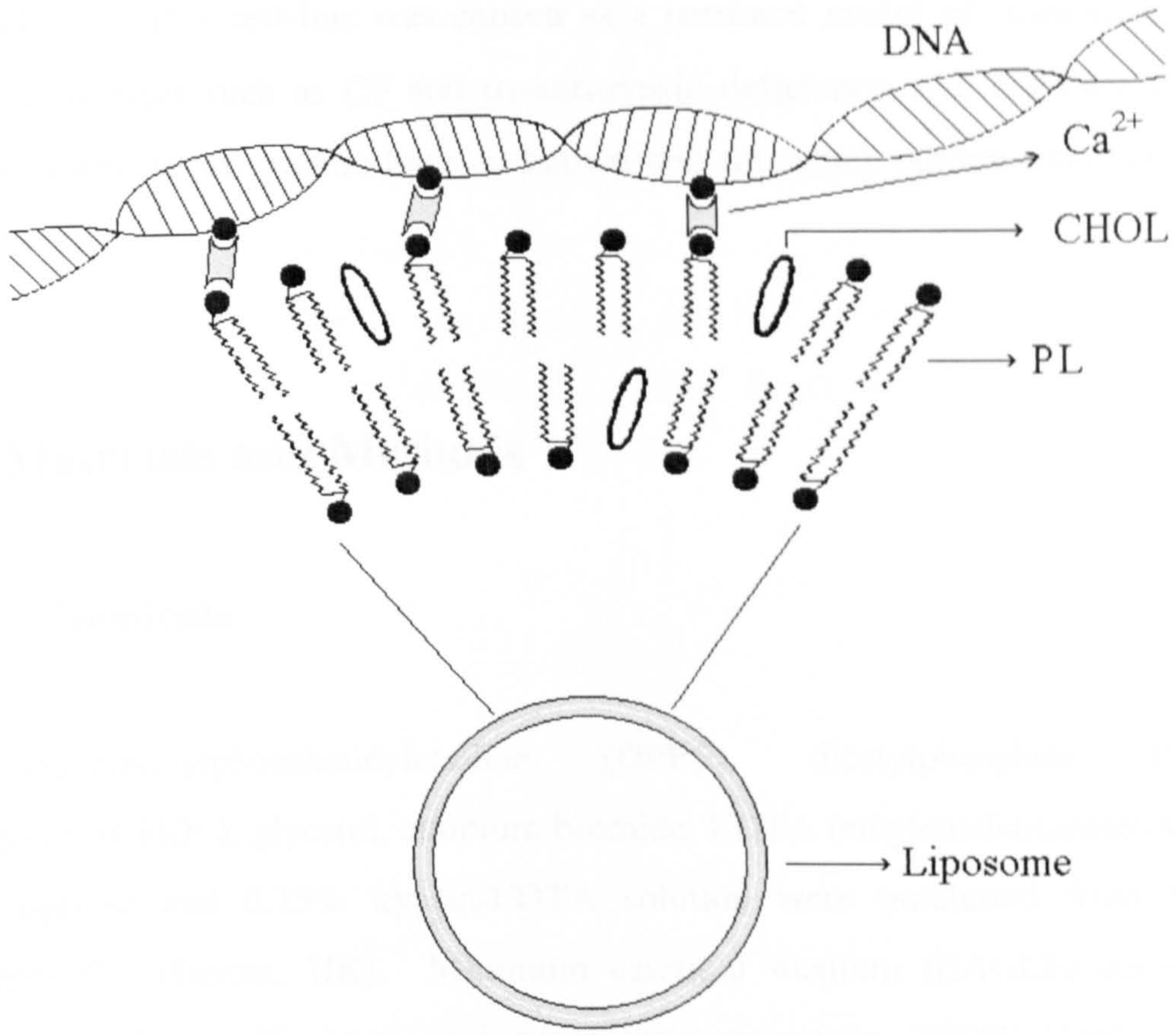


Figure 6.2. Enlargement of a section of the bilayer of a liposome-Ca^{2+}-DNA complex to show a possible mechanism of phospholipid (PL) association with DNA by the mediation of Ca^{2+} ions. The lipid vesicle may or may not contain cholesterol (CHOL).

A concern in the clinical application of the above-mentioned triple complex vectors was the involvement of volatile organic solvents, such as chloroform or methanol, in their preparation. In this study similar complexes were prepared by the heating method (HM) introduced in chapter two of this thesis thus avoiding any harmful chemical or methodology. The physical features of the ternary complex of HM-liposome-Ca^{2+}-DNA were investigated using atomic force microscopy,

transmission electron microscopy, light scattering, electrophoresis and an ethidium bromide intercalation assay. The transfection efficiency of the ternary complex was assessed using a human bronchial epithelial cell line (16HBE14o-) in the presence of serum (10%). This cell-line was chosen as a pertinent model of pulmonary gene therapy challenges such as CF and α_1-antitrypsin deficiency, two common single-gene disorders whose genetic basis is known and for which current treatments are unsuccessful.

6.2. Materials and Methods

6.2.1. Chemicals

Dipalmitoylphosphatidylcholine (DPPC), dicetylphosphate (DCP), Cholesterol (CHOL), glycerol, ethidium bromide, EDTA (ethylenediaminetetraacetic acid), agarose and 0.25% trypsin-EDTA solution were purchased from Sigma Chemical Co. (Dorset, UK). Minimum essential medium (EAGLE) containing Glutamax-1, fetal calf serum, and penicillin/streptomycin (10000 U/mL, 10000 µg/mL) were obtained from GibcoBRL® Life Technologies Ltd (Paisley, UK). DNA ladder marker (1kb) was obtained from Promega (Madison, USA). DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate) was purchased from Roche Diagnostics Ltd (Southampton, UK) at a concentration of 1 mg/ml and used as supplied. Deoxyribonuclease I (DNase I) was obtained from Invitrogen (Groningen, Netherlands). All solvents (chloroform, methanol, Analar grade) were obtained from Sigma Chemical Co. (Dorset, UK). All other chemicals were of commercial analytical grade.

Phosphate-buffered saline (PBS) was prepared as explained in section 2.2.1. and adjusted to pH 7.4.

6.2.2. Experimental methods

6.2.2.1. Liposome preparation

Anionic liposomes were prepared by the heating method as explained in section 2.2.2.1. Sizing of the liposomes was performed by using polycarbonate filters of 100nm or 400nm pore size. For gene transfer and other experiments the final concentration of liposomes was adjusted to provide the liposome/DNA ratios of the complexes as explained in each relevant section. In all other cases the liposomal samples were prepared containing 10mM total lipid.

6.2.2.2. Plasmid DNA

The plasmid pcDNA3.1/His B/*lac*Z (8578 nucleotides) (Invitrogen, Groningen, Netherlands), containing β-glactosidase reporter gene, grown in competent Escherichia coli DH5α cells, prepared by alkaline lysis, and purified by CsCl-EtBr density gradient ultracentrifugation (Sambrook et al 1989) was kindly provided by Dr A. R. Evans (Liverpool John Moores University, England, UK). The purity of plasmid preparations was determined by agarose gel electrophoresis and optical density (the $OD_{260/280}$ ratio was between 1.8 and 1.9). Concentrations of the plasmid samples were measured by UV absorption at 260nm, by multiplying the sample's absorbance by a factor of 50 (Muller et al 1993). The plasmid samples were stored at –20°C until use.

6.2.2.3. Incorporation of DNA to liposomes

The ternary complexes of HM-liposome-Ca^{2+}-DNA (vesicles of which were prepared by the heating method) were constructed by introducing plasmid DNA and then calcium (50mM $CaCl_2$) to the liposomal suspensions followed by incubation of the mixture for 30min under N_2 at room temperature. For gene transfer studies

complexes were made containing liposome/DNA ratios of 4:1, 5:1, 6:1, 7:1 and 8:1 (lipid:DNA, w/w). For other assays, complexes were composed of liposome/DNA ratio of 7:1. Separation of free DNA was carried out by centrifugation twice at 100×10^3 g for 30min at 4°C. DNA concentration was measured spectrophotometrically, by multiplying the absorbance at 260nm as explained in the previous section. Quantification of plasmid DNA was also performed by agarose gel electrophoresis as outlined below.

6.2.2.4. Agarose gel electrophoresis

Agarose gels were made of 0.8% (w/v) molecular biology grade agarose in TAE buffer (40mM Tris-acetate, 1mM EDTA, pH: 7.7). Samples for electrophoresis (20µl) were first mixed with 5µl loading buffer (0.01% (w/v) bromophenol blue, 20% (v/v) glycerol in distilled water) before loading onto the gels. For comparison, 1kb (kilo base) DNA ladder of molecular weight markers was also loaded on the gels. Electrophoresis was carried out at 70 volts for 2.5 hours using TAE as a running buffer. The DNA bands in the gel were stained with ethidium bromide (0.5µg/ml) in water for 20 minutes and then destained in water for 30 minutes to remove excess ethidium bromide from the gel. DNA bands were visualised using UV light on a transilluminator, and the gels photographed with a Kodak DC120 camera and photo system (Kodak digital sciences, New York, USA). The amount of DNA was analysed using the electrophoresis documentation and analysis system fitted with 1D Image Analysis Software (Kodak digital sciences, New York, USA) based on the calibration made utilising known amounts of DNA.

6.2.2.5. Light scattering

The mean particle size and polydispersity index of the HM-liposomes alone, HM-liposome-Ca^{2+}-DNA complexes and HM-liposome-Ca^{2+} (control), were determined by dynamic light scattering as explained in section 2.2.2.4. Calcium

concentration in the ternary complexes as well as the HM-liposome-Ca^{2+} samples was 50mM. The light scattering measurements were performed at least in triplicate.

6.2.2.6. Ethidium bromide intercalation assay

The accessibility of ethidium bromide (EtBr) to the DNA or DNA associated with the HM-liposomes was monitored at room temperature in a Perkin Elmer Luminescence Spectrometer (LS 50B, UK). The fluorescence was read at excitation and emission wavelengths of 500 and 610nm respectively, using 2.5mm excitation and 5.0mm emission slits. The sample chamber was equipped with a magnetic stirring device. The value of fluorescence obtained upon addition of 5µg/ml DNA and 0.2µg/ml EtBr simultaneously was set as 100%. The difference between the fluorescence intensity reading at each stage and that of EtBr-DNA was designated ΔF.I. Each ΔF.I. value was determined as follows:

$$\Delta F.I. = 100 - [(F_\alpha \times 100) / F_{ED}]$$

where F_α is the fluorescence intensity reading at each stage and F_{ED} is the fluorescence intensity of EtBr-DNA complex. The F_α and F_{ED} are average values from three or more experiments. The pattern of the ethidium bromide assay, by adding the reagents in different orders, is presented in Table 6.2.

Table 6.2. Experimental plan of the ethidium bromide intercalation assay.

Assay	Material added at				
No.	0 sec.	280 sec.	420 sec.	520 sec.	760 sec.
1	EtBr + DNA	___	Ca^{2+}	___	___
2	EtBr + DNA	___	Mg^{2+}	___	___
3	EtBr + DNA	LUV* + Ca^{2+}	___	MLV**	___
4	EtBr + DNA	LUV	___	Ca^{2+}	MLV
5	EtBr + DNA	Ca^{2+}	___	LUV	MLV
6	EtBr + DNA	LUV	___	Ca^{2+}	LUV
7	EtBr + DNA	Ca^{2+}	___	MLV	___
8	LUV-Ca^{2+}-DNA	___	EtBr	___	___
9	LUV-Ca^{2+}-DNA	___	EtBr + MLV	___	___
10	LUV-Ca^{2+}-DNA	EtBr	___	DNA	___

* LUV: large unilamellar vesicle.
** MLV: multilamellar vesicle.

6.2.2.7. DNase I protection assay

The procedure used for assessing the resistance of the ternary complexes to nuclease degradation was similar to the method of Faneca et al (2002) using electrophoresis and spectrofluorimetry. Complexes were submitted to DNase I action (5 units DNase I/μg of DNA) at 37°C for 30 min, followed by inactivation of the enzyme with 0.5M EDTA (1 μl/unit of DNase I). The liposomal complexes were precipitated by centrifugation (100×10^3 g, 30min) and the pellets were resuspended

in PBS. After addition of 61.22mM EDTA the samples were incubated at room temperature for 30min in order to release the liposome-associated DNA molecules. Another round of centrifugation was carried out as above and samples from the supernatant were applied to the electrophoresis gels as explained in the previous section. Samples of plasmid DNA without liposomes and Ca^{2+} were also subjected to DNase I treatment under similar experimental conditions (control).

For spectrofluorimetric measurements, following treatment of the complexes with DNase I, the same experimental procedures as described for the EtBr intercalation assay (section 6.2.2.6.) were carried out. The extent of DNA degradation was determined according to the following equation:

$$\textbf{DNA degradation (\%)} = (F_i - F_a) / (F_{100} - F_0) \times 100 \quad \text{(Faneca et al 2002)}$$

where F_i is the fluorescence value emitted by EtBr in the presence of the complexes treated with the inactive enzyme (Dnase I inactivated by chelation with 0.5M EDTA as explained above), F_a is the fluorescence value emitted by EtBr in the presence of the complexes treated with the active enzyme, F_{100} is the fluorescence value emitted by EtBr in the presence of 5µg/ml DNA and F_0 is the residual fluorescence of EtBr (i.e. fluorescence intensity of 0.2µg/ml EtBr).

6.2.2.8. Transmission Electron Microscopy

The triple complex of HM-liposome-Ca^{2+}-DNA was studied under transmission electron microscope (TEM) as explained in section 2.2.2.7.

6.2.2.9. Scanning tunnelling microscopy

For visualisation with scanning tunnelling microscopy (STM) a 20µl suspension of HM-liposome-Ca^{2+}-DNA complex was deposited on highly oriented

pyrolytic graphite (HOPG). The sample was then dried at room temperature under atmospheric pressure in a clean room with a relatively slow drying rate. The STM was operated in air as outlined in section 2.2.2.8. The diameter of liposomes was determined by a Digimatic caliper (Mitutoyo Ltd. England) using the dimensions of the STM images.

6.2.2.10. Atomic force microscopy

In addition to STM another scanning probe microscope, that is atomic force microscopy (AFM), was used to study the ultrastructures of the liposomal vectors. Visualisations were performed using an atomic force microscope model NanoScope IIIa (Digital Instruments Inc., Santa Barbara, California, USA) connected to an Optizoom microscope (0.8 × - 2.0 ×). The images were transferred to a computer via a camera (Sony CCD, Japan). The HM-liposome-Ca^{2+}-DNA samples were deposited on stainless steel discs and dried in air after absorbing excess solution using a filter paper. The AFM was operated in the tapping mode at room temperature using standard silicone nitride probes of 120μm lengths with a spring constant of 0.38 N/m. Data were analysed using NanoScope IIIa analysis software (Version 4.23r3, Digital Instruments Inc., USA).

6.2.2.11. Cells

Immortal human respiratory epithelial cells (16HBE14o-), kindly donated by Dr A. R. Evans (Liverpool John Moores University, England, UK), were grown and maintained as described previously in Chapter three (section 3.2.2.4.).

6.2.2.12. Gene transfer

For gene transfer experiments HBE cells (approximately 50,000 cells/ml) were grown in 24-well plates to almost 50% confluence. The medium was removed from each well and 500µl of the control reagents or liposomal vectors in complete medium (cMEM) was added per well. Cells in each well were transfected with different lipid/DNA ratios of 4:1, 5:1, 6:1, 7:1 and 8:1 (w/w). The liposomal vectors were prepared as explained in sections 6.2.2.1. and 6.2.2.3. Free DNA and DOTAP (with a 6:1 w/w ratio of DOTAP:DNA) were used as control. A constant amount of plasmid DNA (i.e. 1.25µg) was used in a total volume of 500µl/well. The cells were incubated with the transfection reagents for 24 hours at 37°C, 5% CO_2 and 95% humidity, after which the transfection medium was removed and replaced with cMEM (1ml/well). The cells were incubated for another 24 hours to allow time for the expression of the transgene product before analysis (i.e. 48 hours post-transfection).

6.2.2.13. β-galactosidase assay

After the transfection period the medium was removed and the wells were washed twice with PBS (1ml/well). To each well 125µl of low detergent lysis buffer (0.05% v/v Triton X-100, 2mM EDTA, 50mM HEPES, 2mM DTT, pH: 7.5) was added. The 24-well plate was frozen to –80°C for 1 hour following by rapid thawing in a water bath at 37°C. This freeze-thaw cycle was performed twice before analysis of the cell extracts. Three aliquots (20µl each) from each well of the 24-well plate were transferred to wells of a 96-well plate which was used for β-galactosidase analysis, whilst 40µl from each well of the 24-well plate was kept aside for quantification of cellular protein.

For quantification of the β-galactosidase activity in each 20µl aliquot of cell extract, an equal volume of 2× β-galactosidase assay buffer (200mM sodium phosphate buffer with pH:7.3, 100mM β-mercaptoethanol, 2mM $MgCl_2$, 4mg/ml

ONPG) was added. The samples were incubated at 37°C for 2 hours, during which time the β-galactosidase enzyme present in the samples hydrolyse the ONPG substrate to o-nitrophenol, which has a yellow colour. This reaction was terminated by the addition of 40µl sodium carbonate (1M) and the absorbance was determined at 414nm on a spectrophotometric 96-well plate reader (BioRad Microplate Reader Benchmark). The β-galactosidase was quantified on each occasion, by reference to standard curve of purified enzyme (0-160 mU) diluted in low detergent lysis buffer.

The level of β-galactosidase in each sample needed to be related to the size of cell population that expressed the enzyme and so was normalysed to the cellular protein content in each sample. For this, 100µl of ice-cold 10% (w/v) trichloroacetic acid was added to each of the 40µl samples, which were assigned for the protein assay, and the protein content of each sample was precipitated on ice for 10 minute. The precipitated protein was pelleted by centrifugation (13000 rpm, 10 minutes). The supernatant, which included the lysis buffer, was removed and the protein pellet was resuspended in 40µl NaOH (1M). The protein samples were heated to 60°C in a water bath for 1.5 hour and vortexed regularly to ensure complete solubilisation. Protein was quantified using the BioRad DC Protein Assay (Bio-Rad Laboratories, CA, USA) according to the manufacturers instructions. Protein concentration was established by reference to standard curve of bovine serum albumin (BSA, 0-1mg) in NaOH (1M).

6.3. Results

6.3.1. Plasmid DNA analysis

The qualitative (i.e. purity, presence/absence of proteins/RNA) and quantitative (concentration) analysis of the plasmid DNA samples used in this study were determined spectrophotometrically as well as by gel electrophoresis. The absorption spectrum of the plasmid samples was established between 200 and

340nm. A typical example of this scan is demonstrated in Figure 6.3. The purity of the plasmid samples was determined by taking the ratio between the optical densities at 260 and 280nm. The plasmid DNA samples utilised throughout this work had $OD_{260/280}$ ratios between 1.8 and 1.9, indicating the purity of the samples (e.g. see Sambrook et al 1989).

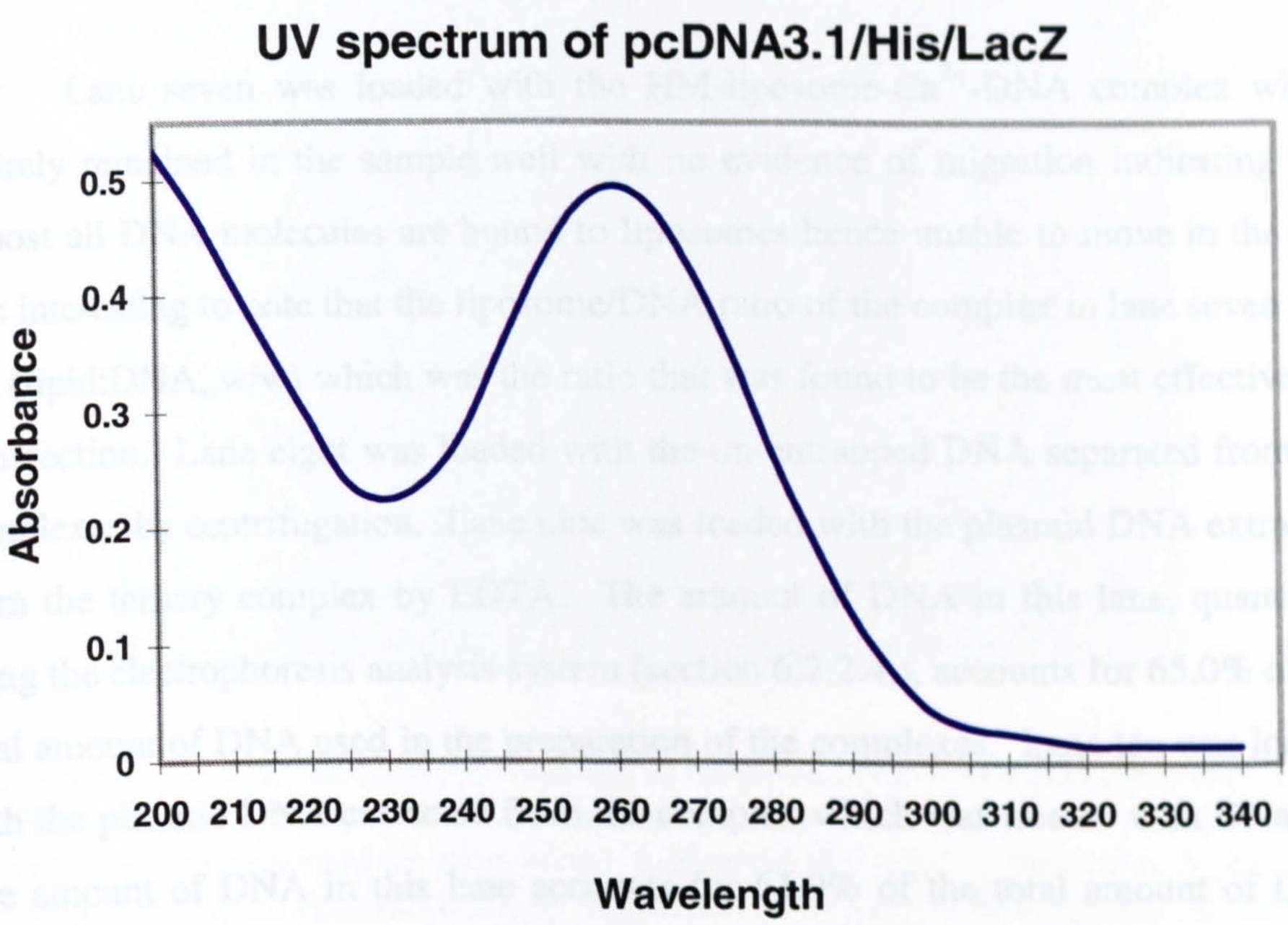


Figure 6.3. The absorption spectrum of the purified plasmid DNA pcDNA3.1/His B/*lac*Z.

6.3.2. Agarose gel electrophoresis

Complexes of HM-liposome-Ca^{2+}-DNA (7:1 lipid:DNA w/w ratio) and samples of plasmid DNA alone were subjected to agarose gel electrophoresis (Figure 6.4.). This figure represents a typical electrophoresis gel of more than ten similar

gels performed during this study. Lane 1 contained DNA ladder as a marker for comparison. The next five lanes were loaded with plasmid samples of known quantities (0.25, 0.5, 1.0, 1.5 and 2.0μg) as a calibration for plasmid quantification. As expected, the majority of the DNA was in the form of supercoiled structures, observed in the band of DNA which had migrated furthest from the sample well. Smaller bands formed from linear plasmid DNA followed behind the supercoiled plasmids.

Lane seven was loaded with the HM-liposome-Ca^{2+}-DNA complex which entirely remained in the sample well with no evidence of migration indicating that almost all DNA molecules are bound to liposomes hence unable to move in the gel. It is interesting to note that the liposome/DNA ratio of the complex in lane seven was 7:1 (lipid:DNA, w/w) which was the ratio that was found to be the most effective for transfection. Lane eight was loaded with the un-entrapped DNA separated from the complexes by centrifugation. Lane nine was loaded with the plasmid DNA extracted from the ternary complex by EDTA. The amount of DNA in this lane, quantified using the electrophoresis analysis system (section 6.2.2.4.), accounts for 65.0% of the total amount of DNA used in the preparation of the complexes. Lane ten was loaded with the plasmid DNA extracted from the complex which was treated with DNase I. The amount of DNA in this lane accounts for 61.0% of the total amount of DNA used in the preparation of the complexes. The sample in lane ten is comparable quantitatively and qualitatively to the sample in lane nine which was not treated with the enzyme. This indicates that the ternary complex is able to protect DNA molecules from the enzymatic degradation. To check the functionality of the enzyme a sample of free plasmid DNA was subjected to DNase I treatment and was found to be completely degraded by the enzymatic action (lane eleven).

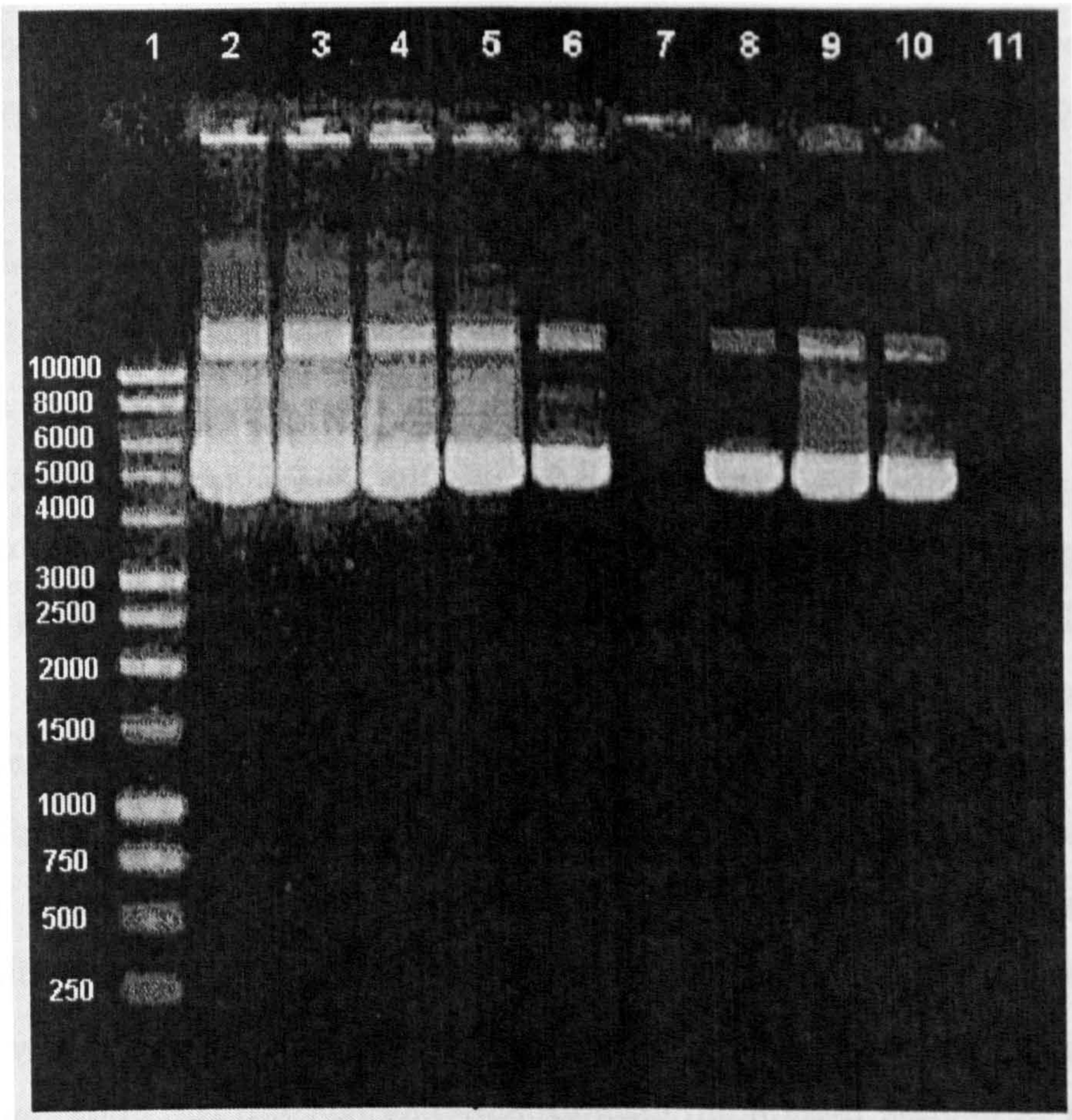


Figure 6.4. An agarose electrophoresis gel of the plasmid DNA and HM-liposome-Ca^{2+}-DNA complex. The contents of each lane are as follows: 1. Ladder with size markers written next to each band (in base pairs), 2. Plasmid (2.0 µg), 3. Plasmid (1.5 µg), 4. Plasmid (1.0 µg), 5. Plasmid (0.5 µg), 6. Plasmid (0.25 µg), 7. HM-lipo/Ca^{2+}/DNA (7:1 lipid:DNA w/w), 8. Un-entrapped DNA, 9. DNA extracted from HM-lipo/Ca^{2+}/DNA, 10. DNA extracted from HM-lipo/Ca^{2+}/DNA (7:1 lipid:DNA w/w) after treatment with DNase I, 11. Plasmid DNA treated with DNase I.

6.3.3. Incorporation efficiency of DNA in HM-liposomes

A variety of different methods have been used for the incorporation/encapsulation of nucleic acids in carrier systems including phospholipid vesicles (Chonn and Cullis 1998; Brown et al 2001). While these methods have succeeded in entrapping nucleic acids in liposomes, they suffer from several disadvantages including: 1) low entrapment efficiency, 2) the requirement

for large amounts of polynucleotides, 3) the limitation of the procedure to one type of phospholipid and 4) involving potentially harsh procedures such as sonication. These deficiencies become important considerations when attempting to incorporate small quantities of valuable DNA or when examining the influence of vesicle lipid composition on cellular delivery.

In this respect, the method explained for the preparation of HM-liposome-based vectors (section 6.2.2.3.) appears well suited for the incorporation of nucleic acids into liposomes. Incorporation of the DNA molecules to the HM-liposomes is achieved merely by incubation of the components (i.e. HM-liposome, plasmid and Ca^{2+}) at room temperature without employing treatments such as vortexing or sonication. In addition, HM-liposomes can potentially be made from almost all phospholipids or lipid mixtures and the procedure not only can be scaled up for industrial purposes but also can be scaled down for using small aqueous volumes (few millilitres) when sample availability is limited. The 50mM concentration of calcium was chosen on the basis of previously published results (Mozafari et al 1998a) indicating that this concentration allowed the highest amount of DNA to be incorporated to the anionic liposomes.

The entrapment efficiency of plasmid molecules was measured both by spectrophotometric measurements as well as using an electrophoresis analysis system as explained in sections 6.2.2.3. and 6.2.2.4. One example of an electrophoresis gel used for the quantification of the plasmid molecules can be seen in Figure 6.4. above. Each quantification assay was performed at least in triplicate and the average (± S.D.) DNA entrapment efficiencies obtained by spectrophotometry and gel electrophoresis were 70.3% (± 1.5) and 67.7% (± 3.8) respectively (Table 6.3.).

Table 6.3. Entrapment efficiency of DNA in HM-liposomes evaluated by UV spectrophotometry and gel electrophoresis techniques. Average data are expressed as mean ± S.D. of three experiments.

	Quantification Method	
Assay No.	UV Spectrophotometry	Gel Electrophoresis
1	69.52	69.71
2	72.18	62.45
3	68.78	71.05
Mean	**70.3% (± 1.5)**	**67.7% (± 3.8)**

6.3.4. Light scattering measurements

The mean particle size and polydispersity index of the HM-liposomes alone, HM-liposome-Ca^{2+}-DNA (7:1 lipid:DNA w/w) complexes and HM-liposome-Ca^{2+}, were determined by a dynamic light scattering technique (Table 6.4.). HM-liposomes were prepared either as multilamellar vesicles (MLV) or sized by using 100nm or 400nm pore-size filters. Upon addition of DNA and calcium, vesicle sizes increased by almost two to four times indicating the presence of two to four vesicles in the ternary complexes. Addition of calcium alone (with no plasmid DNA) did not confer any significant increase in the mean particle sizes of the liposomes. This is an indication of the presence of DNA in the complexes.

Table 6.4. Mean particle size of HM-liposomes with and without DNA and/or Ca^{2+} (see text for details). Data are expressed as mean ± S.D. of three or more experiments.

Sample	Mean Particle Size	Size Increase*
HM-liposome *(prepared using 100nm filters)*	159.6 ± 2.3	—
HM-liposome-Ca^{2+}-DNA *(prepared using 100nm filters)*	317.5 ± 19.3	1.99
HM-liposome-Ca^{2+} *(prepared using 100nm filters)*	174.9 ± 4.8	1.10
HM-liposome *(prepared using 400nm filters)*	510.4 ± 15.8	—
HM-liposome-Ca^{2+}-DNA *(prepared using 400nm filters)*	1658.3 ± 56.9	3.25
HM-liposome-Ca^{2+} *(prepared using 400nm filters)*	523.9 ± 24.9	1.03
HM-liposome *(MLV)*	605.2 ± 3.4	—
HM-liposome-Ca^{2+}-DNA *(MLV)*	2245.6 ± 55.3	3.71
HM-liposome-Ca^{2+} *(MLV)*	611.2 ± 13.4	1.01

*size enlargement values are relative to the sizes of corresponding empty liposomes.

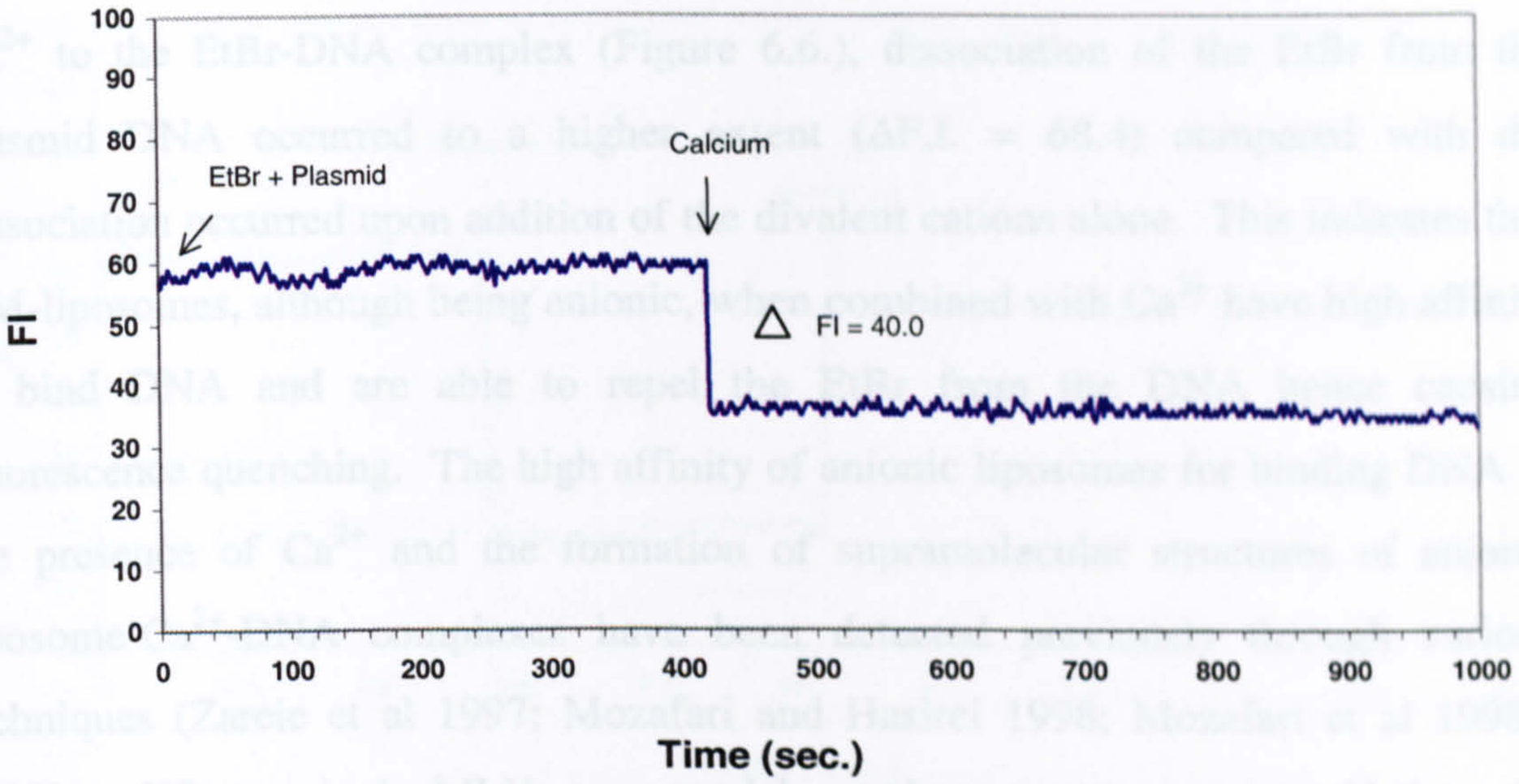
6.3.5. Ethidium bromide intercalation assay

Ethidium bromide (EtBr) is a monovalent DNA intercalating agent whose fluorescence is dramatically enhanced upon binding to DNA and quenched when displaced by higher affinity compounds or by condensation of the DNA structure

(Faneca et al 2002). Therefore, this chemical probe was used to examine the ability of the anionic liposomes prepared by the heating method (HM-liposomes) to protect DNA as well as to study the ability of the liposomal vector to release the DNA upon encountering a model membrane system. The model membrane system used was in the form of anionic micrometer sized multilamellar vesicles (MLV) which can be a model for both the cell membrane and the endosomal membrane.

Figure 6.5.a. shows a typical graph of the effect of calcium ions (50mM) on the fluorescence intensity of EtBr-DNA complex. EtBr-DNA complex exhibited a stable fluorescence intensity during a 420 second period and all ΔF.I.'s reported here are relative to the fluorescence intensity of EtBr-DNA complex. Upon addition of Ca^{2+} ions (arrow at *T*= 420 s) the fluorescence of the EtBr-DNA complex decreased (ΔF.I. = 40.0) instantaneously, indicating the dissociation of the EtBr from the plasmid DNA. This observation could be explained by the fact that Ca^{2+} has two positive charges, twice as much as EtBr, while smaller in size. Consequently Ca^{2+} has more positive charge density than EtBr hence having more affinity for negatively charged DNA molecules and thus it causes the detachment of EtBr from DNA resulting in the fluorescence quenching. Another divalent cation (i.e. Mg^{2+}, 50mM) exhibited a similar effect to Ca^{2+} on the fluorescence of the EtBr-DNA complex (Figure 6.5.b.) but with a slightly higher ΔF.I. (i.e. 43.8).

a



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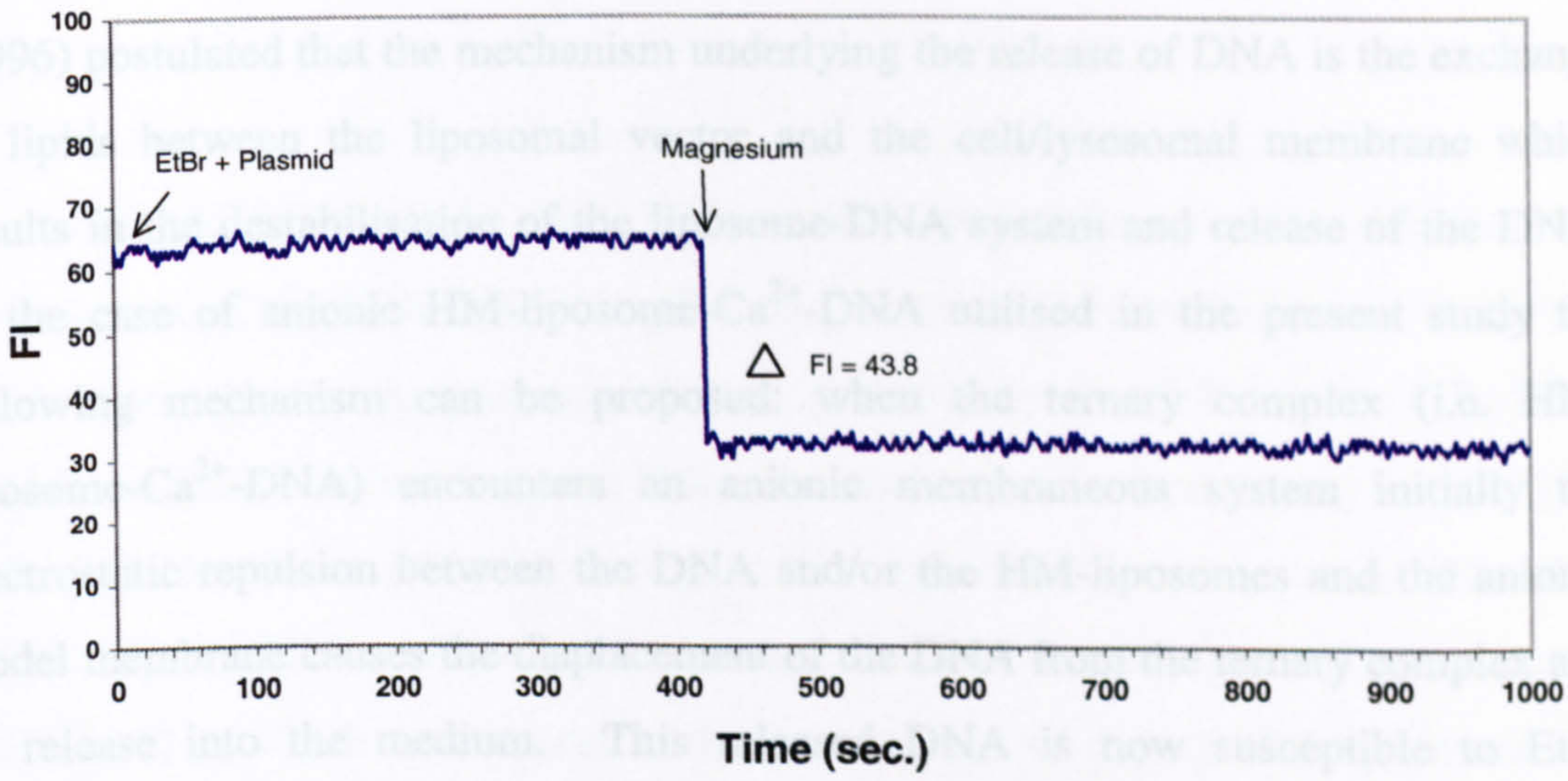


Figure 6.5. a) effect of calcium (50mM) and b) magnesium (50mM) on the fluorescence intensity of EtBr-DNA complex (see text for details).

When anionic HM-liposomes (in the form of LUV) were added together with Ca^{2+} to the EtBr-DNA complex (Figure 6.6.), dissociation of the EtBr from the plasmid DNA occurred to a higher extent (ΔF.I. = 68.4) compared with the dissociation occurred upon addition of the divalent cations alone. This indicates that HM-liposomes, although being anionic, when combined with Ca^{2+} have high affinity to bind DNA and are able to repel the EtBr from the DNA hence causing fluorescence quenching. The high affinity of anionic liposomes for binding DNA in the presence of Ca^{2+} and the formation of supramolecular structures of anionic liposome-Ca^{2+}-DNA complexes have been detected previously through various techniques (Zareie et al 1997; Mozafari and Hasirci 1998; Mozafari et al 1998a, 1998b). When anionic MLV, as a model membrane system, were added to the reaction medium the fluorescence increased (ΔF.I. = 10.6, see section 6.2.2.6.). This phenomenon, which was reported by Xu and Szoka (1996) for a cationic liposomal vector, indicates the ability of the anionic HM-liposome vectors to release their incorporated DNA upon encountering cell or lysosomal membranes. Xu and Szoka (1996) postulated that the mechanism underlying the release of DNA is the exchange of lipids between the liposomal vector and the cell/lysosomal membrane which results in the destabilisation of the liposome-DNA system and release of the DNA. In the case of anionic HM-liposome-Ca^{2+}-DNA utilised in the present study the following mechanism can be proposed: when the ternary complex (i.e. HM-liposome-Ca^{2+}-DNA) encounters an anionic membraneous system initially the electrostatic repulsion between the DNA and/or the HM-liposomes and the anionic model membrane causes the displacement of the DNA from the ternary complex and its release into the medium. This released DNA is now susceptible to EtBr intercalation hence the observed increase in the fluorescence intensity. Once the DNA is released from the ternary complex the anionic HM-liposome of the ternary complex can interact with the model membrane in the form of fusion or aggregation by the mediation of the Ca^{2+} ions. Several groups have reported that Ca^{2+} and other divalent cations can trigger aggregation and/or fusion of phospholipid vesicles (e.g. Lansman and Haynes 1975; Wilschut et al 1980, 1981, 1985; Holland et al 1996; Mozafari and Hasirci 1998).

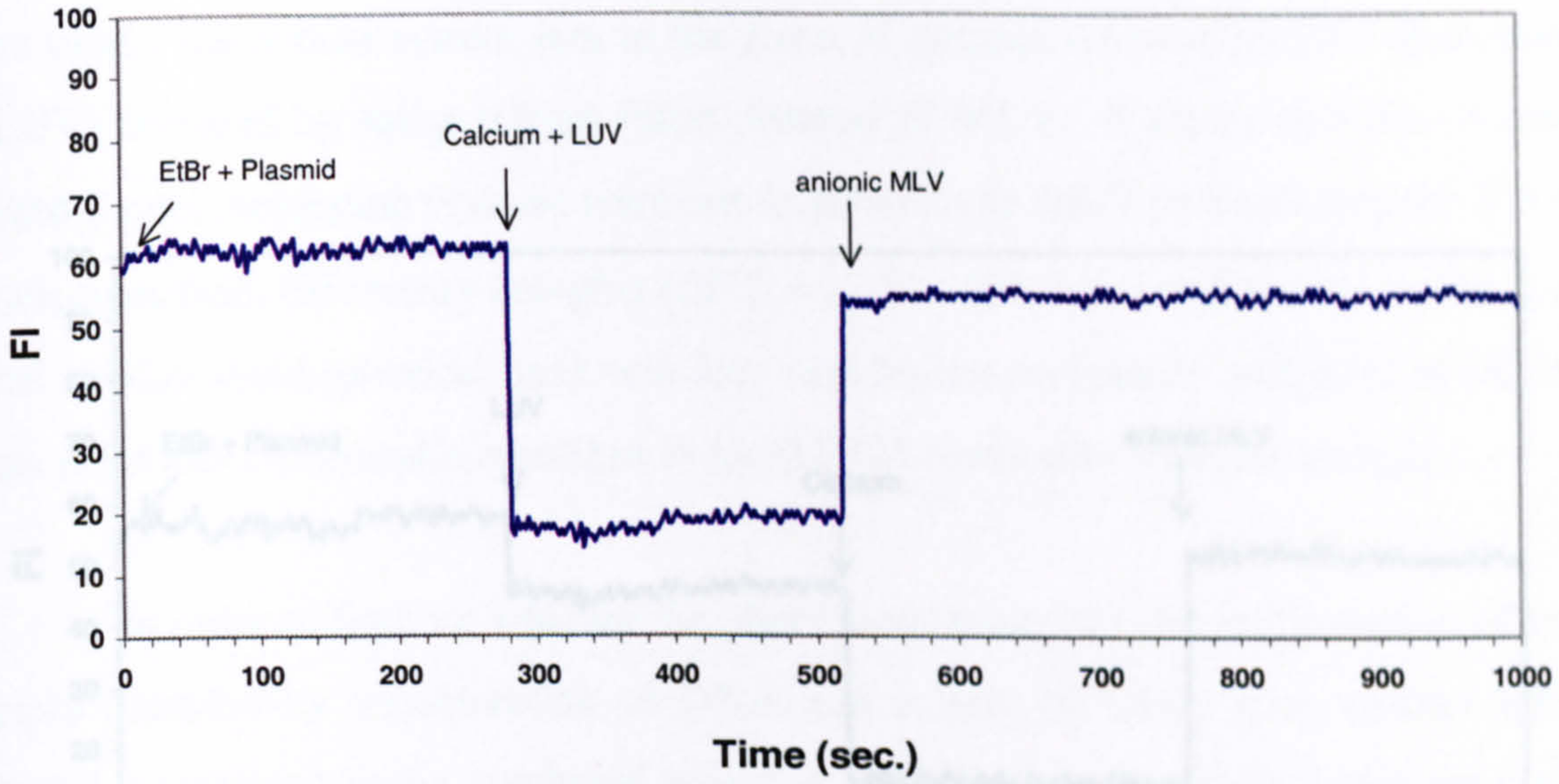
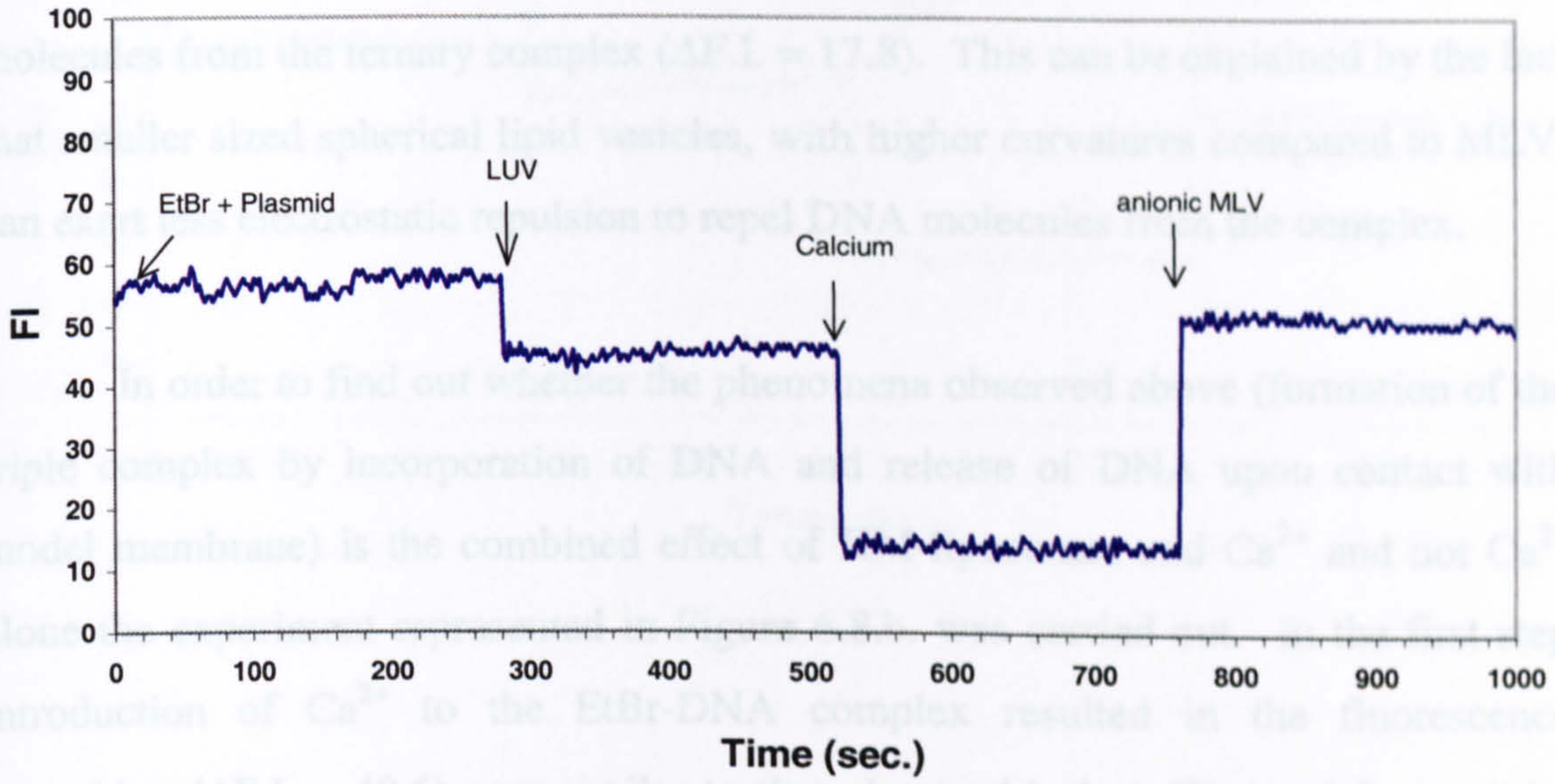


Figure 6.6. Effect of LUV and Ca^{2+} (injected at *T*=280s) and MLV (injected at *T*=520s), on the fluorescence intensity of EtBr-DNA complex.

Figure 6.7.a. shows that anionic HM-liposomes can also decrease the fluorescence of the EtBr-DNA complex on their own (ΔF.I. = 22.0), but to a much lower extent than in the presence of Ca^{2+}, probably by destabilising the EtBr-DNA complexes and/or interaction with EtBr. Interaction of the liposomal phospholipids with EtBr has been detected through a UV spectrophotometric assay (Mozafari 1996). Upon addition of Ca^{2+}, a further quenching of the fluorescence was detected (ΔF.I. = 72.7) which was almost to the same extent of that of HM-liposomes and Ca^{2+} together. Fluorescence quenching again indicates the incorporation of DNA molecules into the HM-liposomes. The ternary complex formed by the action of the Ca^{2+} was able to release the DNA when encountering the model membrane system to a similar extent (ΔF.I. = 12.2) as previously observed. This observation is in agreement with the work of Xu and Szoka (1996) and their finding that anionic liposomes exhibit a strong ability to displace bound DNA. This could be as a result of electrostatic repulsion between negatively charged liposomes and DNA. The

overall process was not dependent on the sequence of introducing Ca^{2+} and HM-liposomes to the reaction medium (Figure 6.7.b.).

a



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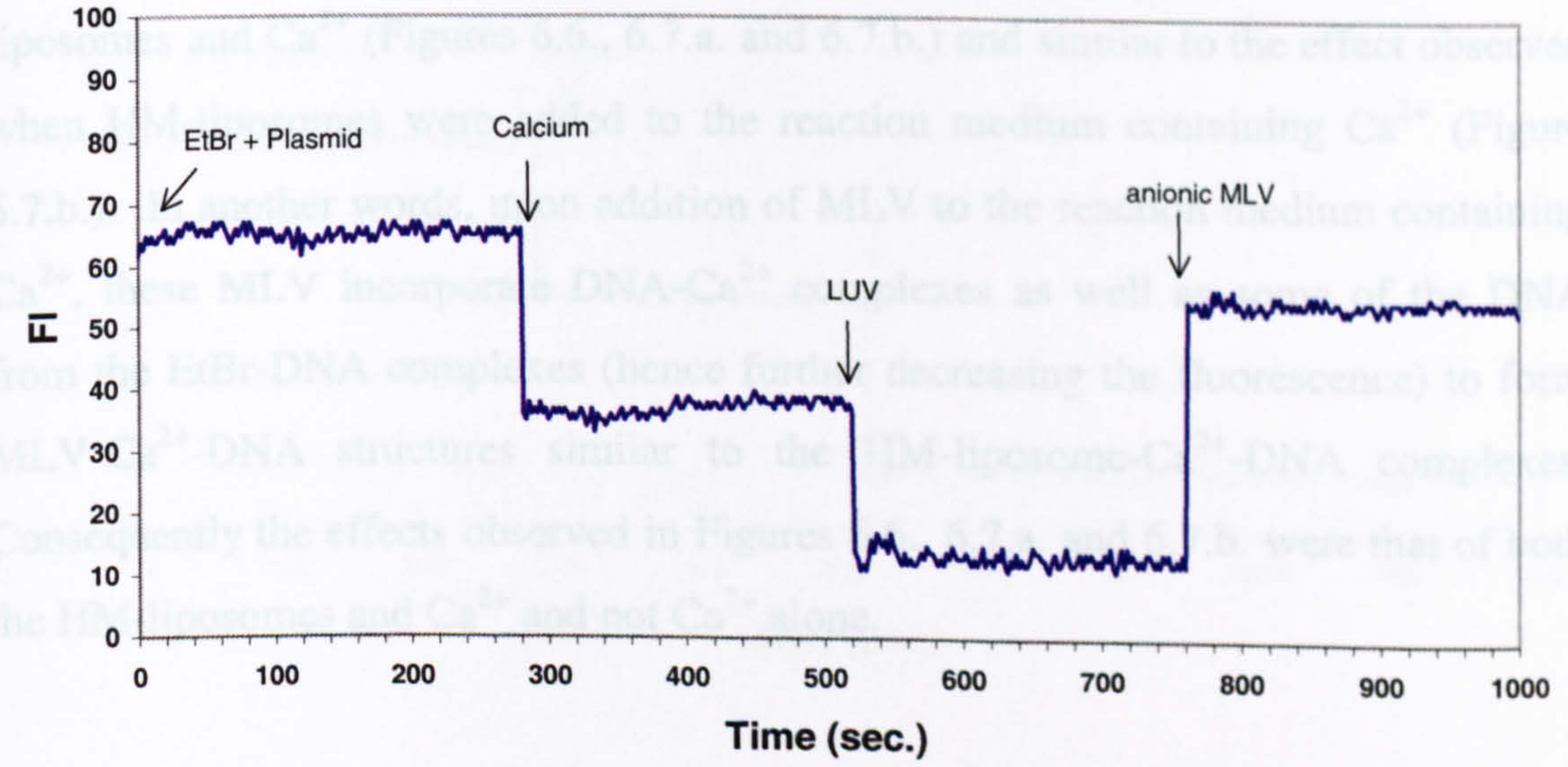
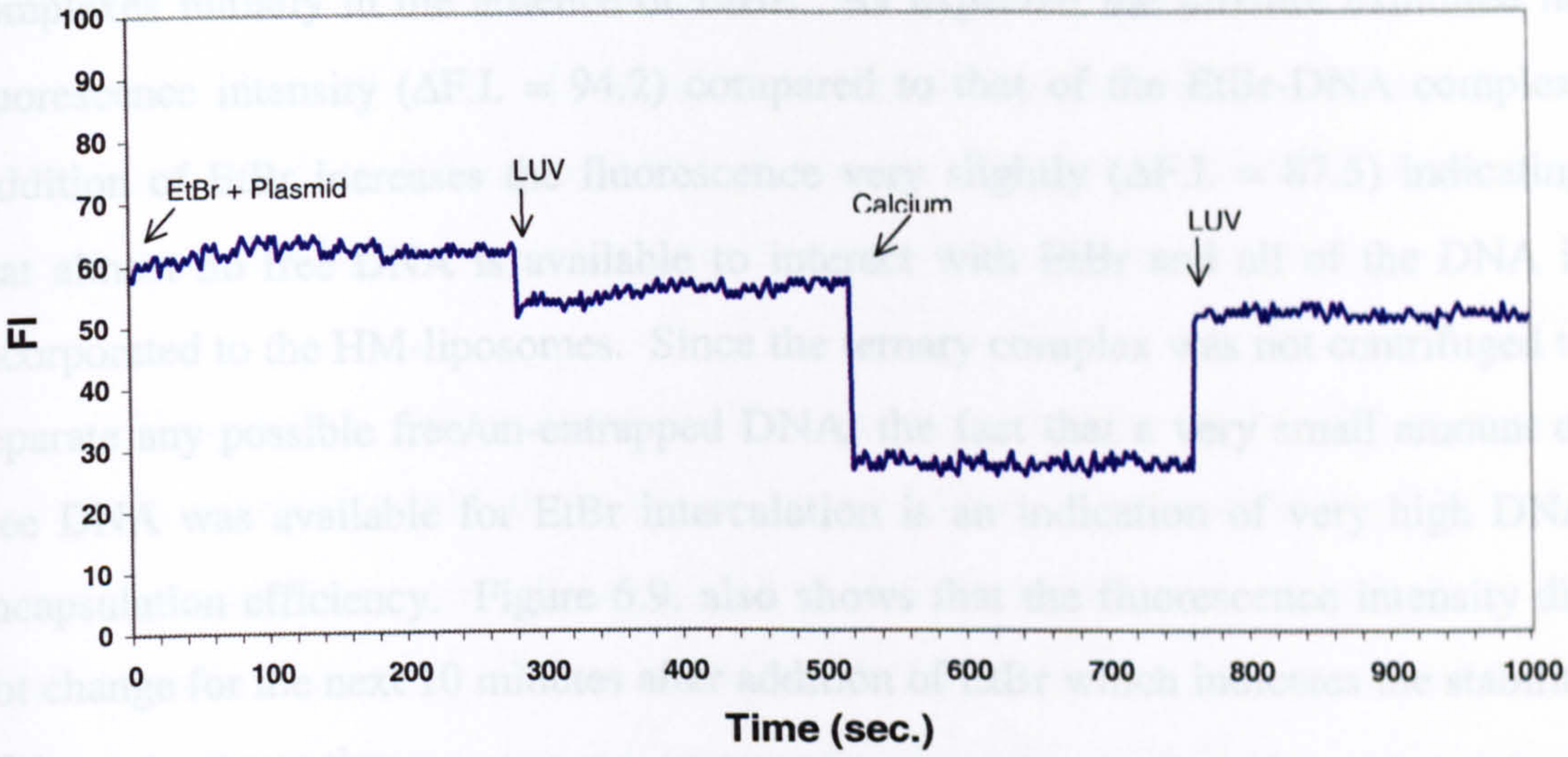


Figure 6.7. a) effect of LUV (injected at *T*=280s), Ca^{2+} (injected at *T*=520s) and MLV (injected at *T*=760s), and b) effect of Ca^{2+} (injected at *T*=280s), LUV (injected at *T*=520s) and MLV (injected at *T*=760s) on the fluorescence intensity of EtBr-DNA complex.

Figure 6.8.a. demonstrates a similar assay to that of Figure 6.7.a. except that the model membrane system was in the form of anionic unilamellar HM-liposomes (LUV) prepared by using 100nm filters instead of MLV. It seems that the smaller sized model membrane vesicles were not as efficient as MLV in displacing the DNA molecules from the ternary complex (ΔF.I. = 17.8). This can be explained by the fact that smaller sized spherical lipid vesicles, with higher curvatures compared to MLV, can exert less electrostatic repulsion to repel DNA molecules from the complex.

In order to find out whether the phenomena observed above (formation of the triple complex by incorporation of DNA and release of DNA upon contact with model membrane) is the combined effect of HM-liposomes and Ca^{2+} and not Ca^{2+} alone the experiment represented in Figure 6.8.b. was carried out. In the first step introduction of Ca^{2+} to the EtBr-DNA complex resulted in the fluorescence quenching (ΔF.I. = 40.6) very similar to that observed before (Figure 6.5.a. and the initial part of Figure 6.7.b.). In the second step MLV was added to the reaction medium and resulted in further fluorescence quenching (ΔF.I. = 82.0). This is opposite to the effect observed when MLV was added to the mixture containing HM-liposomes and Ca^{2+} (Figures 6.6., 6.7.a. and 6.7.b.) and similar to the effect observed when HM-liposomes were added to the reaction medium containing Ca^{2+} (Figure 6.7.b.). In another words, upon addition of MLV to the reaction medium containing Ca^{2+}, these MLV incorporate DNA-Ca^{2+} complexes as well as some of the DNA from the EtBr-DNA complexes (hence further decreasing the fluorescence) to form MLV-Ca^{2+}-DNA structures similar to the HM-liposome-Ca^{2+}-DNA complexes. Consequently the effects observed in Figures 6.6., 6.7.a. and 6.7.b. were that of both the HM-liposomes and Ca^{2+} and not Ca^{2+} alone.

a



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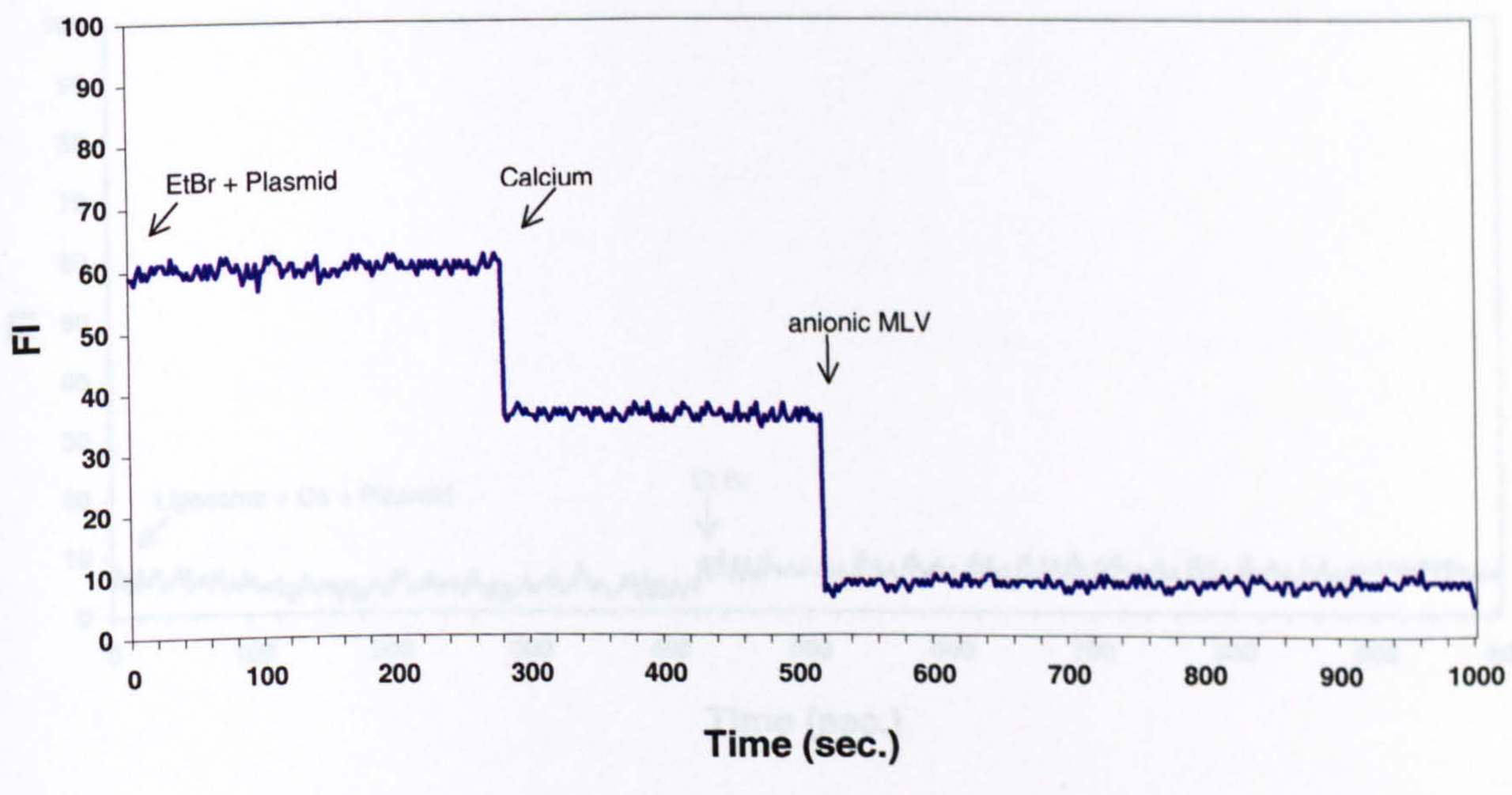


Figure 6.8. a) Effect of LUV (injected at T=280s), Ca^{2+} (injected at T=520s) and LUV (injected at T=760s) and b) effect of Ca^{2+} (injected at T=280s) and MLV (injected at T=520s) on the fluorescence intensity of EtBr-DNA complex (see text for details).

Figure 6.9. shows the fluorescence intensity of the HM-liposome-Ca^{2+}-DNA complexes initially in the absence of EtBr. As expected the mixture exhibited no fluorescence intensity (ΔF.I. = 94.2) compared to that of the EtBr-DNA complex. Addition of EtBr increases the fluorescence very slightly (ΔF.I. = 87.5) indicating that almost no free DNA is available to interact with EtBr and all of the DNA is incorporated to the HM-liposomes. Since the ternary complex was not centrifuged to separate any possible free/un-entrapped DNA, the fact that a very small amount of free DNA was available for EtBr intercalation is an indication of very high DNA encapsulation efficiency. Figure 6.9. also shows that the fluorescence intensity did not change for the next 10 minutes after addition of EtBr which indicates the stability of the ternary complex.

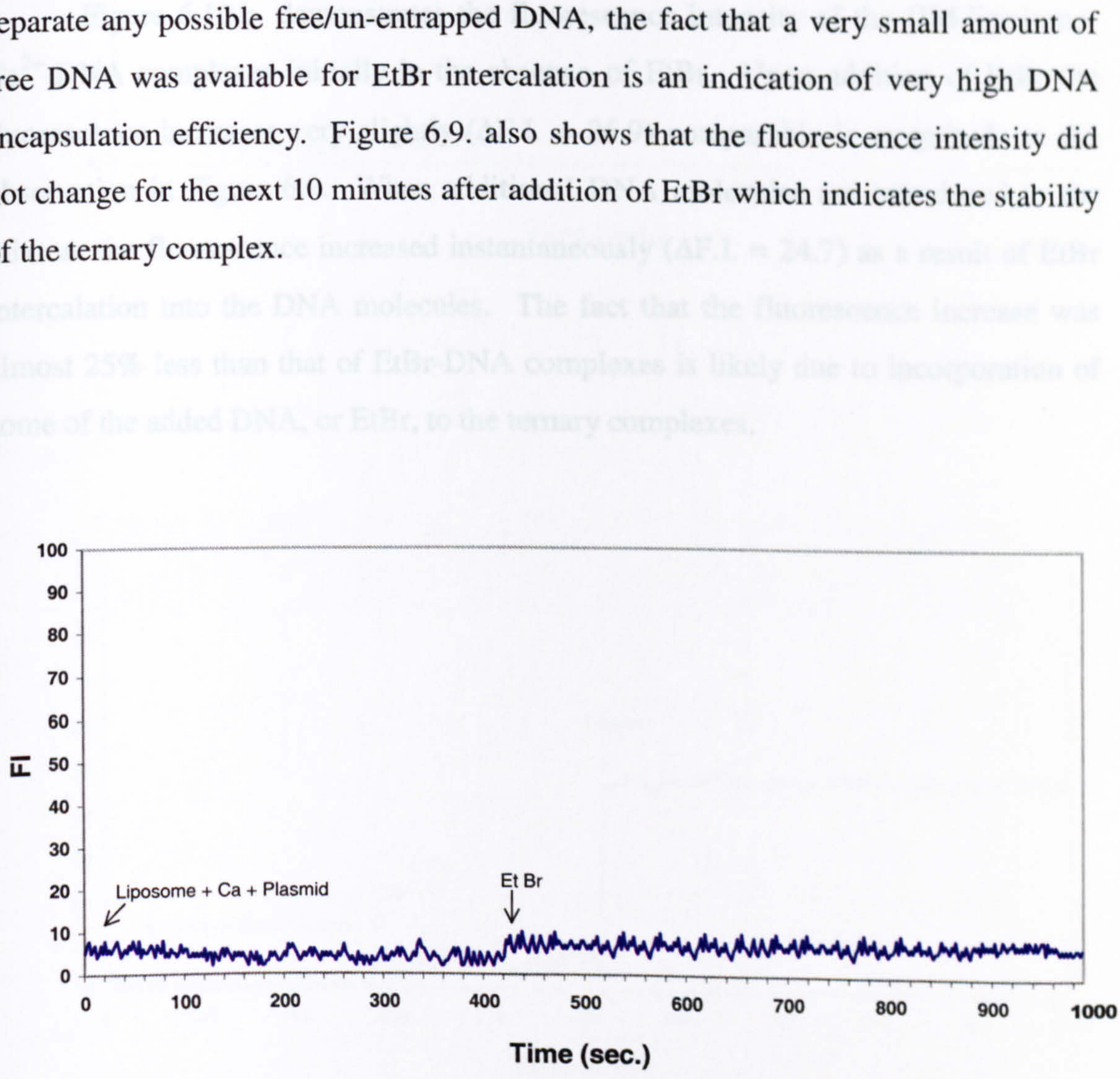
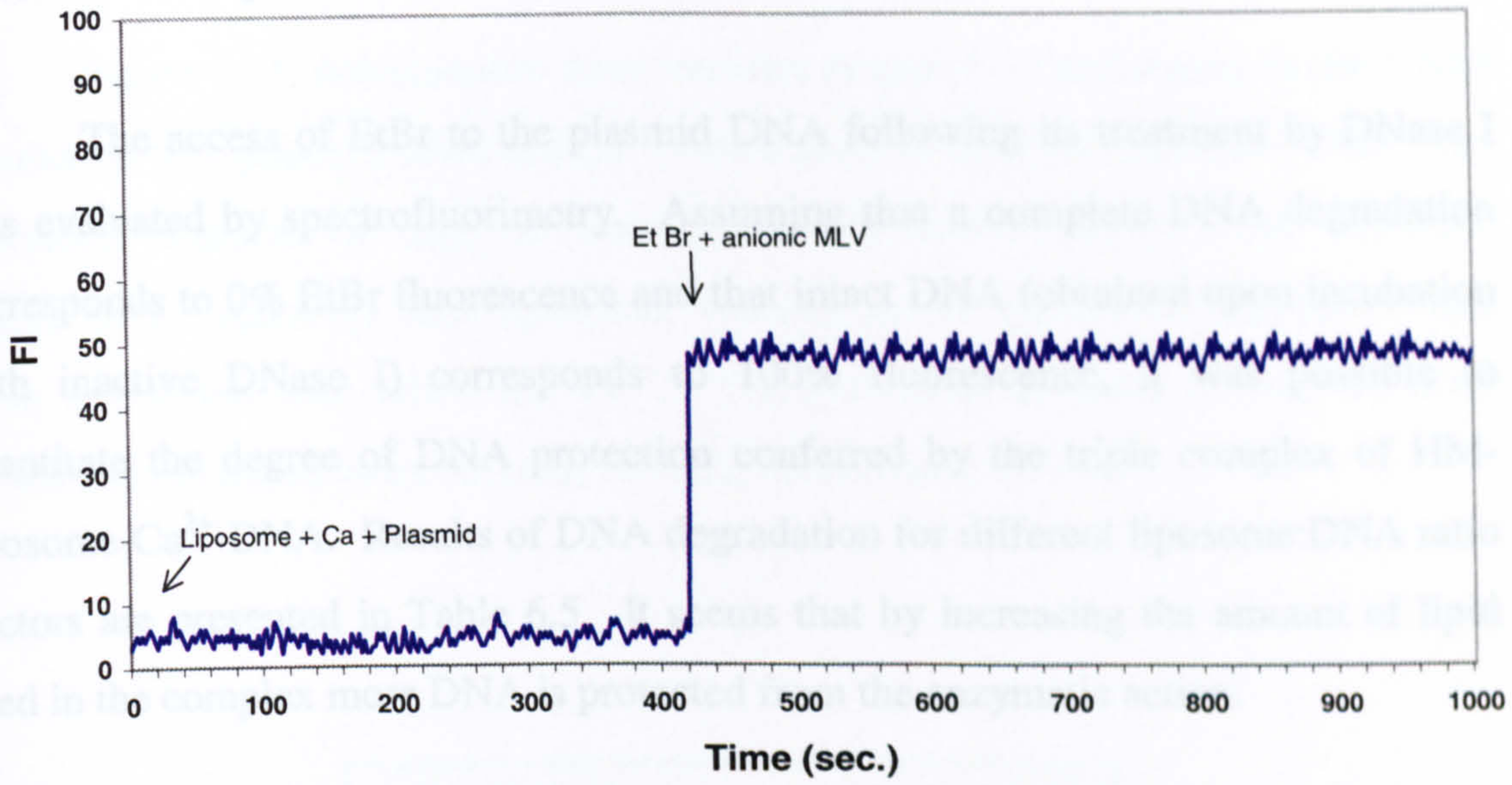


Figure 6.9. Effect of EtBr (injected at *T*=420s) on the fluorescence intensity of HM-liposome-Ca^{2+}-DNA complex.

In another assay EtBr was added simultaneously with MLV to the HM-liposome-Ca^{2+}-DNA complexes (Figure 6.10.a.). This resulted in sharp fluorescence increase (ΔF.I. = 18.7) due to release of DNA from the complexes as a result of electrostatic effect of MLV. In addition to the electrostatic effect, Xu and Szoka (1996) also considered a hydrophobic factor for the release of DNA from their cationic liposome-DNA complexes upon contact with model membranes.

Figure 6.10.b. demonstrates the fluorescence intensity of the HM-liposome-Ca^{2+}-DNA complexes initially in the absence of EtBr. Upon addition of EtBr the fluorescence increases very slightly (ΔF.I. = 86.9) comparable in magnitude to the observation in Figure 6.9. When additional DNA molecules are introduced to the mixture the fluorescence increased instantaneously (ΔF.I. = 24.7) as a result of EtBr intercalation into the DNA molecules. The fact that the fluorescence increase was almost 25% less than that of EtBr-DNA complexes is likely due to incorporation of some of the added DNA, or EtBr, to the ternary complexes.

a



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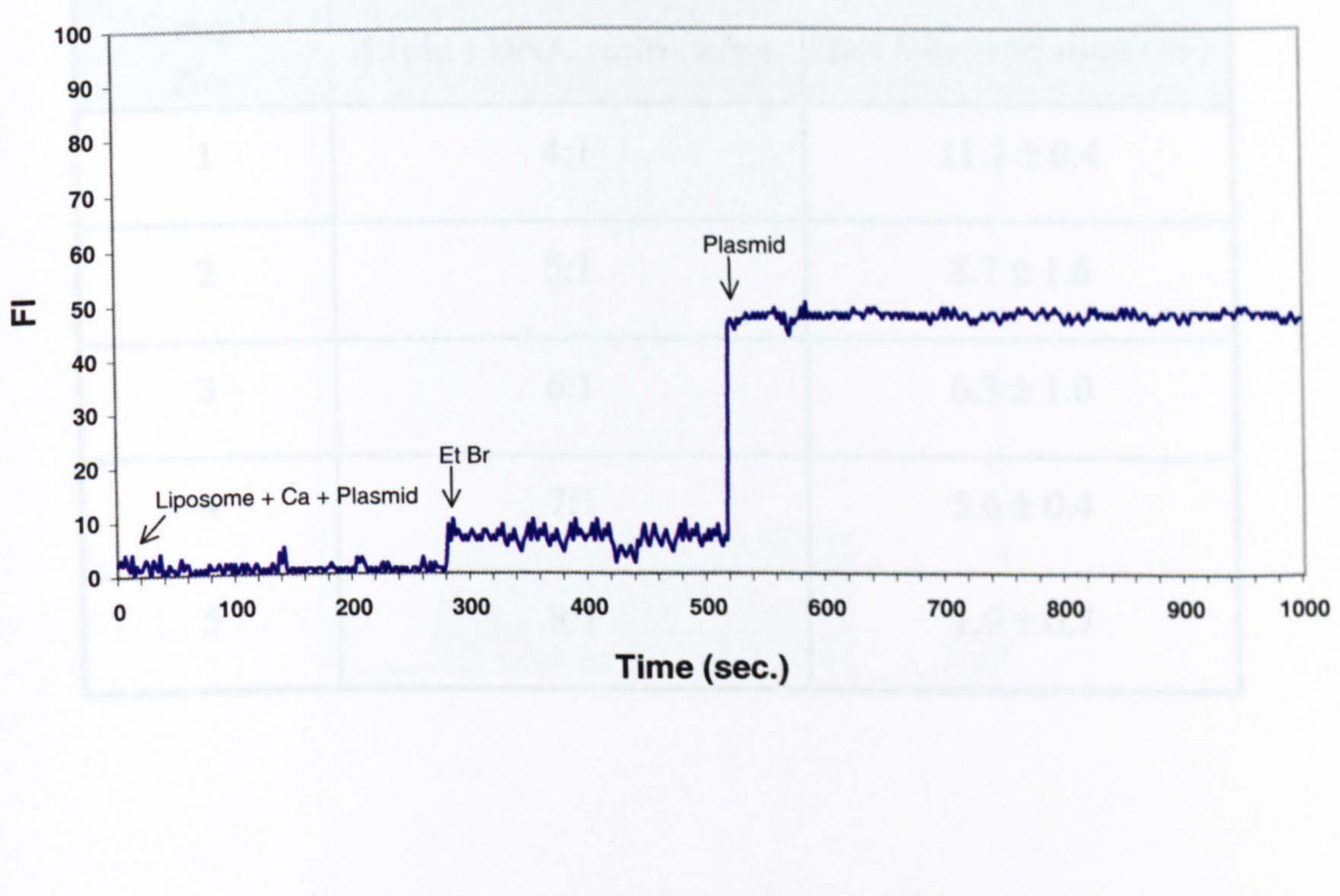


Figure 6.10. a) effect of EtBr and MLV (injected at *T*=420s), and b) effect of EtBr (injected at *T*=280s) and plasmid DNA (injected at *T*=520s) on the fluorescence intensity of HM-liposome-Ca^{2+}-DNA complex.

6.3.6. DNase I protection assay

The access of EtBr to the plasmid DNA following its treatment by DNase I was evaluated by spectrofluorimetry. Assuming that a complete DNA degradation corresponds to 0% EtBr fluorescence and that intact DNA (obtained upon incubation with inactive DNase I) corresponds to 100% fluorescence, it was possible to quantitate the degree of DNA protection conferred by the triple complex of HM-liposome-Ca^{2+}-DNA. Results of DNA degradation for different liposome:DNA ratio vectors are presented in Table 6.5. It seems that by increasing the amount of lipid used in the complex more DNA is protected from the enzymatic action.

Table 6.5. Percent of DNA degradation for liposomal vectors treated with DNase I. Data are expressed as mean ± S.D. of three or more experiments.

Sample No.	Lipid : DNA ratio (w/w)	DNA degradation (%)
1	4:1	11.2 ± 0.4
2	5:1	8.7 ± 1.6
3	6:1	6.3 ± 1.0
4	7:1	3.6 ± 0.4
5	8:1	1.9 ± 0.7

6.3.7. Transmission Electron Microscopy

Figure 6.11. demonstrates three vesicles prepared by the heating method after incubation with plasmid DNA and Ca^{2+} which seem to be in an aggregated state. The individual diameter of each of these vesicles is 80.3nm, 86.0nm and 103.0nm. Figure 6.12. represents four liposomal vesicles in an aggregated/semi-fused state upon interaction with DNA and Ca^{2+}. The individual diameter of each of these vesicles is calculated to be 62.1nm, 66.4nm, 85.3nm and 95.7nm.

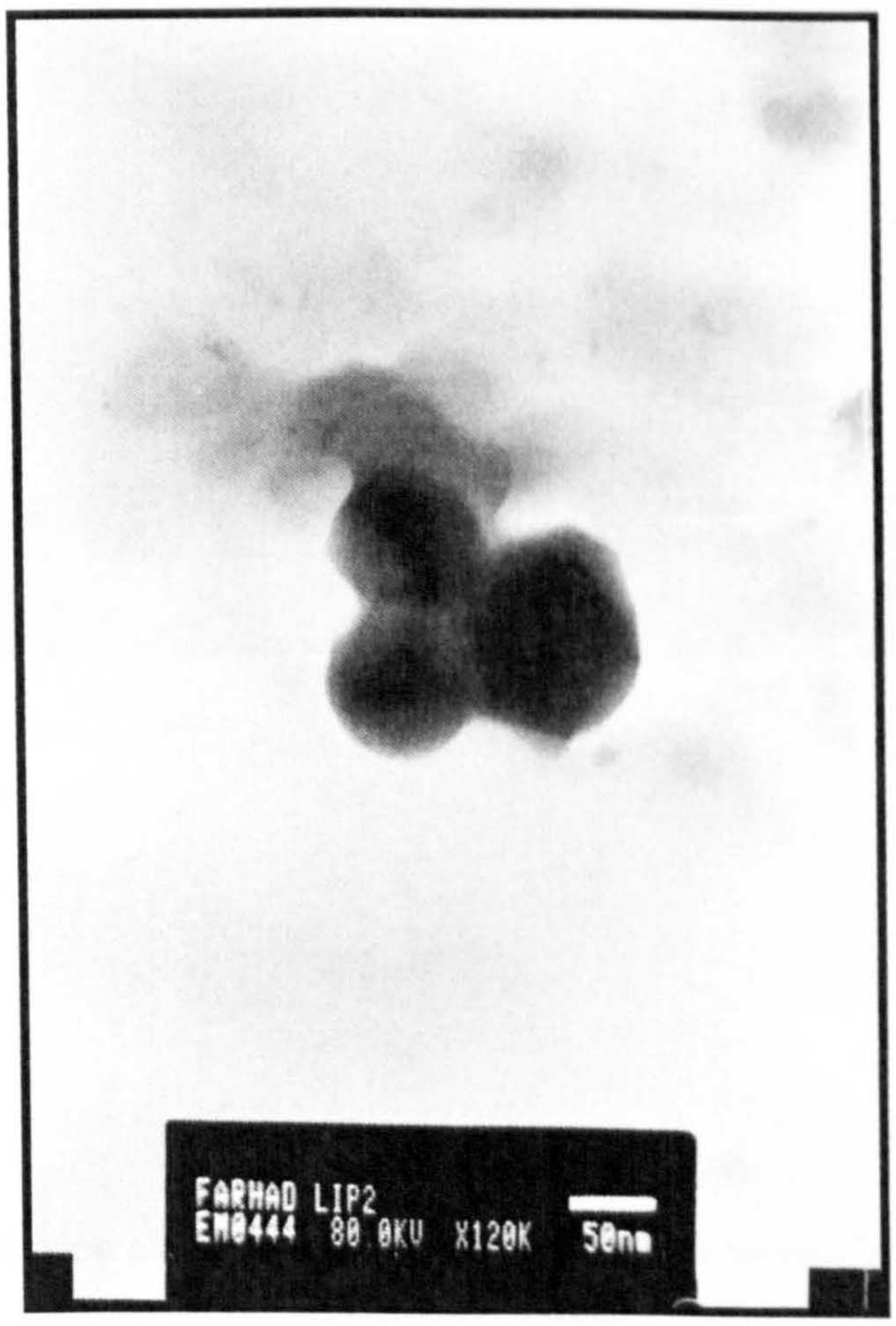


Figure 6.11. Transmission electron micrograph of HM-liposome-Ca^{2+}-DNA complex. Bar represents 50nm.

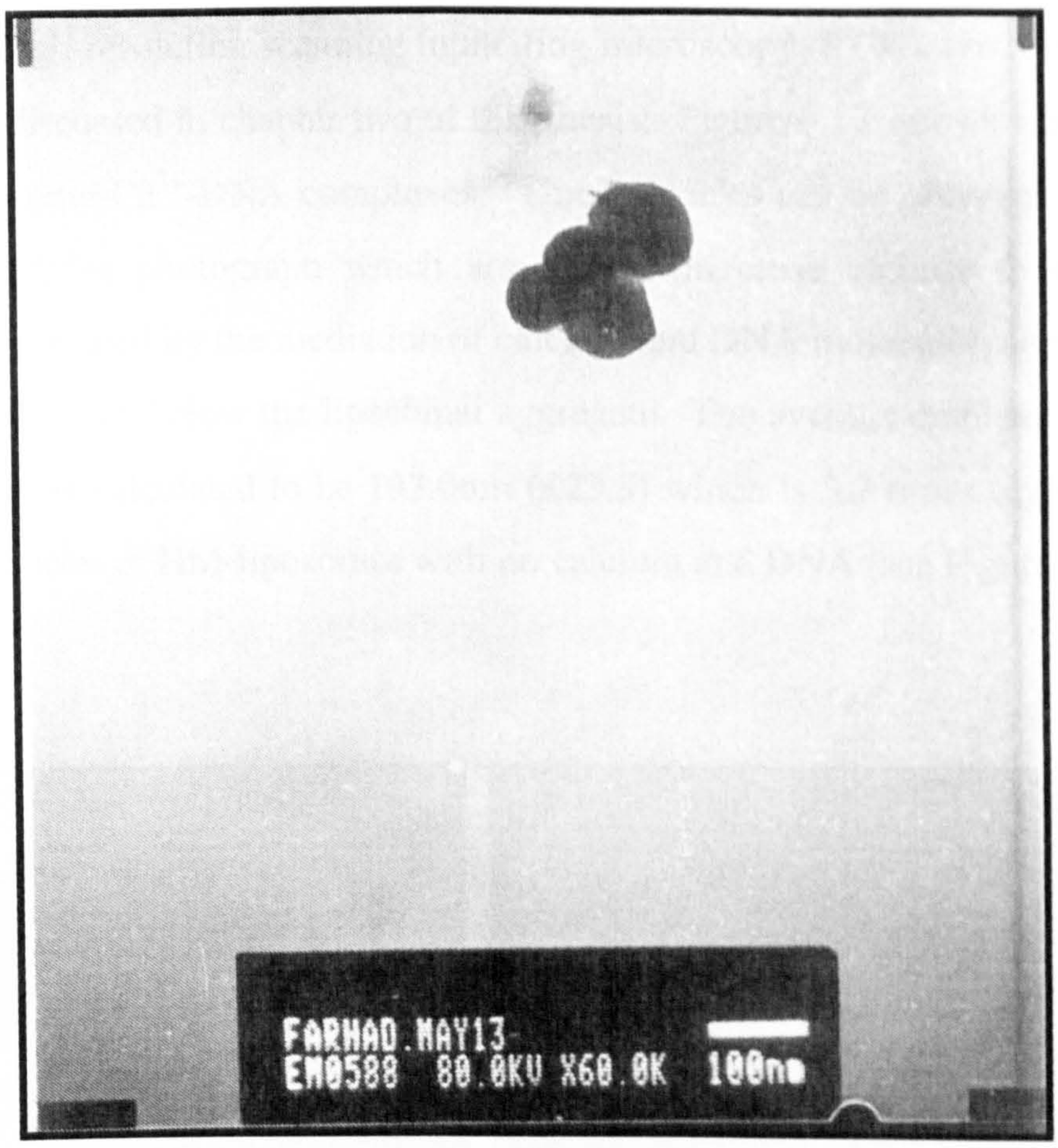


Figure 6.12. Transmission electron micrograph of HM-liposome-Ca^{2+}-DNA complex. Bar represents 100nm.

6.3.8. Scanning tunnelling microscopy

The ultrastructure of the liposomes extruded through 100nm filters was studied by high-resolution scanning tunnelling microscopy (STM), the importance of which was discussed in chapter two of this thesis. Figure 6.13. shows a STM image of HM-liposome-Ca^{2+}-DNA complexes. Three vesicles can be observed on the left hand side of the photograph which are located in close vicinity to each other, probably aggregated by the mediation of calcium and DNA molecules, and one single vesicle can be seen below the liposomal aggregate. The average diameter of vesicles in this figure is calculated to be 193.0nm (±23.3) which is 3.2 times bigger than the average diameter of HM-liposomes with no calcium and DNA (see Figure 2.13.).

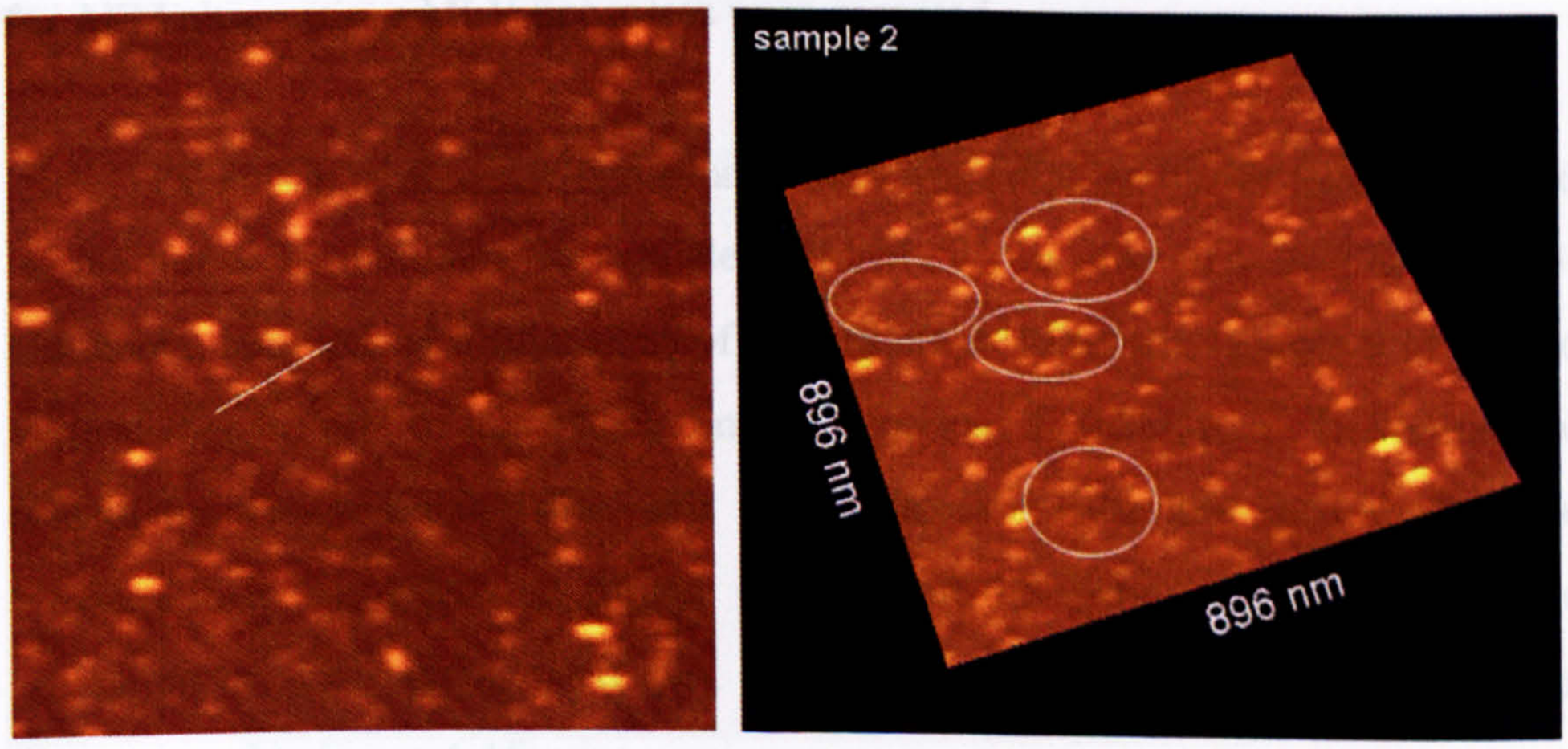


Figure 6.13. Two and three-dimensional scanning tunnelling micrographs of HM-liposome-Ca^{2+}-DNA complexes.

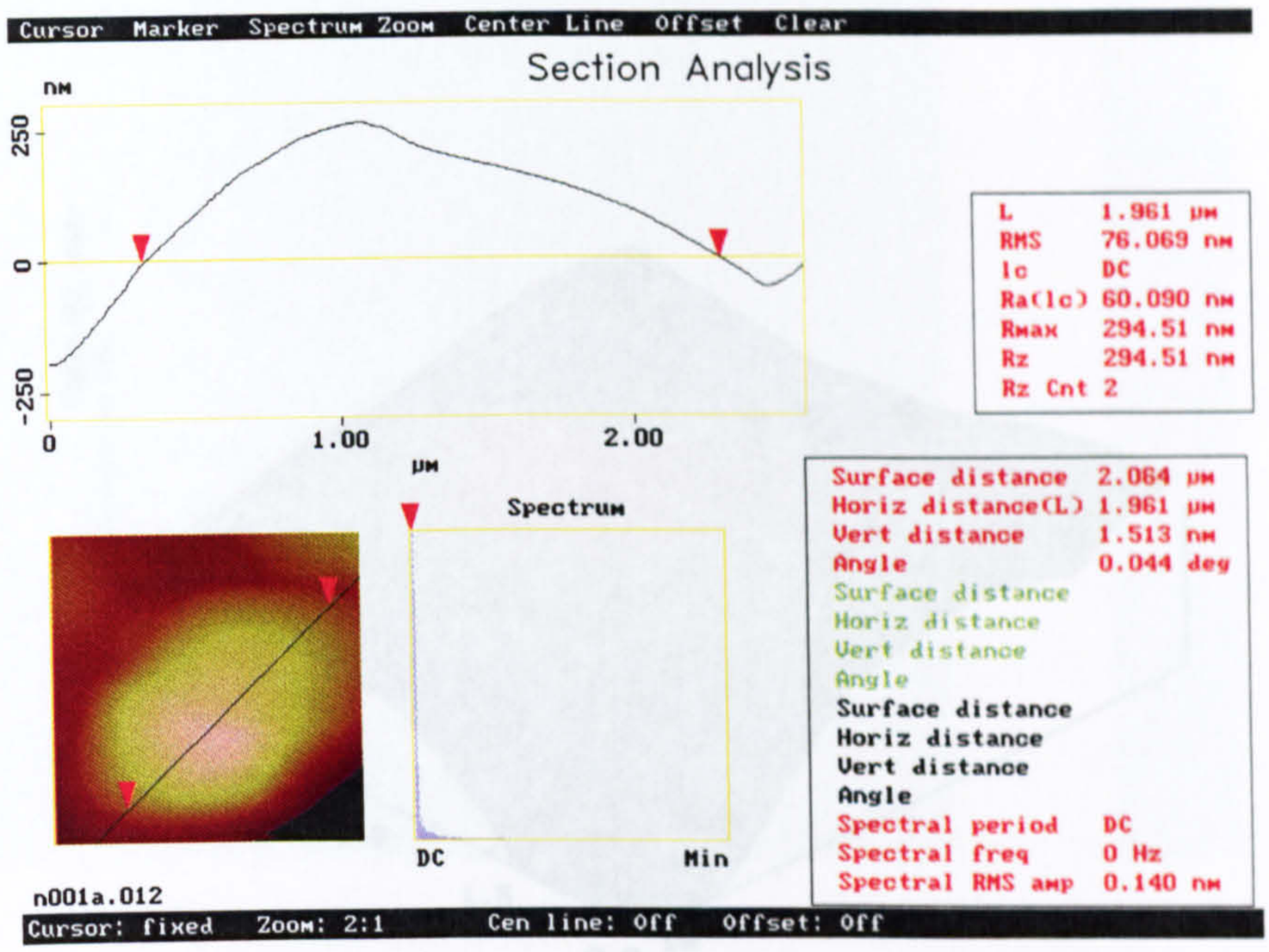
6.3.9. Atomic force microscopy

To gain direct insight into the triple complex structures, they were imaged, in addition to STM (previous section), by AFM as well. Since its introduction in 1986,

the potential of AFM for investigating biological samples on a scale ranging from living cells to single molecules has been recognised (Binning et al 1986). In contrast to conventional biological imaging methods, specimens investigated by AFM can be in a native, unlabeled state and investigated in their native environment for several hours without damage (Muller and Anderson 2002). AFM has been used for many biological specimens such as DNA, proteins, membranes and cells (Lawric et al 1999; Kasemo 2002) in addition to microcapsules (Zimmermann et al 2003) and liposomes (Kawaura et al 1998; Maeda et al 2002; Vermette et al 2002; Almofti etal 2003). However, there has been no report for anionic liposome-Ca^{2+}-DNA complexes. Excellent AFM images of these complexes were obtained and the sizes of the liposomes were measured by AFM. AFM is able to measure the particle sizes (diameters) of individual vesicles with diameters from 10nm to 10μm. The sensitivity of the AFM used in this study was in the micrometer size ranges, hence for AFM observations MLV liposomes were prepared.

Visualisation of a HM-liposome, which was prepared by the heating method in the form of multilamellar vesicle (HM-MLV), is depicted in Figure 6.14. Horizontal long axis size (diameter) of the vesicle is 1.961 μm (Figure 6.14.a.) and vertical (height) size of this vesicle on the same axis is 0.268 μm. The horizontal size of the vesicle on the short axis (perpendicular to the long axis used in the previous image) is 1.368 μm (Figure 6.14.b.) and the vertical (height) size of the vesicle on the short axis is 0.316 μm. A three dimensional image of the vesicle is demonstrated in Figure 6.15.

a



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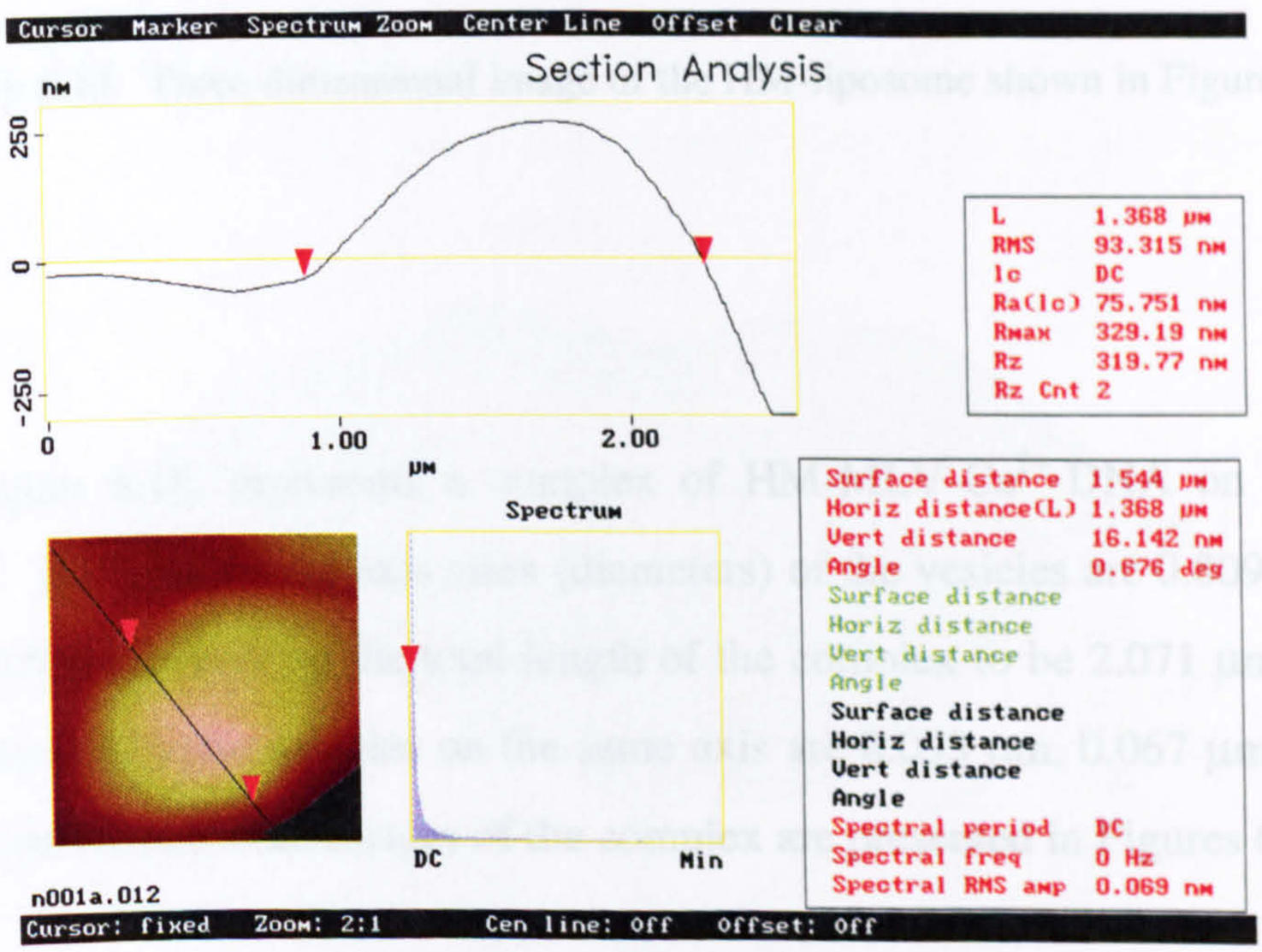

Figure 6.14. Atomic force microscopy image of a multilamellar vesicle prepared by the heating method after interaction with calcium and DNA. Horizonal distance analysis of the vesicle on the long (a) and short (b) axis are presented.

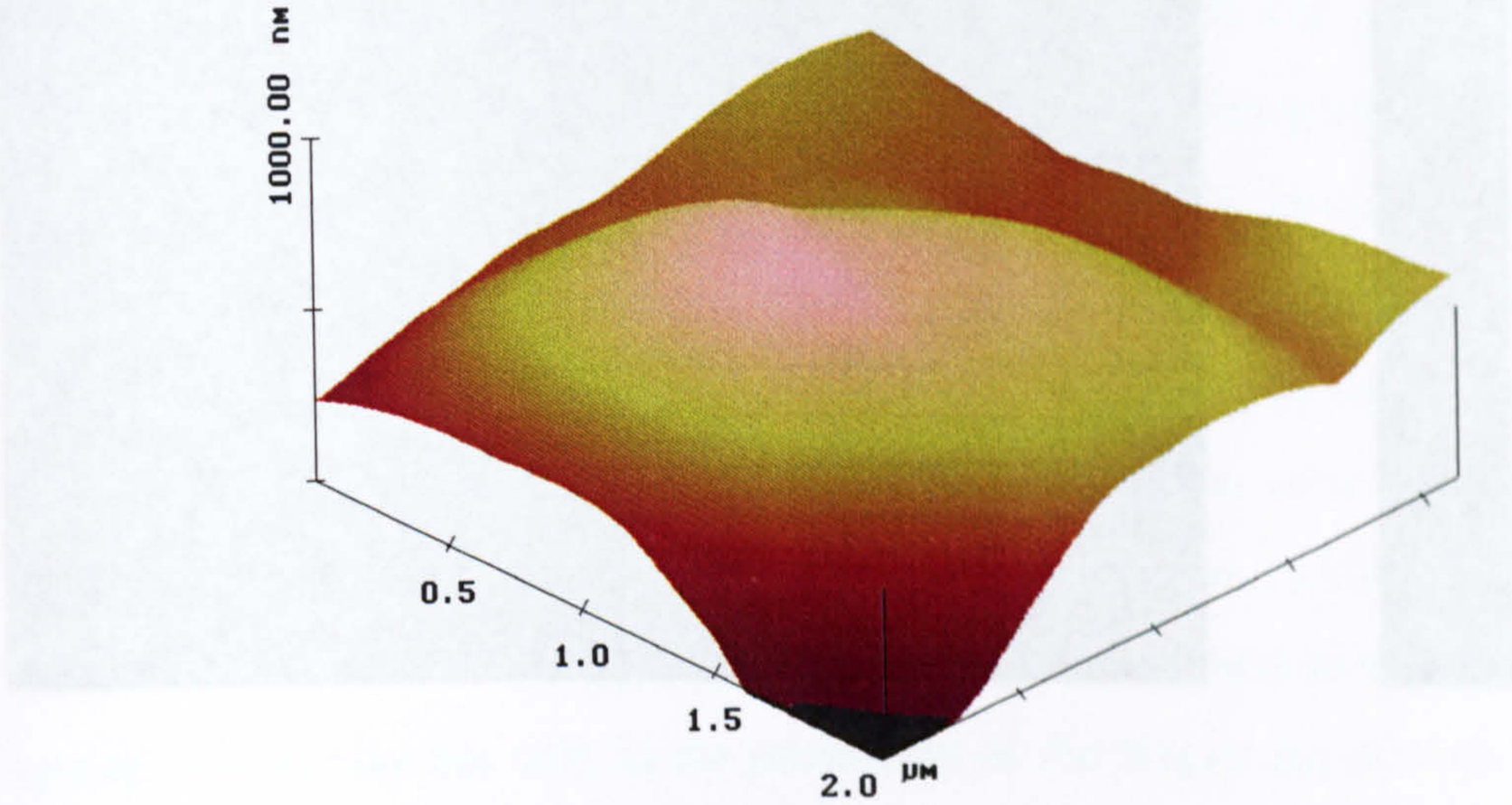

Figure 6.15. Three dimensional image of the HM-liposome shown in Figure 6.14.

Figure 6.16. represents a complex of HM-MLV-Ca^{2+}-DNA on a stainless steel disc. Horizontal long axis sizes (diameters) of the vesicles are 0.809 µm, 0.726 µm and 0.536 µm making the total length of the complex to be 2.071 µm. Vertical (height) sizes of these vesicles on the same axis are 0.093 µm, 0.067 µm and 0.090 µm. A three dimensional images of the complex are presented in Figures 6.17.

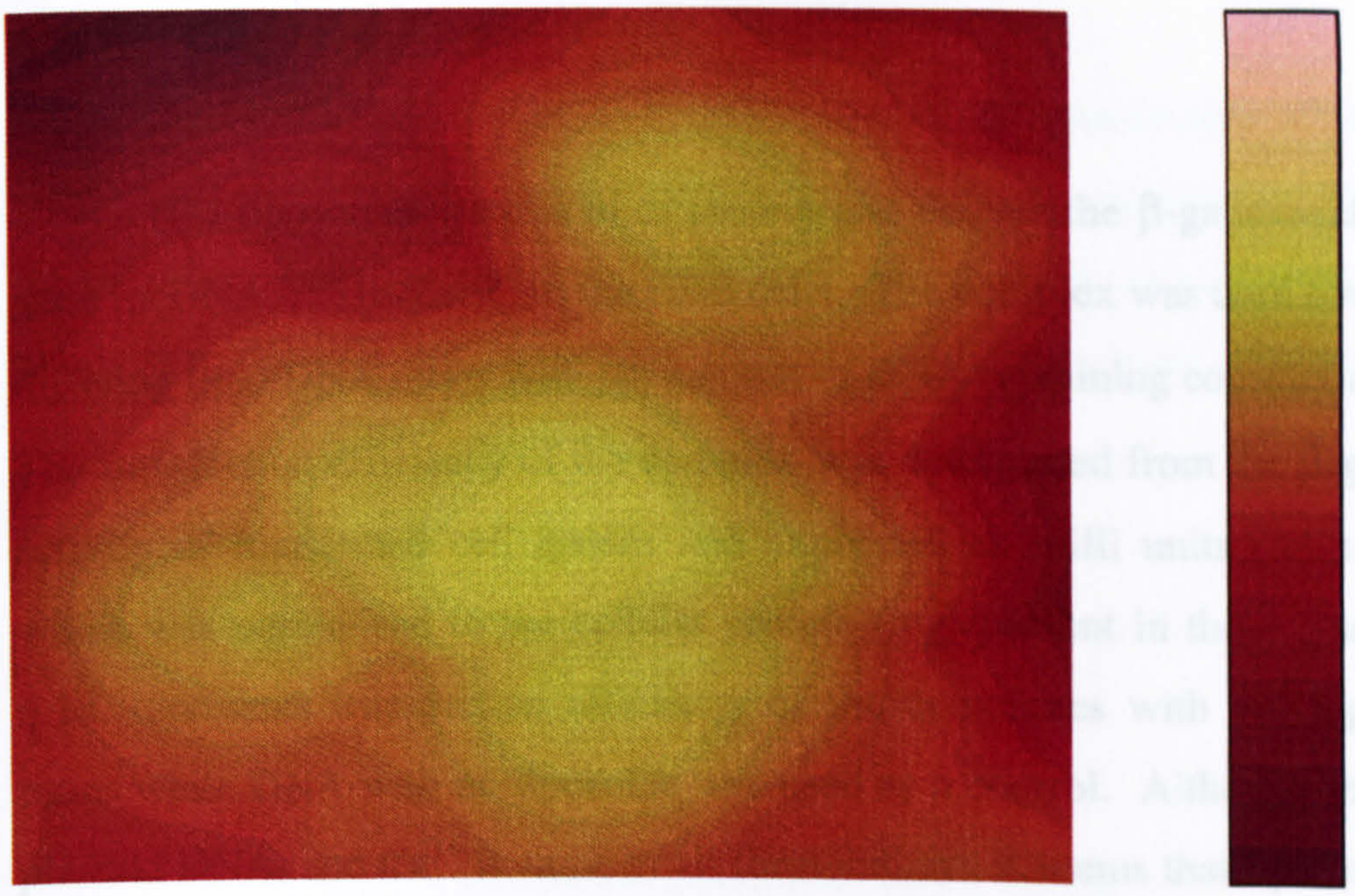

Figure 6.16. Two-dimensional AFM image of a triple complex on stainless steel disc. Scan area 2.5 × 2.5 μm.

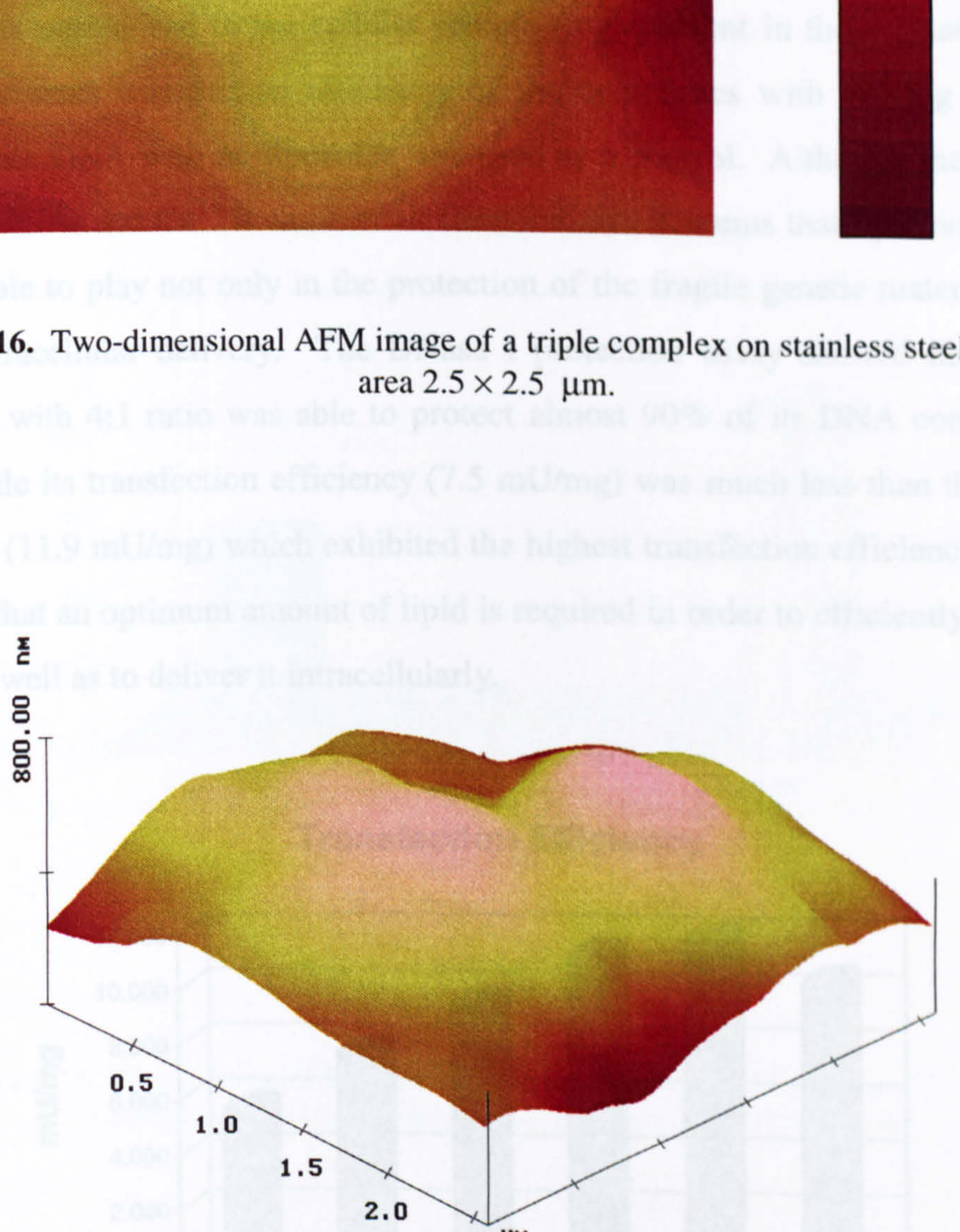

Figure 6.17. Three dimensional image of the triple complex demonstrated in Figure 6.16..

6.3.10. Gene transfer

HM-liposomes were used to protect and deliver the β-galactosidase reporter gene (pcDNA3.1/His/*Lac*Z) to the HBE cells. The complex was used over a range of differing lipid:DNA ratios with the amount of DNA remaining constant at 2.5 μg/ml. The transfection efficiency of the complex was determined from the β-galactosidase activity of transfected cell lysates and expressed as milli units (mU) of enzyme, which was normalised to the cellular protein (mg) present in those lysates. Figure 6.18. represents transfection efficiency of the complexes with varying lipid:DNA ratios while DNA with no liposome was used as a control. Although the amount of plasmid DNA, and Ca^{2+} is same in all formulations, it seems that lipid amount has a crucial role to play not only in the protection of the fragile genetic material but also in its intracellular delivery. The DNase I protection assay showed that even the complex with 4:1 ratio was able to protect almost 90% of its DNA content (Table 6.5.) while its transfection efficiency (7.5 mU/mg) was much less than the one with 7:1 ratio (11.9 mU/mg) which exhibited the highest transfection efficiency. Hence it appears that an optimum amount of lipid is required in order to efficiently protect the DNA as well as to deliver it intracellularly.

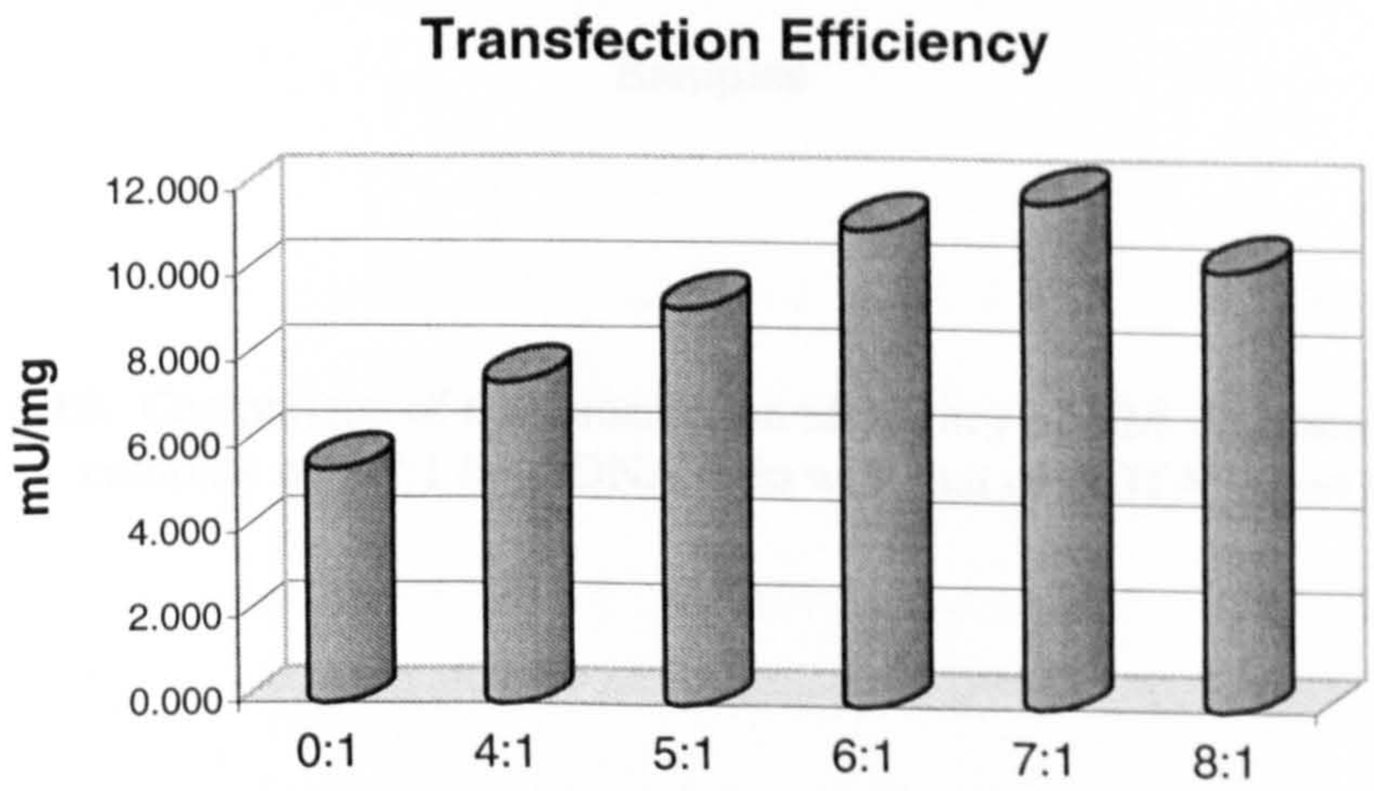

Figure 6.18. Gene transfer efficiency of HM-liposome-DNA-Ca^{2+} complexes with varying lipid:DNA ratios. As a control plasmid DNA was used with no liposome or Ca^{2+} (0:1 ratio) (n=4).

Figure 6.19. demonstrates a comparison of the transfection efficiency of the ternary complex with the highest transfection efficiency, containing 7:1 lipid:DNA ratio, to that of the generally used commercially available cationic lipid DOTAP. It seems that DOTAP was able to transfect the HBE cells more efficiently than the ternary complex (20.8 mU/mg vs. 11.9 mU/mg).

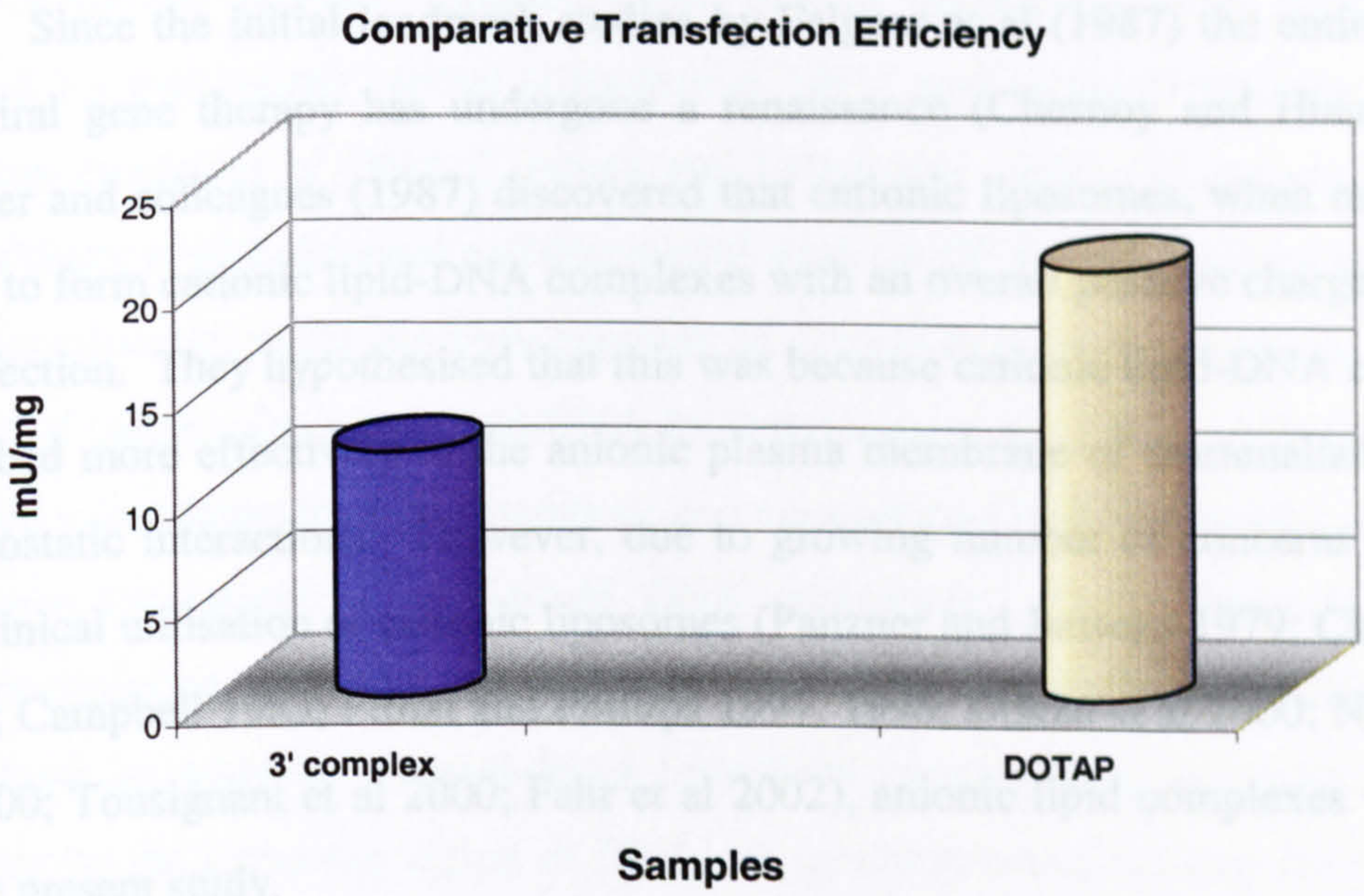

Figure 6.19. Comparison of the transfection efficiency of HM-liposome-DNA-Ca^{2+} complex with 7:1 lipid:DNA ratio with that of DOTAP (n=4).

6.4. Discussion

Viral-based carriers of DNA are presently the most common method of gene delivery. However, non-viral methods are rapidly emerging as alternative carriers, because of their relative ease of production, the absence of viral genes to cause disease and the nonimmunogenicity due to a lack of exposed proteins. Another principal advantage of non-viral vectors is the potential to transfer and express (transfect) large pieces of DNA into cells while viral vectors have a maximum gene-carrying capacity of 40 kilo base pairs (Friedmann 1997).

Since the initial landmark studies by Felgner et al (1987) the entire field of non-viral gene therapy has undergone a renaissance (Chesnoy and Huang 2000). Felgner and colleagues (1987) discovered that cationic liposomes, when mixed with DNA to form cationic lipid-DNA complexes with an overall positive charge, enhance transfection. They hypothesised that this was because cationic lipid-DNA complexes adsorbed more effectively to the anionic plasma membrane of mammalian cells via electrostatic interactions. However, due to growing number of concerns regarding the clinical utilisation of cationic liposomes (Panzner and Jansons 1979; Chawla et al 1979; Campbell 1983; Filion and Phillips 1997, 1998; Dokka et al 2000; Nagahiro et al 2000; Tousignant et al 2000; Fahr et al 2002), anionic lipid complexes were used in the present study.

Despite huge number of *in vitro* and *in vivo* studies employing lipid-DNA complexes compared with other delivery systems, the mechanisms of transfection via these carriers remain poorly understood and are actively being investigated. Much less attention has been given to the anionic liposomal systems in this regard. At present, hundreds of plasmid DNA molecules are required for successful gene transfer and expression. The enhancement of transfection efficiencies using lipid-based methods requires a full understanding of the supramolecular structures of lipid-DNA complexes, their interactions with cell membranes, and of events leading to the release of DNA and its delivery to the nucleus. The precise nature of the supramolecular structures of lipid-DNA complexes in different lipid membrane

systems is beginning to be understood (Koltover et al 1999; Safinya 2001). The transfection efficiencies of non-viral delivery methods may be improved through insights into transfection-related mechanisms at the molecular and self-assembled levels. Therefore, together with gene transfer studies, the supramolecular structure and the mechanism of DNA release from the HM-liposome-Ca^{2+}-DNA triple complex, upon contact with model membranes, was also evaluated in this work.

In order to correctly interpret the structural/morphological data regarding the triple complex, a brief literature review of similar work would be useful. DNA chains dissolved in solution are known to give rise to a variety of condensed and liquid-crystalline phases. Studies show regular condensed DNA morphologies induced by multivalent cations (Bloomfield 1991) and liquid-crystalline phases at high concentrations of DNA (Livolant and Leforestier 1996). Moreover, there has been a flurry of experimental and theoretical work on DNA chains mixed with lipids. Oligolamellar structures has been reported in cryo-transmission electron microscopy studies of cationic liposomes and DNA by Gustafsson et al (1995). A freeze-fracture electron microscopy study by Sternberg and co-workers (1994) revealed two types of structures for cationic lipid-plasmid DNA complexes. They observed fibril-like images depicting isolated DNA molecules coated by a cationic lipid bilayer and/or monolayer in addition to semi-fused liposomes composed of 2-3 vesicles drawn together by the fusogenic action of DNA. A combination of the two structures was also reported in which tubule-like lipid-coated plasmid molecules were connected to the liposomes, a complex which they called *spaghetti-meatball*-complex or assembly (Sternberg et al 1994; Sternberg 1998).

The type of lipids used in the liposome-DNA formulations may, in some cases, affect the structure of the complexes. A commonly used charge-neutral lipid in cationic lipid-DNA mixtures is di-oleoyl phosphatidylethanolamine (DOPE). A recent X-ray diffraction (XRD) investigation have shown that DOPE containing complexes may give rise to an inverted hexagonal H_{II}^{C} liquid-crystalline structure (Koltover et al 1998) as illustrated in Figure 6.20.

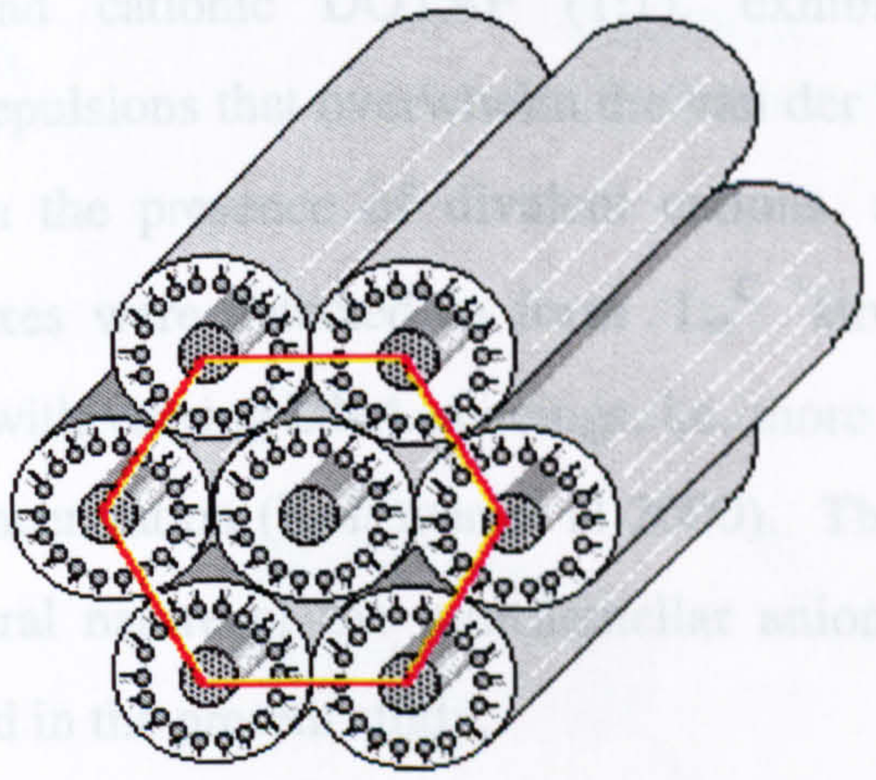

Figure 6.20. Schematic of the inverted hexagonal H_{II}^{C} phase composed of DNA molecules coated with lipid monolayers in the form of cylinders arranged on a hexagonal lattice (redrawn after Safinya 2001).

Studies have revealed that one of the commonly occurring supramolecular assemblies, which forms spontaneously when DNA is complexed with cationic lipids, is a multilayer assembly of DNA sandwiched between bilayer membranes (Raedler et al 1997; Lasic et al 1997; Koltover et al 1999; Safinya 2001). Similar structures have been reported for cationic liposome-DNA complexes in the presence of divalent cations (Koltover et al 2000) or a tumor targeting ligand transferrin (Xu et al 2002). In their seminal paper Felgner et al (1987) pictured the DNA strand decorated with distinctly attached cationic liposomes and proposed the 'bead-on-string' structure. However, the precise structure of the cationic lipid-DNA complexes elucidated by high-resolution synchrotron small-angle X-ray scattering (SAXS) revealed that the structure is different from the hypothesised model (Raedler et al 1997; Salditt et al 1997; Safinya 2001). The SAXS data lead to a model in which the cationic liposome and DNA condense into a multilayer structure, denoted L_{α}^{C}, with

DNA sandwiched between the bilayers (Figure 6.21.). In the absence of DNA, however, the lamellar L_α phase of membranes composed of DOPC (di-oleoyl phosphatidylcholine) and cationic DOTAP (1:1), exhibits strong long-range interlayer electrostatic repulsions that overwhelm the van der Waals attraction (Roux and Safinya 1988). In the presence of divalent cations, such as Ca^{2+}, cationic liposome-DNA complexes were detected to form L_α^C structures, similar to that shown in Figure 6.18., with varying DNA spacings, i.e. more compact DNA packing upon increasing ion concentration (Koltover et al 2000). This finding may provide insight into the structural nature of the multilamellar anionic HM-liposome-Ca^{2+}-DNA complexes utilised in the present study.

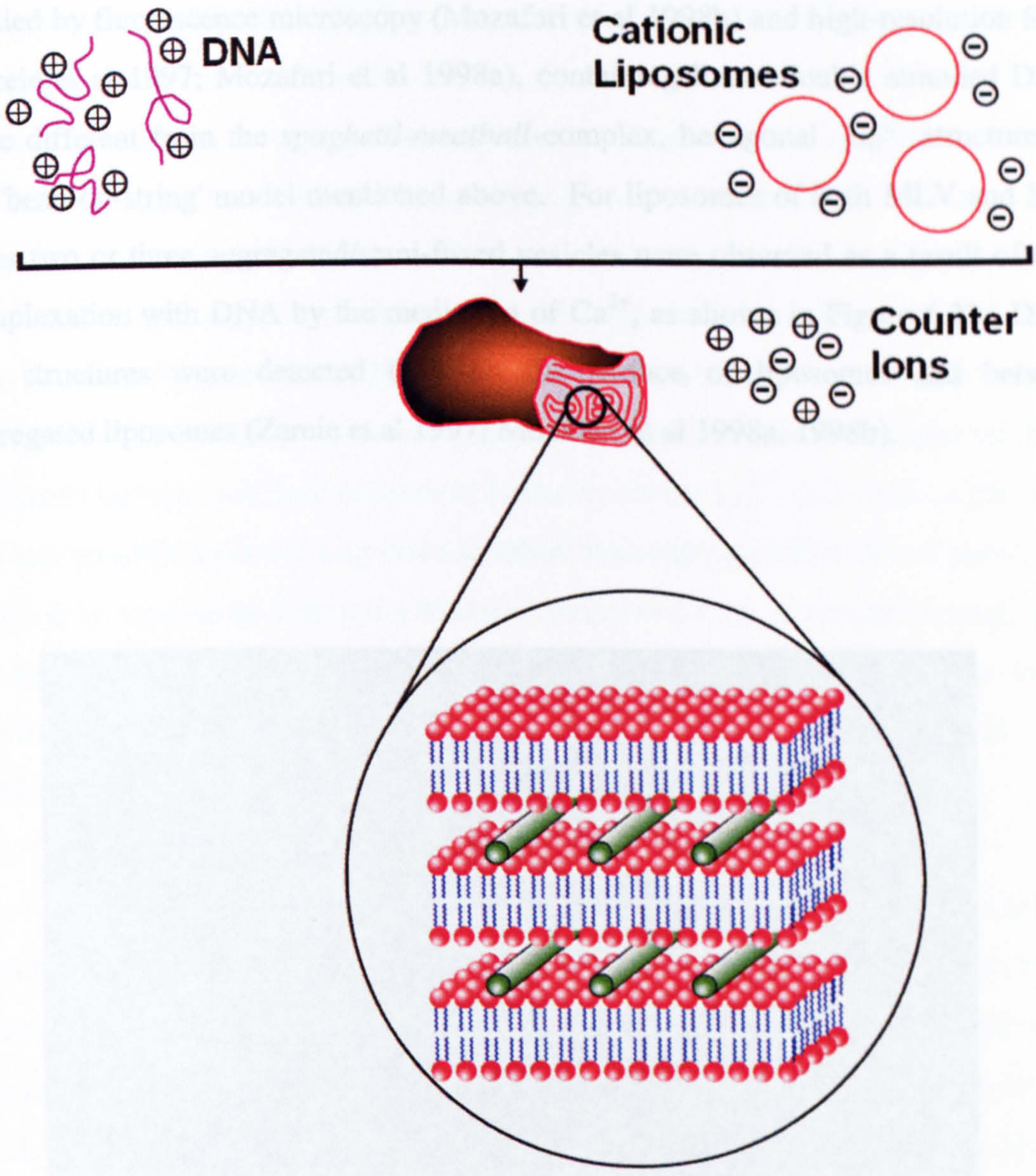

Figure 6.21. Schematic drawing of the collapse of DNA and cationic liposomes into dense multilamellar aggregates. The condensation is driven by the release of bound counterions initially covering the liposomes and DNA molecules. Enlarged section depicts the local structure of the self-assembled DNA-lipid complexes. The semiflexible DNA double-helices are illustrated by cylinders embedded in bilayer lipid membranes. This represents the lamellar L_α^C phase of lipid-DNA complexes with alternating lipid bilayers and DNA monolayers. The scheme is redrawn after Radler et al (1998).

The supramolecular structures of anionic liposome-Ca^{2+}-DNA complexes studied by fluorescence microscopy (Mozafari et al 1998b) and high-resolution STM (Zareie et al 1997; Mozafari et al 1998a), containing linear double stranded DNA, were different from the *spaghetti-meatball*-complex, hexagonal H_{II}^{C} structures or the 'bead-on-string' model mentioned above. For liposomes of both MLV and SUV types two or three aggregated/semi-fused vesicles were observed as a result of their complexation with DNA by the mediation of Ca^{2+}, as shown in Figure 6.22. DNA-like structures were detected both on the surface of liposomes and between aggregated liposomes (Zareie et al 1997; Mozafari et al 1998a, 1998b).

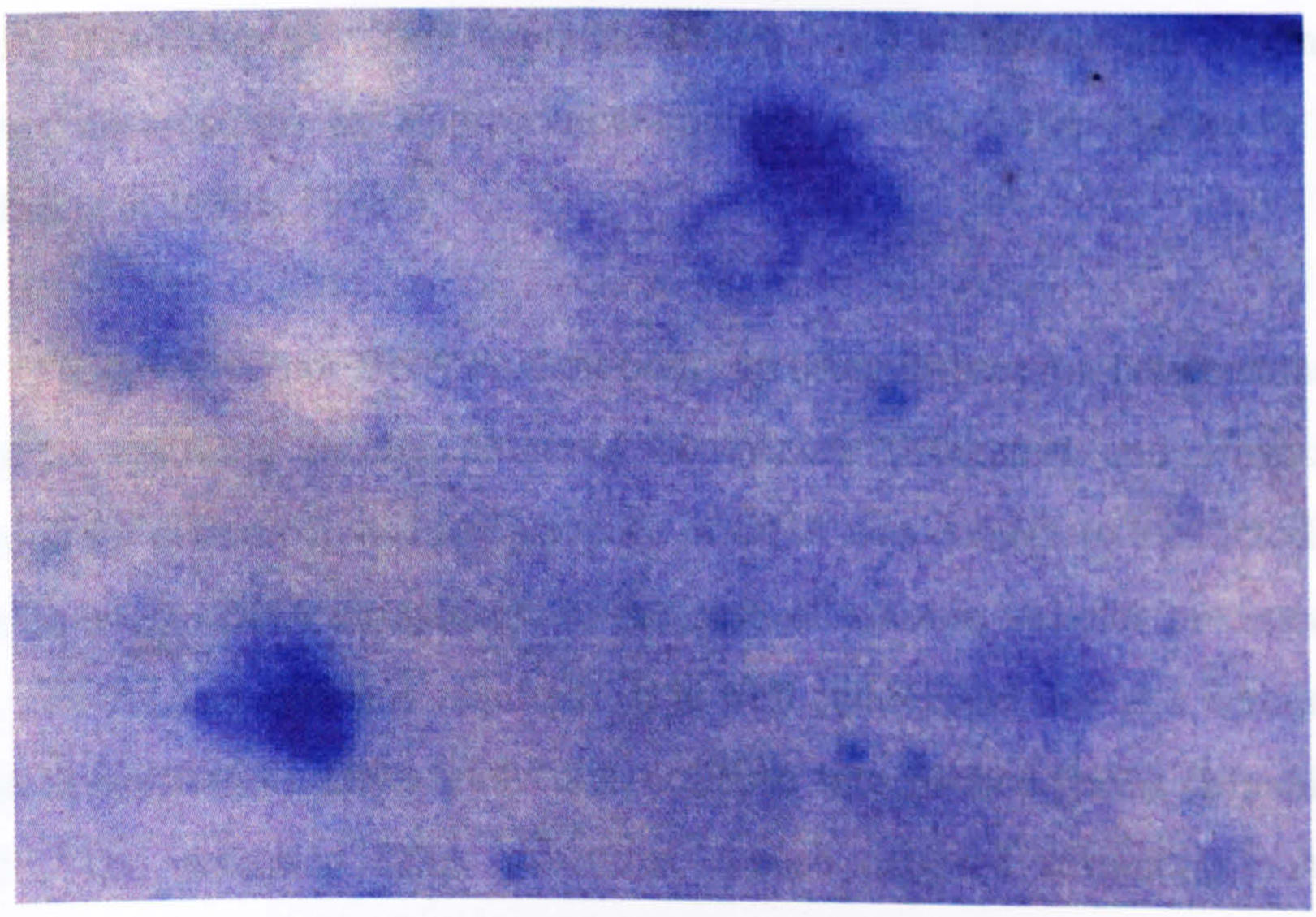

Figure 6.22. A fluorescece microscopy image of the liposome-Ca^{2+}-DNA complexes (from Mozafari et al 1998).

Similar structures were observed in the present work for LUV type liposomes, studied by TEM (Figure 6.11. and 6.12.) and STM (Figure 6.13.), and for MLV liposomes studied by AFM (Figures 6.14. to 6.17.). Three and four aggregated/semi-fused liposomes were detected by TEM. In the STM image three closely located vesicles, possibly in an aggregated state, and one single vesicle are evident while in the AFM images four vesicles in an aggregated/semi-fused state and one single vesicle can be seen. Whether the individual vesicles contain DNA molecules or not is not clear at this stage. The huge size of the single vesicle observed through AFM, both horizontally and vertically (Figure 6.14.), compared to the vesicles in the aggregate (Figure 6.16.), however, may imply that it contains layer(s) of DNA molecules between the lipid bilayers as is the case with $L_\alpha{}^c$ structures. Although it was not possible to detect any plasmid DNA molecule, probably due to their small sizes, it is interesting that quite similar complexes were observed through three different microscopies. This aggregated/semi-fused structures are similar to our previous observations by fluorescence microscopy and STM (Zareie et al 1997; Mozafari et al 1998a, 1998b).

Neither Ca^{2+} nor DNA on their own can bring interaction between the anionic liposomes resulting in the observed complexes (Mozafari and Hasirci 1998). Although for cationic liposomes presence of DNA was enough to lead into formation of the liposome-DNA complexes, in the case of anionic vesicles presence of both DNA and divalent cation is required. It is well established that at a certain level of charge neutralisation, DNA molecules collapse into packed forms (Manning 1980, 1981). The collapsed DNA structures, whose exposed surface is substantially smaller than that of the fully extended forms, can be encapsulated efficiently by liposomes (Gershon et al 1993). The charge neutralisation of DNA molecules can be brought about by divalent cations such as Ca^{2+}. It seems that Ca^{2+} can facilitate DNA incorporation to anionic liposomes by two main mechanisms: *i*) encapsulation of DNA molecules within the lipid vesicles following charge neutralisation and collapse of DNA, *ii*) attachment of the DNA molecules to the surface of the vesicles by electrostatic attraction. While both of these scenarios can happen to, and produce one single liposome, the second case, more possibly, can lead to the formation of the

observed aggregated/semi-fused liposome complexes. These are consistent with the data obtained through the microscopic studies (i.e. observation of both individual and aggregated vesicles) and the EtBr intercalation assay (i.e. fast release of DNA from the complexes upon contact with the model membrane indicates presence of some DNA molecules on the surface of the vesicles as discussed under section 6.3.5.).

A major goal of research on liposome-DNA complexes is to elucidate the key parameters resulting in the different complex structures and to establish the correlation between these structures and transfection efficiency. Interestingly studies on the nucleic acid-lipid interactions inherently have the potential to increase our understanding of many other phenomena in addition to transfection, including bacterial conjugation, nuclear membrane trafficking, viral infection and origin of life. One example of a correlation between the self-assembled structures of cationic liposome-DNA complexes and the transfection efficiency of these vectors came from the work of Lin et al (2000). This group observed that the transfection efficiency of H_{II}^{C} structures of cationic liposome-DNA vectors was higher than the L_{α}^{C} structures in mouse fibroblast L-cells. It is postulated that the reason for varying transfection efficiency in these two structures is that the lipids of the H_{II}^{C} complexes have the ability of fusion with the cell membranes (e.g. endosomal and plasma membranes) whereas L_{α}^{C} complexes remain stable inside cells (Lin et al 2000; Safinya 2001). In the case of the anionic liposomal vectors utilised in this study there are indications of the presence of DNA on the surface of the vesicles, as discussed above. It is known that both DNA and Ca^{2+} can induce fusion of phospholipid vesicles (Lansman and Haynes 1975; Wilschut et al 1980, 1981, 1985; Holland et al 1996; Mozafari and Hasirci 1998). In fact the observed aggregated/semi-fused vesicles are believed to be due to the fusogenic action of DNA/Ca^{2+} since in the absence of DNA and Ca^{2+} only individual non-aggregated vesicles were detected through the microscopic studies (Figures 2.10.-2.14.). One advantage of the vector introduced in this chapter is that due to the presence of DNA and Ca^{2+} in the complex there is no need to use any other fusogenic or endosomolytic agent. This is in comparison with some cases where application of fusogenic or endosomolytic agents in the transfection vector was found to be mandatory (Uduehi et al 1996; Pouton et al

1998). Even if the surface attached DNA molecules, involved in the fusion event, are not delivered to the nucleus the remaining DNA molecules entrapped inside the lipid bilayers can be utilised in the transfection of the cell.

According to a recent report by Lesage and colleagues (2002) for DNA transfer into cells, several important conditions must be fulfilled. The efficiency of the carrier to transport DNA must be high. The DNA has to be protected against enzymes in the extracellular medium. The DNA-carrier complex must be internalised into the cell and the functionality of DNA has to be preserved for the expression of the gene in the cell. Finally, the carrier must not be cytotoxic (Lesage et al 2002). Even if all of the above mentioned criteria are met there is no guarantee for successful transfection. Several researchers and recently Evans (2003) have detected the presence of gene transfer complexes inside the cells but no or little expression of the gene. One reason for this can be that if the complexes are taken up by cells through endocytosis they will be subject to the lysosomal degradation (Brisson and Huang 1999). Hence another important criteria for transfection is the entry of the functional DNA to the nucleus where the transcription machinery is present. The data obtained through the course of this thesis attest that our gene transfer complex meets all the above criteria. The complex was found to be non-toxic as evaluated by two toxicity assays (i.e. NRU and MTT, section 3.3.). Relatively high DNA entrapment efficiency values were found for the complex (i.e. *c.* 70%, section 6.3.3.). Entrapment efficiencies of *c.* 49% and *c.* 44% have recently been reported for plasmid DNA and antisense oligonucleotides incorporated to anionic liposomes (Fillion et al 2001). Bailey and Sullivan (2000) have reported DNA encapsulation efficiencies up to 80% in the neutral liposomes. However, the transfection efficiency of these neutral vesicles have not been evaluated in that study or thereafter to the best of my knowledge. For cationic liposomes, on the other hand, values up to 99% have been reported for DNA entrapment efficiency while again the cationic system was found to exhibit cytotoxicity (Tang and Hughes 1999). The ternary complex was able to efficiently protect its DNA load from DNase I activity (section 6.3.6.). By detecting the products of the reporter gene, transfection studies indicated that the ternary complex was able to transfect the cells (section 6.3.10.).

This means that the complex was internalised into the cell and the structure and function of the DNA was preserved.

Studies have suggested that the ratio of DNA to lipid is an important variable that determines the efficiency of transfection and the cellular toxicity (Felgner et al 1994). Furthermore, the lipid:DNA ratio needs to be optimised in order to avoid excess of non-complexed DNA in the formulation (Schreier 1998). To evaluate the effect of varying the ratio of lipid to DNA, HBE cells were transfected with a constant amount of plasmid DNA complexed with a variable amount of lipid. The optimum lipid:DNA ratio for gene transfer to the cultured HBE cells was found to be 7:1 (Figure 6.18.). Although the complex with 8:1 lipid:DNA ratio possesses the highest value for DNA protection (Table 6.5.), its transfection efficiency was not the maximum observed. This could be as a result of high amount of lipid in the formulation providing more covering for the DNA, hence less available DNA for transfection, or presence of more empty liposomes which compete with DNA-loaded vesicles for entry to the cell. Another possible reason is that high lipid concentration may result in bigger complexes which can not enter the cells efficiently. The transfection efficiency of DNA carrier complexes vary with the cell type used. Therefore transfection abilities of different complexes can be more meaningful when compared directly with those using the same cell type and even same reporter gene. Based on this fact the transfection efficiency of the ternary complex with 7:1 lipid:DNA ratio when compared with data obtained for the same gene and cell line is equal or higher than those obtained for the polymeric reagents polylysine and transferrin-polylysine (Pouton et al 1998) as well as commercially available lipid-based reagents Transfectam (Pouton et al 1998), Perfect Lipids (pFx-1 to pFx-8) and GenePORTER (Evans 2003). The transfection efficiency of GenePORTER in another human bronchial epithelial cells (i.e. IB3.1) evaluated by De Semir and co workers (2002) was also lower than that of the triple complexes. Compared with transfection results obtained using the same reporter gene but in two other human lung cell lines (i.e. A549 and H441 cells) the ternary complexes, even the one with 4:1 lipid:DNA ratio possessing the lowest transfection efficiency, are superior to the cationic lipid-based complexes, EDMPC (1,2-dimyristoyl-sn-glycero-3-

ethylphosphocholine, chloride salt):cholesterol and EDMPC:DOPE studied by Gorman et al (1997). However, the transfection efficiency of the complex with 7:1 lipid:DNA ratio is lower than the transfection efficiency of DOTAP obtained in the present study (Figure 6.19.) and by other groups using the same cell line and reporter gene (Pouton et al 1998; Evans 2003). The reason DOTAP was used as a control is due to its being one of the most applied commercially available lipid-based gene transfer vectors and it has been used for transfection of the HBE cells (Pouton et al 1998; Evans 2003) *in vitro*, and has been applied to the airways of mice (Dokka et al 2000; Vaysse et al 2002) and human nasal epithelium (Porteous et al 1997) *in vivo*. It seems that our liposomal formulation is comparable or better than 14 gene transfer systems mentioned above while inferior to DOTAP in terms of transfection. A recent report on the oligonucleotide delivery to IB3.1 cells, a human airway epithelial cell line relevant to the HBE cells used in this study, may provide a possible explanation for lower transfection efficiency of our formulation when compared with DOTAP. Using laser scanning confocal microscopy it was found that almost 100% of the oligonucleotides were delivered to the cells while majority of them could not reach the nucleus and retained in the cytoplasm (De Semir et al 2002). It may be that lack of sufficient nuclear delivery is responsible for the lower transfection ability of the ternary complex compared with DOTAP. However, recently it has been shown that at the dose used to achieve maximum transfection, i.e. 2.25 × LRD (manufacturer's lowest recommended dose), DOTAP caused almost 14% cell death when tested in the 16HBE14o- cells (Evans et al 2001; Evans 2003). In addition, Filion and Phillips (1997, 1998) have reported up to approximately 85% toxicity for DOTAP containing liposomes toward mouse macrophage cells at the lowest concentration tested (i.e. 10nmol/ml). DOTAP containing liposomes were also found to be cytotoxic in other cells including Chinese hamster ovary (CHO) cells (Tang and Hughes 1999). By comparison almost no cell death was detected for the HM-liposomes even at concentrations up to five times higher than that in the complex exhibiting maximum transfection (see section 3.3.). Considering the fact that the transfection complexes/reagents would have to be administered repeatedly over the lifetime of a patient with a genetic defect (such as cystic fibrosis), the chronic toxicity issue need

to be addressed seriously (Cheng and Scheule 1998). The non-toxic nature of our gene transfer formulation is a big advantage in this respect.

The 16HBE14o- cells are known to be refractory to transfection, probably due to the presence of tight junctions which reduce the area of exposed plasma membrane for DNA entry (Evans 2003). Therefore it is possible that the HM-liposome-Ca^{2+}-DNA complex will have higher transfections efficiencies in other cell types. Transfection has often been reported as being more efficient when carried out on cells in serum free medium (Nchinda et al 2002). In general it has been reported that the transfection by liposomes is sensitive to the presence of serum or blood components (Yi et al 2000; Kim et al 2003). Many cationic lipid based transfection systems are inactivated in as low as 5-10% serum (Zhou et al 1991; Lewis et al 1996; Vitiello et al 1996). This drawback should be considered particularly if the liposomal system is to be employed *in vivo*. Consequently, the transfection efficiency of the HM-liposomes was assessed in the serum containing medium. Presence of Ca^{2+} in our formulation not only facilitates the incorporation of DNA to the anionic vesicles but also improves the transfection. It is known that Ca^{2+} increases the permeability of lipid bilayers (Mandersloot et al 1981) and also helps to overcome the transfection-inhibiting effect of serum (Haberland et al 2000). In summary these studies using an *in vitro* model of airway epithelia may help guide attempts to develop *in vivo* gene transfer for airway diseases such as CF and potentially for other diseases of the pulmonary epithelium including surfactant protein B deficiency and α_1 -antitrypsin deficiency (Fasbender et al 1995).

6.5. Conclusions

A non-toxic lipid-based gene transfer vector is introduced which possesses high DNA entrapment efficiency and ability to protect the incorporated DNA from enzymatic degradation. Incorporation of DNA to the liposomes prepared by the heating method is through a very mild procedure which does not involve any potential harm to the DNA structure/function. The methodology of preparing the

HM-liposome-based nucleic acid delivery systems not only can be scaled up for industrial applications but also can be scaled down when sample availability is limited. Moreover, the vector was detected to be able to transfect cultured human lung epithelial cells, in the presence of serum, probably by a mechanism involving fusion of the ternary complex to the plasma membrane and/or the endosomal membrane.

7: GENERAL DISCUSSION AND CONCLUDING REMARKS

7.1. Introduction

Liposomes are closed, continuous bilayered structures made primarily of lipids/phospholipids. Pulmonary application of liposomes has generated promising results with respect to both prolonged and targeted delivery to the lung and reduced systemic toxicities, resulting in enhanced therapeutic efficacies. However, only recently has the field matured to the clinical level although preformulation and formulation, and later preclinical studies, have been conducted and reported in the literature on a regular basis since the mid-1980's. The major activities in the field have been in the following three categories: 1) infectious diseases (antibiotics), 2) asthma (corticosteroids) and 3) lung injury (antioxidants). In addition to these classic areas a fourth has emerged recently which is genetic diseases of the lung (e.g. cystic fibrosis, α_1-antitrypsin deficiency) and their treatment by gene therapy using liposomes (Davies et al 2001). Progress in the field has been documented in a series of review articles beginning with Mihalko and colleagues (1988), continuing with Kellaway and Farr (1990), Schreier et al (1993), Gonzalez-Rothi and Schreier (1995), Schreier and Sawyer (1996) and Brown et al (2001) who reviewed the status of liposomes for cystic fibrosis gene therapy. In addition, a more recent review article by Stecenko and Brigham (2003) compares liposomal vectors with adeno-associated viral vectors in the gene therapy of α_1-antitrypsin deficiency.

As to the technological advances in the field, milestones have been the series of papers on the physical characterisation of liposome aerosols by Taylor and co-workers (1990), Niven and Schreier (1990) and Niven et al (1991, 1992). These were followed by development of dry liposome powder aerosols by Schreier and colleagues (1994) as well as dry liposome:DNA powder aerosols (Allon et al 1997) and the development and characterisation of nebulised cationic liposome:DNA

complexes by Schwarz and co-workers (1996), Eastman et al (1997) and Gagne and Schreier (1997). The majority of *in vivo* pulmonary liposomal gene and drug delivery have been performed directly to the lung by instillation, nebulisation or aerosol inhalation (Schreier 1998). Interestingly, gene delivery to the lung has also been reported to be achieved by intravenous administration of a cationic lipid-DNA complex (lipoplex) (Barron et al 1999).

Preclinical studies established the innocuousness of inhaled liposomes by a number of measures including macrophage function and lung histology in mice (Gonzalez-Rothi et al 1991; Myers et al 1993) and lung function studies in sheep (Schreier et al 1992). Pharmacokinetic and deposition studies in rats (Vyas et al 2004), sheep (Schreier et al 1992), dogs (Bennett et al 1994) and in human volunteers (Farr et al 1985; Walldrep et al 1997) showed that liposomes are retained in the lungs for prolonged periods of time and could potentially serve as sustained release carriers. Normal lung function and no toxic side effects were documented in some studies employing human volunteers (Gilbert et al 1988; Thomas et al 1991). However, many reports on the toxicity and other complications associated with some liposomes in lung and other cells/tissues also exist (Panzner and Jansons 1979; Chawla et al 1979; Campbell 1983; Filion and Phillips 1997, 1998; Dokka et al 2000; Nagahiro et al 2000). Despite the intensive work in the field of liposomal drug delivery to the pulmonary system, which have been conducted for more than two decades, there are no liposomal products approved for application to human or animal airways as yet. It seems that issues including safety, stability, cost and efficiency are responsible for this shortcoming.

In the foregoing circumstances, the primary aim of this thesis was to develop liposomal formulations suitable for efficient gene and drug incorporation, protection and delivery. Particular attention was given to the lungs as an ideal route for both local and systemic delivery of the therapeutic agents. Areas of lung injury, cancer and nucleic acid therapy have been addressed by incorporation of glutathione, 5-fluorouracil and DNA into the liposomes respectively.

7.2. Summarising Discussion

This study has focused on addressing some major problems in the manufacture of liposomes in a pharmaceutically acceptable manner. These problems include safety issues, efficiency in stable incorporation and release of the entrapped agents, as well as length of the time required to produce the lipid vesicles. Safety concerns are mainly due to the application of harmful solvents or potentially toxic ingredients in the preparation of liposomes. An initial objective was to explore ways of making liposomes without application of the potentially toxic solvents. If it would be possible to omit application of harmful solvents, the requirement for performing additional steps to remove these solvents from the liposomal preparation would be eliminated as well. This in turn would decrease the time and consequently cost of liposome preparation as well as removing the safety concerns both for the producer and the consumer of the liposomal products.

As a result of intensive investigations in an attempt to overcome the above-mentioned problems, a new method has been developed which employs heating and small amount of a physiologically acceptable solvent (i.e. glycerol) to prepare the lipid vesicles. By this "Heating Method", liposomes can be produced within few hours in a reproducible and efficient way. Liposomes prepared by the heating method are designated as HM-liposomes. Application of glycerol in the preparation of the HM-liposomes have the following advantages:

- Glycerol is a bioacceptable, non-toxic agent already in use in many pharmaceutical products and can serve as an isotonising agent in the liposomal preparations.

- Unlike the volatile organic solvents employed in the manufacture of conventional liposomes, there is no need for the removal of glycerol from the final preparation.

- It serves as dispersant and prevents coagulation or sedimentation of the vesicles thereby enhancing the stability of the liposome preparations.

- It also improves the stability of the liposome preparations against freezing, thawing etc. Therefore, HM-liposomes are also ideal for freeze-drying e.g. in the preparation of dry powder inhalation products.

The initial part of this thesis provides a concise overview of the history and general properties of liposomes (chapter 1). Preparation of HM-liposomes, in both micrometric and nanometric size distributions, and their physicochemical characterisation by various techniques are explained thoroughly (chapter 2). The cytotoxicity profiles of the HM-liposomes, with and without DNA, evaluated by using two different toxicity assays are presented in comparison with that of the liposomes prepared by a conventional technique (chapter 3). In addition, the characteristics of HM-liposomes containing 5-FU as a model anticancer drug (chapter 4), GSH as an antioxidant agent (chapter 5) and plasmid DNA as a genetic material (chapter 6) are also explained. The biological and pharmaceutical characterisation of the HM-liposomes were carried out employing a human lung epithelial cell line (HBE cells) as an ideal relevant *in vitro* model of the human airways. Using this cell line, the effect of encapsulation into the HM-liposomes on the cytotoxicity of 5-FU (chapter 4) as well as the transfection efficiency of a gene transfer vector constructed using the HM-liposomes (chapter 6) were studied.

In order for the results obtained in different chapters to be comparable, a single liposomal composition was used throughout this thesis. Liposomes prepared, both by the heating method and a conventional technique, were composed of DPPC:DCP:CHOL (7:2:1 molar ratio). However, it should be noted that the heating method is not confined to this lipid composition. The liposome composition selected in the present study was based partially on the fact that DPPC (the main ingredient of the HM-liposomes prepared in this study) is a natural lung component. This selection was also based on the results obtained by different groups previously

(Papahadjopoulos et al 1977; Jurima-Romet et al 1992; Kahveci et al 1994; Borucu et al 1995; Zareie et al 1996a; Mozafari 1996; Filion and Phillips 1997; Zareie et al 1997; Mozafari and Hasirci 1998; Mozafari et al 1998a; Mozafari et al 1998b; Banerjee and Bellare 2001). In accordance with the findings of these literature reports, results obtained in the present work attest that HM-liposomes composed of DPPC:DCP:CHOL are stable for many months after their preparation (chapter 2) and possess high drug and DNA encapsulation efficiencies as well as permeability and release properties (chapters 4 to 6). The stability of the HM-liposomes relies on the fact that DPPC has a relatively high phase transition temperature (i.e. 41°C). Presence of glycerol further augments this stability while presence of cholesterol also provides ideal permeability and release properties. Additionally, employment of the negatively charged lipid DCP into the HM-liposome structure prevents liposomal aggregation and sedimentation by providing electrostatic repulsion between the vesicles. The reason for using a negatively charged lipid, and not cationic lipid(s), in the formulation of the HM-liposomes, is the toxicity and other complications reported for the cationic lipids (chapter 3). In terms of safety issues the HM-liposome formulation was detected to be superior to the liposomes prepared by conventional techniques using volatile organic solvents. Cytotoxicity studies revealed that HM-liposomes are completely non-toxic towards the HBE cells while conventional liposomes exhibited significant levels of toxicity (chapter 3). This was confirmed by employing two generally used cytotoxicity tests, namely NRU and MTT assays. It was found that presence of calcium and DNA do not affect the cytotoxicity results.

Incorporation of drugs into the HM-liposomes can be achieved by several routes: *i)* adding the drug to the reaction medium along with the liposomal ingredients and glycerol; *ii)* adding the drug to the reaction medium when temperature has dropped to a point not lower than the transition temperature (T_C) of the lipids; *iii)* adding the drug to the HM-liposomes after they are prepared e.g. at room temperature (incorporation of DNA to the HM-liposomes was performed by this route as explained in chapter 6). It is known that formation of liposomes requires heating the liposomal components at temperatures not lower than the Tc of

the lipids. This is because below Tc lipids are in the gel state and can not usually form bilayered structures. When cholesterol (or any other sterol) is used as a liposomal component (as was the case in the present study), liposomes are prepared successfully at 120°C (chapter 2). Since the majority of the phospholipid molecules employed as liposomal constituents have transition temperatures below 60°C, in the absence of cholesterol (or other sterols) HM-liposomes can be prepared for example at 60-70°C. The antineoplastic drug 5-FU was incorporated to the HM-liposomes efficiently at two temperatures of 60°C and 120°C (chapter 4). This indicates that, even in the presence of sterols, incorporation of drugs sensitive to high temperatures to the HM-liposomes can be achieved with high efficiencies by adding the drug to the reaction medium when the temperature has decreased to 60°C or 70°C for example. It seems that once sterols are dissolved at high temperatures, at a lower temperature (i.e. 60°C which is above the Tc of the lipids) liposomes are either still not formed or are highly dynamic and able to incorporate drug molecules efficiently.

Incorporation of plasmid DNA molecules, which are highly sensitive to high temperatures, to the HM-liposomes was carried out at room temperature by incubation of DNA with the empty HM-liposomes. Since both DNA molecules and the HM-liposomes prepared in this study are anionic, incorporation of DNA to the HM-liposomes was performed in the presence of calcium. Morphological studies revealed either one huge vesicle or two to four aggregated/semi-fused vesicles for liposomes of both MLV and LUV types as a result of their complexation with DNA by the mediation of Ca^{2+} (chapter 6). It was detected that HM-liposomes have a high DNA encapsulation efficiency and are able to protect the incorporated DNA molecules from the enzymatic action of DNase I. Furthermore, it was observed that HM-liposomes are able to release the incorporated DNA upon encountering a model membrane system. It was established that the optimum lipid:DNA ratio for the transfection of the HBE cells is 7:1. However, the transfection efficiency of the HM-liposomes was less than that of the commercially available cationic lipid DOTAP.

7.3. Prospective Work

A distinct feature of the heating method compared with the conventional techniques of liposome preparation is the absence of potentially toxic detergents and organic solvents. It was suspected that the presence of trace amounts of the volatile organic solvents, employed in the manufacture of the conventional liposomes, is responsible for their toxicities. This needs to be further confirmed by employing sensitive analytical instruments such as mass spectrometry.

In future, animal studies are necessary to determine the efficacy of the developed system as a means of obtaining information on the *in vivo* characteristics of the HM-liposomes intended for delivery of therapeutic agents. Investigation of approaches to prevent the opsonisation and increase the circulation time of the HM-liposomes, e.g. by insertion of polymers such as poly(hydroxyethyl L-asparagine) (PHEA) (Metselaar 2003) or poly(ethylene glycol) (PEG). Additionally, employment of targeting strategies to increase localisation of the drug at the target site in the body and avoid harm to healthy tissues are among the prospective points to be considered.

The transfection efficiency of the HM-liposomes needs to be established in other cell types *in vitro* as well as in animal models *in vivo*. Means of improving the transfection efficiency of the HM-liposomes, for example by optimising the concentration and type of the divalent cation and/or the lipids used in the formulation, needs to be explored. Furthermore, optimum ways of delivering HM-liposomes, containing DNA or other drugs, to the airways should be established e.g. by investigating the physicochemical and pharmaceutical characteristics of nebulised/aerosolised formulations *in vivo*.

7.4. Conclusions

A novel method for producing liposomes, without employing any toxic chemical or hazardous process, was developed during the course of this thesis. This '*heating method*' enabled preparation of stable liposomes with ease, good reproducibility and different size distributions. Liposomes manufactured by the new method (HM-liposomes) were completely non-toxic when tested in a human lung epithelial cell line. The antineoplastic drug 5-FU and the antioxidant agent GSH were encapsulated in the HM-liposomes with high efficiencies. HM-liposomes sustained the release of these drugs for prolonged periods and reduced the cytotoxicity of the 5-FU *in vitro*. A new non-viral, and non-cationic gene transfer vector was constructed by using the HM-liposomes. Incorporation of DNA to the HM-liposomes was achieved with high efficiency through a very mild procedure, which does not involve any potential harm to the DNA structure/function. High-resolution electron and scanning probe microscopic studies revealed either one huge vesicle or two to four aggregated/semi-fused vesicles for HM-liposomes of both MLV and LUV types as a result of their complexation with DNA and calcium. The HM-liposomes were detected to be able to protect the incorporated DNA from enzymatic degradation. Moreover, the vector was detected to be able to transfect cultured human lung epithelial cells, in the presence of serum, probably by a mechanism involving fusion of the ternary complex to the plasma membrane and/or the endosomal membrane.

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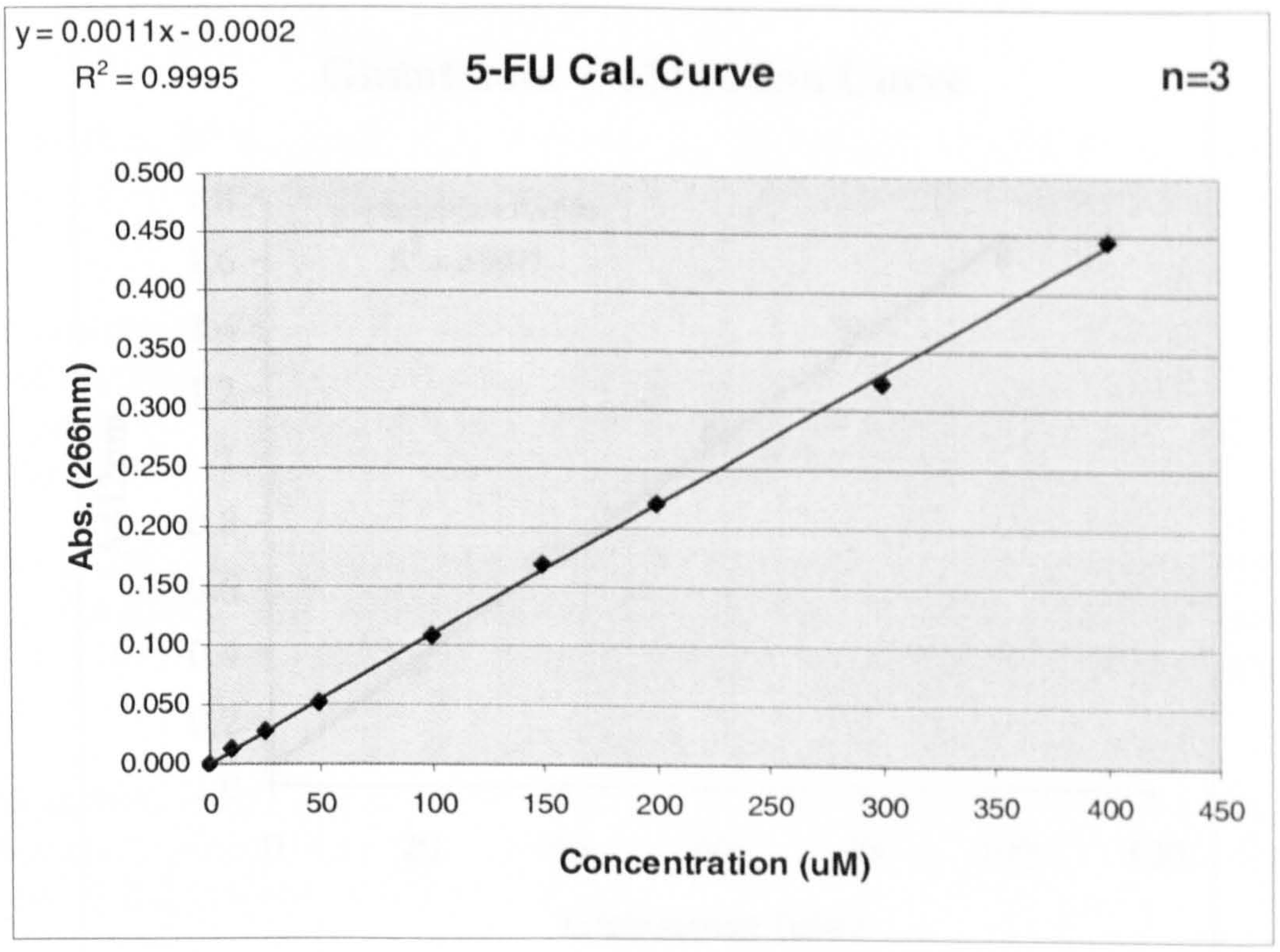
Appendix 1.

Structural details of Scanning Tunnelling Microscopy

Mechanically, scanning tunnelling microscopy (STM) consists of two main modules, a scanner and a coarse positioner. These modules can be separated to perform distinct measurements, performance tests and experiments. Even different modules for coarse positioning can be installed to the scanner module. The scanner part is constructed with a piezo tube (PZT) (EBL #3 PZT-5H, Staveley Sensors INC, CT) glued to an aluminium body. Electrical connection of PZT are through a special connector. The tip holder is as well glued to the interior of the PZT tube but isolated electrically. Rough approach unit is a magnetically driven slider which is fastened to the scanner using two screws and the whole system is mounted on a vibration-isolation stage. Electrical connections to the system is through a printed circuit board that is placed near the vibration-isolation stage. Very thin wires are used between the STM and this board to reduce the vibrational coupling between them.

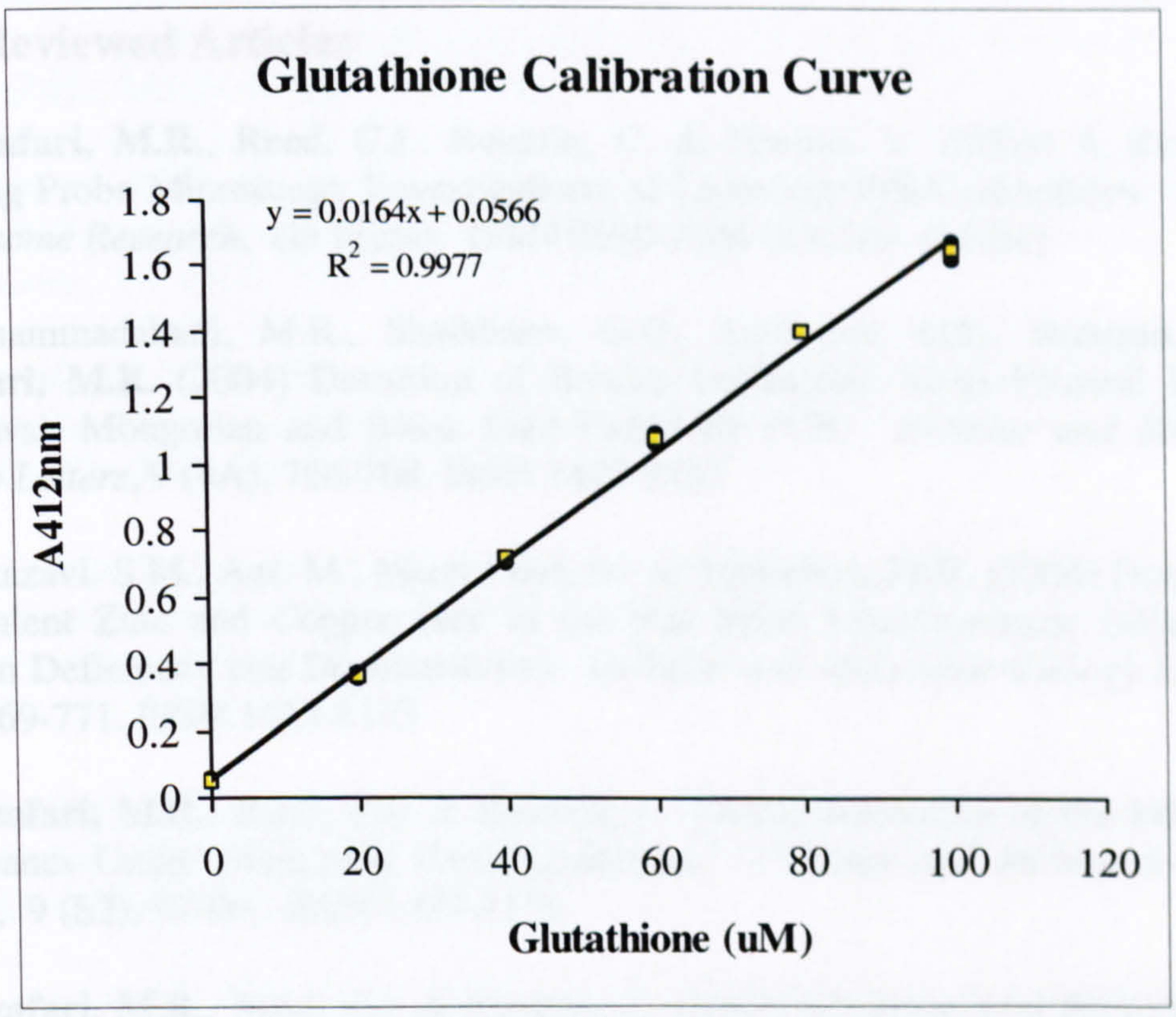
Appendix 2.

Example of 5-Fluorouracil Calibration Curve



Appendix 3.

Example of Glutathione Calibration Curve



Appendix 4.

Publication List of the Author of this Thesis

(up to February 2005)

Peer-Reviewed Articles

1. **Mozafari, M.R.**, Reed, C.J., Rostron, C. & Hasirci, V. (2004) A Review of Scanning Probe Microscopy Iinvestigations of Liposome-DNA complexes. *Journal of Liposome Research*, (In Press). ISSN 0898-2104 (Review Article)

2. Mohammadabadi, M.R., Shaikhaev, G.O., Sulimova, G.E., Rahman, O. & **Mozafari, M.R.** (2004) Detection of Bovine Leukaemia Virus Proviral DNA in Yaroslavsl, Mongolian and Black Pied Cattle by PCR. *Cellular and Molecular Biology Letters*, 9 (4A), 766-768. ISSN 1425-8153

3. Mortazavi, S.M., Ani, M., Mesri Pour, M. & **Mozafari, M.R.** (2004) Distribution of Divalent Zinc and Copper Ions in the Rat Brain Synaptosomes: Influence of Calcium Deficiency and Depolarisation. *Cellular and Molecular Biology Letters*, 9 (4A), 769-771. ISSN 1425-8153

4. **Mozafari, M.R.**, Reed, C.J. & Rostron, C. (2004) Formation of the Initial Cell Membranes Under Primordial Earth Conditions. *Cellular and Molecular Biology Letters*, 9 (S2), 97-99. ISSN 1425-8153

5. **Mozafari, M.R.**, Reed, C.J. & Rostron, C. (2004) Ultrastructural Architecture of Liposome-Entrapped Glutathione: A Cryo-SEM Study. *Cellular and Molecular Biology Letters*, 9 (S2), 101-103. ISSN 1425-8153

6. **Mozafari, M.R.** (2004) Micro and Nano Carrier Technologies: High Quality Production Within Pharmaceutical Standards. *Cellular and Molecular Biology Letters*, 9 (S2), 44-45. ISSN 1425-8153

7. Yurdugul, S. & **Mozafari, M.R.** (2004) Recent Advances in Micro- and nano-Encapsulation of Food Ingredients. *Cellular and Molecular Biology Letters*, 9 (S2), 64-65. ISSN 1425-8153

8. **Mozafari, M.R.**, Reed, C.J., Rostron, C. & Martin, D.S. (2004) Transfection of Human Airway Epithelial Cells Using A Lipid-Based Vector Prepared By The Heating Method. *Journal of Aerosol Medicine*, 17 (1), 100. ISSN: 0894-2684

9. **Mozafari, M.R.**, Reed, C.J. & Rostron, C. (2004) 5-Fluorouracil Encapsulated In Colloidal Lipid Particles: Entrapment, Release and Cytotoxicity Evaluation in an Airway Cell Line. *Journal of Aerosol Medicine*, 17 (1), 100. ISSN: 0894-2684

10. **Mozafari, M.R.**, Reed, C.J., Rostron, C., Kocum, C. & Piskin, E. (2002) Construction of Stable Anionic Liposome-Plasmid Particles Using the Heating Method: A Preliminary Investigation. *Cellular and Molecular Biology Letters*, 7 (3), 923-927. ISSN 1425-8153

11. **Mozafari, M.R.**, Reed, C.J., Rostron, C., Kocum, C. & Piskin, E. (2002) Formation and Characterisation of Non-Toxic Anionic Liposomes for Delivery of Therapeutic Agents to the Pulmonary Airways. *Cellular and Molecular Biology Letters*, 7 (2), 243-244. ISSN 1425-8153

12. **Mozafari, M.R.**, Reed, C.J. & Rostron, C. (2002) Development of Non-Toxic Liposomal Formulations for Gene and Drug Delivery to the Lung. *Technology and Health Care*, 10 (3&4), 342-344. ISSN 0928-7329

13. **Mozafari, M.R.**, Yamout, N. & Rajabi-Siahboomi, A.R. (1998) Factors Affecting the Liposomal Delivery of Nucleic Acids. *Iranian Journal of Chemistry*, 11 (1), 24-27. ISSN 1015-2849

14. **Mozafari, M.R.**, Zareie, M.H., Piskin, E. & Hasirci, V. (1998) Formation of Supramolecular Structures by Negatively Charged Liposomes in the Presence of Nucleic Acids and Divalent Cations. *Drug Delivery*, 5 (2), 135-141. ISSN 1071-7544/98

15. **Mozafari, M.R.** & Hasirci, V. (1998) Mechanism of Calcium Ion Induced Multilamellar Vesicle-DNA Interaction. *Journal of Microencapsulation*, 15 (1), 55-65. ISSN 0265-2048/98

16. Zareie, M.H., **Mozafari, M.R.**, Hasirci, V. & Piskin, E. (1997) Scanning Tunnelling Microscopy Investigation of Liposome-DNA-Ca2+ Complexes. *Journal of Liposome Research*, 7 (4), 491-502. ISSN 0898-2104

Other Publications

17. **Mozafari, M.R.**, Reed, C.J., Rostron, C., Martin, D.S., Kocum, C. & Piskin, E. (2004) Scanning probe microscopy investigations of nano and microscale bioactive carrier complexes. *UKSPM 2004, UK Annual Scanning Probe Microscopy Meeting*, Nottingham, England.

18. **Mozafari, M.R.**, Reed, C.J. & Rostron, C. (2004) Non-toxic liposomal formulation for gene delivery. *PRISM Conference (Postgraduate Researchers in Science Medicine)*, Sherrington Buildings, Liverpool, UK.

19. **Mozafari, M.R.**, Reed, C.J. & Rostron, C. (2004) Microstructural study of lipid-based carrier systems using atomic force microscopy. *PRISM Conference*

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20. Yurdugul, S. & **Mozafari, M.R.** (2004) Application of Nano-Encapsulation Technology in the Food Industry. *12th Iranian Researchers Conference in Europe (IRCE 2004)*, UMIST, Manchester, UK, pp 257.

21. **Mozafari, M.R.**, Reed, C.J., Rostron, C., & Martin, D.S. (2003) Second Generation of Liposome/Ca2+/DNA Complexes: A Microstructural Study Using Atomic Force Microscopy. *6th International Conference, Liposome Advances, Progress in Drug and Vaccine Delivery*, London, England, pp 114.

22. **Mozafari, M.R.**, Reed, C.J., Rostron, C., & Martin, D.S. (2003) Transfection of Human Airway Epithelial Cells Using a Lipid-Based Vector Prepared by the Heating Method. *14th Drug Delivery to the Lungs Conference*, London, England, pp 54-58. ISBN 0-0544567-1-8.

23. **Mozafari, M.R.**, Reed, C.J., Rostron, C., & Martin, D.S. (2003) 5-Fluorouracil Encapsulation in Colloidal Lipid Particles: Entrapment, Release and Cytotoxicity Evaluation in an Airway Cell Line. *14th Drug Delivery to the Lungs Conference*, London, England, pp 180-183. ISBN 0-0544567-1-8.

24. **Mozafari, M.R.**, Reed, C.J., Rostron, C., Kocum, C. & Piskin, E. (2002) Pharmaceutical Applications of Nanotechnology- Implications in Drug Targeting and Gene Therapy. *Proceedings of Pharmacy & Pharmacology, 9th Iranian Students Seminar in Europe (ISS-2002)*, Birmingham, England, pp 7.

25. **Mozafari, M.R.**, Reed, C.J., Rostron, C., Kocum, C. & Piskin, E. (2002) Formation and Characterisation of Non-Toxic Anionic Liposomes for Delivery of Therapeutic Agents to the Pulmonary Airways. *9. Liposomes. From Models to Applications*, Wroclaw, Poland.

26. **Mozafari, M.R.**, Reed, C.J. & Rostron, C. (2002) Safety and Efficacy of Anionic Liposome Mediated Gene Transfer to the Human Airway Epithelial Cells. *Genes as Medicines*, London, England.

27. **Mozafari, M.R.**, Reed, C.J. & Rostron, C. (2002) Cystic Fibrosis Gene Therapy by Liposomes: Prospects and Problems. *The Ian Ward Members Papers Evening, The Association of Clinical Biochemists North West Region*, Liverpool, England.

28. Yamout, N. & **Mozafari, M.R.** (2002) Plant Proteins and Bones. *Medicine Today Journal*, 11 (417) 1 & 9.

29. **Mozafari, M.R.**, Reed, C.J. & Rostron, C. (2001) Reduced Cytotoxicity of Anionic Liposome/Calcium/DNA Complexes Prepared by the Heating Method. *British Pharmaceutical Conference Science Proceedings 2001*, pp 100 (Pharmaceutical Press, London, UK). ISBN 0 85369 512 1.

30. **Mozafari, M.R.**, Reed, C.J. & Rostron, C. (2001) Non-Toxic Liposomal Formulations for Gene and Drug Delivery to the Lung. *8th Biomedical Science and Technology Symposium*, Ankara, Turkey, pp O16.

31. **Mozafari, M.R.**, Reed, C. & Rostron, C. (2001) The Influence of the Preparation Method on the Toxicity of Anionic Liposomes. *Proceedings of Pharmacy & Pharmacology, 8th Iranian Students Seminar in Europe (ISS-2001)*, UMIST, Manchester, England, pp 3.

32. **Mozafari, M.R.** (2000) Effect of Ca2+ on the Formation of Liposome-DNA Complex: Implications in Liposomal Gene Transfer. *Proceedings of Pharmacology & Dentistry, 7th Annual Iranian Students Seminar in Europe (ISS-2000)*, UMIST, Manchester, England, pp 6-7.

33. **Mozafari, M.R.** & Negarestani, S. (1998) The Necessity of Understanding Epilepsy Correctly. *Medicine Today Journal*. 7 (234), 7-8.

34. **Mozafari, M.R.**, Zareie, M.H., Piskin, E. & Hasirci, V. (1997) Morphological Observations of Anionic Liposome/DNA/Ca2+ Complexes by Scanning Tunnelling Microscopy and Fluorescence Microscopy. *Proceedings of the Fourth International Symposium on Biomedical Science and Technology*, Istanbul, Turkey, pp 41-42.

35. **Mozafari, M.R.** & Hasirci, V. (1997) Effect of Metal Cations on the Formation of Anionic Lipid-DNA Complex: Implications in Liposomal Gene Transfer. *First Kizilirmak Symposium on Pure and Applied Sciences*, Kirikkale University, Kirikkale, Turkey, pp 54-59.

36. **Mozafari, M.R.** & Hasirci, V. (1996) Study of the Ca2+ Induced Anionic Liposome-DNA Interactions. *Liposome Advances, Progress in Drug and Vaccine Delivery*, London, England, pp 114.

37. **Mozafari, M.R.** & Hasirci, V. (1996) Anionic Multilamellar Vesicle-Mediated Gene Transfer. *Proceedings of the Third National Symposium on Biomedical Science and Technology*, Bursa, Turkey, pp 109-110.

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39. Kahveci, G., **Mozafari, M.R.**, Rouhvand, F., Altay, G. & Zhdanov, R.I. (1994) Importance of Nucleic Acid-Lipid Interactions in Functioning of the Initial Cell. *12th National Congress of Biochemistry*, Istanbul, Turkey, pp C-338.

Patent Application

Mozafari, M. R. (2004) A New Method and Apparatus for Fast Production of Carrier Complexes on Small and Large Scale. *Patent application No 0404993.8*