Processing and Characterisation of Liposomes for Use in Gene Delivery.

Leigh Anthony Maguire.

University College London

Department of Biochemical Engineering

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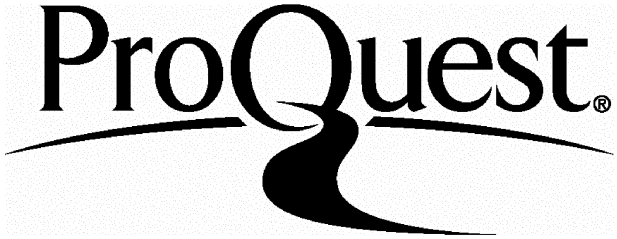

Abstract.

In recent years liposomes have received much attention for use in gene therapy and DNA vaccines. This project aims to investigate issues involved with the processing of liposome based gene delivery vectors and in doing so propose a controllable, reliable and scaleable method for processing such a vector. With this in mind a high velocity jet homogeniser was investigated for use in the preparation of small unilamellar vesicles (SUV). The device was able to produce SUV with a distribution having a mean of approximately 140nm following a single pass at 103.4 mNm^{-2}: this size is suitable for sterilisation by filtration and *in vivo* gene delivery. By increasing either the number of passes through the device or the operating pressure it was possible to control the final size of the SUV between 80 and 140nm.

The liposomes produced were used to encapsulate plasmid DNA that had been condensed with poly-L-lysine (PLL). Charge ratio was determined to be a key processing criteria with a liposome: PLL/DNA charge ratio of 4 or above being required to form stable complexes. The liposomes encapsulated between 85 and 95% of the PLL/DNA with smaller liposomes being more effective.

Targeting moieties are often attached to the surface of liposomes in order to improve their delivery. In order to investigate the effects of such molecules upon processing of liposomes and PLL/DNA/liposome complexes a Fab' antibody fragment was attached to the liposome. The presence of the Fab' resulted in increased size and reduced negative

charge. These effects were shown to result in reduced PLL/DNA encapsulation and stability.

Polyethylene glycol (PEG) can be used to improve the stability of complexes. The effects of PEG with a molecular weight of 2000 KDa upon processing included improved stability of PLL/DNA/liposome complexes and successfully shielded the zeta potential of the particles. However its presence also resulted in a reduced PLL/DNA encapsulation efficiency.

Acknowledgements.

The acknowledgements page is often the most read part of a thesis and is therefore a source of much distress to the author. With this in mind I would firstly like to thank those whom actually read on. If you enjoy what you read why not tell your friends.

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Tables.

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A3. Standard curve for protein assay.

Abbreviations.

CF,	Cystic fibrosis
Chol,	Cholesterol
ctDNA,	Calf thymus DNA
DNA,	Deoxyribonucleic acid
DOPC,	Diolyoil phosphotidylcholine
DOPE,	Diolyoil phosphotidylethanolamine
EIAV,	Equine Infectious-Anaemia Virus
ELISA,	Enzyme linked Immunosorbant Assay.
EMCA,	European Medicines Control Agency
FDA,	American food and drug administration
HA,	Haemagluttinin
HIV,	Human Immunodeficiency Virus
humAb4D5	Humanised version of the monoclonal antibody 4D5 .
LUV,	Large Unilamellar Vesicle.
MCA,	Medicines Control Agency
MLV,	Multilamellar Large Vesicle.
munAb4D5	Murine version of the monoclonal antibody 4D5,
OA,	Oleic acid
PCS,	Photon Correlation Spectroscopy.
p DNA	Plasmid DNA
PAGE.	Poly acrylamide gel electrophoresis

PE, Phosphotidylethanolamine

PEG, Polyethylene glycol

PE-MPB, Phosphotidylethanolamine-maleimido-phenyl butyrate

PLL, Poly-L-lysine

RES, Reticuloendothelial system

RNA, Ribonucleic acid

SDS, Sodium dodecyl sulphate

SIV, Simian Immunodeficiency Virus

SMPB, N-succinimidyl (-4-[P-maleimido-phenyl] butyrate)

SUV, Small Unilamellar Vesicle

Tc, Transition temperature

tEM, Transmission electron microscopy .

Notation.

ε_0 extinction coefficient of proteins at A_{280nm}

γ surface/interfacial tension (mNm^{-1})

ζ zeta potential (mV)

η Kolmogoroff length scale (m)

μ viscosity (Kgm^{-1})

ρ density ($Kgm^{-1}s^{-1)}$

ε energy dissipation rate (m^2s^{-3})

a_1, a_2 radii of primary particles 1 and 2 (m)

A	Hamaker constant (J)
c	ionic concentration (M)
e	charge of an electron (1.6×10^{-19} C)
H	separation distance between two primary particles (m)
k	Baltzmann's constant (1.381×10^{-23} J/K)
T	absolute temperature (K)
V_A, V_R, V_T	interaction energies (Van der Waal's attraction, electrical repulsion and total), (J)
x	dimensionless ($= (H/a_1 + a_2)$
y	dimensionless ($= a_1 + a_2$)
z	valence (charge number) of the ionic species
γ_1, γ_2	dimensionless functions of zeta potentials of particles 1 and 2 in Eq 5.3
p	permittivity, dimensionless
κ	Debye-Huckel parameter ($0.329 \times 10^{10} \sqrt{cz^2}$ (m))

1. Introduction and Literature Review.

1.1. What is Gene Therapy?

Gene therapy is defined as a technology aimed at modifying the genetic component of cells for a therapeutic benefit (Kirn *et al.,* 2002). In the case of monogenic heritable disorders, such as cystic fibrosis (CF), if the normal gene product could be expressed the abnormal biological phenotype of the individual could be corrected (Crystal, 1995).

1.1.1. Potential of Gene Therapy.

Currently clinical trials are underway in areas as diverse as HIV infection, venous leg ulcer treatment, screening for genetic disorders, several different cancers, neuroblastoma, Gaucher's disease and sickle cell disease to name a few (see http://clinicaltrials.gov/ct/gui).

The Human Genome Project has highlighted the potential of gene therapy in the treatment of human disease. It is becoming more apparent that many diseases have a genetic component. Table 1.1 shows some of the 4000 genes known to be involved in monogenic conditions.

Table 1.1. Single gene disorders under investigation for somatic gene therapy.

Disease	**Affected Gene Product (Heredity Type)**	**Incidence Rate Live Births**
Hypercholesterolaemia	Low density lipoprotein receptor (Dominant)	1:500
Cystic Fibrosis	Cystic fibrosis transmembrane conductance regulator (Recessive).	1:2500
Duchenne muscular dystrophy.	Dystrophin	1:8000
Haemophilia A/B	Factor VIII/IX (X Chrom Recessive)	1:10,000
Gaucher's disease	Glucocerebrosidase receptor (recessive)	1:40,000
Phenylketonuria	Phenylamine hydrolase	1:12,000

Adapted from Gottschalk & Chan (1998).

Cancers are caused by mutations in genes involved in the regulation of the cell cycle. The main treatment for many, for example colorectal cancer (CRC), is the surgical removal of the primary tumour, but this is often unsatisfactory due to advanced disease or the development of metastases. Despite advances in cancer therapy the five year survival rate for colon cancer patients has remained static and new measures are urgently needed (Guy *et al.*, 2000). Gene therapy offers a rational new approach to cancer therapy, which could complement conventional treatment. Gene therapy strategies that are being investigated for the treatment of cancer include; gene directed enzyme prodrug (suicide gene) therapy (Kirn *et al.*, 2002); therapy designed to boost the immune response against the tumour; oncocyclic virus therapy; transfer of therapeutic genes (e.g. tumour suppressor genes); and anti sense therapy (Engelhard, 2000).

Mutations of the tumour suppressor gene p53 are among the most common in many human cancers. Due to its critical involvement in the cell cycle and apoptotic signalling p53 is the most extensively studied tumour suppresser gene and therefore appears to be an appealing target for gene therapy trials (Zeimet *et al.*, 2000). The re-expression of functional p53 in tumour cells has been shown to suppress tumour growth and induce apoptosis.

One of the more recent uses of gene therapy is in the treatment of cardiovascular disease. Of the 36 protocols currently registered with the National Institutes of Health Office of Biotechnology Activities the vast majority involve the delivery of vascular endothelial growth factor or fibroblast growth factor to enhance angiogenesis for the treatment of coronary artery and peripheral vascular diseases (Liau *et al.*, 2001).

1.1.2. DNA Vaccines.

Traditional vaccines consist of primarily killed, weakened or components of pathogens. They function by priming the immune system to fight dangerous viruses, bacteria and parasites before they can get a foothold by tricking the immune system into behaving as though the body has already been attacked by the pathogen.

The immune response is composed of two arms. The first element, the humoral response is carried out by B-lymphocytes. These cells recognise antigens expressed on the surface of pathogens and produce antibodies, which bind to and neutralise their pathogenicity.

The second element, the cellular response, is carried out primarily by cytotoxic T-lymphocytes. These recognise antigens expressed on the surface of cells infected by a pathogenic organism and kill the cell. Once an antigen has been encountered by the immune system some B-lymphocytes differentiate into "memory cells" which remember the encounter so that if the pathogen is encountered again the response will be quicker and more effective.

For an efficient immune response both arms are required. However, with traditional vaccines composed of killed pathogens or their components, only the humoral response is generated, as there is no cellular infection. Using live attenuated pathogens does generate a cellular response in addition to a humoral one, however, they can still generate disease in immune deficient individuals and there is also a small chance that a mutation may occur potentially resulting in the organism regaining its pathogenicity.

Genetic vaccines under development are composed of plasmids containing genes that encode antigenic proteins. They are delivered to cells, usually muscle cells, by microinjection and once in the nucleus synthesise the antigen which may be either secreted out of the cell, resulting in a humoral response, or are displayed on the surface of the cell thus generating a cellular response. In this way DNA vaccines can possess the advantages of attenuated vaccines without the risks (Weiner & Kennedy, 1999).

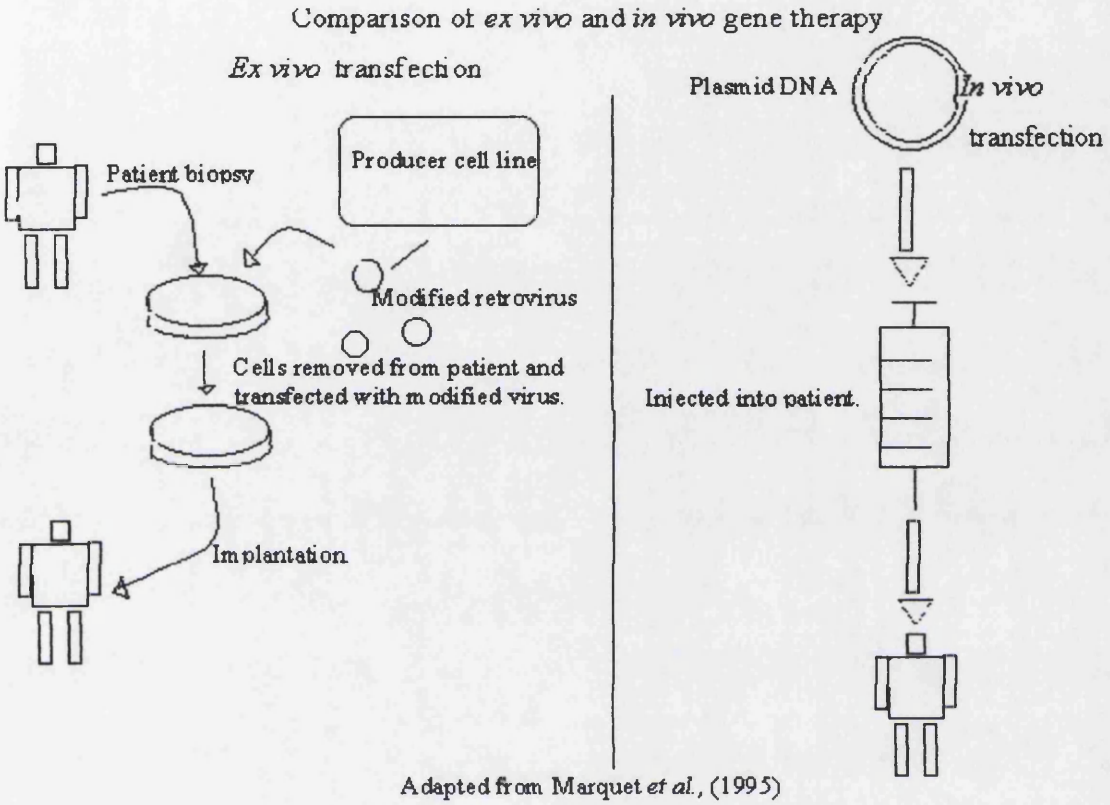
However, DNA vaccines do have several disadvantages over traditional vaccines especially for the prevention of bacterial and parasitic infections. Many micro-organisms

possess outer capsules composed of polysaccharides. As DNA only codes for proteins, DNA vaccines cannot be substitutes for polysaccharide based vaccines (e.g. pneumococcus) (Robinson *et al.,* 1996). Many proteins are modified during their synthesis (e.g. by glycosylation) and these modifications are specific to the type of organism. Therefore animal cells may not be able to correctly manufacture the proteins of parasites and bacteria. However, as viruses use the host cells machinery for protein synthesis DNA vaccines will result in the correct production of the viral protein (Robinson *et al.,* 1996).

1.2. Methods of Delivery.

Gene therapy requires the successful delivery and expression of an "expression cassette" incorporating the gene plus promoter and regulation sequences, to the target cell (Gottschak & Chan, 1998; Crystal, 1995). Two methods have been developed, *in vivo* and *ex vivo*. In *ex vivo,* affected cells are removed from the body, transfected with the therapeutic gene and replaced (Mulligan, 1993) (see Fig 2.1.). *In vivo* gene delivery involves the injection of the expression cassette into the patient, which must then find the correct tissue to transfect (see Fig 2.1.). *Ex vivo* gene delivery has the advantage of higher transfection levels than *in vivo* while also offering the propagation of higher cell doses. However, *ex vivo* delivery is patient specific and more costly than *in vivo* due to cell manipulation and quality control difficulties (Mountain, 2000).

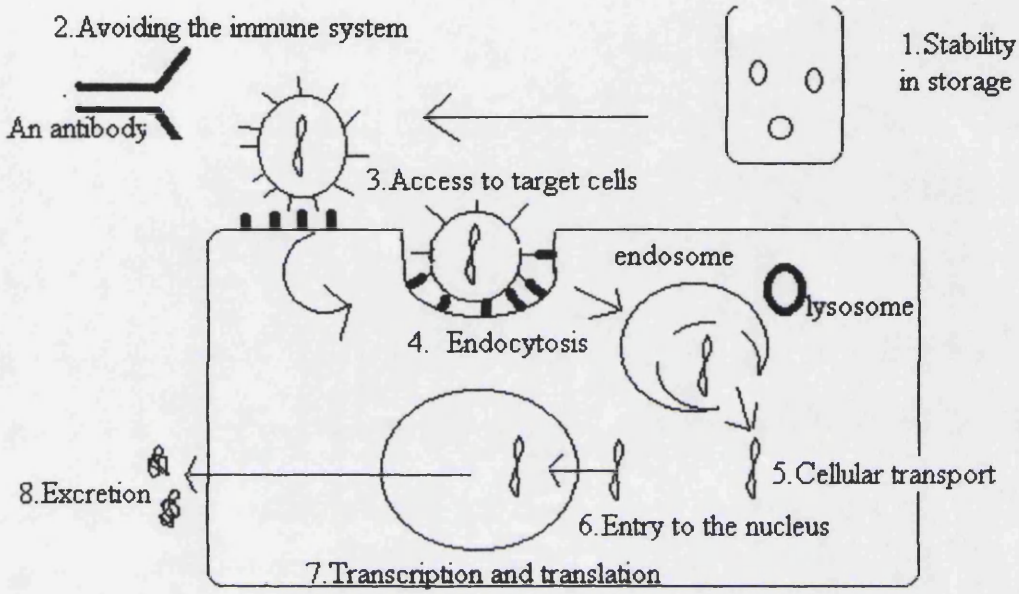
Fig1.1.



1.3. Obstacles to the Effectiveness of Gene Delivery Vectors.

The delivery of DNA to cells usually requires a vector (see 1.4 and 1.5). The purpose of vectors is to improve the therapeutic activity of the gene by aiding in overcoming barriers that block the eventual successful expression of the therapeutic gene in the desired cells. These barriers include agents that will damage the DNA, such as nucleases; access to target cells; the plasma membrane; endosomes; nuclear transport; transcription and translation (see fig 1.2.).

Fig 1.2. Barriers to gene delivery.



1.3.1. Access to Target Cells.

If a vector is intended for *in vivo* gene delivery, in addition to binding to the cells it must also be able to travel through the patients body avoiding inactivation by the immune system. Surface charge and size are probably the two most important characteristics of the vector that must be taken into account. In addition, the site of administration can also influence the efficacy of the gene (Bally *et al.*, 1999).

1.3.1.1. Surface Charge.

Studies have shown that the surface charge (or zeta potential) of a vector is important in determining its efficacy. Positive charges have been shown to facilitate binding to cell membranes, which carry a net anionic charge (Bally *et al.*, 1999). This is advantageous in *ex vivo* gene delivery, however in the case of *in vivo* gene delivery it may result in binding to non-target cells. A net cationic surface charge may also result in the binding of serum proteins resulting in alterations in the surface charge characteristics of the vector. Protein binding is also associated with increased plasma elimination rate; increased non specific cell binding; increased up take by phagocytes and activation of the complement system (Bally *et al.*, 1999). Possessing a net anionic or cationic charge has been shown to be associated with rapid elimination from the body by the reticuloendothelial system (RES). It would therefore be advantageous to produce vectors carrying a net neutral charge or which have their charge shielded by, for example polyethylene glycol (PEG). Recently it has been shown that liposomes carrying a net neutral rather than negative charge absorb significantly less fibronectin, a protein abundant in the plasma, which plays an important role in cell adhesion and formation of blood clots (Price *et al.*, 2001). However, in an experiment involving diluted plasma the same research team reported similar absorption profiles for neutral and negatively charged liposomes.

1.3.1.2. Size.

The most obvious effect of size is that vectors of a large size (>200 nm) will be trapped in the small capillaries of organs such as the lungs and liver, resulting in their accumulation in them. Also vectors greater than 200 nm in size will be rapidly taken up by the cells of the RES following administration by intravenous injection (Bally *et al.*, 1999). For vectors taken up into cells by endocytosis the size of the endosome is of importance. The endosome of lymphocytes is approximately 100 nm. However it has been shown that *in vitro* larger lipoplexes (DNA/cationic liposome complexes) tend to associate and be taken up by cells more efficiently than smaller lipoplexes resulting in greater levels of transfection (Ross & Hui, 1999).

1.3.1.3. Site of Administration.

The site of administration of the vector can also influence the efficacy of the gene. Administration may be, intramuscular, intraperitoneal, subcutaneous, intrathecal, intratracheal or intratumoural (Bally *et al.*, 1999). For example, positively charged macromolecules will accumulate in tumours because of strong ionic interactions with the negatively charged tumour cells (Mahato *et al.*, 1997). Therefore, local administration of a cationic vector into a tumour will aid transfection of the gene. However, systemic administration may result in the vector being taken up by the RES. Systemic administration by way of intravenous injection is suitable for treatment of the liver and lungs, which have been shown to accumulate vectors administered in this way (Mahato *et*

al., 1997). Tumours with leaky blood vessels will also accumulate systemically administered vectors especially if they are long circulating.

1.3.2. The Complement System.

The complement system is group of blood proteins involved in the lysis of foreign cells and the removal of particles by phagocytes, which may pose a barrier to gene delivery by increasing vector elimination from the blood stream. The action of the complement system is through an enzyme cascade triggered either by antibodies attached to the surface (the classical pathway) or certain initiating surfaces. The complement system can be activated by cationic materials such a poly-L-lysine, dendrimers, and polyethyleneimine. The potency of the activation is chain length dependent. Coating vectors with polyoxyethylene has been shown to prevent activation of the complement system (Pouton & Seymour 2001).

1.3.3. Crossing the Plasma Membrane

Once a vector has encountered its target cell it must next penetrate its plasma membrane. The efficiency of a vector can be greatly increased by incorporating a ligand for a specific receptor found on the target cell onto the surface of the vector. Receptor mediated gene transfer takes advantage of the ability of receptors on the surface of a variety of differentiated cells to efficiently bind and internalise a ligand (Perales *et al.*,

1994). A variety of such ligands are currently under investigation for use in gene therapy (see Table 1.2.).

Table 1.2. Ligands currently being investigated for use in receptor mediated gene delivery.

Ligand	**Target Cells**
Asialoglycoprotein	Hepatocytes *Hepatoma HepG2*
Transferin	Various
Insulin	Hepatoma
Polymeric Immunoglobulin	Respiratory Hepatic epithelium
EGF[b]	Carcinoma

Adapted from Mahato *et al.*, (1997).

The process by which the ligand/DNA complexes are internalised is by receptor mediated endocytosis into clathrin coated vesicles (Perales *et al.*, 1994). Receptor mediated gene transfer is not completely selective. Specific receptors are often found on more than one type of cell. This means that any strategy using receptors should consider the possibility of expression of the gene in tissues other than the desired target tissue (Perales *et al.*, 1994) and therefore the possibility of side effects.

1.3.4. Endosomes.

Most molecules taken up by cells *via* receptor mediated endocytosis are subject to the cell's degradation mechanisms. This bottleneck in gene delivery can be responsible for the degradation of over 99 % of internalised DNA (Ogris & Wagner 2002). The cell internalises the molecule into clathrin coated endosomes. Lysosomes fuse with the endosome and the enzymes within them enter the endosome and digest its contents. The optimum pH of lysosomal enzymes is pH 5. In order to lower the pH of the endosome to pH 5, protons (H^+) enter the endosome causing its internal pH to fall. Stability to lysosomal enzymes and an ability to escape the endosome have been found to be critical for the expression of the therapeutic gene (Perales *et al.*, 1994).

The study of viral escape mechanisms from endosomes may help in the design of synthetic vectors. Wagner and co-workers (Wagner *et al.,* 1992) have demonstrated that incorporating the haemaglutinin (HA) subunit HA-2 from Influenza viruses into a DNA-transferin-poly-L-lysine) complex increases gene expression. However, a potential drawback to using viral proteins in non-viral vectors is the possibility of introducing immunogenicity to the vector complex (Perales *et al.*, 1994).

Several groups have demonstrated the potential of pH sensitive liposomes. Huang and co-workers (Conner *et al.,* 1984; Wang & Huang, 1987) and Düzgünes and co-workers (Düzgünes *et al.,* 1985) have demonstrated the production of liposomes containing oleic acid (OA) that become unstable under acid conditions and did not require the presence of

proteins or other macromolecules for fusion to other membranes. The fusion of the liposome and endosomal membrane is triggered when the pH in the endosome is about pH 5 and is believed to be due to changes in the surface charge of the liposome enabling it to bind to and disrupt the endosomal membrane allowing release of its contents into the cytoplasm (Wang & Huang, 1987). Poly-L-lysine) complexes have been shown to act in a similar way (Perales *et al.*, 1994).

1.3.5. Vector Unpacking and Stability in the Cytoplasm.

Plasmid unpacking has recently been shown to be a limiting factor in gene expression. Short term expression has also been shown to be significantly enhanced by using short chain polycations that dissociate from the plasmid DNA more quickly than larger molecular weight polymers (Schaffer *et al.*, 2000).

Once the DNA has escaped from the endosome it must cross the cytoplasm before entering the nucleus of the cell where translation of the gene and synthesis of the therapeutic protein may begin. Recently it has been shown that the cytoplasm itself offers a barrier to transfection in the form of nucleases present within the cytoplasm, the origins of which are currently unknown (Lechardeur *et al.*, 1999). Macromolecules able to condense the plasmid DNA such as cationic liposomes or PLL were also shown to convey protection to the DNA from enzymatic attack within the cytoplasm.

1.4. Transfection Vectors.

The ideal vector should be:

1. Stable *in vitro* and *in vivo.*
2. Efficient at delivering the gene.
3. Able to protect the gene from degradation.
4. Specific to the target cell.
5. Non toxic and non immunogenic
6. Available in large quantities.
7. Susceptible to control.

Unfortunately no vector developed to date fulfils all of these requirements (Deshmukh & Huang, 1997). Two strategies have been developed, viral and non-viral gene delivery.

1.4.1. Viral Vectors.

Viruses can be regarded as "natures own vectors". Two main groups of virus have been used in gene therapy trials, retroviruses and adenoviruses however, other groups are also being investigated for their potential such as adeno associated viruses and herpes simplex viruses.

1.4.1.1. Retroviruses.

Retroviruses such as murine leukeimis virus are RNA viruses that possess the enzyme reverse transcriptase. Retroviruses transfer their genetic material directly into the genome of the host cell. This makes them useful for the treatment of chronic disorders. However, it also makes them potentially oncogenic (Crystal, 1995). Because they incorporate DNA into the host genome, retroviruses require DNA replication (and therefore dividing cells) for the integration of the provirus genome. For this reason they have mostly been used in *ex vivo* trials (Perales *et al.,* 1994). Research on the development of vectors based on lentiviruses, a sub family of retroviruses which do not integrate their DNA into the host genome and can infect non dividing cells, (such as Human Immunodeficiency Virus (HIV), Simian Immunodeficiency Virus (SIV) and Equine Infectious-Anaemia Virus (EIAV)) may allow *in vivo* use of retroviral vectors (Robbins *et al.,* 1998).

Retroviruses are made suitable for use as vectors by the removal of the key structural genes, gag, pol and env from the genome and replacing them with the gene of interest (Gottschalk & Chan. 1998). The vector genome is introduced into a packaging cell line that has been engineered to produce gag, pol and env proteins but is unable to replicate the complete viral genome. The resulting virons are therefore capable of infecting new cells but unable to replicate within them.

1.4.1.2. Adenoviruses.

Adenoviruses are DNA viruses and deliver their genome in an episomal form (i.e. they do not incorporate their genome into host chromosomes), they therefore do not require cells to be dividing in order for the genome to be expressed. They can also be produced by cell lines in grater titres than retroviruses (Crystal, 1995). Adenoviruses have a high tropism for cells of the respiratory system making them attractive as vectors for these cells (Gottschalk & Chan, 1998).

The genome of adenoviruses can be classified into early (E1-E4) and Late (L1-L5) genes. The early genes are involved in regulating viral and host cell gene expression, viral replication and inhibition of apoptosis while the late genes are required for encapsulation of the virus (Ribbins *et al.*, 1998). The gene required by the virus to direct production of other adenoviruses is E1. For the production of adenoviral vectors the E1 gene is deleted and replaced by the therapeutic gene and the replication defective virus can be propagated in a packing cell line that provides the E1 gene polypeptides.

Replication-competent adenovirus can also be used for cancer gene therapy. The E1 gene can be inserted into a first generation virus under the regulation of a tumour specific promoter. In theory if this vector is injected straight into a tumour it could replicate specifically in the tumour cells but not healthy surrounding tissues and could be used to carry a suicide gene to the tumour cells (Ribbins *et al.*, 1998).

1.4.1.3. Other Viral Vectors.

Adeno associated viruses have also been studied for use as gene therapy vectors. These viruses are attractive as they insert their genome into a specific region of chromosome 19. However, the size of the expression cassette that can be delivered is small, ~5kb. Also in order to infect cells they require co-infection by adenoviruses or herpes viruses (Ribbins *et al.*, 1998).

Herpes-simplex virus type I and II are also of interest in gene therapy. Herpes-simplex viruses are large DNA virus that can infect a wide variety of cells including neurones, muscle, tumours, liver and pancreatic islets. One advantage of herpes-simplex is that it has a large capacity for the insertion of the therapeutic gene (up to 50kb) (Ribbins *et al.*, 1998).

1.4.1.4. Disadvantages of using Viral Vectors.

There are several disadvantages to using viral vectors. These include:-

1. Generation of an immune response resulting in an inability to administer more than once.
2. Random integration of viral genes into host chromosome presenting risk of oncogenesis.
3. Clearance of systemically delivered viruses by the immune system.
4. Difficult to engineer viral envelopes or capsids so that they will target cells other than their natural host cells.

5. Possible recombination of viral vector DNA with wild type viral DNA resulting in generation of infectious viruses.
6. High cost of producing large quantities.
7. Can only deliver small expression cassette, approximately 10 kbp due to the large amount of DNA that must remain for the virus to still be able to infect cells.

(Templeton & Lasic, 1999).

1.4.2. Non Viral Vectors.

Non-viral vectors are man made and include chemical (calcium phosphate precipitation); mechanical (micro-injection); membrane fusion (liposomes) and direct uptake into cells by receptor mediated endocytosis (Morgan & Anderson, 1993).

The advantages of non-viral vectors include:-

1. Reduced immunogenicity;
2. Reduced clearance by complement system;
3. Can deliver a larger expression cassette;
4. Greater potential to perform multiple administrations *in vivo* than viral vectors;
5. Potential to target cells;

(Templeton & Lasic, 1999).

The main disadvantages of non viral vectors are their relatively low levels of delivery and short duration of gene expression compared to viral vectors (Templeton & Lasic, 1999), while many are not suitable for clinical applications due to their lack of regulated and sustained expression of foreign genes in targeted cells (Gottschalk & Chan, 1998).

Even though non-viral vectors are regarded as being potentially safer than viral vectors they still posses some safety concerns. Most of the health risks surrounding non-viral gene therapy are to do with the potential to induce cytotoxic and immune reactions in the recipients. Both the DNA itself and its vector may have the potential to be responsible. The unmethylated CpG motifs of bacterial DNA (plasmids are produced in bacteria) have been shown to trigger an immune response and production of cytokines (Mclachlan *et al* 2000). Some liposomal drugs have been shown to trigger a hypersensitivity reaction, which includes cardiopulmonary distress. Studies on miniature pigs have shown that the reaction is complement mediated and has been termed "complement activation-related pseudoallergy" (Szebeni *et al.*,1999). Cationic lipids are not found in nature and cationic liposome/DNA complexes remain quite cytotoxic, as they appear to be incompatible with the physiological environment, which contains mostly anionic molecules (Lee & Huang, 1999).

1.4.3. Plasmids.

Before discussing non-viral vectors further it is worth introducing plasmids, as it is these entities that the majority of non viral vectors have been developed to deliver to the target cells. Plasmids are circular duplex DNA molecules found in bacteria which encode extra genes for none essential proteins but which may convey an advantage upon the possessor such as antibiotic resistance. Plasmids can be easily manipulated to encode none bacterial products. At present most gene therapy trials with plasmid DNA (pDNA) are using plasmids in the order of 10 kbp. However, it is reasonable to predict that this size will increase over the coming years to several hundred or more kilo base pairs enabling delivery of larger genes, multiple genes or even whole pathways.

1.4.3.1. Production of Plasmid DNA.

The main problem that is encountered in the production of plasmids is that these molecules are sensitive to shear forces. This sensitivity increases with increase size of the plasmid. Shear forces encountered during down stream processing affects the tertiary structure of the plasmid, which is important for its proper functioning (Levy *et al.*, 1999). Laboratory scale production of plasmids involves the fermentation of a suitable recombinant bacterial strain usually *Escherichia coli.* The biological, properties of *E. coli* production of plasmid DNA mean that the plasmid will be contaminated by molecules of very similar structure, charge and physical behaviour i.e. RNA, DNA and lipopolysaccharides, reducing the number of procedures that can be used to isolate the plasmids (Varley *et al.*, 1999).

Because of the effects of shear, chemical lysis of the cells to release the plasmids is usually used in preference to mechanical methods. A typical method is the lysis of cells using a solution of SDS and sodium hydroxide (Schleef, 1999). This alkaline suspension is subsequently neutralised by addition of acidic potassium acetate (pH 5.5). This high potassium acetate concentration causes renaturation of the plasmids and aggregation of chromosomal DNA, proteins, membrane components and SDS, which forms a floc (Schleef, 1999). Heat treatment for 5 min at 95 °C has also been shown to give DNA release comparable to alkaline lysis. This method has the added advantage of producing a lysate that is less viscous than that obtained by alkaline treatment (Wang *et al.*, 2002).

Centrifugation can be used in the laboratory to remove the floc or alternatively purification can be carried out using a caesium chloride/ethidium bromide density gradient ultra centrifugation. This method however is unacceptable for production of clinical material because it uses mutagenic materials and is unscaleable (Marquet *et al.*, 1997). Plasmid DNA can be separated from the precipitated debris by bag filtration. The resulting filtrate can then be captured directly by expanded bed anion exchange chromatography without the need for further clarification. The eluent can be further purified and buffer exchanged by size exclusion chromatography to yield pharmaceutical grade pDNA. This method has been shown to be scaleable (Varley *et al.*, 1999). Other scalable purification techniques include the use of a short chain polymeric alcohol such a PEG, which can be added to the DNA to condense it and aid its isolation during column chromatography (Horn *et al.*, 1998).

1.4.3.2. Characterising pDNA

In order to proceed to advanced clinical trials the pharmaceutical development of gene therapy products will have to pass stringent characterisation criteria. The American Food and Drug Administration (FDA) have prepared guidelines that can be found at their web site (http://www.fda.gov/). Important points to consider include the levels of contaminants such as bacterial chromosomal DNA, RNA, Proteins, endotoxins/pyrogens, viruses as well as contaminants picked up during the fermentation and purification processes (some of the testing methods for these contaminants are summarised in Table 1.3.). Other characteristics include the stability and the physical properties of the pDNA itself.

Table 1.3. Characterisation of Process Contaminants.

Contaminant	*Test Method*
Protein	• Colorimeter assay • UV Spectroscopy • Amino acid comparison analysis, • Electrophoreses, • ELISA, • HPLC.
Nucleic Acids	DNA hybridisation • Southern blot • Dot blot Polymerase chain reaction
Pyrogens	• USP pyrogen test • LAL • Endogenous pyrogen

Residuals	assay Volatile substances • Residual Moisture Solvents • Gas chromatography mass spectroscopy Metals/Salts • plasma emission spectroscopy. • atomic absorption spectroscopy.

Adapted from Marquet *et al.*, 1997b

It is recommended that the final plasmid DNA product should contain no more than 100 pg cellular DNA per dose as determined by a method with a sensitivity of 10 pg (DiPaolo *et al.*, 1999). The monitoring of nucleic acid contamination is generally carried out by hybridisation analysis. The levels of host cell DNA can be monitored by Southern blot (Marquet *et al.*, 1997b).

The traditional method for the monitoring of protein contaminants is based upon developing and screening antibodies to different process streams, which can then be used for the development of Western blot and ELISA type assays. This is a long and demanding process (Marquet *et al.*, 1997b). Once proteins that purify together have been identified they can be isolated and used for reference standards and as antigen for the production of more antibodies with greater specificity. The assays produced by this method are ideal for monitoring the removal of protein during the process and the consistency of the process.

Pyrogens are molecules that induce the histamine response resulting in symptoms such as fever and chills. Endotoxins are pyrogenic lipopolysaccharides derived from gram negative bacteria. *E. coli* is a gram negative bacteria and any system using it must therefore be assessed for contamination by endotoxins. The usual assays for endotoxin levels are the United States Pharmacopeia rabbit pyrogen assay and the *limulus* amebocyte lysate assay. The rabbit pyrogen assay will detect any pyrogen and often gives false results, so both assays are used (Dipaolo *et al.*, 1999).

Any process that utilises materials derived from animal or especially human sources poses the risk of contamination by viruses, which may be harmful to the recipient. Virus removal can be accomplished by chromatography or filtration while viruses can be inactivated by low pH. Viral contamination can be assessed by transmission electron microscopy (tEM), polymerase chain reaction, cytopathic effect and reverse transcriptase assays (Dipaolo *et al.*, 1999). It is necessary for the purification to be shown to be able to clear more virus than is actually estimated to be present in a single dose. Viral removal must still be monitored in processes that do not include animal products due to risk of contamination by personel.

Shear forces can convert the desirable supercoiled and circular structures into undesirable forms such as open circular and linear DNA (Levy *et al.*, 2000). The effect of shear on the structure of the plasmids can be studied by agarose gel electrophoresis. Other physical changes that may occur during processing include aggregation. Aggregation can be monitored by, native PAGE, size exclusion chromatography and light scattering

(Dipaolo, *et al.*, 1999). The FDA in "points to consider on plasmid DNA vaccines for preventative infectious disease indications" point out that different plasmid forms may be less effective than the supercoiled form and that a minimum amount should be agreed.

1.4.4. Types of Non-Viral Vectors.

The majority of non-viral vectors utilise molecules with the ability to condense DNA into small particles able to enter cells through endocytosis, for example with poly cations such as poly-L-lysine (PLL) (Choi *et al.*, 1999; Deshmukh & Huang, 1997). Cellular membranes are either negatively or neutrally but never positively charged. Therefore such positively charged complexes will bind unspecificly to cellular membranes (Deshmukh & Huang, 1997). One advantage of PLL over other polycations is that it can be cross-linked to a ligand or antibody so that the complex can target specific cells (Perales *et al.*, 1994). This is known as receptor mediated gene transfer. However, the polydispersity of PLL synthesis leads to variable DNA delivery and difficulty in forming DNA complexes (Lou & Saltzman, 2000).

Many types of polycation have been evaluated for their potential as gene therapy vectors. Polyethyenimines (PEI) are among the most promising and have been shown to effectively deliver genes to cells without the need for additional endosomal disruption components due to its intrinsic endosome buffering properties. PEI has also been used *in vivo via* lung instillation, kidney perfusion and intra-venous administration. Different molecular weights or isomers of PEI differ in their toxicity (Li & Huang, 2000).

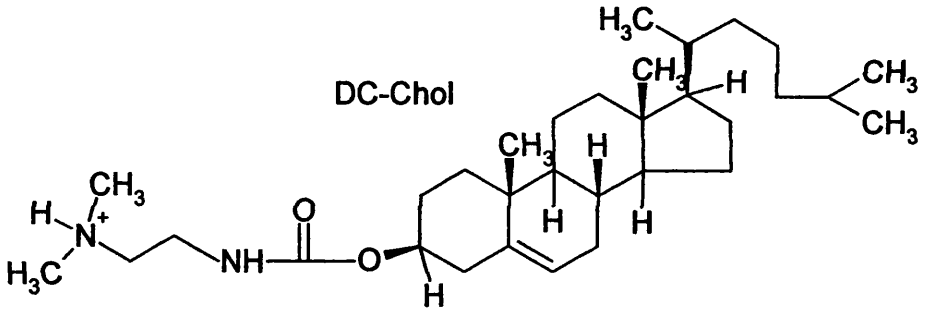
Imidazole containing polymers such as polyhistadine have also shown some promise (Pack *et al.*, 2000).

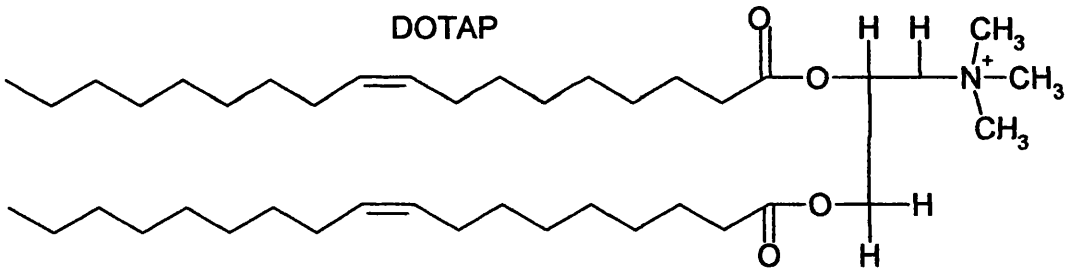
1.4.4.1. Cationic Lipids.

Cationic lipids have also been extensively studied and have reached clinical studies for cancer and cystic fibrosis. They have proven to be safe in relatively low doses, however no long term safety studies have been carried out (Li & Huang, 2000). The invention of cationic lipids has lead to the development of cationic liposomes, which are of great interest in gene therapy. The cationic charge of the liposome neutralises the anionic charge of the DNA condensing the DNA into a more compact structure. Cationic liposomes form complexes with, rather than encapsulate DNA (see 1.5.3.).

Cationic lipids are composed of four functional groups namely a positively charged head group, a spacer of varying length, a linker and a hydrophobic anchor. The lipids can be classified into one of three groups based on the structure of their hydrophobic anchor: those containing cholesterol, single chain hydrocarbons or double chain hydrocarbons. The single chain hydrocarbon cationic lipids are more commonly known to as detergents and are generally regarded as being too toxic for use in transfection however, in the presence of DOPE this toxicity is dramatically reduced. The cationic lipids, currently under investigation for use in gene delivery are mostly of the cholesterol and double hydrocarbon anchor type. Fig 1.2 below gives examples of both of these types of cationic lipid (Li & Huang, 1999).

Fig 1.3. Cationic lipids.



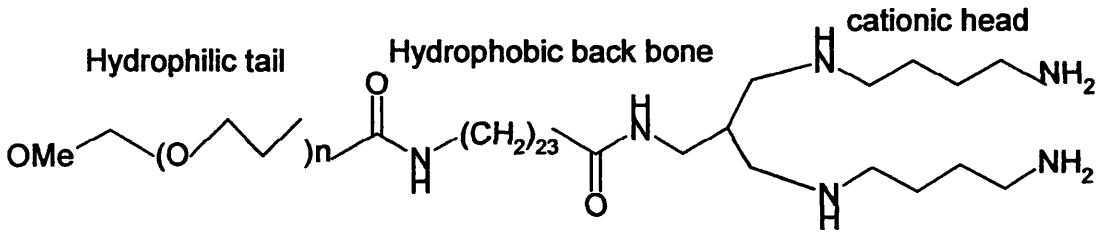


1.4.4.2. Self Assembling Complexes.

A self assembling vector is attractive for a number of reasons. Firstly, self assembly implies the innate ability to form discrete ordered structures of a homogenous population of defined units. Secondly such properties are compatible with a reproducible and scaleable process. A novel range of bipolar amphiphilic (BOLA) lipids (Eaton *et al.*, 1999) have recently been devised that incorporate a cationic head group, a hydrophobic backbone and a hydrophilic tail. These BOLA lipids self assemble with polyanions such

as plasmid DNA, to form stable complexes in aqueous solutions. The lipid forms a hydrophobic layer to condense the DNA and stabilise the resulting particle while the hydrophilic group provides a shell around the particle, which reduces non specific interactions. The hydrophilic head may be PEG, which is often used to sterically stabilise vectors (see 1.8.1.) or a polysaccharide. An example of a BOLA lipid can be seen in fig 1.3. below. In addition the BOLAsome can be targeted to specific cells by the attachment to the distal end of the hydrophilic tail of a targeting moiety such as an antibody fragment.

Fig 1.4.



An example of a pegylated BOLA lipid

The advantage of the BOLA lipid over other cationic lipids, which can also form self assembling complexes, is that the BOLA is water soluble allowing DNA condensation to take place in a single phase without the need to form liposomes (Eaton *et al.*, 1999).

1.5. Liposomes.

Liposomes are colloidal particles that can entrap macromolecules such as nucleic acids (Templeton & Lasic, 1999). They are composed of lipids (most commonly phospholipids) arranged in bilayers enclosing an aqueous space. Liposomes are able to encapsulate a wide variety of water soluble molecules ranging in size from small ions to macromolecules such as proteins and polynucleotides (Sulivan *et al.*, 1986).

A large number of applications for liposomes are currently being researched and this reflects the ability to alter their characteristics. The uses for liposomes include, medical such as drug delivery and in the study of cell membranes, as well as non medical such as cosmetics (Lasic, 1998). Table 1.4. shows some of the liposome drug delivery formulations currently on the market or under development.

Table 1.4. Adapted from Flourence, (1999)

Company	Product	Drug	Disease	Status
NeXstar Pharmaceuticals Boulder CO	AmBisome	Amphortericin B	Systemic fungal infections	Approved in US and 29 other countries.
SQUUS Pharmaceuticals (formally liposome technology INC) Menlo Park CA	Doxil	Doxorubicin	Kaposi's sarcoma	Approved in US and 18 other countries
The Liposome company Inc Preston NJ	Abelcet	Amphotericin Doxorubicin	Systemic Fungal infections Metastatic Breast cancer.	Approved Us and 19 other countries. Phase III
Araonex pharmaceuticals Inc. the woodlands Texas	Nyotran	Nystatin	Candidemia; Systemic fungal infections	Phase II Phase III
IGI Vineland Laboratories Vineland NJ	Newcastle disease vaccine	Newcastle disease killed virus	Newcastle disease	Licensed by U.S.D.A.
Novovax Inc Rockville MD	*E.coli* 0157:H7 vaccine	*E.coli* 0157:H7 Killed	*E.coli* 0157:H7 infection	Phase I

A wide variety of lipids can be used to form liposomes. Some examples are shown in Table 1.5. Phospholipids form stable structures that undergo a characteristic gel-liquid crystalline phase transition at a lipid specific transition temperature (Tc). Below this temperature the liposomes are solid while when above the Tc they are considered fluid. The Tc of a phospholipid is a function of its chain length. In forming liposomes it is necessary to hydrate the lipid layer (see 1.5.2) above the temperature of the phospholipid with the highest Tc. This is because it can be difficult to remove the lipid layer from the sides of a vessel (Szoka & Papahadjopoulos, 1980). Preparation of liposomes with a defined Tc is important especially in drug delivery, as the liposomes will have a lower permeability below the Tc of the bilayer (Szoka & Papahadjopoulos, 1980).

Cholesterol is often included in liposome formulations. Cholesterol has several effects upon phospholipid membranes such as the elimination of Tc at a 33% molar ratio to phospholipid; the concentration of the area of phospholipid molecules in monolayers; increased width of bilayers composed of short chain phospholipids; and the increased perpendicular orientation of acyl chains. Of particular importance to drug delivery is the fact that the inclusion of cholesterol in the liposome reduces its permeability to entrapped ions and polar molecules while also reducing the ability of the drug to penetrate and cause disorder in the bilayer (Szoka, & Papahadjopoulos, 1980).

Table 1.5. Properties of some communally used lipids in liposome manufacture.

Lipid	***Abbreviation***	**Charge/mol**	**Tc (at pH7)**
Egg phosphatidylcholine	EPC	0	-15 to –7
Dimyristoylphosphatidy choline (C16:0)	DMPC	0	23
1-palmitoyl-2-myristoyl phosphotidylcholine (C16:0 14:0)	PMPC	0	35
Dioleoylphosphatidylch oline (C18:1)	DOPC	0	-22
Dipalmitoyl phosphatidylethanolami ne	DPPE	(partially titrated at pH7)	60
Dioleoylphospotidylgly cerol	DOPG	-1	-18
Dimyristoyl phosphatic acid	DMPA	-2	45
Dipalmitoyl phosphotidylserine	DPPS	No data	38
Dipalmitoyl sphingomylin	DPSP	0	41
Oleic acid	OA	-1 (conveys pH sensitivity)	
Cholesterol	Chol	-1	

Table adapted from Szoka & Papahadjopolous, (1980).

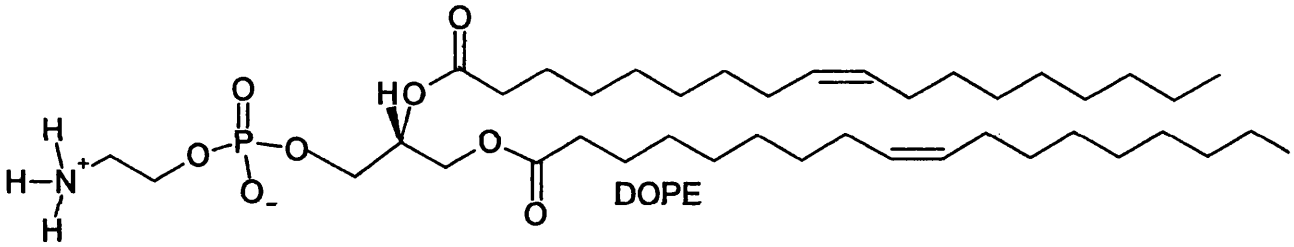
1.5.1. Types of Liposomes.

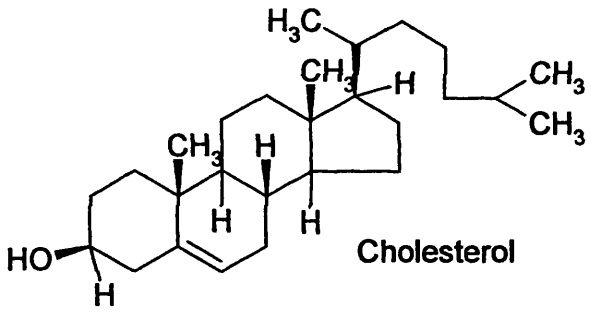
Liposomes are classified according to their size and number of bilayers. *Multilamellar large vesicles* (MLV) (the terms liposome and vesicles are often used interchangeably in the literature) are composed of many bilayers, ranging in size from a half to several microns, with an entrapped aqueous space between each bilayer of between 1 and 4 l/mol of lipid. *Small unilamellar vesicles* (SUV) possess a single bilayer, are approximately 100 nm and have an entrapping volume of 0.2 to 1.5 l/mol lipid. *Large unilamellar vesicles* (LUV) are also composed of a single bilayer, however they are greater than 100 nm and have an entrapping volume of 9 to 15 l/mol lipid (Sulivan *et al.*, 1986).

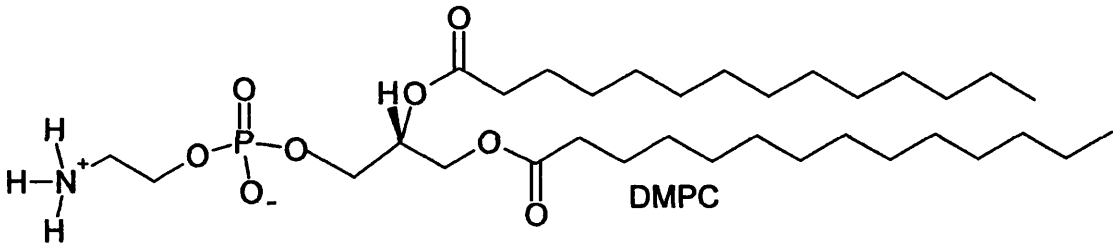
One of the characteristics of liposomes that give them such potential for use in gene therapy is the ability to alter their characteristics by changing their lipid composition or through incorporation of other molecules into their structure. This has led to an additional classification as defined below.

Conventional liposomes can be defined as liposomes composed only of neutral or negatively charge phospholipids and cholesterol (cholesterol is often present in liposome formulations as it conveys rigidity to the bilayers). They can be characterised *in vivo* as possessing a short blood circulation time and show a strong tendency to accumulate rapidly in phagocytic cells of the RES, the liver and the spleen (Storm & Crommelin, 1998). Fig 1.4. Shows the structure of some lipids commonly used in the formation of liposomes.

Fig 1.5. Lipids commonly used in liposome formulation.







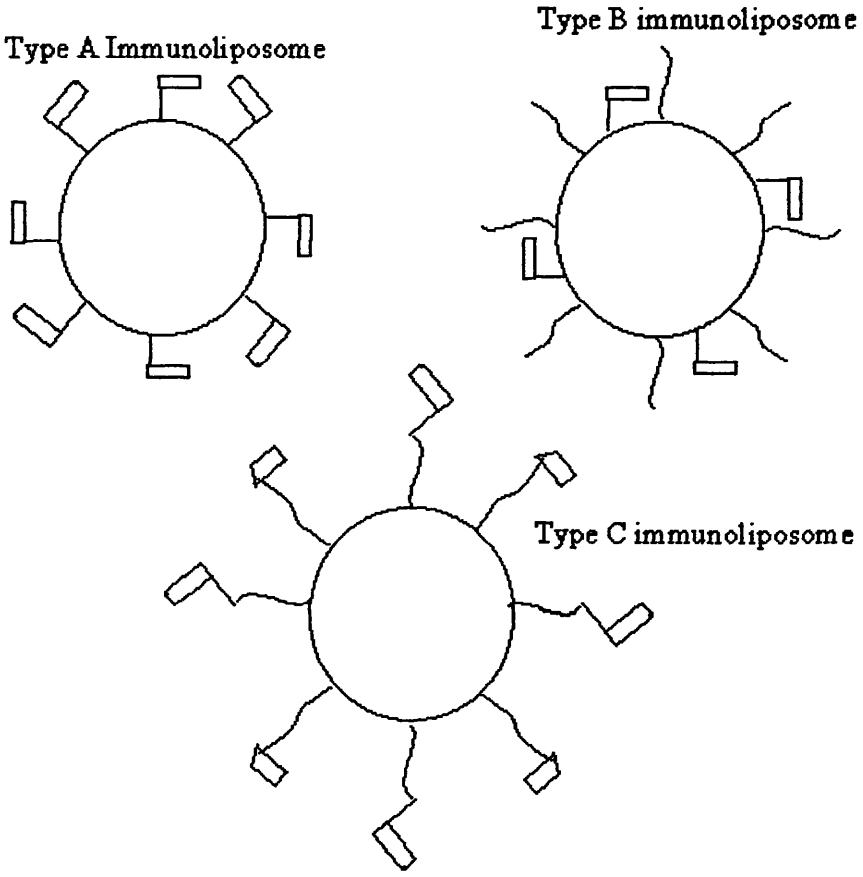
Sterically stabilised or stealth liposomes can persist in the blood stream for prolonged periods of time improving the therapeutic efficiency of liposome encapsulated drugs. The most important feature of these liposomes is that they are able to penetrate areas of high vascular permeability such as found in solid tumours. The most popular method for the production of stealth liposomes is through the attachment of the hydrophilic polymer polyethylene glycol (PEG) covalently to the surface of the liposome. Steric stabilisation

results from the local surface concentration of highly hydrated PEG groups that creates a steric barrier (see 1.8.) against interactions with molecular and cellular components in the biological systems (Storm & Crommelin, 1998).

Targeted liposomes possess ligands on their surface which enables the liposome to target the cells of a specific tissue. Antibody or antibody fragments may also be attached to the liposome (immunoliposomes). In order to increase the circulation time of immunoliposomes, attachment of the antibody (or antibody fragment) to the end of PEG molecules already incorporated into the structure has been demonstrated (Allen *et al.*, 1995). Immunoliposomes can be referred to as being either type A (has only antibody attached to liposome), B (PEG and antibody attached to liposome or C (Antibody attached to the distal terminus of PEG) (see Fig 1.5.).

Cationic liposomes (see section 1.4.4.1).

Fig 1.6. Types of Immunoliposomes.



Adapted from Allen *et al.*, (1995)

1.5.2. Preparation of Liposomes

Several methods have been developed for the production of liposomes of various types. The emphasis of these methods is not on the formation of the bilayer itself, as phospholipids will form bilayers spontaneously as a result of unfavourable interactions with water, but on forming liposomes of the desired size and number of bilayers. The methods can be broken down into three or four phases: the drying down of lipids from organic solvents; dispersion of the lipids in aqueous media; purification of liposomes and analysis of the final product. The simplest form of dispersion is mechanical (New, 1990). MLV can be formed by drying down the lipids on the surface of a round-bottomed flask followed by hydration by the addition of an aqueous medium. The liposomes are formed when the flask is agitated. Small glass beads can be added to the flask to aid in the agitation. The resulting MLV can be converted to SUV by exposing them to a high amount of energy. Several methods have been utilised for converting MLV to SUV including the use of sonication, membrane extrusion and exposing the MLV to a high pressure change, e.g. in a French press or homogeniser (New, 1990).

1.5.2.1. Sonication.

Bath and probe sonication are classical methods for the down sizing of MLV to SUV. Although they are convenient methods at laboratory scale they do not produce liposomes of uniform distribution and are poorly reproducible (Tsai, 1999). Bath sonication cannot

be scaled up while probe sonication, although potentially scaleable, is problematic due to metal contamination, lipid degradation and generation of heat and aerosols (New, 1990).

1.5.2.2. Homogenisation.

Homogenisers can be used to down size liposomes in a reproducible and scaleable manner. It has been reported that the maximum size reduction of cholesterol containing formulations resulted after 5-10 passes through a mini lab 8.30 high pressure homogeniser (Bachmann *et al.*, 1993). Further cycles through the device resulted in re-growth of the liposomes. A similar situation was observed by Tsai, (1999), in experiments using the Mini Lab 40 homogeniser. Here it was observed that after 5 passes liposomes were smaller than after 8 passes through the device. The reason for this regrowth has not yet been determined. A single step method of liposome preparation that avoids the need for MLV preparation *via* the film method has been developed using the Gaulin micron lab 40 high pressure homogeniser (Brandl *et al.*, 1990). This method involves the mixing of lyophilised lipids in aqueous media followed by homogenisation. However it has proven difficult to reproduce in studies at UCL (Tsai, 1999).

1.5.2.3. Microfluidisation.

The microfluidiser uses the principles of fluid dynamics to produce liposomes in a continuous process, which is compatible with pharmaceutical and good manufacturing practices (Sorgi & Huang, 1996). The apparatus consists of an interaction chamber in

which pressurised streams of MLV are directed through each of two micro-channels and accelerated to a laminar velocity of 100 m/s. The two streams are made to collide and the resulting turbulence and cavitation forces cause the disruption of the liposomes (Watwe & Bellare, 1995). The average size of the resulting liposomes can be controlled by altering the operating pressure used and/or the number of passes through the device. It has been found that a maximum reduction is reached after 5 to 10 passes in the case of cholesterol containing liposomes (Sorgi & Huang, 1996). One of the major advantages of this technique over other methods of liposome down sizing, e.g. sonication and extrusion, is that high lipid concentrations (up to 400 μmol lipid/ml) may be processed (Talsma *et al.*, 1989).

1.5.2.4. Extrusion

Extrusion through a polycarbonate membrane under pressure can be used to generate a homogenous suspension of liposomes in a reproducible manner. This method requires less pressure than the French press method and the upper-limit of the distribution is controlled by the pore size (New, 1990). It has been shown that passing the liposomes through large pore sizes results in liposomes that are smaller than the pore size while passing though pores below 50 nm results in liposomes which are larger than the pore (Mayer *et al.*, 1986). It has also been found that extruded liposomes are only produced above a lipid dependent minimum pressure associated with the lysis pressure of the lipid bilayer, and that the polydispersity of the liposomes is dependent upon the extrusion pressure (Hunter & Friskin, 1998).

1.5.3. Complexing DNA with Liposomes

Cationic liposomes form liposome/DNA complexes. They have been shown to condense plasmid DNA in the same way as cationic polymers. This can be shown by ethidium bromide fluorescence experiments used to show DNA condensation profiles (Hope *et al.*, 1998). These liposome/DNA complexes form by way of a self assembly process and involve the large scale rearrangement of the liposomes into a variety of polymorphic structures such as non-bilayer arrangements e.g. *honeycomb* type structures as well as bilayer structures which resemble *spaghetti and meatball* type structures, spherical and invaginated particles, oligolamellar structures and *map-pin* structures (Sternberg *et al.*, 1994; Sternberg *et al.*, 1998). The biological significance of this has yet to be understood (Li & Huang, 2000).

Anionic liposomes have been shown to be able to encapsulate a variety of macromolecules including proteins and plasmid DNA. The entrapment usually takes place during the actual formation of the liposomes. When the liposomes form some of the solute is entrapped in the lumen (Hug & Sleight, 1991). Water soluble materials may then be encapsulated however, the efficiency of this entrapment is generally poor. DNA has been shown to exhibit a size dependent encapsulation into negatively charged LUV (Szoka, & Papahadjopoulos, 1980). Several methods have been developed to improve the encapsulation efficiency of DNA into anionic liposomes. The encapsulation volume of MLV can be improved by increasing the hydration time. 20 h of gentle shaking has been found to trap 50 % more of the hydration solute than a 2 h hydration (Szoka &

Papahadjopoulos, 1980). Condensing the DNA with a protein or small organic molecules prior to encapsulation has also been shown to improve encapsulation (Hug & Sleight, 1991).

The drying down of the lipids by rotary evaporation followed by the hydration of the resulting lipid film is not the only method for the preparation of liposomes. These alternative methods are mostly suitable for the production LUV of various sizes and have been developed to improve encapsulation of macromolecules. However, together with one of the methods of downsizing described above they can also be used to form SUV. Detergent removal, ethanol infusion, ether infusion, reverse phase evaporation and calcium induced fusion are examples that have been extensively reviewed in the literature (New, 1990; Szoka, & Papahadjopoulos, 1980).

The problem of these methods is that they form vectors which are regarded as being too large for gene therapy at least *in vivo* (see section 1.3.1.2.) and the majority of the methods used for the downsizing of liposomes such as sonication and homogenisation would probably damage the DNA. However, anionic liposomes are known to entrap positively charged DNA/polycation complexes *via* charge interactions. Electron microscopy studies on PLL/DNA/anionic liposomes by Lee & Huang (1996) showed that the resulting particles appear spherical with a high electron dense core surrounded by low density coating. Fig 1.6. shows a possible mechanism for the formation of these particles.

Fig 1.7. Possible mechanism for the encapsulation of cationic PLL/DNA complexes by excess anionic liposomes.

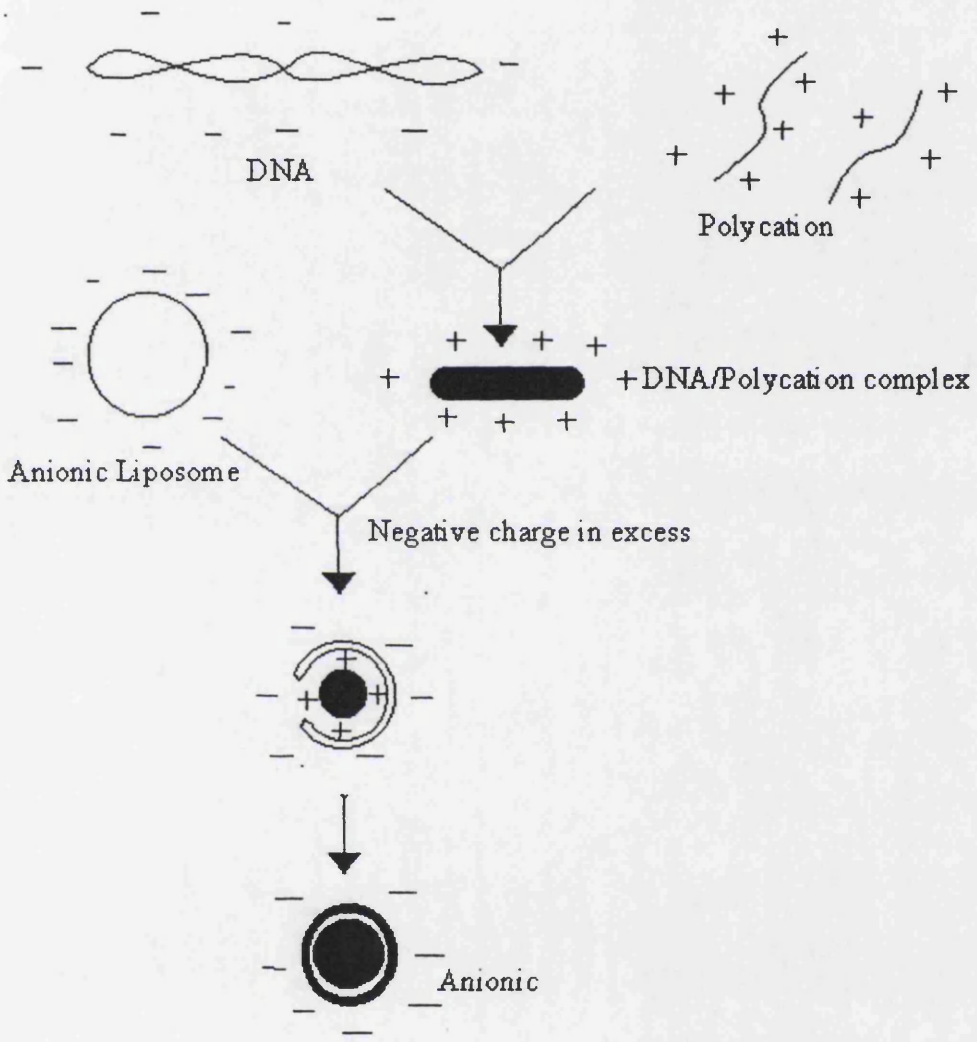


Fig modified from Lee & Huang, (1996).

1.6. Immunoliposomes.

In order to improve the ability of liposomes to target specific cells it is possible to couple receptor ligands or antibodies on the liposome surface. Liposomes possessing antibody or antibody fragments are known as immunoliposomes.

1.6.1 Antibody Fragments.

Paul Ehrlich first proposed the concept that antibodies could be used as "magic bullets" for the treatment of disease almost 100 years ago. However, it was not until 1975, with the development of hybridoma cell technology that the concept became possible in principle (Holliger & Hoogenboom, 1998; Abrams, 1995).

The basic structure of an antibody is a Y shaped tetramer of polypeptides composed of two heavy and two light chains joined by disulphide bonds. See Fig 1.7, for structure.

Because hybridoma cell lines generate antibodies of rodent origin they would evoke an immune response. By applying genetic engineering "humanised" antibodies can be produced, in which several sequences are replaced by their human equivalent (Abrams, 1995). Generating large quantities of humanised antibody in a mammalian expression system can be very expensive. A viable alternative is to express antibody fragments in a bacterial system (Peters & Sikorski, 1999).

Most antibody fragments are derived from the Fv and Fab region (see fig 1.7). Fv fragments are composed of the V_H and V_L domains and possess the entire antigen binding site. They are quite unstable having a tendency to dissociate upon dilution (Glockshube *et al.,* 1990). However their stability can be improved by covalently attaching the two domains together, for example by a polypeptide to form single chain Fv fragments (scFv) or by a disulphide bond to form disulphide Fv fragments (dsFv).

Fig 1.8. An Antibody.

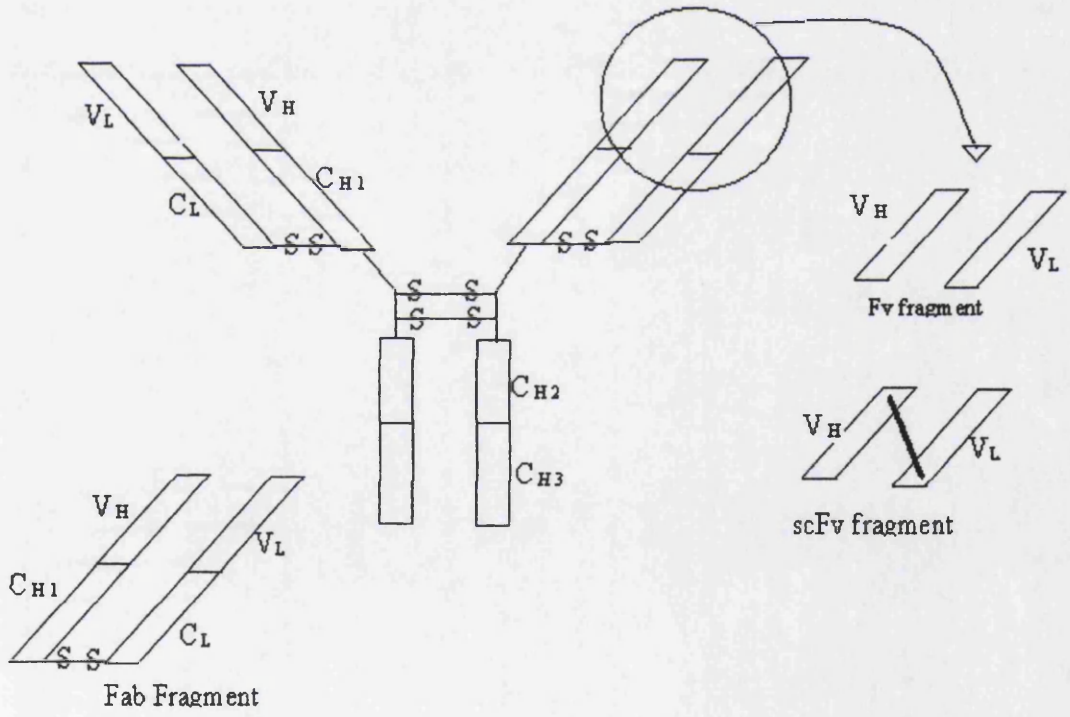


Fig adapted from Kelly *et al.*, (1992)

Fab fragments consist of the entire light chain plus the variable and first constant domain of the heavy chain. The presence of disulphide bonds increases the stability of the Fab compared to Fv fragments. Fab' fragments have been extended to include one or more hinge cystine residues, while $(Fab)'_2$ contain two Fab' arms linked by a hinge cystine. Due to the presence of the cystine residue Fab' possess a free thiol group in their hinge region. This group provides a singular site for the covalent attachment of the antibody fragment to the surface of liposomes and ensures correct orientation of the Fab' (see 1.6.2.).

The Fab' used in this project is based upon the humanised version of the monoclonal antibody 4D5 (humAb4D5) which is a recombinant of the murine monoclonal antibody, munAb4D5. This antibody is directed against the growth factor tyrosine kinase receptor, $p185^{HER2}$, which is the product of the HER2 protooncogene. This receptor is found over-expressed in 25-30 % of human primary breast and ovarian cancers and appears to contribute to cell transformation and tumour progression (Slamon *et al.*, 1989).

1.6.1.1. Fermentation of Antibody Fragments.

Antibody fragments can be produced by proteolytic digestion of whole antibodies. However, generating large quantities from mammalian systems may make them costly. A possible solution is to produce them using recombinant bacteria (Chapman *et al.*, 1999; Peters and Sikorski, 1999). Several systems have been studied for the production of

antibody fragments in *E. coli* including, cytoplasmic, periplasmic, cell surface and extra cellular expression.

Initial experiments used cytoplasmic expression. However, this technique has the problem of forming insoluble, inactive inclusion bodies. Even though refolding strategies have been developed recovery remains inefficient (Buchner & Rudolf, 1991).

Recombinant proteins may be expressed in the periplasm of gram negative bacteria such as *E. coli,* by coupling it to a bacterial protein expressed in this way or by adding the appropriate signal sequence. Periplasmic expression has many advantages over cytoplasmic and other expression systems. This is because the periplasm can be regarded as being functionally equivalent to the lumen of the endoplasmic recticulum of eukaryotic cells (Skerra & Pluckthun, 1988), which provide a reducing environment necessary for correct disulphide bond formation. This means that recombinant proteins expressed in this way are more likely to possess their correct folding configuration therefore their full activity. An additional advantage of periplasmic expression is that fewer proteases are found in the periplasm when compared to the cytoplasm. The problem with periplasmic expression is the risk of leakage from the periplasm into the extracellular medium.

An expression system for the production of a humAb4D5 Fab' fragment containing a hinge thiol group has been used at UCL. The process uses the *E. coli* strain W3110 (wild type *E. coli* ATCC 27325) transformed with plasmid pAGP-4. This plasmid encodes a resistance gene for chloramphenicol. Transciptional control was provided by the *E. coli*

tac promoter. In order to direct secretion to the periplasm each antibody chain was proceeded by the *E. coli* omp A signal sequence (Bowering, 2000).

1.6.1.2. Downstream Processing of Fab'.

A variety of methods may be used for the recovery and purification of product from fermentations as summarised in Table 1.6. The methods used will depend on where the product has been expressed.

The procedure for the extraction of Fab' from the periplasm used in this project involves incubation of cell paste in lysis buffer at 60 °C for a period of 3 h. A high temperature was used for the lysis due to the high thermal stability of the Fab' (Kelly *et al.*, 1992), while having the added advantage of degrading the majority of bacterial proteins resulting in a purer Fab' preparation. Further purification was carried out by centrifugation to separate cell debris followed by affinity chromatography (Bowering, 2000).

Table 1.6. The stages and methods involved in the down stream processing of a recombinant protein.

Stage	location	Step	Typical Methods
Primary recovery	Extracellular medium	Cell removal.	Centrifugation • Tubular, • Bowl, • Multichamber • Disk. Microfiltration • Dead-end • Cross-flow filtration.
	Cell associated proteins.	Cell recovery.	Centrifugation Microfiltration.
		Cell *disintegration*	Mechanical disruption • Homogenisation • Bead milling. Non mechanical • Osmotic shock, Chemical lysis • Enzymatic lysis.
		Cell Debris removal.	Centrifugation Microfiltration
Purification	All		Chromatography • Affinity, • Ion exchange, • Hydrodynamic interaction, Gel filtration.

Adapted from Lee, (1989).

1.6.1.3. Characterisation of Fab'.

As with plasmid DNA the concentrations of bacterial DNA, proteins, pyrogens/endotoxins, etc, need to be assessed. The same assay methods as outlined in section 1.4.3.2, may be used for the monitoring of such contaminates.

It is important to show that the Fab' posses the correct immunological folding configuration in order for it to be able to bind to its target protein. This can be determined by circular dichromic spectroscopy (Kelly *et al.*, 1992). Differential scanning calorimetry has been used to show that the humanised 4D5 Fab' is thermodynamically stable at a temperature of up to 76°C (Kelly *et al.*, 1992).

1.6.2. Covalent Attachment of Fab' to Liposomes.

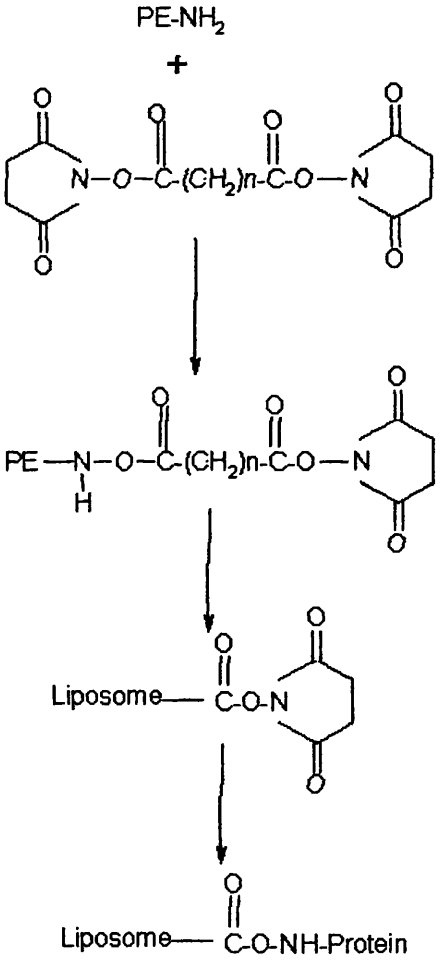
As mentioned in section 1.6.1, Fab' is composed of the entire light chain plus the variable and first constant chain of the heavy chain along with a free cystine residue. This sulphur containing amino acid residue can be reduced, e.g. by 2-mercaptoethylamine, in order to produce a free thiol group. This free thiol group can be grafted to liposomes by covalent attachment to, for example a malimide (to produce a thioether bond) or pyridyldithiol (to produce a disulphide bond) terminated lipid (linker lipid) (Martin *et al.*, 1990).

Linker lipids are usually produced by reacting phosphotidylethanolamine (PE) derived lipids (e.g. DOPE) with a heterobifunctional cross-linking reagent. For example to

produce a malemide terminated linker lipid, N-succinimidyl (-4-[P-maleimido-phenyl] butyrate) (SMPB) is reacted with PE to give PE-MPB (Martin *et al.,* 1990). Fig 1.8, below shows the reaction for the formation of a linker lipid incorporating a reactive carbonyl group suitable for complexing proteins (Martin *et al.,* 1990).

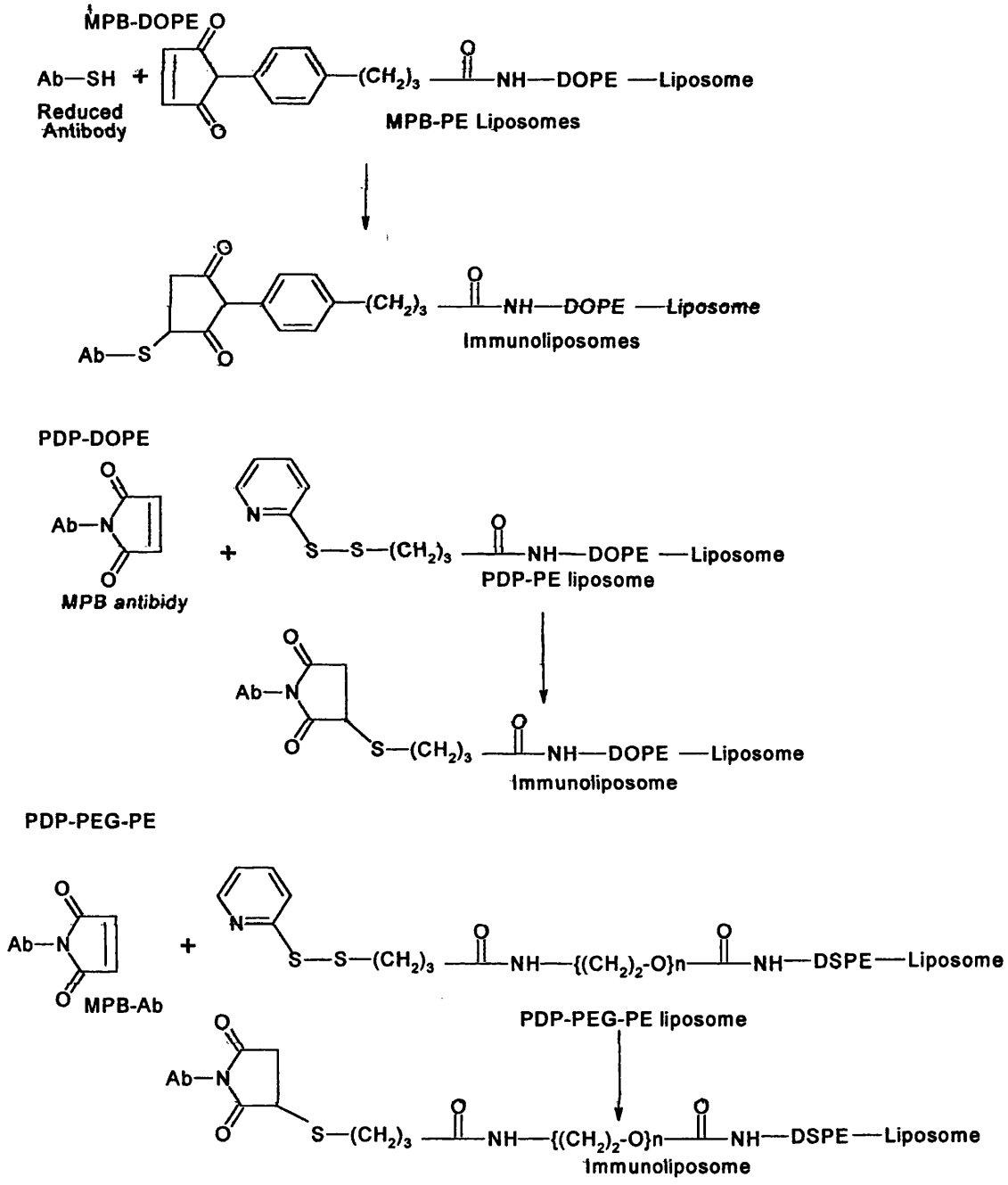
In order to improve *in vivo* half-life of liposomes, PEG has been attached to the surface of liposomes (see chapter 6). PEG can also be incorporated into the structure of immunoliposomes. Fab' can either be attached to the liposome surface as described above with PEG attached in a similar way or Fab' can be attached to the distal terminus of PEG molecules already attached to the liposome, using similar chemistry described above (Allen *et al.,* 1995; Hansen *et al.,* 1995; Maruyana *et al.,* 1995; Park *et al.,* 1995; Kirpotin *et al.,* 1997). Studies have shown that the PEG does not effect the binding of the Fab' to its target molecule. Several other coupling methods have been studied for the covalent attachment of antibodies to PEGylated liposomes (see Fig 1.9, adapted from Hansen et al 1995).

Fig 1.9. Reaction scheme for the formation of a linker lipid.



Adapted from Martin *et al.*, (1990)

Fig 1.10. Methods for the attachment of antibody fragments to liposomes.



1.7. Characterisation of Liposomes and Immunoliposomes.

The physiochemical properties of liposomes strongly influence their behaviour. Characterisation in terms of, Fab' coupling efficiency (in the case of immunoliposomes), particle size, zeta potential and long term stability is required if they are to be successful (Nässander *et al.,* 1995). Also encapsulation of pDNA needs to be well characterised.

Important factors that should be taken into consideration when preparing immunoliposomes for use as vectors are;

1. A sufficient quantity of antibody must bind to liposomes in a reproducible way;
2. The coupling process must not affect the integrity of the liposome;
3. The liposome antibody complex should be stable on storage and administration;
4. The homing capacity of the antibody should be preserved after binding to liposomes;

(Nässander *et al.,* 1995).

1.7.1. Size and Zeta Potential

The size and zeta potential of liposomes are not only important to their behaviour *in vivo* (see 1.3.1.), but they also influence their processing. For example the charge of the liposomes will effect whether they will form complexes with DNA or encapsulate DNA/polycation complexes (see 1.5.3). In the literature most liposomal formulations are in the order of 100 nm, which would suggest that mostly the biological consequences of

size are being considered. However, size will effect processing. Smaller liposomes will possess a reduced aqueous volume, which would restrict the loading capacity and reduce drug encapsulation efficiency. Another factor that should be taken into account is that the bilayers of small liposomes have a greater degree of curvature. This would result in increased instability (Sullivan *et al.*, 1986). Size and zeta potential can both be studied by dynamic light scattering.

1.7.2. Lamellarity and Chemical Composition.

The Lamellarity of liposomes can be assessed by NMR spectroscopy using ^{31}P labelled phospholipids. The addition of external Mn^{2+} quenches the ^{31}P NMR signal of the labelled phospholipids in the outer most monolayer. Thus unilamellar vesicles (which have two monolayers) will show a 50 % reduction in the ^{31}P NMR signal (Mayer, *et al.*, 1986). The radius of curvature of SUV can result in the asymmetric distribution of different components in the inner and outer monolayers. This is thought to be due to the inability of unsaturated acyl chains to pack as closely as saturated chains, thus the head group of an unsaturated chain occupies a larger area and therefore prefers the outermost monolayer (Szoka, & Papahadjopoulos 1980). The lipid composition of the outer surface can be assessed using an assay which will not cause break up of the bilayer and compared with results after the bilayer has been broken down. Barenholz *et al.*, (1979) gives an example for assaying the outer and total PE content of a liposome.

It is important to prevent the chemical degradation of the lipid components of the liposome. The oxidation of phospholipids may be prevented through the handling and storage of the lipids under an inert atmosphere such a nitrogen or argon (Szoka, & Papahadjopoulos, 1980). If for example DOPE is hydrolysed the permeability of the liposome could be increased which would manifest itself as increased instability and release of encapsulated contents.

1.8. Stability.

Aggregation of gene therapy vectors is one of the major problems hindering their development. Formulations tend to aggregate under physiological conditions, which severely reduces their effectiveness. Recently the aggregation of PLL/DNA complexes has been analysed theoretically using established colloidal theory here at UCL (Lee *et al.*, 2001). They show that aggregation is a complex function of the physiochemical properties of a system with initial distribution, solution pH, ionic strength and temperature being the most important parameters. Low pH, low ionic strength, low temperature and a narrow initial size distribution are process conditions that may help in the formation of stable complexes (Lee *et al.*, 2001).

Aggregation is also of concern from an industrial perspective especially in terms of long term storage. Several methods are available to improve the stability of DNA complexes including, using sucrose gradients to produce heterogeneous populations of complexes, frozen formulations and dehydration formulations which are reviewed by Anchordoquy

et al., (2001). The most favoured method from the literature would be steric stabilisation, usually through the incorporation of polyethylene glycol (PEG) onto the surface of the vector.

1.8.1. Steric Stabilisation.

Two methods have been developed for the stabilisation of colloidal suspensions. Electrostatic stabilisation involves the absorption of ionic species on the surface of the particle. Steric stabilisation is the stabilisation of a suspension of colloidal particles through the addition of a hydrophilic polymer to the surface of the particles, which prevents the particles from aggregating. Steric stabilisation is exploited both industrially and biologically because it offers many advantages over electrostatic stabilisation (Napper, 1983). For example steric stabilisation is relatively insensitive to the presence of electrolytes while the addition of electrolytes, such as salts, to an electrostatically stabilised suspension will result in the aggregation of the particles (Napper, 1983). This is undesirable in preparations designed for introduction into the human body, which has relatively high concentrations of electrolytes. Several polymers have been investigated for use in the steric stabilisation of colloidal gene delivery vectors however the majority in the literature exploit polyethylene glycol (See Chapter 6). In addition to improving the stability of the liposome PEG has also been shown to shield the liposome against damage *in vivo* due to the actions of serum proteins. For example phosphotidylserine (PS) based liposomes react aggressively with blood coagulation proteins and activate the

complement system. The addition of 15 mol % of PE-PEG_{2000} has recently been shown to abolish over 80 % of the clotting activity of PS liposomes (Chiu *et al.*, 2002).

1.9. Project Background and Aims.

For gene therapy and DNA vaccination to become a reality the development of vectors capable of the delivery of the therapeutic gene to the required cells in an effective and safe manner is necessary. There are two schools of thought as to what will make the best vectors. The viral camp believe the best vectors will be viruses which have been modified in such a way as to remove their ability to replicate in host cells but which are able to infect and deliver the therapeutic gene to the nucleus of the target cell. However there are a number of major health risks involved with viral gene delivery which have been highlighted by the recent death in 1999 of the American teenager Jessie Gelsinger during a clinical trial with an adenovirus vector, and more recently by the case of a boy in France being treated for X-SCID with a modified retrovirus using the *ex vivo* gene delivery method developing leukaemia.

Such concerns have increased interest in the development of non-viral methods of gene delivery. The current interest in non-viral gene therapy has led to the development of numerous different vector formulations ranging from polycations to liposomes. What has become clear from the literature is that no single vector formulation will be used due to the large array of different diseases that gene therapy will be used to treat and the different requirements of each. For example, in the requirements of cystic fibrosis and those of cancer. In the former a population of localized cells needs to be targeted with a single corrective gene, while in the treatment of cancer the aim is to target a diffusely spread population of cells with the aim of killing them. Hence the requirement in cancer

therapy for accurate delivery is greater than in cystic fibrosis where transfection of non-targeted cells will be less likely to cause severe side effects (Miller & Vile, 1995). Each different target disease treated by gene therapy will therefore require the development of a specific method and vector formulation tailored to the specific needs of that disease. However what is evident from the literature is that these vectors will follow a basic plan, which will be modified for the target.

The aims of this project are to investigate the issues involved with the processing of a multi-component non viral gene delivery vector complex and in doing so propose a controllable, reliable, and reproducible method of processing such a vector with the view of future scale up. There are many vector formulations in the literature with significant inconsistencies between them. However it is generally agreed that the characteristics of a general vector include, a plasmid in which the therapeutic gene or genes are encoded, a means of condensing the plasmid DNA, protection for the DNA both *in vivo* and *in vitro*, incorporate a means of cell targeting and be easy to produce in large quantities.

With this in mind a model system formulation for an anionic liposome will form the basis for the work discussed in the project. The liposome is composed of dioleoylphosphotidylcholine (DOPC) a common naturally occurring phospholipid which is a component of cell membranes and readily forms bilayer structures, cholesterol (CHOL) which has been shown to help in the stability and integrity of the bilayer and oleic acid (OA) which is a helper lipid which conveys a negative charge to the particles (2/2/1). As targeting and stabilising moieties are often added to the surface of liposomes,

a total lipid concentration of 1% dioleoylphospotidylethanolamine (DOPE) will also be included in the formulation (see 1.7). This will enable the effects of the attachment of anti $p185^{HER2}$ Fab' to liposome for targeting and PEG for steric stabilization to be investigated. The liposomes produced will then be used to encapsulated plasmid DNA which has been condensed using poly-L-lysine to form the final gene delivery vector complex. The method of production of the liposomes will use the standard drying down procedure, which involves the mixing of the lipid components in organic buffer and the subsequent drying down of the solution to form a lipid cake. The film is then hydrated in an aqueous buffer to form MLV. The potential of an ultra high velocity jet homogeniser will be investigated as a means of downsizing the MLV to form SUV in a scaleable and reproducible manner. The biophysical properties of the liposomes produced will be measured during their processing. Key biophysical characteristics that will form the focus of the work will include size distribution and zeta potential, which will be measured by photon correlation spectroscopy (PCS).

2. Materials and Methods.

Unless otherwise stated all materials were obtained from Sigma-Aldrich Ltd (Pool, Dorset, UK).

2.1. Plasmid DNA.

Plasmid pSVβ was obtained from frozen cell paste of *E. coli* DH5α kindly donated by A. Kay (UCL Biochemical Engineering). The plasmid DNA was purified using a Qiagen Giga Prep Kit (Qiagen Ltd, West Sussex, UK), suspended in TE Buffer pH 8 at a concentration of 1.5 mg/ml and stored until required at –20 °C.

2.2. Pico Green Double Stranded DNA Fluorescence Analysis of Plasmid DNA.

Pico green double stranded DNA quantitation reagent (Molecular Probes Inc, Eugine, OR, USA) was diluted 1/400 in TE buffer and 100 µl added to 100 µl of sample or standard in a 96 well plate. The fluorescence was measured using a 96 well Fluorimeter (Fluorocount; Packard Bioscience, Pangbourn, Burks, UK) controlled by plate reader software (Packard bell: NEC Computers (UK) Ltd, White Waltham, Maidenhead, Berks, UK) with excitation filters of 485 nm and emission filters of 530 nm. A standard curve was prepared on each plate by serial dilution from an initial concentration of 1000 ng DNA /ml (see appendix 1 Fig A1).

2.3. The Fab' Antibody Fragment.

Fab' was obtained from frozen cell paste of *E. coli* W3110 (wild type ATCC 27325) transformed with the plasmid pAGP-4 kindly donated by N. Murrell (UCL Biochemical Engineering). The strain and plasmid were originally supplied by N. Weir, Celltech Therapeutics Ltd, Slough, UK. The Fab' is expressed in the periplasm of the bacteria. To purify the Fab' cell paste was incubated with cell lysis buffer pH 7.4 (100 mM TRIS amino methane plus 10mM EDTA) at a concentration of 143 g/l for 3 h at 60 °C mixing thoroughly. The lysis solution was clarified by centrifugation at 10,000 rpm for 1 h in a Beckman J2 MI centrifuge (Beckmann Instruments, Palo Alto, CA, USA) fitted with a JA 10 rotor. Glycine was added to the clarified lysate to a final concentration of 1M and its pH adjusted to 7.5 before being loaded onto a 5ml column of streamline A (Amersham Biosciences, St Albans, UK,) equilibrated with 1M glycine buffer pH 8 (150 g glycine, 4.5 g sodium glycinate in 2 l). Bound Fab' was eluted from the column with a 0.1 M trisodium citrate/citric acid buffer pH 3 (5.35 g citric acid and 0.61g citrate in 300 ml). Following chromatography the purified Fab' was dialysed into a storage buffer pH 5.5 (82.03 g sodium acetate, 96 ml 0.1 M acetic acid, 29.22 g NaCl, 0.4 g sodium azide in 2 l), filtered using a 0.2 μm sterile syringe filter and the concentration assessed at 280 nm (ε_o = 1.43). The purified Fab' was stored at 4 °C until required.

2.4. ELISA Assay for Fab' Quantification.

96 well plates (Maxisorp, life technologies Ltd, Paisley, UK) were filled with 100 µl of the anti Kapa light chain antibody HP6045 (originally supplied by N. Weir, Celltech Therapeutics Ltd, Slough, UK) at a concentration of 2 µg/ml in PBS buffer pH 7 and incubated over night at 4 °C. The following day the plates were washed with PBS buffer containing 0.05% tween 20 (PBS/t) and into each well 100 µl of sample conjugate buffer pH 7 was added (6.05 g TRIS, 2.92 g NaCl, 0.1 ml tween 20 1 g casein in 500 ml). Into the top row of wells either 100 µl of standard or sample was added and a 1 in 2 serial dilution made down each column. Initial concentrations of standard and sample were 1 µg/ml. The plates were then incubated for 1 h at RT. Following incubation the plates were washed again with PBS/t and 100 µl of an anti Kapa heavy chain antibody conjugated to a peroxidase (GD12 peroxidase [The Binding Site, Birmingham, UK) was added to each well at a 1 in 2000 dilution of stock in sample conjugate buffer. The plates were incubated for a further 1 h at RT, washed again in PBS/t before 100 µl of substrate solution (0.1 M Sodium acetate/citrate pH 6, 100 µg/ml TBP, H_2O_2 0.02 % (v/v)) was added to each well and the A_{630nm} recorded using a dynatech MR 7000 microplate reader (Dynex Technologes, Billinghurst, UK). A standard curve was prepared by plotting A_{630nm} against $\log_{10}$ Fab' concentration (see appendix 1 Fig A2).

2.5. Preparation of Poly-L-lysine Condensed Plasmid DNA Complexes (PLL/DNA).

Equal volumes of plasmid PSVβ and PLL (average molecular weight 29 KDa unless other wise stated) were mixed together at a charge ratio of 2 (see appendix 2 table A1) using a PDH 2000 infuse/withdraw syringe pump (Harvard apparatus, Holliston, MA, USA). The device uses a microcontroller, which controls a small step angle motor driving lead screw and pusher block. 2 ml disposable syringes were fitted to a three way luer lock adapter and 0.2 cm silicon tubing the outlet of the tubing from the syringes were joined together by a t-connector which was also the site of mixing. Unless other wise stated the infuse rate used was 6 ml/min.

2.6. Preparation of MLV.

Dioleoylphosphotidylcholine (DOPC), Dioleoylphosphotidylethanylamine (DOPE), Cholesterol (Chol) and Oleic acid (OA) were dissolved in chloroform/methanol (4/1 vv) in a 50 ml round bottom flask at a molar ratio of 2/0.05/1.95/1. When necessary 10 μg of the fluorescent cholesterol analogue, Chol BODIPY®FL (Molecular Probes Inc) was added to provide a rapid quantitative method of lipid concentrating monitoring (see appendix 1 fig A3). The organic solvent was removed by evaporation using a rotary evaporator (Buchi rotavapor R-114; Buchi labortechnic, Flawil, SW) operating at 80 rev/min under a low vacuum at 60 °C until a thin lipid film formed on the bottom of the round bottom flask. The film was further dried by

placing the flask in a Sanyo Gallenkamp vacuum oven (Loughbourgh, Leis, UK) at 60 °C. Following drying the film was hydrated with a appropriate volume of 10 mM HEPES buffer pH8 at 60 °C. To facilitate the hydration process 2 mm glass beads were added to the flask, which was placed on the rotary evaporator without the vacuum at 240 rev/min until all the film was removed from the bottom of the flask.

In the preparation of Immunoliposomes and PEGylated liposomes DOPE was substituted with DOPE-MPB or DOPE-$PEG_{500, 1000}$ or $_{2000}$ (Avanti Polar Lipids Inc, Alabaster, USA) at the equivalent molarity in order to maintain the correct molar ratio of the lipids.

The concentration of lipid was monitored by recording the fluorescent marker using a 96 well plate fluorimeter (Fluorocount) using excitation and emission filters of 485 and 530 nm respectively.

2.7. Downsizing of MLV by Sonication.

The liposomes where downsized using a Gallenkamp SANYO Soni prep (Gallenkamp, Loughborough, Leis, UK). 5 ml of MLV suspension was dispensed into a test tube, which was placed into an ice filled beaker. The probe of the sonicator was then placed halfway into the suspension and the MLV sonicated for 1 min at 15 kHz followed by a 2 min rest. The cycle was repeated 7 times unless other wise stated.

2.8. Downsizing MLV using The High Velocity Jet Homogeniser.

More details on the method of operation of the high velocity jet homogeniser are given in chapter 3. The device was operated according to the standard operating procedure. All suspensions to be down sized using the device were stored in glass rather than plastic containers in order to avoid the possibility of particles clogging the nozzle of the machine. Each pass of suspension through the device was followed by a wash of 200 ml RO water in order to remove any residue from the previous run.

2.9. Mixing SUV with PLL/DNA.

Liposomes were mixed with PLL/DNA complexes using the syringe pump in the same way as for the preparation of PLL/DNA complexes previously described. Because in some experiments the size distribution of the PLL/DNA was recorded prior to mixing with the liposomes 15 min was allowed between PLL/DNA formation and mixing with the SUV in order to prevent any time dependent differences from occurring.

2.10. Preparation of Immunoliposomes.

Purified Fab' was first concentrated to 10 mg/ml and buffer exchange into a 0.1M sodium acetate buffer pH 5.8 containing 2mM EDTA using an Amicon Ultrafiltration

Cell (WC Grace & Co, Beverly, MA, USA). Concentrated Fab' was incubated with 5mM 2-mercaptoethylamine at a final concentration of 5 mM, for 30 min at 37 °C. The reduced Fab' was then desalted in 10mM HEPEs Buffer pH 8 using PD 10 columns (Amersham Biosciences) before being incubated with liposomes at 50 μg/ml Fab' per μmol total lipid overnight at room temperature. Following incubation 6 mM 2-mercaptoethanol at a final concentration of 6 mM, was added for 30 min at room temperature followed by gel filtration chromatography using sepharose CL-4b (Amersham Biosciences) to separate immunoliposomes from unbound Fab' and 2-mercaptoethanol. Fluorescence and a modified Lowy total protein assay kit (Sigma, Pool, UK) was used to confirm the presence of liposomes and Fab' and calculate the binding efficiency of the liposomes (see appendix 1 Fig A4).

2.11. Size Determination

The size of particles was determined of photon correlation spectroscopy using the zetasizer 3000 (Malvin Instruments, Malvin, UK). 4 ml disposable cuvettes were filled with 2 ml sample and placed in the instrument. Size distribution plots were obtained using the monomodel method of data analysis.

2.12. Zeta Potential Determination

Zeta potential measurements were taken by laser Doppler spectroscopy also using the zetasizer 3000. Samples were injected using disposable syringes into the *in situ*

cuvette. Prior to measuring the zeta potential of the sample the cuvette was flushed with H_2O until the count rate of the instrument was less than 10, and the instrument calibrated with polystyrene standards with zeta potential of –50 mV (supplied by the manufacturer).

2.13. Encapsulation Efficiency

0.5 ml Of PLL/DNA/Liposome complex was incubated in an eppindorf with 100 µl of trypsin at a concentration of 100 units/ml for 30 min at 37 °C. This process results in the release of DNA from unencapsulated PLL/DNA complexes. Released DNA was separated from liposomes PLL and trypsin by adding an equal volume Phenol:Chloroform:Isomylalcohol (Amersham Pharmacia Biotech, Rainham, Essex, UK) and centrifuging at 13,000 rpm using a bench top centrifuge. The aqueous upper phase contains any released DNA. The DNA in the upper phase was then further purified by ethanol precipitation and suspended in 1ml of TE buffer and assayed by Picogreen (see 2.2.). The percentage of free and encapsulated PLL/DNA were calculated in terms of total DNA present in the complex, which was maintained constant at 2 µg in all experiments.

2.14. Determination of Liposome Surface Tension.

The surface tension of the liposomes was determined by sedimentation volume (Vargha-Butler *et al.,* 1989). 1 ml of MLV was centrifuged to form a pellet which

was resuspended into a relevant n-propanol/water solution and placed into graduated Windrobe sedimentation tube with 1 ml total volume. The tubes were sealed and shaken to distribute the particles evenly n the liquid column. The reading of the sedimentation volume was taken until no further changes occurred. The surface tension of the MLV was equal to the surface tension of the suspending liquid in which maximum sedimentation occurs i.e. the lowest sedimentation volume.

The n-propanol/water binary mixtures were prepared by mixing various volume ratios of water and n-propanol between 0 and 50 % n-propanol. This gave solutions with a range of surface tensions between 72 mjm^{-2} (=100 % water) and 27 mjm^{-2} (50% water) (see appendix 4). Before use the surface tension of the buffers was confirmed using a Torsion Balance (Torsion Balance Supplies, Malvern Wells, Worcs, UK).

3. Characterisation of an Ultra High Velocity Jet Homogeniser for the Downsizing of MLV.

3.1. Introduction.

The development of methods for the down sizing of multilamellar large vesicles (MLV) to form small unilamellar vesicles (SUV) in a controllable, scaleable and reproducible manner is one of the greatest hurdles that needs to be overcome if the potential of liposomes in gene therapy is to be realised. The problems associated with many of the commonly used lab scale methods such as sonication have already been discussed in chapter 1 section 1.5.2 of this report however, it is sufficient to say here that most suffer from poor reproducibility and lack the potential for scale up which will be required to supply clinical trials and eventually the medical market place.

Of the methods discussed in chapter 1 homogenisation (1.5.2.2) potentially shows the greatest promise from an industrial perspective. With this in mind the use of an ultra high velocity jet homogeniser (The Constant Systems, Constant Systems Ltd, Northants, UK.) was investigated for its capacity in MLV disruption.

This device was originally designed for the disruption of bacterial cells (Lovitt *et al.*, 1996) however a recent investigation demonstrated its potential in the formation of oil/water emulsions of defined size and distribution (Soon *et al.*, 2001). Given the success of the jet homogeniser in this area and the similarity of emulsions to liposome

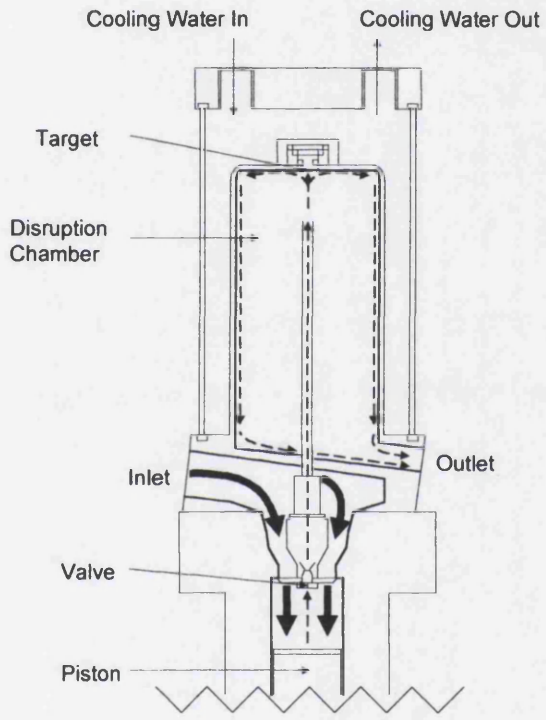
dispersions, the potential of the ultra high velocity jet homogeniser was investigated for liposome disruption.

In addition a parallel study has been carried out here at UCL upon the operation of the jet homogeniser in which Computational Fluid Dynamics (CFD) has been used to investigate key processing data such as the velocity of the jet and the energy dissipation within it. Using information obtained from this study the mechanism by which the jet homogeniser downsizes MLVs may potentially be ascertained.

The main parts of the jet homogeniser are shown in fig 3.1. A typical run starts with a down ward stroke of the piston, which draws fluid into the piston chamber. An upward stroke of the piston forces the crude dispersion through a small orifice at high speed forming the jet, which travels through the disruption chamber before impinging the target. The disrupted material is then cooled by contact with the walls of the disruption chamber, which are held at a low temperature by a re-circulated flow of coolant. The operating range of the device is in the order of 35-207 mNm^{-2} and orifices are available between 0.1 and 0.18 mm. In this study data has been obtained for pressures between 103.4 and 172.3 mNm^{-2} and an orifice of 0.18 mm.

The results from this chapter along with results from chapter 4 have been used to form the basis of the paper shown in Appendix 5a.

Fig 3.1. Schematic of the Ultra High Velocity Jet Homogeniser.

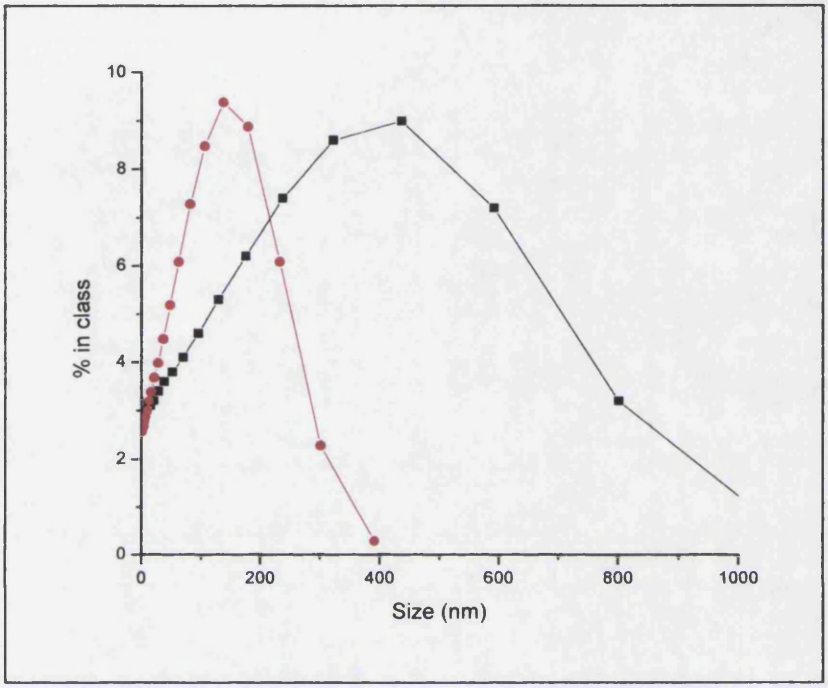


3.2. Suitability of the Jet Homogeniser for the Preparation of SUV Suspensions.

MLV were prepared using a standard laboratory scale method which involves dissolving and mixing the selected lipids in the required ratio (DOPC/DOPE/OA/Chol 2/0.05/1.95/1/1) in an organic solvent buffer of chloroform/methanol (4/1 v/v) and the subsequent removal of the solvent by way of rotary evaporation to form a "lipid cake" on the bottom of a round bottom flask. The film was then hydrated with agitation resulting in the formation of a suspension of MLVs, which was milky in appearance. This method of MLV preparation resulted in the formation of a suspension, which had a high level of heterogenicity in the size distributions of the particles formed as can be seen from the size distribution plot in Fig 3.2. This data shows that the particle mean size of the MLV suspension distribution was in the order of 500 nm and the polydispersity was of 0.4 or above indicative of a broad distribution and heterogeneous particle population.

In Fig 3.2 a size distribution plot for a suspension of liposomes after a single pass through the jet homogeniser is also presented at an operating pressure of 15 KPSI equivalent to 103.4 mNm^{-2}. This distribution plot demonstrates that a single pass through the jet homogeniser at this operating pressure was sufficient to produce a suspension of SUV with a distribution having a mean particle size of approximately 140 nm and a polydispersity of 0.3.

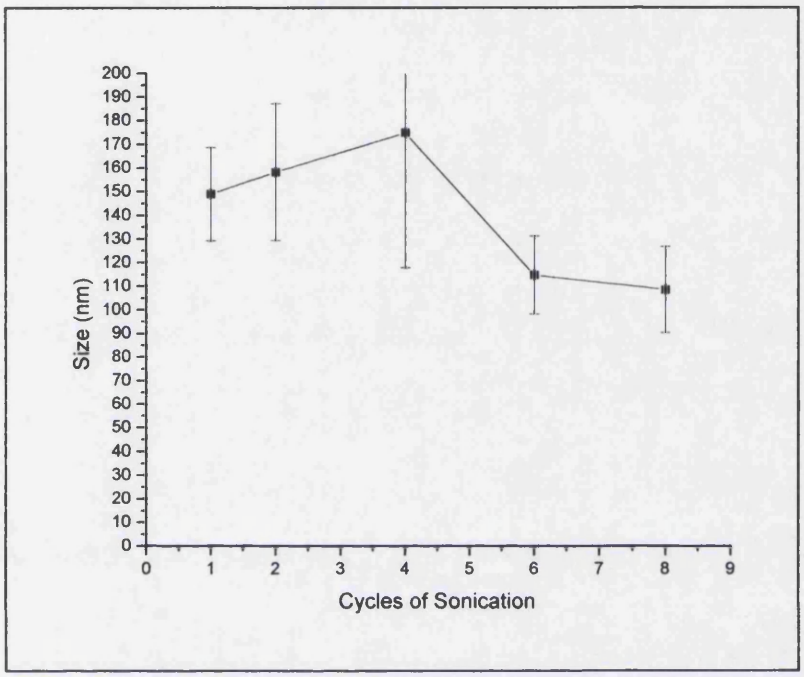
Fig 3.2.



Typical size distribution profiles for a MLV suspension (■) prepared using the standard drying down and re-suspension method previously described and a suspension of SUV produced from a single pass through the jet homogeniser at an operational pressure of 103.4 mNm^{-2} (●).

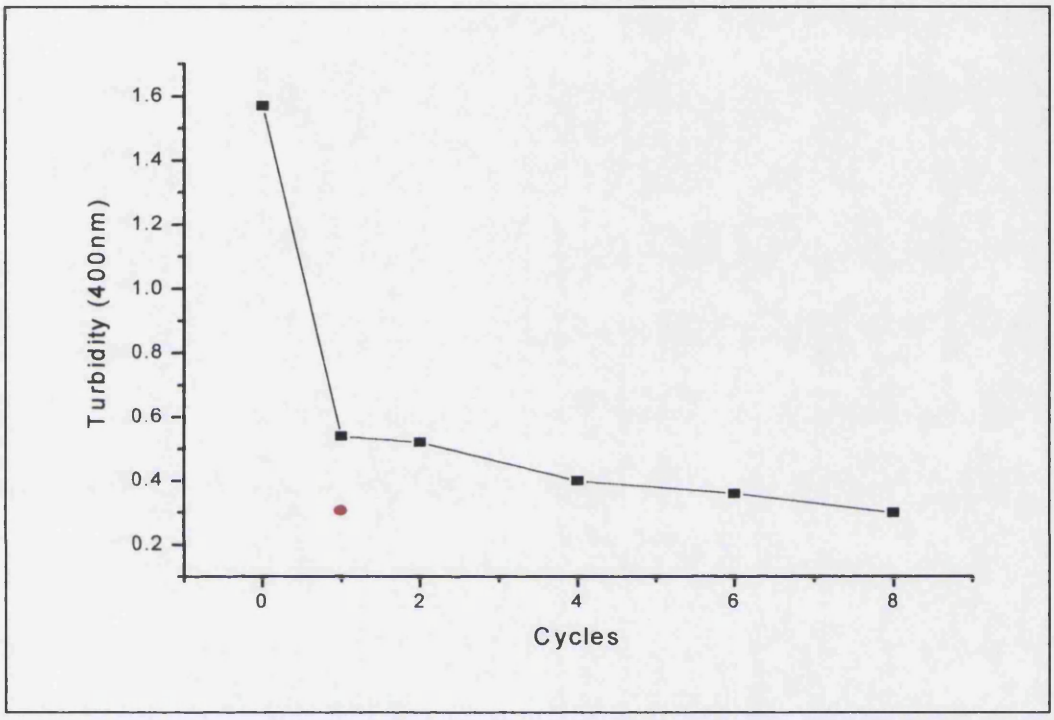
The ability of the jet homogeniser to form SUV of a size within the range suitable for *in vivo* gene delivery offers advantages over conventional methods of MLV downsizing, which often require multiple passages or cycles. Fig 3.3 shows the mean particle size of a distribution of liposomes as function of the number of cycles of sonication. The MLV suspension was subjected to cycles of sonication at a frequency of 15 KHz for a period of 1 min followed by 2 min of rest. As can be seen a single cycle resulted in a suspension with a distribution having a mean similar to that obtained with the jet homogeniser. Additional cycles of sonication had little effect upon the mean particle size of the distribution but resulted in a gradual clarification of the suspension (see Figure 3.4). However, the sonicated suspensions obtained were noticeably more turbid than those obtained from homogenisation with 8 cycles of sonication being required to obtain the equivalent turbidity measurement at 400 nm obtained from a single pass through the jet homogeniser. MLV suspensions are by nature turbid having a milky appearance and the clarification of a liposome suspension is consistent with the replacement of MLV with SUV in a suspension (Templeton & Lasic, 1999). This would therefore suggest that sonication is a less efficient method of MLV disruption than the jet homogeniser.

Fig 3.3.



The mean particle size of liposomes following successive cycles of sonication at a frequency of 15 KHz for intervals of 1min followed by 2 min rest. Error bars represent standard errors (n=5).

Fig 3.4.

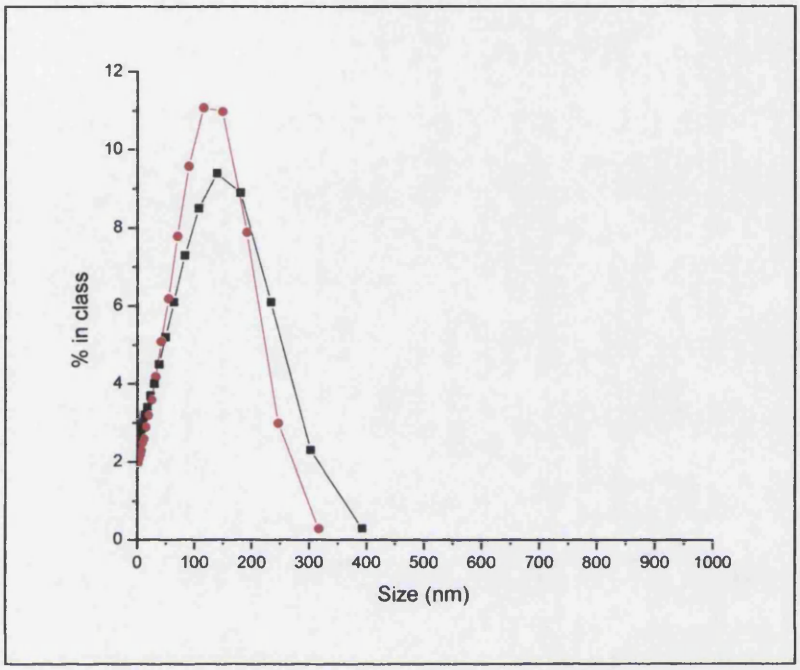


Turbidity of a SUV suspension generated by successive cycles of sonication (■) at an amplitude of 15 KHz for cycles of 1 min exposure and 2 min rest. Also shown is the turbidity of a SUV suspension generated following a single pass through the jet homogeniser (●) at a pressure of 103.4 mNm^{-2}. The turbidity measurements were taken at a wavelength of 400 nm.

Sterility along with the control of pyrogens and endotoxins are issues that are of utmost importance in the production of any drug, especially those intended for administration via systemic injection. Many of the methods commonly in use for sterilising biopharmaceutical products have proven unsuitable with liposomes and when DNA is also taken into account the number is reduced still further. γ-Ray and heat sterilisation have both been shown to damage liposomes (Watwe & Bellare, 1995) while autoclaving has been reported to adversely affect DNA (Zuidam *et al.,* 1993). Probably the most suitable method for the production of sterile liposomes will therefore be the use of sterile filtration combined with an aseptic technique. A noticeable advantage of the jet homogeniser is that even after just a single pass the resulting SUV suspension had a size distribution suitable for sterilisation with a 200 nm sterile filter medium without causing excessive loss of liposomes from the suspension (Fig 3.5).

Following filtration, some reduction in the polydispersity of the distribution was seen. The polydispersity was reduced from 0.3 to 0.2, which is notable from fig 3.5. There was a slight reduction in the mean particle size of the distribution, which would be consistent with the removal of the largest unilamellar vesicles from the suspension. However the narrow distribution of the suspension prior to filtration resulted in this lipid loss being low. Typically the recovery of lipid following filtration was in the order of 88 %.

Fig 3.5.



Typical size distribution profiles of SUV suspensions generated by a single pass through the jet homogeniser at 103.4 mNm^{-2} prior to (■) and following a single pass through a 200 nm sterile syringe filter medium (●).

3.3. Control of Liposome Size.

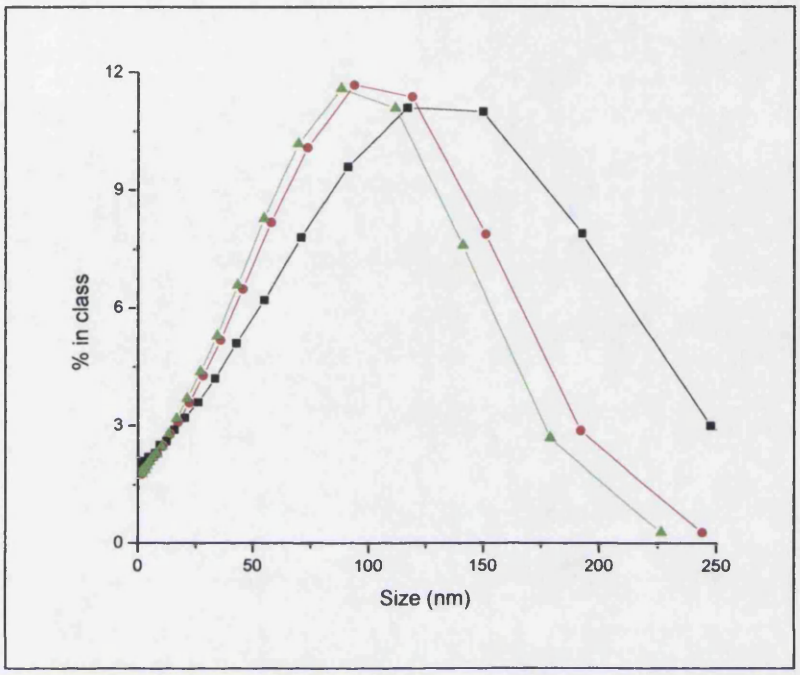
The ability to control in a reproducible manner the size of the liposomes would be extremely useful. The reproducibility of sonication was poor and this has also been reported elsewhere (Tsai, 1999). Also increasing the number of cycles of sonication was shown to have little effect upon the overall size of the liposomes produced only aiding in the removal of MLVs from the suspension. In order to investigate the controllability and reproducibility of the jet homogeniser the size distributions of samples were taken after each pass for up to five passes, at operating pressures of 103.4 mNm^{-2} to 172.4 mNm^{-2}. Typical size distributions for each operating pressure after 1, 3 and 5 passages through the jet homogeniser are shown in Fig 3.6-3.8.

Together these three figures demonstrate that it was possible to control the size of the resulting SUV between approximately 80 to 130 nm by altering either the number of passes through the device or the operating pressure used. The mean particle size derived from distributions obtained from three replicate experiments are shown in fig 3.9. The small standard errors demonstrate that the reproducibility of the jet homogeniser is good especially when compared to the results for a method such as sonication.

This is a powerful tool. It is likely that different cellular targets will require complexes with different sizes. For example as has been discussed in chapter 1 section 1.3, *ex vivo* gene delivery has been shown to favour complexes of a larger size than *in vivo* gene delivery. The ability to control the size of the liposomes produced reproducibly will

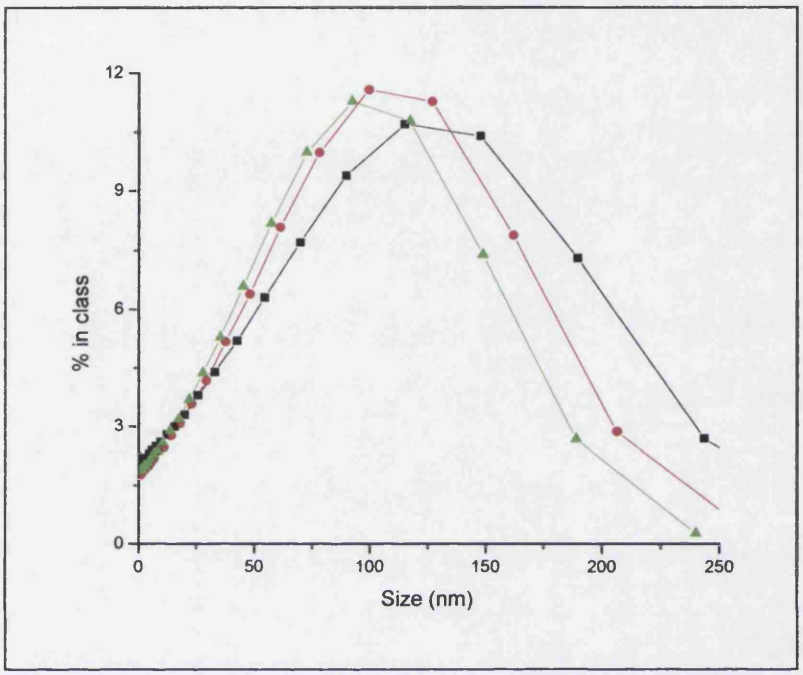
enable one device to produce liposomes for a variety of targets rather than needing several different methods which will also be of value in validation of processes.

Fig 3.6.



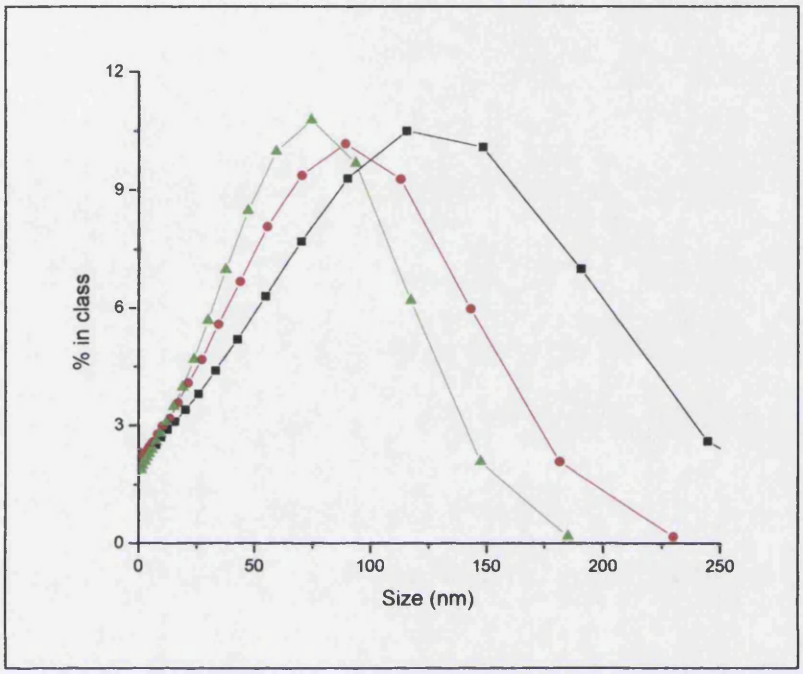
Typical size distribution profiles of SUV suspensions formed following a single pass (■), three (●) and five passes (▲) through the jet homogeniser at an operating pressure of 103.4 mNm^{-2}. All samples were filtered using a 200 nm sterile syringe filter prior to recording the size distributions.

Fig 3.7.



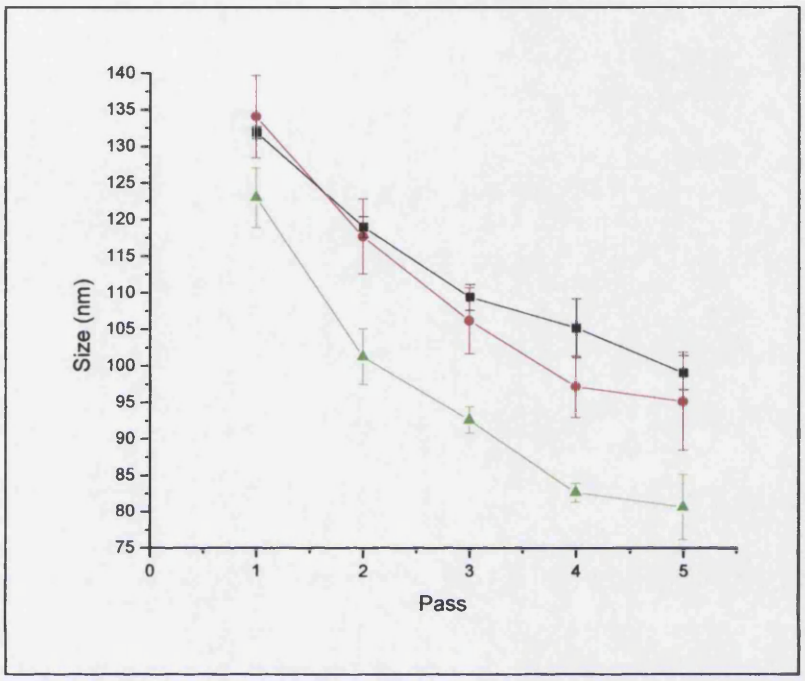
Typical size distribution plots for SUV formed by downsizing MLV using the jet homogeniser at 137.5 mNm^{-2} for 1 pass (■) three passes (●) and 5 passes (▲). Prior to recording size distributions the samples were filtered using a 200 nm filter.

Fig 3.8.



Typical size distribution profiles obtained by downsizing MLV using the jet homogeniser at an operating pressure of 172.4 mNm^{-2} for a single pass (■) three passes (●) and five passes (▲). Prior to recording size the samples were filtered using a 200 nm filter.

Fig 3.9.



Mean particle size of SUV suspensions generated by downsizing MLV using the jet homogeniser at operating pressures of 103.4 (■) 137.5 (●) and 172.4 mNm^{-2} (▲) as a function of the number of passages through the device. Prior to recording the size distributions of the suspensions the samples were filtered using a 200 nm filter medium. Standard errors are for three replicate experiments.

3.4. Potential Drawbacks of the Ultra High Velocity Jet Homogeniser.

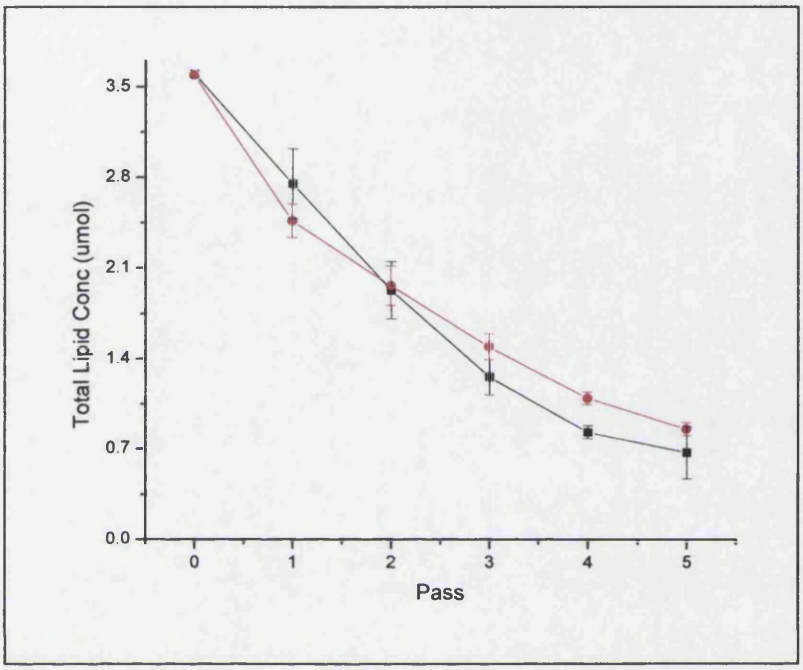
Trace quantities of the fluorescent cholesterol analogue Chol BODIPY®FL was incorporated into the liposome formulation in order to provide a quick and simple quantitative method for the assessment of total lipid concentration during the processing of the liposomes. Fig 3.10 shows the concentration of total lipid after each pass through the jet homogeniser at operating pressures of 103.4 and 172.4 mNm^{-2}. Each pass through the jet homogeniser resulted in a reduction in the lipid concentration of the suspension. Why this should be has not been ascertained as part of this study, however it is possible that the lipid loss was due to the dilution of the suspension from water remaining in the system following the wash steps (see chapter 2 section 2.5.2). The loss of lipid in this way has also been seen in the operation of the microfluidiser (See chapter 1 section 1.5.2.3 (Sorgi & Huang, 1996)). If this is indeed the case simply increasing the lipid concentration of the MLV suspension should rectify the problem however viscosity may eventually become an issue. Operating the homogeniser in a continuous rather than discontinuous mode may also be a potential solution. It should also be noted that there is no significant difference between the lipid concentrations at the two pressures shown from which is can be concluded that pressure has no influence upon this phenomenon.

The leakage of cooling water into the disruption chamber may also be potentially to blame. This has been reported in the use of the Lab 8.3 high-pressure homogeniser (Bachmann *et al.,* 1993) but has never been reported with the jet homogeniser, which received several services during the cause of this research. An additional problem that

has also been reported with other devices in use for liposome disruption is metal contamination, for example in the operation of the microfluidiser (Talsma *et al.*, 1989). Due to the high pressures involved in the operation of the jet homogeniser this may warrant future investigation.

Loss of lipid concentration is a problem encountered often in the downsizing of MLV. Even sonication, which occurs within a confined space, will result in some loss of lipid due to the formation of aerosols. What is more important is that any lipid loss is proportional for each component lipid. This was assessed by measuring the zeta potential of the liposomes after being passed through the jet homogeniser. This showed that the zeta potential of the suspensions was unaffected by either sonication or passing through the jet homogeniser, remaining at approximately –45 mV. This would therefore suggest that no single lipid component of the formulation was being lost disproportionately during the processing of the liposomes. If this were occurring then it would be expected that the zeta potential of the particles would be altered in some way.

Fig 3.10.



Total lipid concentration of liposome suspension following each pass through the jet homogeniser at 103.4 (■) and 172.4 mNm^{-2} (●). Error bars are for standard errors following three replicate experiments at each pressure.

3.5. Mechanism of MLV Breakage.

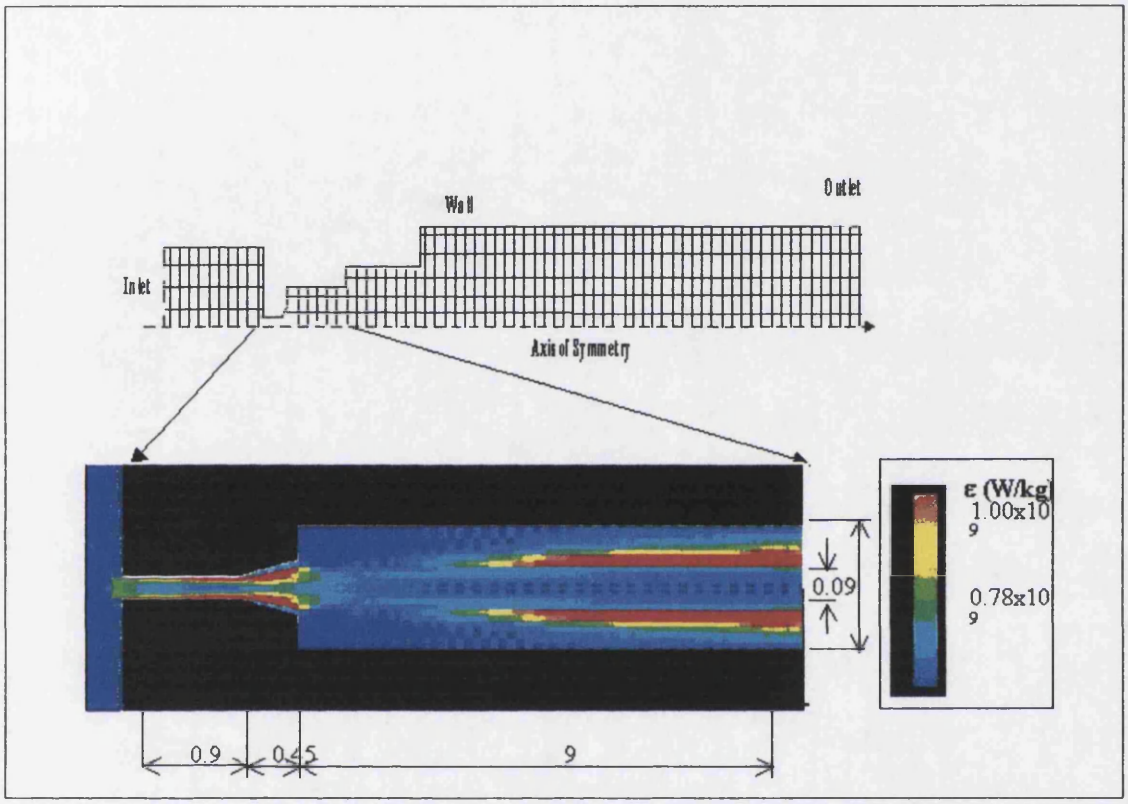
The bioprocess industries are increasingly using Computational Fluid Dynamics (CFD) as a way of investigating the flow of fluids through complex environments such as fermenters, centrifuges and homogenisers. Key process information including fluid velocity and energy dissipation rate profiles can be obtained allowing a rational basis for the scale up of a given device.

A parallel study at UCL Biochemical Engineering has been investigating the liquid flow field within the jet homogeniser by CFD to simulate the flow of the jet. These simulations have shown that at an operating pressure of 137.5 mNm^{-2} 1.06 s is the predicted time taken by the jet to reach the target plate positioned 0.17 m away. These predictions were confirmed by comparing them with high-speed video films of the jet taken during the operation of the homogeniser (Soon *et al.*, 2001). CFD predictions for the volume distribution of the jet indicated that the liquid did not fill the chamber but rather formed a discrete jet. This would therefore mean that the liquid making up the jet would not interfere with the walls of the disruption chamber. Therefore the effect of stress forces can be discounted in having a role in the downsizing of the liposomes.

CFD simulations have also been used to predict the energy dissipation rates of the jet as a function of the axial position for the central core of the jet (see fig 3.11). These simulations showed that the energy dissipation rates were generally high throughout the jet however, it is notable that the highest energy dissipation rates occur at the air water

boundary (being 5.2^{10} m^2s^{-3}) of the jet and not where the jet impinged the target as might otherwise have been expected.

Fig 3.11.



CFD simulation of the jet of the homogeniser showing the energy dissipation rate within the jet as a function of axil position. The CFD was kindly computed by Hu Zang (UCL Biochemical Engineering) as part of a parallel study using the CFX 4.3 package (Heat technology, Oxfordshire, UK).

This sort of CFD simulation can be used to help in the identification of the mechanism by which the MLVs are downsized by the jet homogeniser. Studies on the breakage of oil drops in an emulsification operation may provide useful insights since there has been little published on the mechanism of breakage of MLV to date. According to such studies the mechanism of drop breakage in a turbulent flow field, such as that occurring in the jet can be determined by the ratio of drop diameter to the Kolmogoroff length scale, η, defined by the following expression (Calabrese *et al.*, 1986; Ayazi Shamlou and Titchener-Hooker, 1993; Boye *et al.*, 1996; Soon *et al.*, 2001):

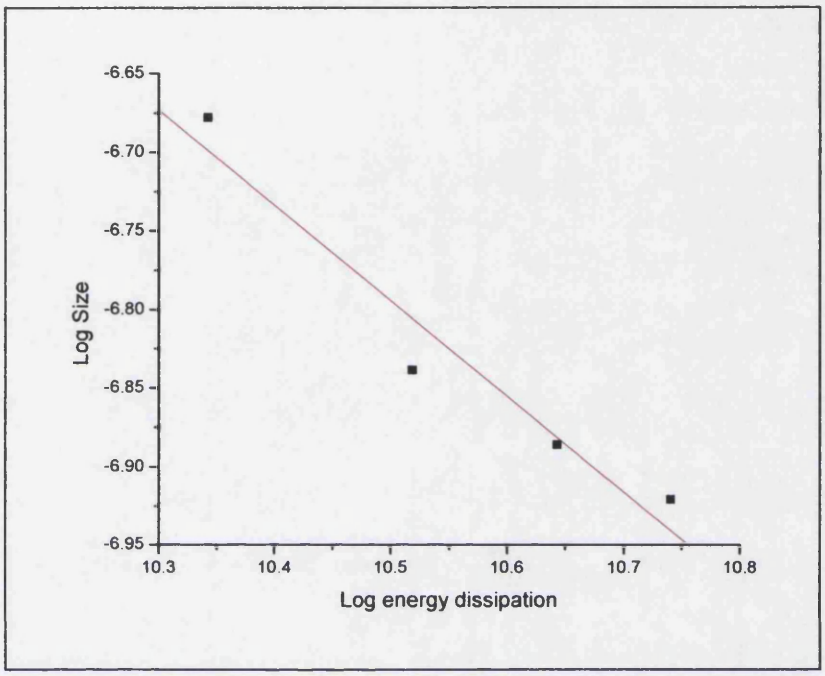
$$\eta = \left[\frac{\left(\mu_c / \rho_c \right)^3}{\varepsilon} \right]^{1/4} \qquad \text{(Equ 3.1)}$$

Where μ_c and ρ_c are the viscosity and density of the continuous phase respectively and ε is the energy dissipation rate. When the maximum stable drop diameter is larger than the Kolmogoroff's length scale the breakage of drops is assumed to occur by inertial forces that act on the surface of the drops. However, if the Kolmogoroff length scale is larger than the maximum drop size it is thought that breakage occurs by viscous forces which loose energy as heat.

The continuous phase viscosity and density of the MLV dispersions were measured and correspond to the values of water, thus, $\mu_c/\rho_c = 1 \times 10^{-6}$ $m^2 s^{-1}$. The energy dissipation values were obtained directly from CFD predictions shown in Fig 3.11. Substituting

these values into Eqn 1 predicts a Kolmogoroff length scale in the range of about 66 nm and 94 nm. With the mean size of downsized liposomes at this pressure being between 80 nm and 140 nm accurate prediction from this result is difficult. However the results would suggests that inertial forces may be responsible. Additional evidence can be obtained by further theoretical analysis of these results. When inertial forces are responsible for breakage, the size of the drops is expected to be proportional to $\varepsilon^{3/5}$. However when viscous forces are responsible the size of the drops is expected to be proportional to $\varepsilon^{1/2}$. Fig 3.12 shows a plot of LOG_{10} ε for each pressure studied as a function of LOG_{10} maximum drop size obtained for that pressure. The gradient of this graph is -0.6 therefore confirming the prediction of the Kolmogoroff length scale that inertial forces are responsible for MLV disruption.

Fig 3.12.



Maximum liposome size as a function of the energy dissipation required to generate it from the jet homogeniser. Equation for the straight line is y = -0.61x - 0.41.

3.6. Stability

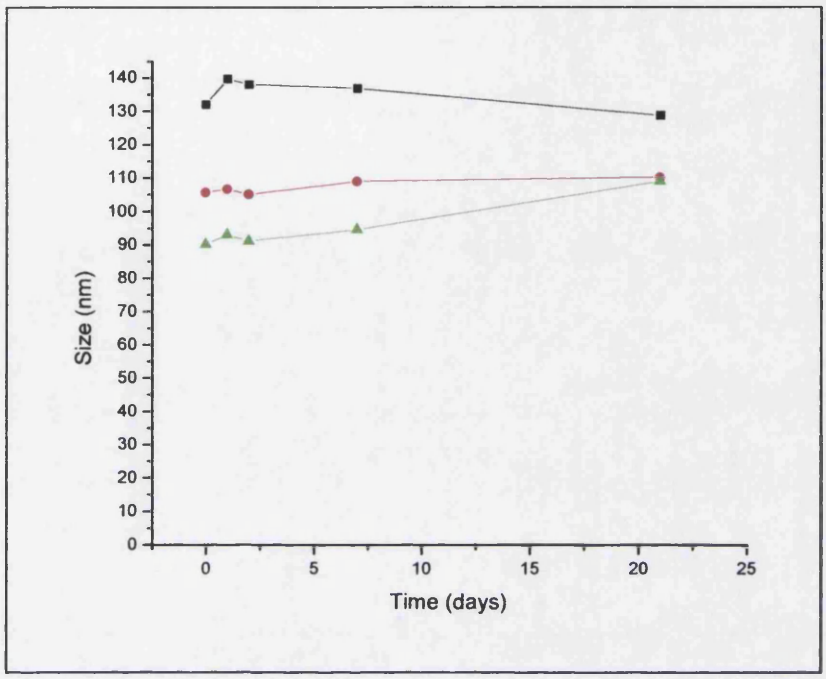
The stability of the liposomes was investigated in order to ascertain whether or not downsizing using the jet homogeniser adversely affected the vesicles. The liposomes were stored at 4 °C and the size was recorded as previously described over a period of three weeks. Due to the ability of the jet homogeniser to control the size of the liposomes produced it was possible to compare liposomes suspensions with distributions having different initial mean sizes.

Fig 3.13 shows the mean particle size for distributions of liposomes over 21 days. The initial mean size of the liposomes was approximately 130 nm, 100 nm and 90 nm. All three suspensions were prepared at an operating pressure of 137.4 mNm^{-2} by increasing the number of passes through the jet homogeniser. The graph shows that each of the liposome samples had a high degree of stability, with the 90 nm liposome sample being the most unstable. However the degree of size increase was small (being only approximately 20 nm) especially if it is compared to PLL/DNA, which aggregates to over 1 μm within several hours following formation (Lee *et al.*, 2001).

This instability of the smaller liposomes may be because liposomes possess thermodynamic, as well as colloidal instability. This is a consequence of the fact that membranes prefer to be flat rather curved and therefore possess kinetic energy in the same way as a loaded spring (Lasic 1999). The greater the degree of curvature the higher the kinetic energy is possessed by the membrane and the greater the thermodynamic

instability. This would therefore make smaller liposomes less stable than larger liposomes as they fuse together in an attempt to reduce the degree of curvature of their membranes.

Fig 3.13.



Stability of SUV generated using the jet homogeniser. Graph shows data for SUV suspensions with initial size distribution having a mean particle size of approximately 90 nm (▲), 100 nm (●) and 130 nm (■).

3.7. Summary.

In this chapter a method for the downsizing of MLV in a scaleable, reproducible and controllable manner has been introduced. This method utilises an ultra high velocity jet homogeniser (the constant systems). It was shown that a single pass through this device was sufficient to form liposomes with a narrow size distribution that was suitable for sterilisation by way of sterile filtration through a 200 nm filter medium without excess loss of liposomes from the suspension.

Controllability of the jet homogeniser was demonstrated. It was shown that by either increasing the number of passes through the jet homogeniser or by increasing the operating pressure used that the size of the resulting liposomes suspension could be reduced further and controlled. It was also demonstrated that using the jet homogeniser to downsize liposomes did not result in disproportional loss of single lipid components of the formulation and that the homogeniser also did not adversely effect the stability of the liposomes.

The mechanism by which the liposomes were downsized within the jet homogeniser was investigated by theoretical analysis using results obtained from CFD of the device (The CFD simulation were kindly ran by Hu Zang Biochemical Engineering UCL). From these simulations it was possible to ascertain that the mechanism by which the jet homogeniser downsized the liposomes was probably by inertial forces.

4. Liposome Encapsulated Poly-L-lysine Condensed Plasmid DNA Complexes.

4.1 Introduction.

The delivery of pDNA to cells for gene therapy although possible is none the less a difficult and far from efficient process. This is in part due to the large size of plasmids, which are several hundred times the size of proteins. For this reason the majority of non-viral vectors for the delivery of plasmids usually incorporate a means of condensing the DNA. The simplest way to achieve DNA condensation is by mixing cationic polymers such as poly-L-lysine (PLL) with the DNA. DNA is a large anionic polymer therefore it will interact and form condensed particles, which behave as colloids, when mixed with cationic polymers.

These particles are extremely unstable and aggregate soon after formation. Recently this has been investigated in the case of PLL/DNA complexes in physiological conditions using the DVLO theory of colloidal stability (Lee *et al.*, 2001). This work has shown that the instability of these complexes is particularly marked under physiological conditions. This is an undesirable characteristic in a system the aim of which is to deliver the plasmid to target cells *in vivo*.

Liposomes possess greater stability than PLL/DNA complexes as was shown in chapter 3 (see Fig 3.13). It is therefore rational to assume that encapsulating pDNA into conventional liposomes may help to form stable vector complexes. However, partly due

to the like charges involved and the large size plasmids the encapsulation of plasmid DNA into liposomes is generally poor and rarely exceeds 20 %, despite efforts to rectify the problem (Szoka & Papahadjopoulos 1980). Recently Lee & Huang (1996) have demonstrated the encapsulation of poly-L-lysine condensed DNA complexes by anionic liposomes, which form complexes with high levels of DNA encapsulation. It is this approach that will be applied in this project.

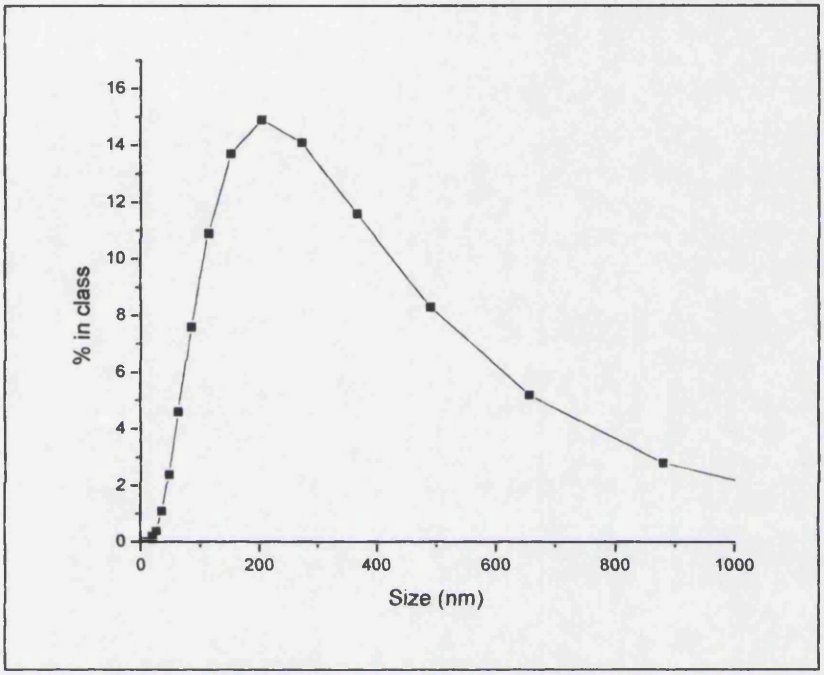
In this chapter the liposomes formed in chapter 3 will be used to encapsulate PLL/pDNA complexes and the effect of process conditions upon the biophysical characteristics investigated.

4.2. Effect of Charge Ratio on Complex Formation.

In the preparation of the complexes investigated in this project mixing was carried out using a syringe pump the method of operation of which is outlined in chapter 2 section 2.3. This approach had the advantage of improving reproducibility which is an innate problem associated with mixing by hand. Appendix 2 shows how the charge ratios were calculated. Previous studies (Tsai *et al.*, 1999) have already shown that the optimum charge ratio for the preparation of PLL/DNA complexes is at a PLL: DNA charge ratio of 2 and at an infusion rate of 6 ml per min using the syringe pump mixing device. Using this information PLL/DNA complexes were produced in 10 mM HEPES buffer pH 8 and resulted in the formation of complexes that had a broad distribution with a mean particle size of 200 nm or more and a polydispersity generally in the order of 0.5 (see fig 4.1). The zeta potential of the complexes was measured to be +20 mV. The complexes had poor stability and would form aggregates of particles over 1 μm in size within hours of mixing.

10 min after the mixing of PLL and DNA the resulting complexes were mixed with various total lipid concentrations of SUV depending on the desired charge ratio also at an infusion rate of 6 ml/min. The 10 min interval between the preparation of PLL/DNA and the mixing together of SUV and the complexes allowed time for the PLL/DNA complexes to form as well as any potential time dependent variations to be ignored (see section 4.4). The size and zeta potential of the complexes were then measured as previously described.

Fig 4.1.

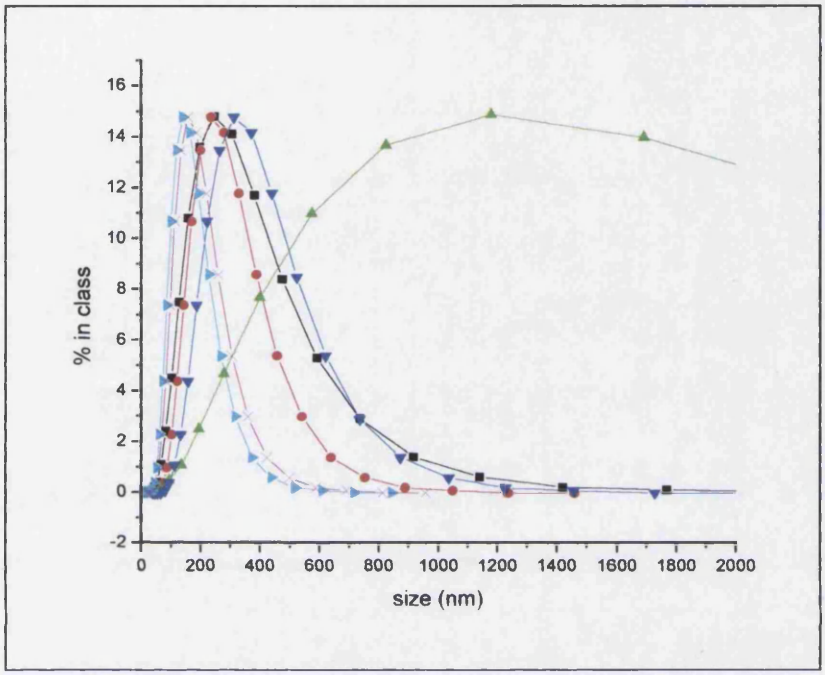


A typical size distribution plot for a suspension of PLL/DNA complexes generated by mixing PLL and DNA at a charge ratio of +2 in 10 mM HEPES buffer pH 8 and at an infusion rate of 6 ml/min.

Fig 4.2 shows size distribution profiles for the PLL/DNA/liposome complexes generated by mixing PLL/DNA and SUV at various charge ratios 10 min post mixing. What is evident is that at charge ratios equal to and close to unity the size of the complexes produced were large. Fig 4.3 shows the mean of the distribution for each charge ratio studied as a function of time post mixing. At the charge ratios close to unity the resulting complexes were extremely unstable and aggregated soon after mixing. However, at charge ratios sufficiently larger or smaller than 1 the resulting complexes possessed high levels of stability and significantly narrower size distributions. This is because at a charge ratio close to 1 the system would be at or near to its isoelectric point, i.e. charges are in equilibrium (Lee & Huang, 1996). There would therefore be little or no charge repulsions between particles resulting in their aggregation. At charge ratios greater or less than one the system possessed an overall positive or negative charge and the resulting charge repulsions will make aggregation less likely to occur.

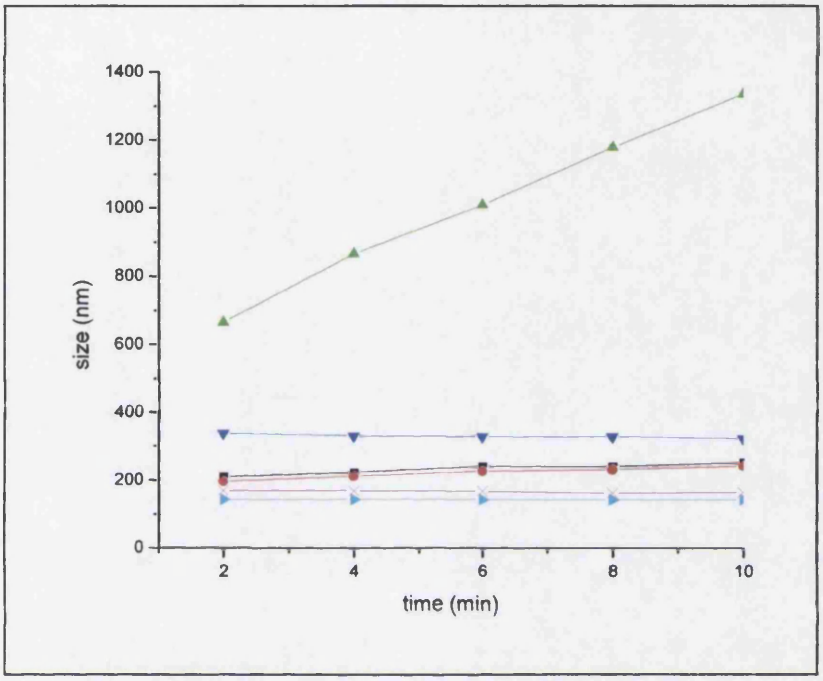
This is demonstrated in Fig 4.4. This figure shows the corresponding zeta potentials for the charge ratios studied. As can be seen at low charge ratios the complexes generated possessed a net positive charge, while at charge ratios greater than 1 the complexes carried a net negative charge. From this result it is evident that the optimum charge ratio for the preparation of these complexes is 4, which is equivalent to a lipid: DNA ratio of 8 (see appendix 3 which shows fig 4.4. as a function of lipid: DNA ratio rather than of charge).

Fig 4.2.



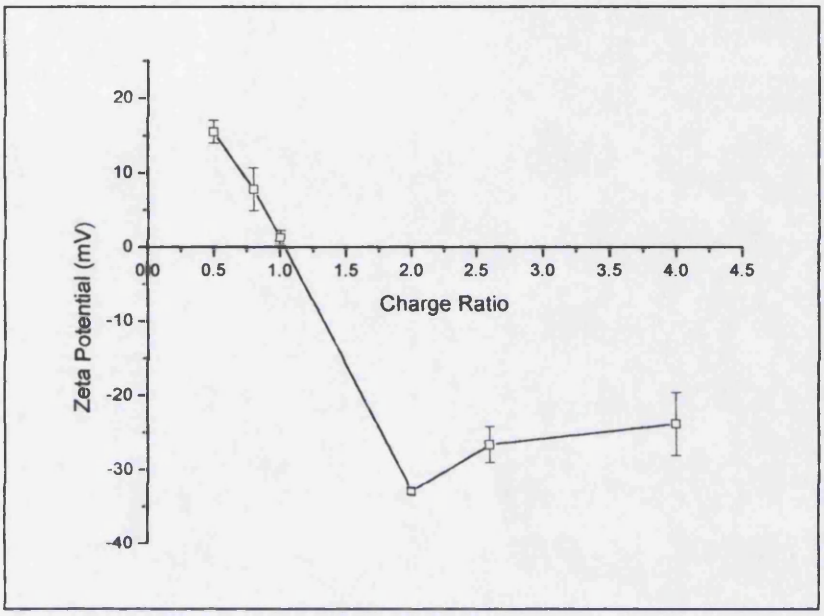
Typical size distribution plots for PLL/DNA/liposome complexes at various charge ratios 10 min following initial mixing at an infusion rate of 6 ml/min. The results shown are for charge ratios of 0.4 (■), 0.8 (●), 1 (▲), 2.5 (▼), 4 (►) and 8 (X).

Fig 4.3.



Mean particle size of PLL/DNA/liposomes generated by mixing PLL/DNA with liposomes at various lipid: PLL/DNA charge ratios as a function of the time post mixing. Results shown are for charge ratios of 0.4 (■), 0.8 (●), 1 (▲), 2.5 (▼), 4 (►) and 8 (X).

Fig 4.4.



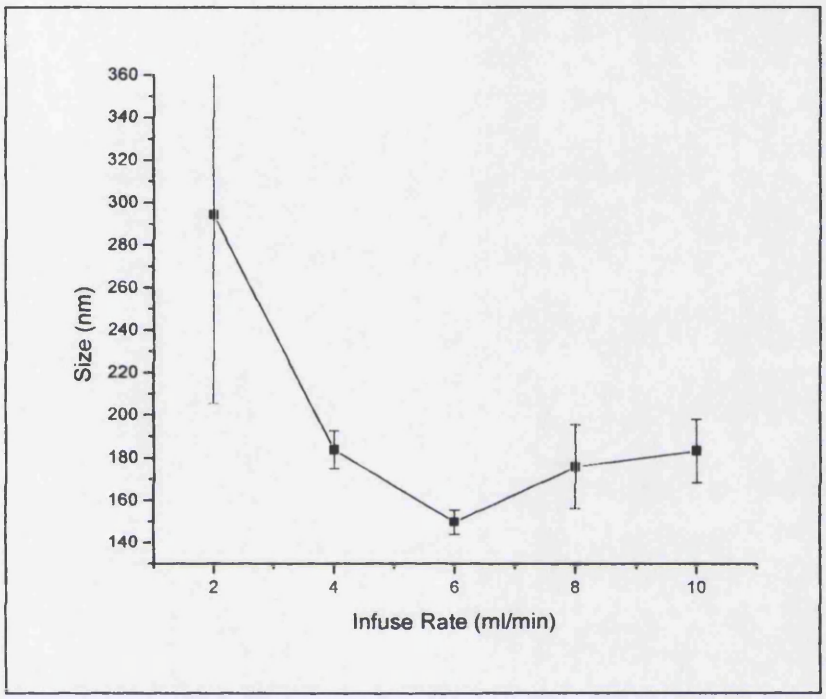
Zeta potential of PLL/DNA/Liposome complexes prepared by mixing Liposomes and PLL/DNA at various charge ratios of Liposome to PLL/DNA. The zeta potential of PLL/DNA is +20 mV while that of empty liposomes is approximately –45 mV.

4.3. Mixing Speed.

All of the complexes that have been studied in this chapter have been prepared using the syringe pump. This method was first devised for the preparation of cationic liposome/DNA complexes (Zelphati *et al.,* 1998) but has also been shown to be suitable for the preparation of PLL/DNA complexes (Tsai, 1999). It was therefore used here. Using the syringe pump has several key advantages over manual mixing. Firstly it removes the inherent lack of reproducibility associated with hand mixing especially between different experimenters, secondly it removes the technical problems associated with the order of mixing and thirdly it lends itself well to scale up.

Previous work using the syringe pump for the preparation of PLL/DNA complexes had shown that an infusion rate of 6 ml/min was suitable for the preparation of these complexes (Tsai 1999) when using a tubing with a cross sectional area of 0.8 mm. This results in a linear velocity of 0.2 m/s. However given the different nature of PLL/DNA complexes and liposomes compared to DNA and PLL (i.e. the mixing of polymers compared to the mixing of colloids) it was decided to carry out mixing at various infusion rates at a charge ratio of 4. Fig 4.5. The result of this experiment shows that the optimum infuse rate was also 6 ml/min and that slower or faster infusion rates the resulting complexes were of a marginally larger size. For all future experiments therefore an infusion rate of 6 ml/min was used.

Fig 4.5.



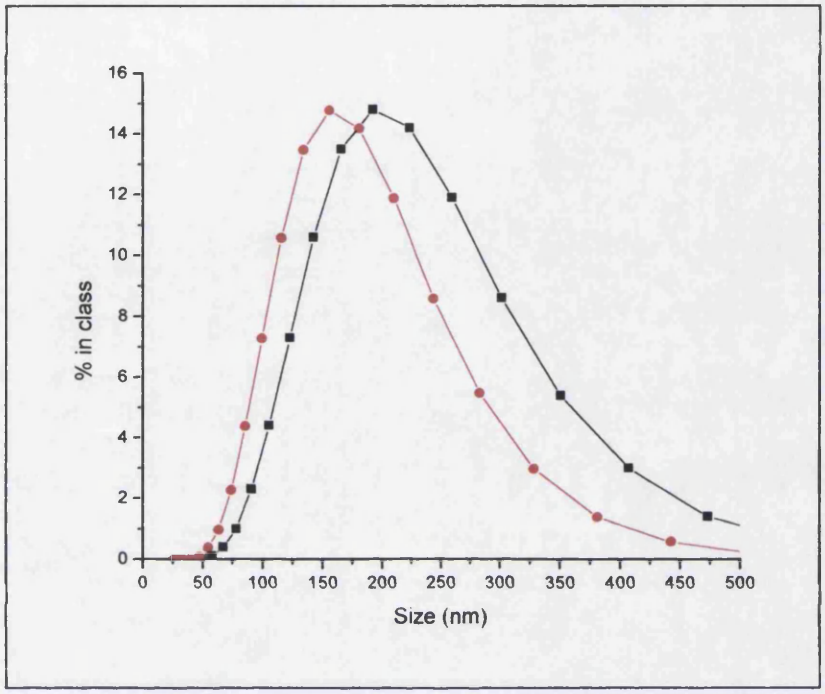
Mean particle size of PLL/DNA/liposomes generated my mixing PLL/DNA and liposomes at a lipid: PLL/DNA charge ratio of 4 as a function of the infusion rate of the syringe pump. Standard errors are for three replicate experiments. The diameter of the tubing was 0.8 mm.

4.4. Maturation Effects.

As previously described above (see section 4.2) in the experiments discussed so far PLL/DNA complexes were mixed with liposomes approximately 10 min following their preparation. This was due to the time required to measure the size of the PLL/DNA complexes prior to mixing in early experiments and to maintain continuity throughout the course of the project. In order to determine whether time was required for PLL/DNA complexes to mature prior to mixing with liposomes experiments were carried out in which PLL/DNA was mixed with liposomes either immediately following PLL/DNA preparation (allowing sufficient time for the syringe pump set up to be altered) or 20 min later.

Fig 4.6 shows typical size distribution profiles for complexes generated immediately following PLL/DNA preparation and complexes prepared 20 min later. Comparing these distributions it can be seen that the PLL/DNA/liposome complexes produced as soon as possible following PLL/DNA formation were larger than those prepared 20 min after the mixing of the PLL with DNA. In both cases it is therefore evident that some period of time is required for the PLL/DNA complexes to mature prior to their mixing with liposomes. Comparing these distribution plots with that shown in fig 4.3 for a charge ratio of 4 shows that the time that had been used in previous experiments is sufficient.

Fig 4.6.



Typical size distribution plots for PLL/DNA/liposomes prepared at a charge ratio of 4 and an infuse rate of 6ml/min either immediately (■) or 20 min (●) following the initial preparation of the PLL/DNA complex.

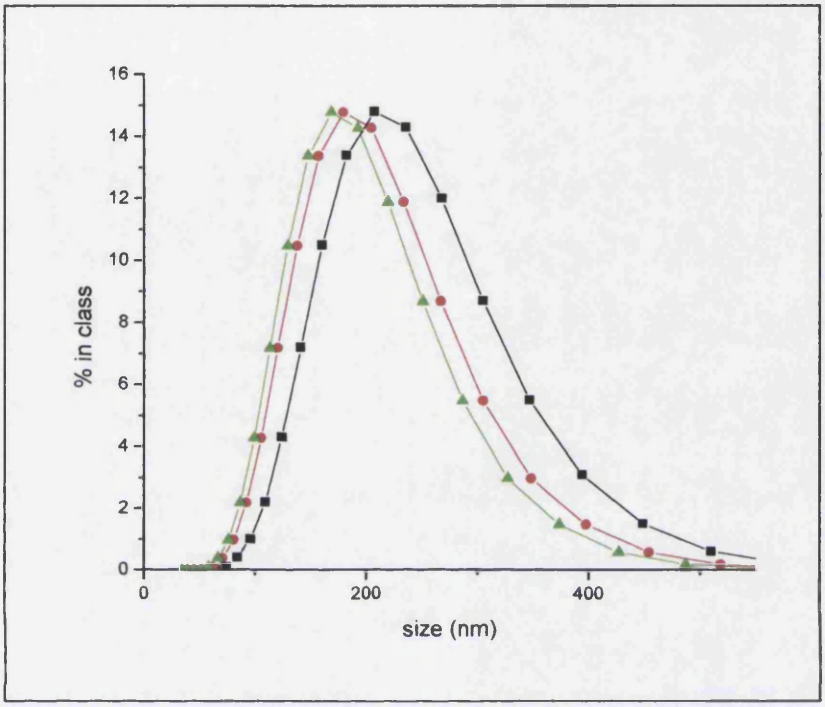
Research using time resolved multi-angle laser light scattering has been carried out to study in real time the kinetics of the formation of PLL/DNA complexes such as those used in this study (Lai & Zanten, 2001) and also the formation of cationic liposome/DNA complexes (Lai & Zanten, 2002). These studies showed that in the formation of both types of complex, especially at charge ratios close to unity, there is a growing phase were the complexes form and aggregate until they eventually reach a stable state. In the case of the cationic liposomes and DNA this aggregation is rapid and is completed within several minutes of mixing however, the time scale observed in the case of the PLL and DNA system was significantly longer. This need for the formation of particles by aggregation that eventually form a stable system may be the cause of the time dependent phenomenon observed here.

4.5. Liposome Size.

As has been mentioned in chapter 3 one of the main advantages of using the high velocity jet homogeniser for the downsizing of multilamellar vesicles (MLV) was that the resulting suspension of unilamellar vesicles may be controlled in terms of their size in a reproducible manner. Size is known to be of importance in the delivery of the gene to target cells however the way in which size effects processing of gene therapy vector complexes has received little attention. Studying the influence of liposome size upon the biophysical nature of the resulting complexes also enables investigation of how altering the process conditions of one step directly affects the outcome of another step downstream in the formation of gene delivery complexes.

Small unilamellar vesicles (SUV) with initial distributions having a mean size of 130 nm, 100 nm or 80 nm were mixed with PLL/DNA at a charge ratio of 4. Fig 4.7 shows the effect of unilamellar vesicle size upon the size distribution of the resulting PLL/DNA/liposome complexes. Perhaps unsurprisingly mixing PLL/DNA with the larger liposomes resulted in complexes of a larger size. This therefore gives a simple method for controlling the properties of the final complex within predefined set limits by altering the operational pressure of the jet homogeniser. The effects of the size of complexes upon gene delivery have been discussed in chapter 1 section 1.3.1.2.

Fig 4.7.

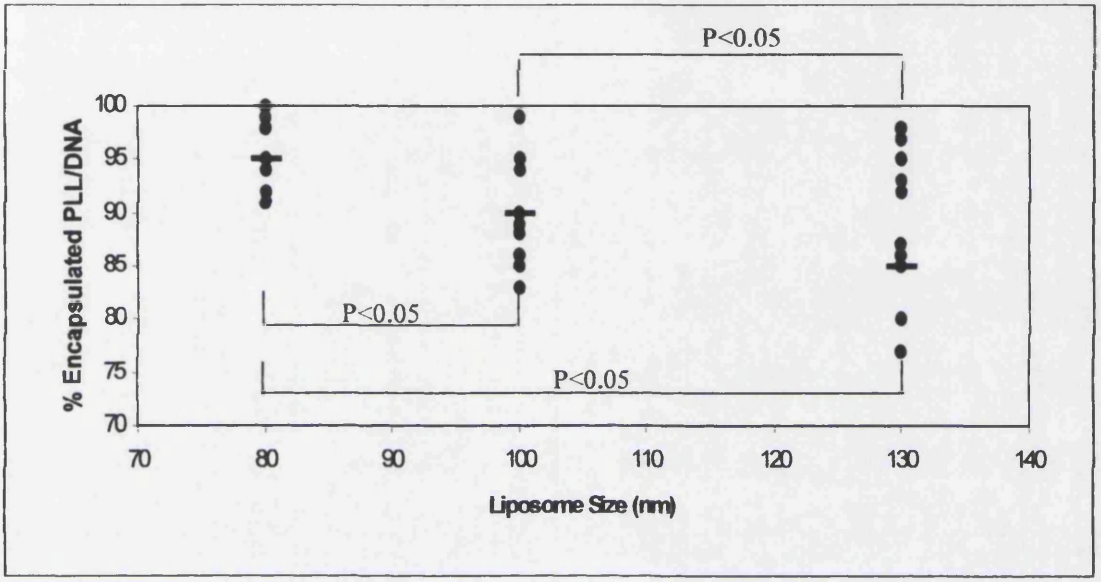


Typical size distribution plots for PLL/DNA/liposome complexes generated by mixing PLL/DNA with liposomes of various initial size distributions at a charge ratio of 4. Initial size distributions had means of 130 nm (■), 100 nm (●) and 80 nm (▲).

As mentioned previously the complexes that are the subject of this work are similar in many ways to those first proposed by Lee and Huang (1996). Electron microscopy showed that the liposomes encapsulated the PLL/DNA. The proposed mechanism for the formation of these complexes is shown in Figure 1.6. In order to ascertain the encapsulation efficiency of the liposomes it was necessary to treat the PLL/DNA/liposome complexes with the enzyme trypsin. Trypsin has the ability to digest lysine-lysine bonds therefore treating PLL/DNA complexes with this enzyme will result in the release of DNA from the complex. As previous studies have shown that liposomes have the ability to protect encapsulated DNA from the action of DNAases (Tsai 1999) it is reasonable to assume that they should also shield the PLL component of the complexes from attack by trypsin. Any non-encapsulated PLL/DNA will therefore be subject to attack by the enzyme and by measuring the quantity of DNA released the encapsulation efficiency of the liposomes can be calculated.

Fig 4.8 shows the effect of liposome size upon the encapsulation efficiency of the liposome. Previous work with drug encapsulation has shown that larger liposomes are more efficient than smaller ones due to their greater aqueous volume however, here it can be seen that the smaller sized liposomes have marginally greater encapsulation efficiency. However, in all three cases studied encapsulation efficiency was close to 90 % and compares well with previous studies.

Fig 4.8.



The efficiency of SUVs to encapsulate PLL/DNA plotted as a function of the mean size of SUVs.

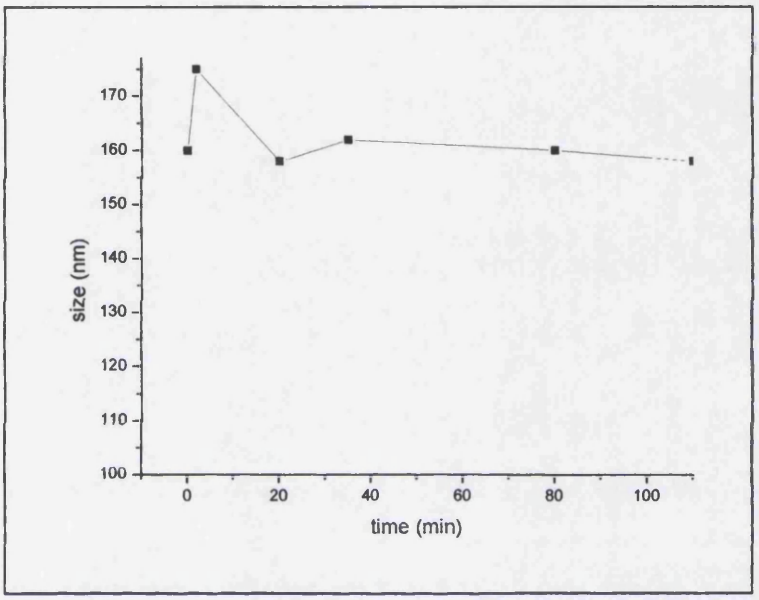
The size distributions of the complexes are shown in Fig 4.7 above. In control experiments in which PLL/DNA was treated with trypsin near 100 % release of the pDNA was recorded while pDNA released from PLL/DNA or PLL/DNA/liposomes, which had not been treated with trypsin was below detectable levels using the pico green assay. The statistical P values were calculated using a t-test. The bars represent the mean valves.

4.6. Factors Affecting the Stability of PLL/DNA/Liposome Complexes.

The stability of the complexes under storage conditions will be discussed in section 5.5 where they have been compared with immunoliposomes and their corresponding complexes. Here the effect of exposure of these complexes to serum proteins was investigated by incubation of the suspensions at 37 °C in the presence of 10 % mouse serum in order to ascertain how the complexes might behave *in vivo*.

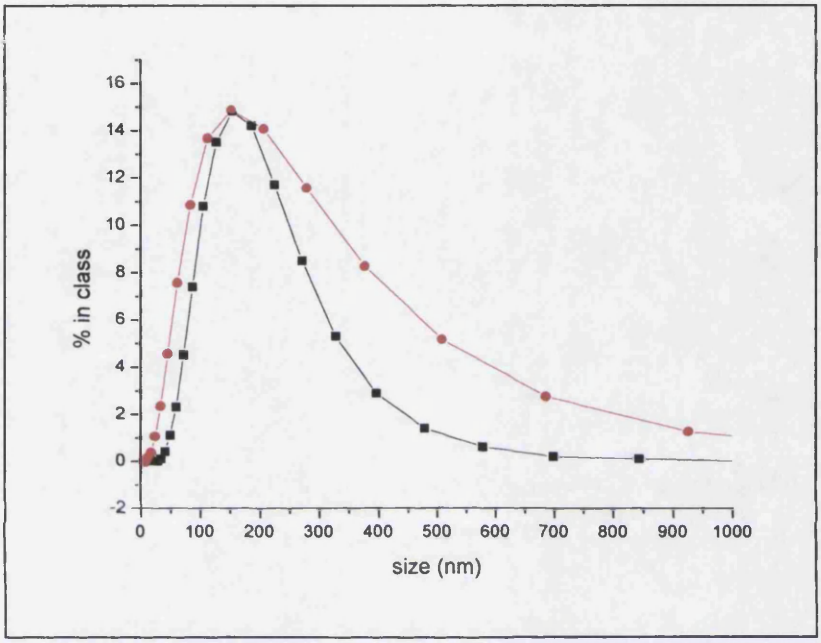
As can be seen in Fig 4.9 the addition of the serum resulted in little if any increase in the overall size of the particles and not a run away aggregation. Even following two hours incubation at 37 °C the complexes remained stable. The result is promising from an *in vivo* transfection point of view. However addition of the serum also resulted in an increase in the polydispersity of the distributions from 0.27 prior to the addition of the serum to 0.7 after addition of serum, which is shown as a broadening of the distribution plots in Fig 4.10. Like the increase in size this remained stable throughout the experiment.

Fig 4.9.



Affect of the addition of mouse serum to a suspension of PLL/DNA/liposomes at a temperature of 37 ˚C over a period of 110 min. Fig shows data for the mean particle size of typical size distributions plots.

Fig 4.10.



Typical size distribution plots of PLL/DNA/liposomes prior to (■) and following 110 min incubation (●) with 10 % mouse serum at 37 °C.

It is possible that this increase in polydispersity may be a consequence of the serum proteins associating with the complexes, a process known as opsonisation. Opsonisation is a process in which foreign particles in the blood stream are coated with specific plasma proteins. These proteins prepare the particle for clearance from the circulation by fixed macrophages, part of the RES. Typically 80-90 % of foreign particles will be opsonised within a few minutes after systemic delivery (Pouton & Seymour 2001). It therefore represents a major barrier to the gene delivery. What is also promising from these results is that it would also seem that incubating the suspension at 37 °C does not appear to adversely affect the complexes at least in the short term.

4.7. Summary.

The preparation of liposome encapsulated PLL/DNA complexes was studied. The key processing criteria was determined to be charge ratio. With a charge ratio of one resulting in the formation of complexes that aggregated soon after mixing while those formed at charge ratios significantly higher or lower than one forming stable complexes. The optimum charge ratio was shown to be equivalent to a lipid: DNA ratio of approximately 8.

The syringe pump was used to mix the components together to form the complexes. It was determined that the optimum infusion rate was 6 ml/min. It was also shown that time for the PLL/DNA complexes to form is required before mixing with liposomes in order to form the smallest complexes. 10 min was shown to be sufficient.

Also investigated in this chapter was the effect of liposome size upon the processing of the complexes. This showed that the smallest of the SUV (initial mean size 80 nm) was marginally better at encapsulating the PLL/DNA compared to larger SUV (initial mean size 130 nm). However results in chapter 3 (see figure 3.13) showed that these smaller liposomes are less stable.

The stability of the PLL/DNA/liposome was studied in the presence of serum in order to ascertain their potential stability *in vivo*. It was found that, although there was little

change in the size of the complexes the polydispersity of the distributions increased dramatically which is an indication of a poorly stabilised system. The increase in polydispersity may be due to the proteins in the serum binding to the complexes a process known as opsonisation, which results in rapid removal of particles from the blood stream by the RES.

5. Attachment of HumAb4D5 Fab' Antibody Fragments to Anionic SUV.

5.1. Introduction.

In order to improve the efficiency of gene delivery to specific target cells the addition of targeting moieties to the surface of a vector has been greatly investigated in the literature. Several different groups of biological molecules have been assessed for their potential in the targeting of liposomes including, sugar residues (Hasida, *et al.*, 2001), Integrin targeting peptides (Hart *et al.*, 1998) and even viral membrane proteins in the formation of virosomes (Imazu *et al.*, 2000; Kaneda, 2000). Here the effects of an antibody fragment and the processing of so called immunoliposomes (Slamon *et al.*, 1989) will be discussed.

The advantages which targeting molecules such as antibody fragments convey upon liposomes for use in drug and gene delivery have been extensively reviewed in the literature (Parales *et al.*, 1994) while little is known about how these molecules affects key processing parameters such as size, distribution, zeta potential, stability and encapsulation efficiency. Such knowledge is vital if these vectors are to be manufactured on a scale suitable to satisfy the demands of clinical trials and the potential market place.

The antibody fragment used here is of the Fab' class and its structure and processing have been discussed in detail in chapter 1 section 1.6.1. It is composed of the entire light chain plus the variable and first constant domain of the heavy chain. The fragment is stabilised

by the presence of disulphide bonds absent from Fv fragments. The presence of a free cystine residue in the Fab' hinge region provides a thiol group which offers a singular site for the covalent attachment of the antibody fragment to the surface of liposomes via functionalised phosphotidylethylamine (PE) lipids e.g. terminated with a malimide group (Hanson *et al.*, 1995) (see chapter 1 section 1.6.2). In this chapter the effects of the attachment of Fab' upon the processing of the liposomal gene delivery vectors discussed in the previous chapters will be investigated.

5.2. Effect of Fab' Upon the Size of Liposomes and their Complexes with PLL/DNA.

The production of immunoliposomes requires the substitution of DOPE with its malimide terminated equivalent DOPE-MPB. The liposomes were produced as previously described using the method of forming a lipid film on the bottom of a round bottom flask and the subsequent hydration with agitation with 10 mM HEPES buffer pH 8. In order to attach the Fab' to the malimide groups the SUV suspensions were incubated overnight with Fab', which had had its cystine resides reduced (to form a thiol group) by incubation with the reducing agent 2-mercaptoethylamine. Gel filtration was then used to separate the immunoliposomes from unattached Fab'

Fig 5.1 compares typical size distributions for conventional liposomes and immunoliposomes during their processing. The first thing that is evident from these distribution plots is that the need to replace DOPE with its malimide terminated equivalent (DOPE-MPB) resulted in both MLV and SUV with larger size distributions than the corresponding conventional liposome equivalents. The larger size of the modified SUV was most likely a direct consequence of the increased MLV size distribution however, what caused this is unknown at this time but may be a consequence of the change in the zeta potential of the modified liposomes (see section 5.3 below).

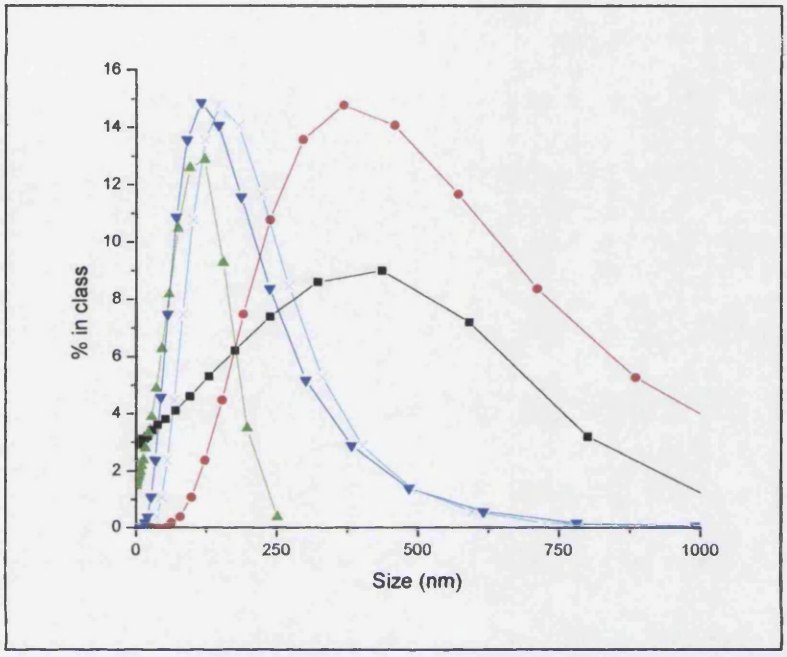
The resulting immunoliposomes were also larger than would have been expected from the effects of a molecule the size of Fab' being anchored to the surface of a SUV alone.

A single Fab' protein is approximately 5 nm in length, therefore it would be expected that Fab' attachment should result in an increase in the diameter of the SUV of approximately 10 nm. However as Fig 5.1 shows the actual size difference between that of the DOPE-MPB SUV and immunoliposomes seen here was actually in the order of 30 nm. The rest of this size increase may possibly have been a consequence of the gel filtration step in the process which was included in order to separate from the immunoliposomes any unbound Fab' and 2-mercaptoethanol. Gel filtration chromatography separates particles on the basis of their size using porous beads in a column. Large particles are eluted first whereas smaller particles having a larger volume of liquid accessible to them move through the column more slowly eluting later. This process may therefore result in a bias in the pooled fractions for larger particles, resulting in the distribution of the suspension shifting towards larger sizes as is seen.

The size of any vector will have consequences upon its efficiency in delivering the therapeutic gene to the target cells, both *in vivo* and *ex vivo*. As has already been discussed in chapter 1 vectors delivered by the *in vivo* method will if above approximately 200 nm in size become entrapped within the lumen of capillaries, accumulating within organs such as the spleen, lungs and liver. Being of a larger size will also increase the likelihood of the vector being taken up by the RES (Bally *et al.*, 1999). Therefore, even though the presence of Fab' may improve the delivery of genes to cells when the complex reaches the target organ, the percentage of the original dose actually making it there may be reduced due to their increased size.

In the case of *ex vivo* delivery it has been shown that larger vectors will have a tendency to associate with and be taken up by cells more efficiently resulting in greater levels of transfection (Ross & Hui, 1999). The larger size plus the increased interaction due to the Fab' of the immunoliposomes may therefore be advantageous here. However, if increased size is a general trend of targeting it should be taken into consideration that some of the benefits attributed to the targeting moiety may in fact be due to this increase in size.

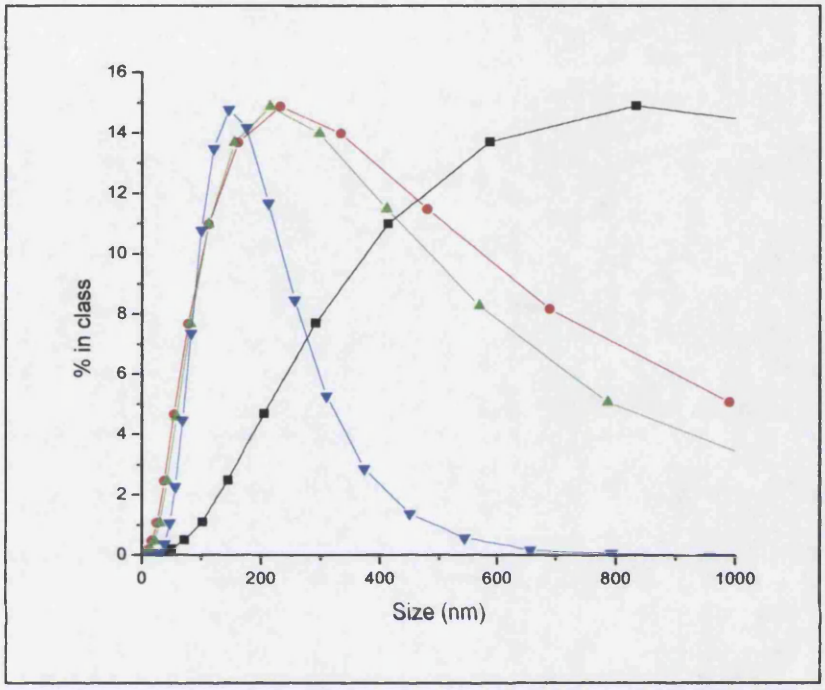
Fig 5.1.



Typical size distribution plots for conventional liposomes and modified liposomes during the processing of immunoliposomes via the attachment of Fab' to their surface. Shown are distributions for conventional MLV, (■) Conventional SUV (▲), MPB modified MLV (●), MPB modified SUV (▼), and the final immunoliposomes (X).

The optimum ratio of liposome to PLL/DNA was examined in the same way as explained previously in chapter 4. However due to the presence of the Fab' the data has been expressed in terms of lipid: DNA concentration ratio rather than charge ratio. Fig 5.2 shows typical size distributions for complexes 10 min after mixing of the components. What was most surprising was that the lipid: DNA concentration ratio which generated the particles with the distribution with the lowest mean was the same as that for the conventional liposomes (see appendix 3) being a lipid: DNA ratio of 8. Whether this was an equivalent charge ratio cannot be determined here. Another surprising observation was that despite the larger size of the initial size distribution of the immunoliposomes compared to the conventional liposomes (see chapter 3 fig 3) the resulting distributions of the corresponding complexes were similar.

Fig 5.2.



Typical size distribution plots for PLL/DNA/immunoliposome complexes taken 10 min following mixing. The distribution plots are for various lipid: DNA ratios of 1 (■), 2 (●), 4 (▲) and 8 (▼).

5.3. Effect of Fab' upon Zeta Potential.

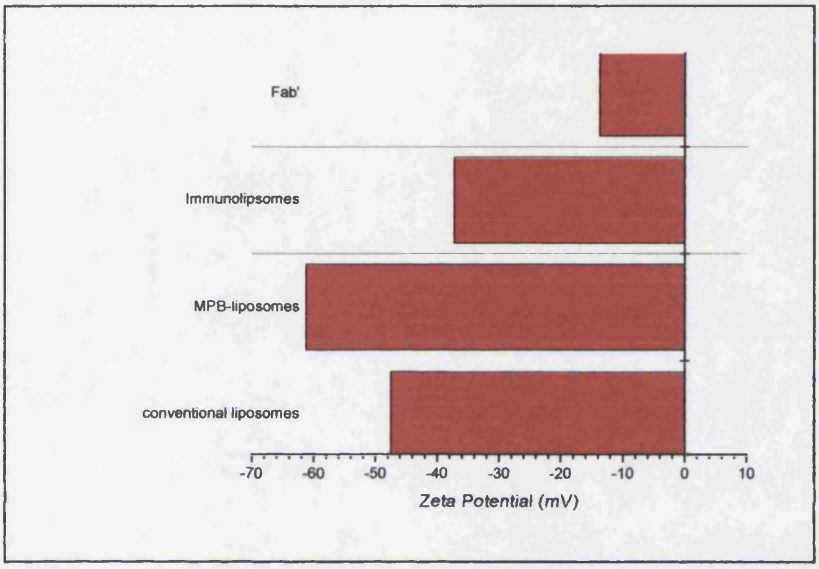
The modified DOPE (DOPE-MPB) that was used to replace DOPE in the formulation for immunoliposomes carries a charge of –1, while DOPE itself is in fact a neutral phospholipid. It would therefore be expected that the modified MLV and SUV should have a different zeta potential than conventional MLV and SUV. This was indeed shown to be the case as can be seen in Fig 5.3. The replacement of DOPE with DOPE-MPB had the effect of increasing the negative zeta potential of the liposomes, from approximately –45 mV for conventional liposomes to –60 mV for modified liposomes.

Fab' is a protein and will therefore be likely to carry a net anionic charge at the pH used in this work (pH8). This was found to be –13.7 mV ±3.6. From the observation that substituting DOPE with DOPE-MPB resulted in a fall in zeta potential it would be intuitive to speculate that the effect of Fab' attachment would also have resulted in a further drop in zeta potential to approximately –75 mV. However as can be seen in Fig 5.3 this was not the case and the presence of the Fab' resulted in a rise in the zeta potential of the liposome. What in fact would seem to have occurred is that the measured zeta potential is in fact the average of the potential from the liposome and Fab' ([-13 + -60]/2=-36.5) rather than the sum potential.

Fig 5.4 shows the corresponding zeta potentials for the PLL/DNA/immunoliposome complexes shown in Fig 5.2. The presence of the Fab' would appear to have no effect upon the mechanism by which the PLL/DNA/liposome complexes are formed. The

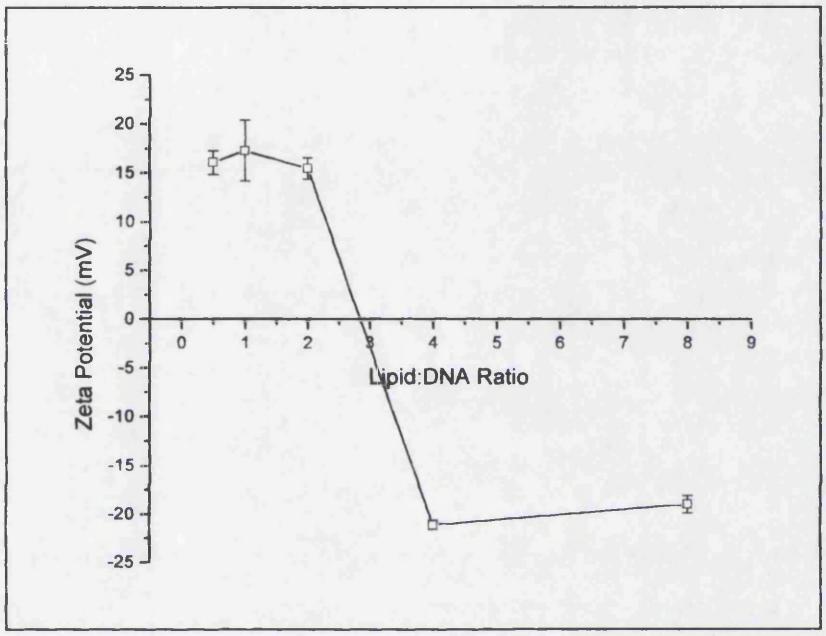
interaction of oppositely charged particles was shown in chapter 4 to result in the formation of the complexes and an excess of either cationic or anionic particles resulted in the formation of a stable system.

Fig 5.3.



Comparison of the zeta potentials for conventional anionic liposomes and immunoliposomes during their processing.

Fig 5.4.



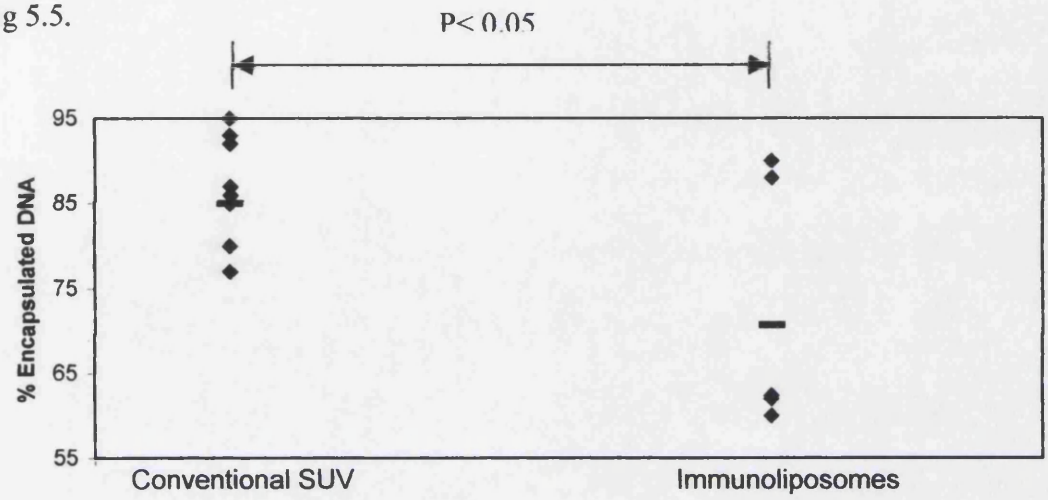
The zeta potential of PLL/DNA/immunoliposomes as a function of the ratio between lipid and plasmid DNA. The corresponding size distributions for the points shown can be seen in Fig 5.2.

5.4. Effect of Fab' upon Encapsulation Efficiency.

The above discussed results show that attaching Fab' to the surface of a liposome alters the surface characteristics of the particle. However they do not help to uncover how these changes may effect the processing of the gene delivery complex. In order to gain greater insight experiments were carried out to identify possible areas of potential concern or advantage that the presence of Fab' may convey. These included stability, which is discussed in section 5.5, and encapsulation of PLL/DNA discussed here.

The method for the determination of encapsulation efficiency has already been discussed in chapter 4 section 4.5. Fig 5.5 shows the encapsulation efficiency for immunoliposomes compared to conventional SUV. The figure shows that the efficiency of PLL/DNA encapsulation was significantly reduced by the presence of the Fab', falling by 15 %. However, it should be noted that this encapsulation efficiency was still significantly greater that reported with many conventional methods of DNA encapsulation, which as mentioned previously rarely exceed 20 %. This result would therefore mean that either additional liposomes will be required to encapsulate the available PLL/DNA or that a means of removing empty immunoliposomes and free PLL/DNA from the complexes may have to be incorporated into the process.

Fig 5.5.



Comparison between the efficiency of encapsulation of PLL/DNA by conventional and Immunoliposomes. Line shows average result. SUV n= 10 and Immunoliposomes n =7. The statistical P value was calculated using a t-test.

In chapter 4 it was shown that the size of the liposome affects the encapsulation efficiency with the larger liposomes having a poorer, although marginally so, encapsulation efficiency than the smaller ones. As the uncomplexed immunoliposomes possess a larger size than the equivalent conventional liposome it is possible that this result is a consequence of that phenomenon. It may however be a result of the lower zeta potential of the immunoliposomes. With the difference in zeta potential between the PLL/DNA and the liposomes being reduced the particles may not interact as aggressively as the more negative conventional liposomes with the cationic PLL/DNA complexes. Alternatively the Fab' may be acting as an actual physical barrier to the interaction between the immunoliposomes and the PLL/DNA hindering the efficiency of the encapsulation process. The precise reason is difficult to ascertain as little is known about how complexes such as these PLL/DNA/ liposome forms.

5.5. Effect of Fab' Upon Stability.

Stability of any new drug formulation is of utmost importance to agencies, which monitor pharmaceuticals in development and in the market place such as the FDA, MCA and the EMCA. Such agencies generally recommend that drugs have a shelf life of approximately two years. To this end the effect of the Fab' was studied upon the stability of the complex.

The stability of PLL/DNA complexes has recently been studied here at UCL Biochemical Engineering, using the DVLO theory of colloidal stability (Lee *et al.*, 2001). Using these same equations, theoretical energy plots have been prepared for both the conventional liposomes and immunoliposomes and their corresponding complexes formed with PLL/DNA, which are shown in fig 5.6.

The total interaction energy V_T between two particles is the sum of the Van Der Waals attractive V_A and repulsive forces V_R, which are obtained from the equations below.

$$V_A = \frac{A}{12}\left[\frac{y}{x^2+xy+x} + \frac{y}{x^2+xy+x+y} + 2\ln\left(\frac{x^2+xy+x}{x^2+xy+x+y}\right)\right] \qquad \text{(Eqn5.1)}$$

Where $x = (H/a_1 + a_2)$ and $y = a_a/a_2$, A is the Hamaker constant assumed to have a value of 5×10^{-21} J (Lee et al., 2001), H is the separation distance between two particles of radius a_1 and a_2.

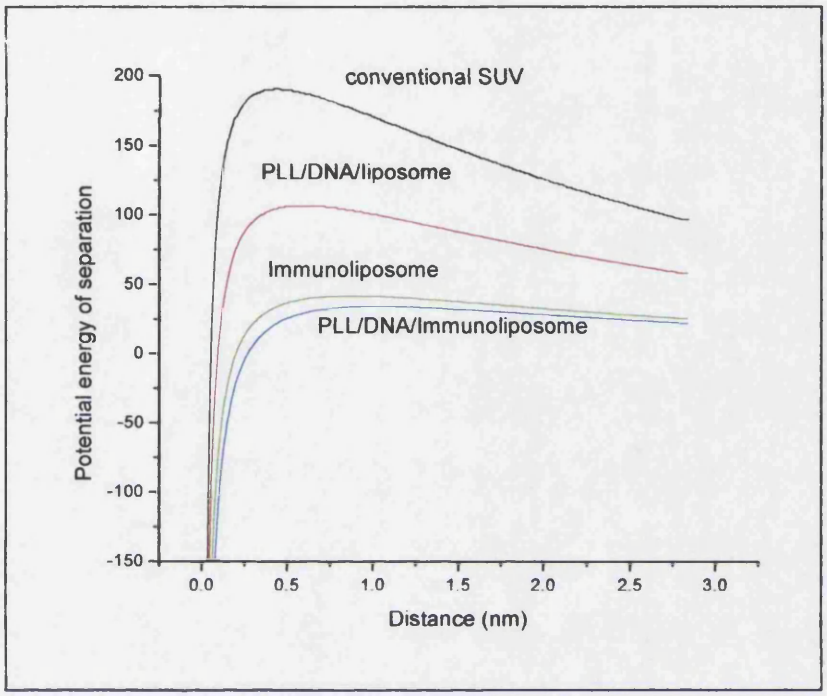
$$V_R = \frac{64\pi p a_1 a_2 k^2 T^2 \gamma_1 \gamma_2}{(a_1 + a_2) e^2 z^2} \exp[-\kappa H] \qquad \text{(Eqn5.2.)}$$

Where p is the permittivity of the medium, k is the Boltzmann constant, T absolute temperature and e the elementary charge and κ is the Debye-Huckel reciprocal length given by $0.329 \times 10^{10} \sqrt{cz^2}$ (m^{-1}) where c is the ionic strength and z the valency of the ions (a symmetrical z-z electrolyte was assumed). The dimensionless functions γ_1 and γ_2 of the zeta potentials ζ_1 and ζ_2 are given by:

$$\gamma_i = \frac{\exp[ze\zeta/2kT] - 1}{\exp[ze\zeta/2kT] + 1} \qquad \text{(Eqn5.3.)}$$

The theoretical energy plots for the liposomes and their complexes with PLL/DNA are shown in Fig 5.6. These plots predict that firstly conventional liposomes and their corresponding PLL/DNA/liposome complexes are more stable than the immunoliposomes and their corresponding PLL/DNA/immunoliposome complexes.

Fig 5.6.

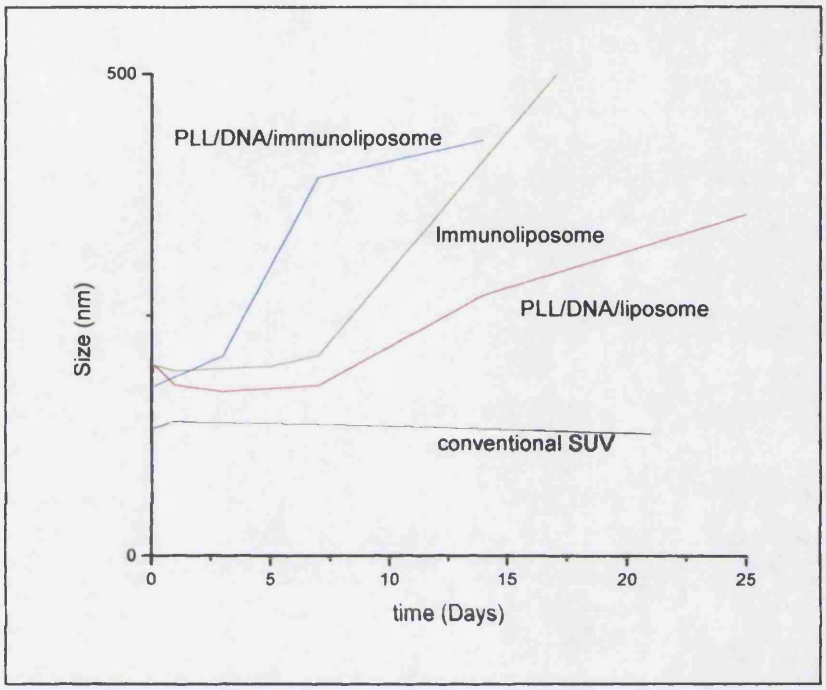


Effect of Fab' addition to liposomes upon the dimensionless total energy of interaction (V_T/kT) between two particles of equal diameter as a function of the distance between their surfaces. The size and zeta potential of each the different formulations are as shown in figure 5.1, 5.2, 5.3 and 5.4. The plots have been calculated for a pH 8, an electrolyte concentration of 10 mM and a temperature of 4 °C.

These predictions were confirmed by experimental observations in which the stability of the suspensions were studied over a period of 21 days at 4 °C (see Fig 5.7.) In this experiment the order of stability exactly matched that which was predicted by the theoretical energy plots. Both the theoretical and experimental data show that the addition of the Fab' to the formulation results in a dramatic drop in the stability of the complexes produced.

This drop in stability was a consequence of the presence of Fab' which as can be seen in Fig 5.3 and 5.4. had the effect of lowering the negative zeta potential of liposomes and the complexes. This results in a reduction in charge repulsions between particles as has been discussed in detail elsewhere (chapter 4 section 4.2). This observation and the effect upon encapsulation efficiency discussed in section 5.4. raise questions of whether or not the advantages of the addition of Fab' to the formulation (i.e. improved delivery) out way these problems caused to their processing.

Fig 5.7.

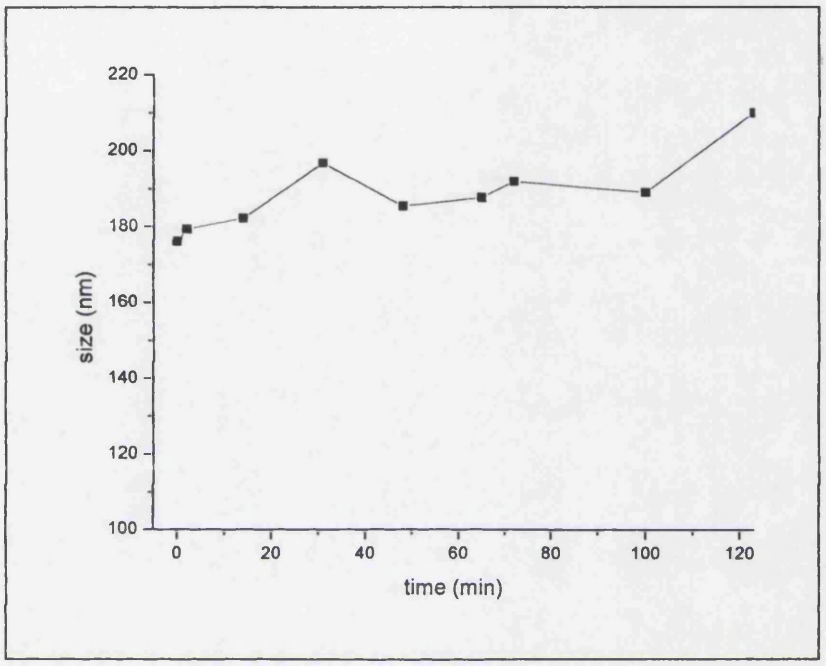


Experimental observations of the stability of PLL/DNA complexes with liposomes and immunoliposomes. All complexes were prepared in 10mM HEPES buffer pH 8 and stored at 4 °C for the duration of the study.

5.6. Effect of Fab' upon the Stability of the Complexes in Serum.

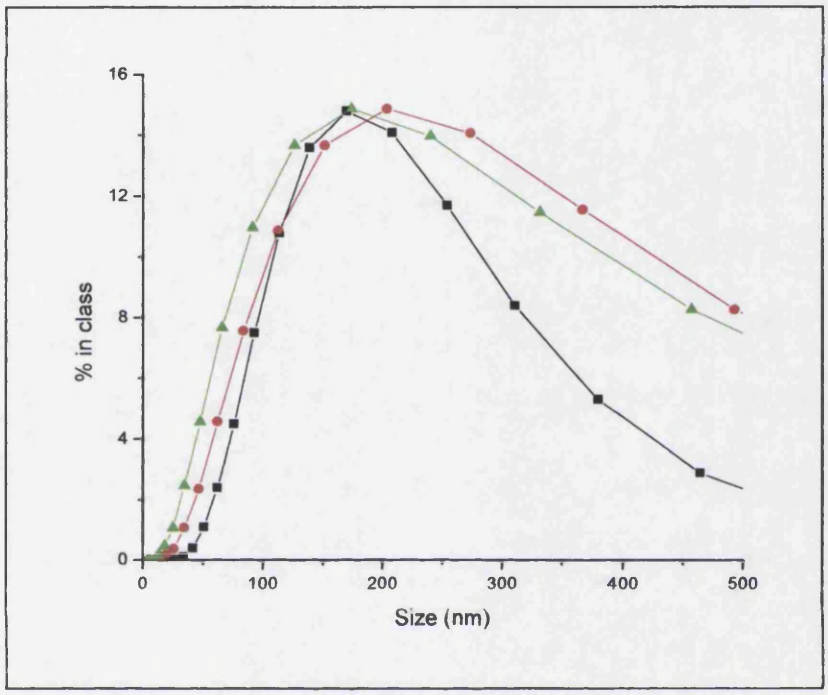
The effect of *in vivo* conditions upon immunoliposomes was also investigated by incubation of the liposomes with 10 % mouse serum at a constant temperature of 37 °C. Fig 5.8. shows the mean particle size of distribution plots over a period of 2 hours following addition of mouse serum resulting in 10% serum in the sample. The result shows that the complexes were surprisingly stable under these conditions however, they show some increase in size after addition of the serum, which is more marked than that which occurred in the case of their conventional equivalents (see fig 4.9.). Again there was also an increase in the polydispersity of the distribution of the particles after the serum was added. As in the case of the conventional liposomes it would appear that opsonisation would be likely to occur upon *in vivo* delivery of these complexes. However the distribution plots in Fig 5.9 do not seem to suggest that aggregation was occurring once the serum has been added to the suspension and that the increase in the size of the liposomes was a result of a broadening of the polydispersity of the suspensions. It has been shown that suspensions of colloidal particles with large polydispersities are significantly less stable than those with low polydispersities. It would therefore be unlikely that these complexes would remain at their present size but would aggregate especially given the results discussed above. This observation would therefore potentially cast doubt upon whether or not the presence of the Fab' would in fact significantly improve the delivery efficiency of the complexes if delivered systemically.

Fig 5.8.



Mean particle size of typical distributions of PLL/DNA/immunoliposome complexes as a function of time after the addition of 10 % mouse serum to the suspension. The immunoliposome complexes were incubated with the mouse serum at a temperature of 37°C over a period of two hours.

Fig 5.9.



Typical distribution plots of PLL/DNA/immunoliposomes. Showing the effects of incubation at 37 °C with 10 % mouse serum. Distribution plots are shown for t=0 (■), t= 2 min (▲) and t= 2 h (●) following addition of the serum.

5.6. Summary.

The work in this chapter has focussed upon the consequences which the attachment of a Fab' antibody fragment have upon the processing of liposomes for the delivery of plasmid DNA. The effect of the Fab' upon biophysical properties such as size, distribution, zeta potential and stability of the liposomes during their processing and the plasmid complexes were studied.

It was shown that the presence of the Fab' upon the liposomes resulted in two major changes to the biophysical properties of the SUV. Firstly the corresponding immunoliposomes were shown to be larger in size and secondly the immunoliposomes possessed a lower zeta potential. The effect of these changes was through theoretical analysis predicted to result in the immunoliposomes possessing a significantly poorer stability compared to conventional liposome even under conditions that lead to high levels of stability such as low ionic strength and temperature. These predictions were confirmed by experimental observation.

The presence of Fab' was also shown to hinder the encapsulation of PLL/DNA within the liposomes. The reason for this effect is unclear but may be due to the Fab' forming a physical barrier to the PLL/DNA. However it should be noted that encapsulation efficiency was still higher than has been reported with many other methods.

Finally experiments were performed to try to determine the behaviour of the complexes *in vivo*. These experiments suggest that the immunoliposomal complexes are likely to interact with serum proteins.

6. Effects of the Addition of Poly ethylene Glycol to Liposomes.

6.1. Introduction.

Many of the problems that researchers have encountered in developing non viral vectors for gene therapy such as aggregation, fast clearance from the blood stream and cytotoxicity have been improved through the incorporation into the vector of PEG. PEG is a non toxic water soluble polymer that not only increases water solubility (making many substances injectible) but also resists the immune response of the body prolonging the half life in the bloodstream (Choi *et al.*, 1999). PEG is a hydrophilic poly (alkenylene alcohol) with the chemical formula $[(CH_2)_2\text{-}O]_n$. The molecular weight can vary between 500 and 20,000 Da.

PLL binds strongly to DNA to form condensed complexes. However, a soluble and neutrally charged complex is rarely obtained because strong charge neutralisation usually results in the formation of insoluble precipitates (Katayose & Kazunori, 1997). Using a PLL-PEG block copolymer leads to the formation of a water soluble complex of ~50 nm with a higher stability than that of PLL/DNA alone (Katayose & Kazunori, 1997).

It has also been shown that PEGylation reduces the interaction of blood components with the vector. Non PEGylated DNA/transferin-PEI complexes are positively charged and have been shown to bind plasma proteins such as, IgM, fibrinogen, fibronectin, and complement C3 (Ogris *et al.*, 1999). Also erythrocytes have been shown to aggregate in

the presence of the complexes at concentrations of DNA relevant for *in vivo* use. PEGylation of these complexes reduces the binding of plasma proteins and aggregation of the erythrocytes (Ogris *et al.*, 1999).

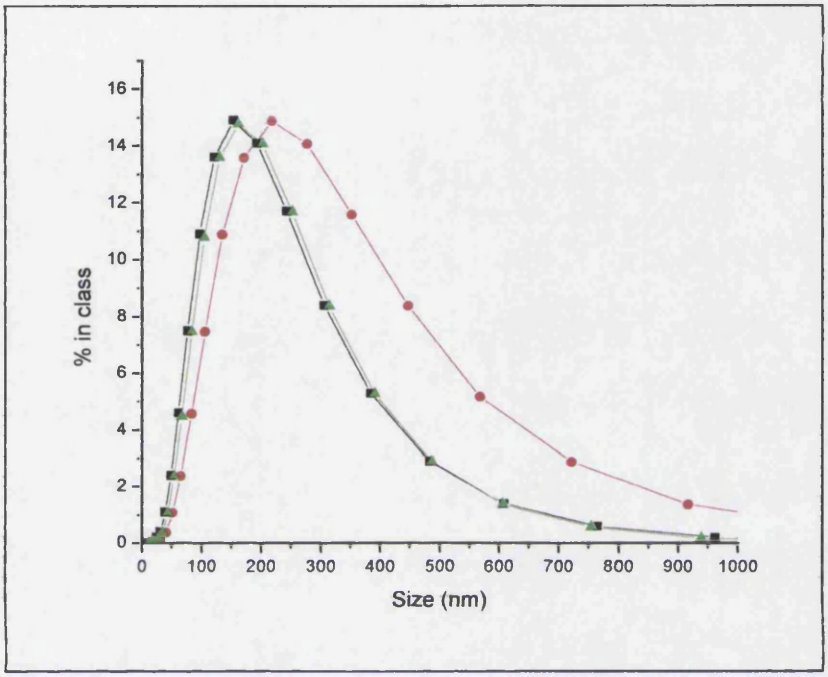
PEG has also been attached to liposomes in the formation of stealth liposomes. These show increased circulation in the blood stream due to lower levels of uptake by the RES. Such long circulating immunoliposomes of ~100 nm in diameter show high levels of extravasation and accumulation into solid tumours *in vivo* which have leaky blood vessels (Maruyama *et al.*, 1995). The mechanism by which the PEG prevents uptake by the RES is not well understood. Recently Price *et al.*, (2001) have shown PEG had no effect upon the amount of plasma proteins absorbed by neutral and negatively charged liposomes. It may therefore be that the PEG acts as a steric barrier to the macrophages rather than the opsonising proteins found within the plasma.

Despite these advantages there have been to date no reports regarding the effects of the addition of PEG into a formulation upon its processing. Several of the experimental observations made during this work have suggested that the addition of PEG into the formulation may be advantageous. It was therefore decided to investigate the effects that PEG incorporation would have upon the biophysical characterisation and processing of the complex. In order to carry out these experiments the DOPE component of the liposome was substituted with DOPE that had been modified by the attachment of PEG of a molecular weight of either 550, 1000 or 2000 Da.

6.2. Effect of PEG upon the Size of Liposomes.

It is reasonable to speculate that the addition of PEG to the surface of a colloidal particle such as a liposome would result in an increase in its size. However the size of the MLV prepared were comparable with those of conventional multilamellar vesicles and those modified for Fab' attachment. This may have been due to the inherent heterogenicity of MLV suspensions. The size of SUV after downsizing was however larger than the conventional SUV. Fig 6.1 shows typical size distribution profiles for liposomes that have been modified with either PEG 550, 1000 or 2000 Da. The distributions are all very similar despite the different size of the PEG being used. Heldt *et al.*, (2001) recently investigated the effect of PEG upon liposome size. However this group reported that the presence of the PEG resulted in the formation of smaller particles with a narrower distribution than conventional liposomes. This was hypothesised to be due to an alternative mechanism by which the PEGylated liposomes formed involving the formation of a polymer aggregate of PEGylated lipid followed by incorporation of none PEGylated lipids that becomes surrounded by a bilayer of free lipids, rather than the formation of bilayer in the case of conventional liposomes. It should be noted that both the formulation used (having a much higher percentage of DOPE) and the mechanism of liposome production were significantly different to that used in this study and the two systems may not be directly comparable.

Fig 6.1.

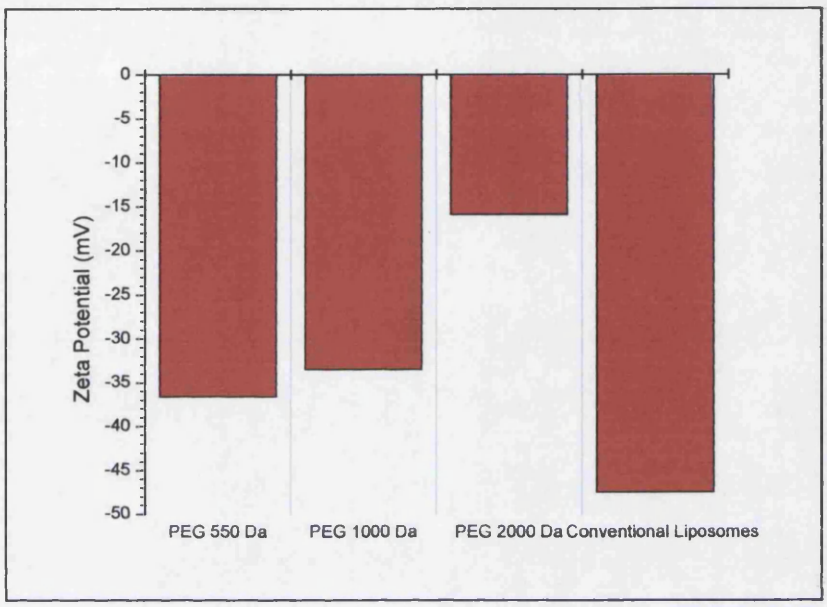


Typical size distribution plots for PEGylated liposomes generated by a single pass through the jet homogeniser at 134 mN/m. The liposomes have been modified by the addition of Poly Ethylene Glycol of 550 (■), 1000 (●) or 2000 Da (▲).

6.3. Zeta Potential.

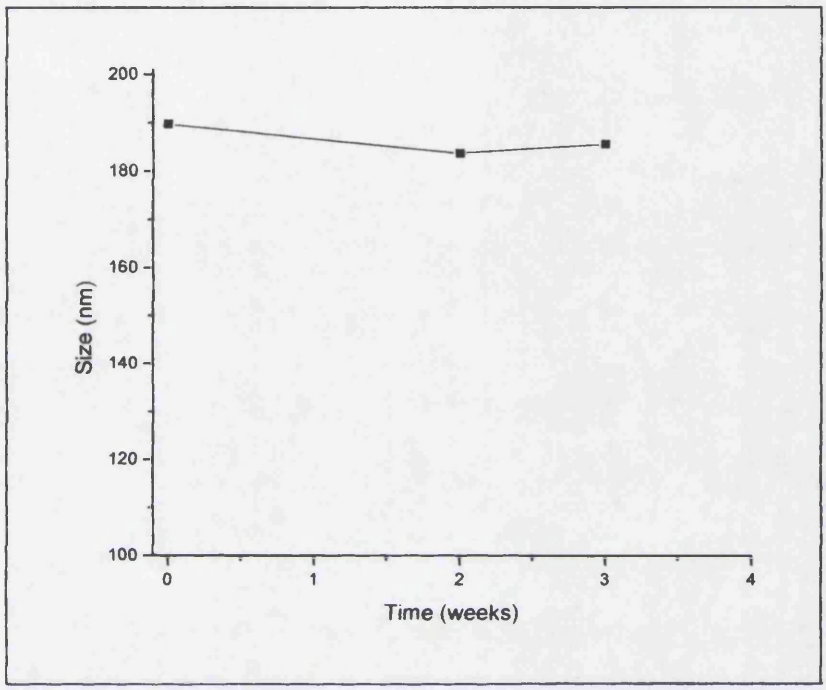
One of the main reasons for the incorporation of PEG into a liposome or any other drug or gene delivery vector is that it has the effect of screening the zeta potential of the particle. The adverse effects of having a strong zeta potential, especially cationic, on the efficacy of the complex has been discussed in chapter 1 section 1.3. These included non-specific binding to cells other than the target cells (which may result in undesirable side effects), triggering the complement system and increased removal from the blood stream by the RES. All of these factors will reduce the likelihood of transfection and expression of the therapeutic protein. The effects of PEG 550, 1000, and 2000 Da upon the zeta potential of the liposomes are shown in Fig 6.2. The result shows that the smaller PEG 550 has little effect upon the liposomes having a zeta potential close to that of the conventional liposomes. However PEG 1000 and 2000 both resulted in a substantial alteration of the measured zeta potential due to the charge screening effect of the molecule with PEG 2000 resulting in liposomes with a zeta potential of –15 mV compared to the typical –45 mV of conventional liposomes. This effect would be expected to result in the increased likelihood of these partials aggregating according to the theories discussed in chapter 5. This however was not the case as can be seen in Fig 6.3. PEG 2000 is in fact the most commonly used molecular weight PEG in the literature.

Fig 6.2.



The affect of PEGylation on the zeta potential of liposomes.

Fig 6.3.



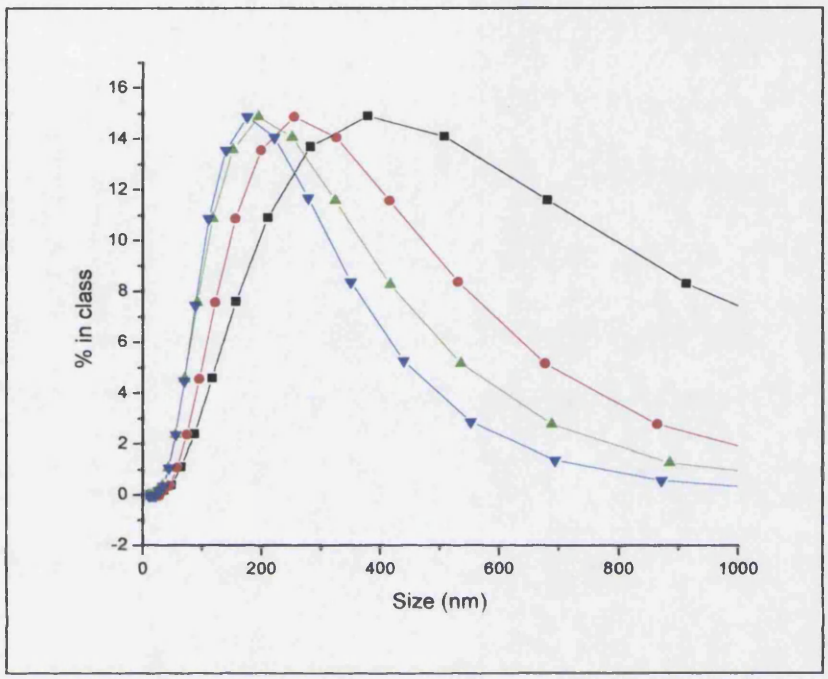
Mean particle size of PEG 2000 Da liposomes over a period of 3 weeks following processing. The suspension was stored at 4 °C.

6.4. Effect of PEG upon the Formation of Liposome Encapsulated PLL/DNA Complexes.

In the majority of the papers in the literature that discuss the use of PEG, the advantages to delivery by both *in vivo* and *ex vivo* methods are the focus. Whether these are advantageous or disadvantageous upon processing has so far been ignored. Fig 6.4. shows the effects of lipid: DNA ratio upon the size distribution profiles of PLL/DNA/liposome complexes containing PEG 2000. The size distribution results are similar as those produced for the conventional and immunoliposomes with low ratios producing larger complexes than the higher ratios. What is also interesting is that again the optimum lipid: DNA ratio is 8.

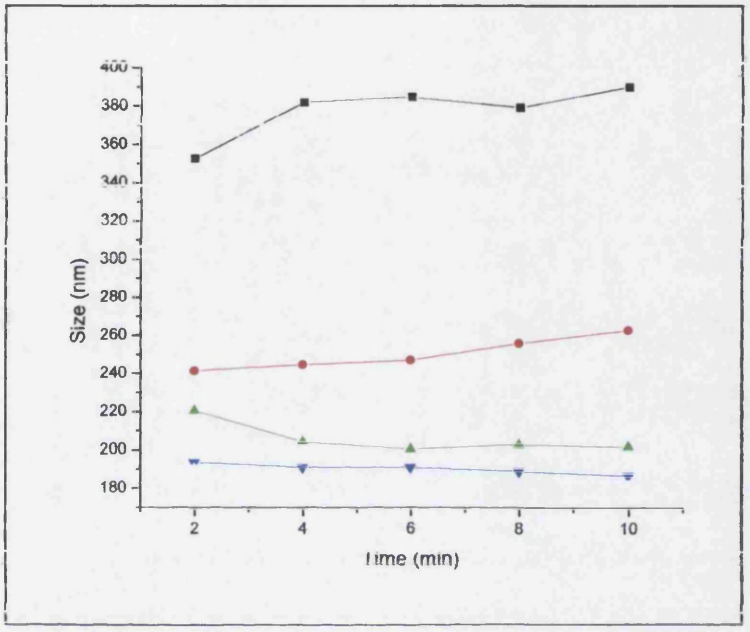
In the case of the conventional liposomes and the immunoliposomes studied, aggregation was seen to occur at the lower lipid: DNA ratios studied due to these ratios being close to the isoelectric point of the system. However as shown in fig 6.5 this was not seen, here demonstrating the stabilising properties of the PEG under conditions that would otherwise result in the rapid aggregation of the particles. Fig 6.6, in which the stability of the complexes is observed for an extended period, confirms this observation further showing that even at low lipid: DNA ratios the complexes were able to maintain their size while even the most stable PLL/DNA/liposome was only stable for approximately fourteen days under optimum storage conditions.

Fig 6.4.



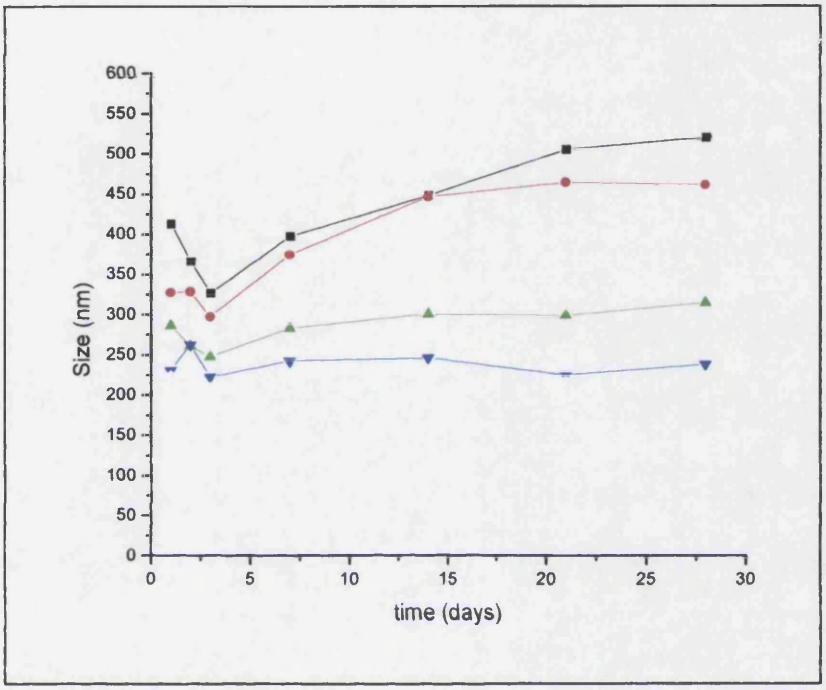
Typical size distributions for PLL/DNA/PEG 2000 Da liposome complexes 10 min following mixing of PLL/DNA with PEG 2000 Da liposomes at lipid: DNA ratios of 1 (■), 2 (●), 4 (▲) and 8 (▼).

Fig 6.5.



Short term stability of PLL/DNA/liposome complexes generated by mixing PEG 2000 liposome and PLL/DNA at lipid: DNA ratios of 1 (■), 2 (●), 4 (▲) and 8 (▼).

Fig 6.6.



The mean particle size of distributions of PLL/DNA/PEG2000 liposome complexes generated by mixing PLL/DNA and liposomes at Lipid: DNA ratios of 1 (■), 2 (●), 4 (▲) and 8 (▼) as a function of time. The complexes were stored at 37 °C.

Another difference between conventional and PEGylated liposomes is that the zeta potential of all of the complexes has been virtually completely masked by the presence of the PEG 2000 (Fig 6.7.). This is of great importance, as it would suggest that the circulation time within the body of these complexes would be greatly improved. However it should be noted that the molecular weight of the PEG is important. The use of PEG 550 and 1000 Da although marginally masking the charge of the uncomplexed liposomes had no detectable effect upon the charge of the DNA complexes both producing complexes at a Lipid: DNA ratio of 8 with a zeta potential of approximately -25mV, while the PEG 2000 liposomes produced near neutrally charged complexes at this ratio. This is a highly desirable result, in chapter 1 section 1.3.1.1 it was stated that the ideal vector formulation would be either neutral or have its charge masked as this would aid in improving its circulation time within the blood stream.

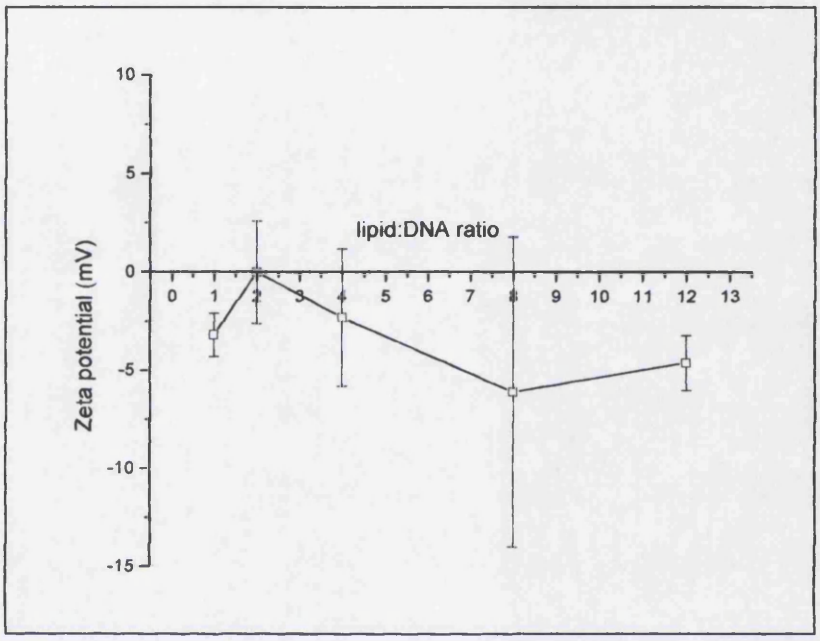
The formulation that has been used here has been shown in chapter 4 to have high levels of DNA encapsulation. Fig 6.8 shows the effect of the presence of PEG upon the efficiency of PLL/DNA encapsulation by the liposomes. The figure shows that the presence of PEG 550 has little effect upon encapsulation efficiency of the liposomes while PEG 1000 and 2000 Da hinder encapsulation efficiency with the PEG 2000 Da resulting in a drop of approximately 20 % when compared to the conventional liposomes with a mean particle size of approximately 130 nm.

The reason for this is probably a consequence of the steric barrier that the PEG conveys upon the liposome. It is this barrier that prevents the liposomes from aggregating.

However, this same barrier will also prevent liposomes and the PLL/DNA complexes from interacting and therefore reduce the efficiency of encapsulation.

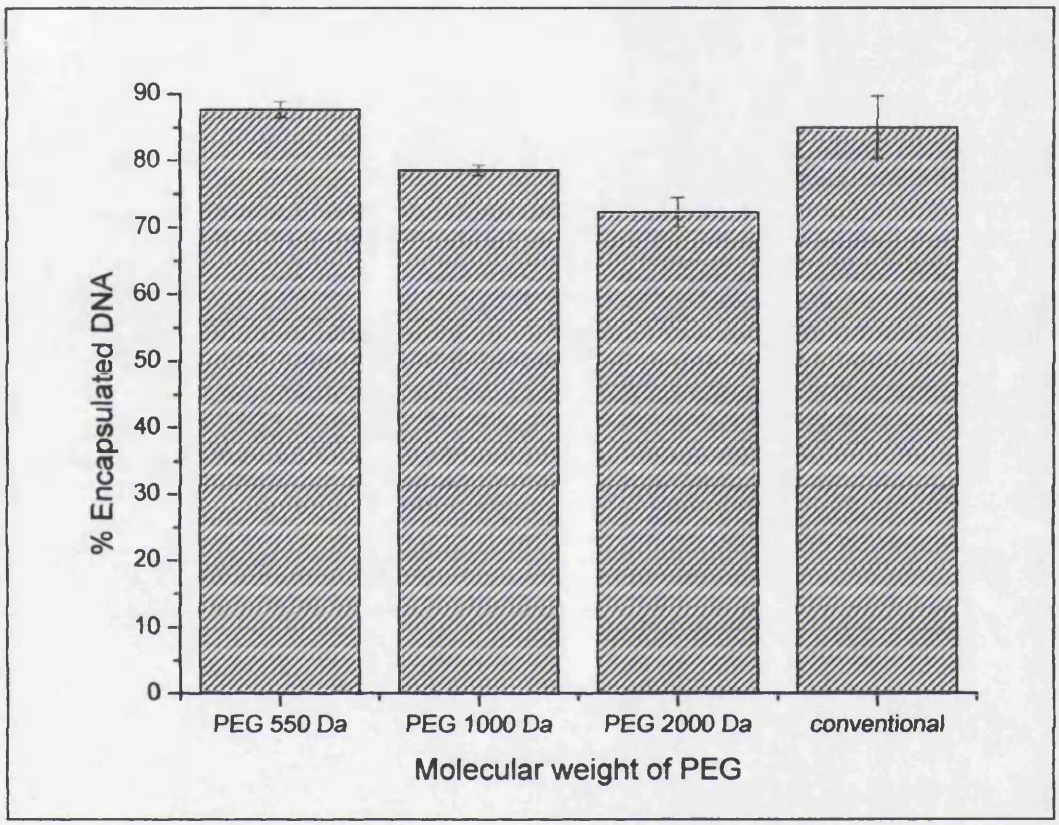
This result is of course highly undesirable and shows a direct relationship between potential problems in processing of the complex and the presence of PEG in a formulation. Unfortunately due to the cost and lack of time it was not possible to investigate the effect of the presence of PEG upon the attachment of Fab' to the surface of the liposomes. However it is speculated that this would be hindered.

Fig 6.7.



Zeta potential of PLL/DNA/Liposomes possessing PEG 2000 Da as a function of Lipid: DNA ratio.

Fig 6.8.



Effect of the molecular weight of PEGylated liposomes on the encapsulation efficiency of the liposomes. Standard errors are for 14 replicates in the case of the PEG 550, 9 for the PEG 1000 and 16 for the PEG 2000 liposomes. Also shown is the result for conventional liposomes with an initial mean particle size of 130 nm (see Fig 4.8).

6.5. Effect of PEG Upon the Surface Tension of Liposomes.

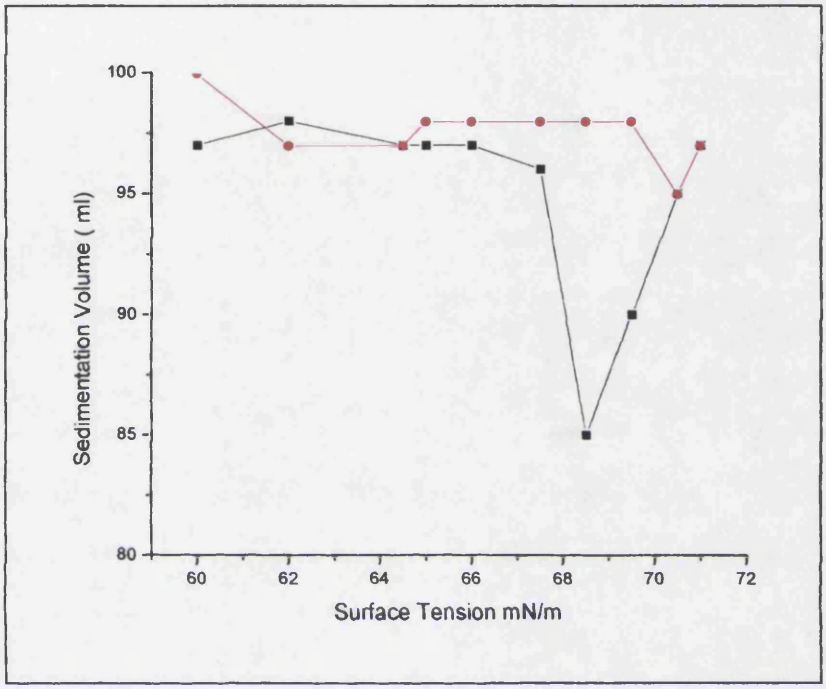
Surface/interfacial tension may have several effects upon the characteristics of the liposomes, for example, affecting downsizing, stability and transfection rates by altering the likelihood of the complex fusing with biological membranes.

In order to investigate the effect of PEGylation upon the surface tension of the liposomes the method of sedimentation was used (Vargah-Butler *et al.,* 1989). This technique is often used in the study of the stability of dispersions of particles in liquid media. The method works due to the fact that when the surface tension of the suspending liquid is equal to that of the particles the free energy of cohesion (ΔF^{coh}) will be 0 as;

$$\Delta F^{coh}=-2\gamma_{PL} \qquad \text{(Equ 6.1)}$$

where $-2\gamma_{PL}$ is the particle/liquid interfacial tension. It therefore follows that under such conditions a maximum or minimum in sedimentation of the particles would be seen.

Fig 6.9.



Sedimentation volume as a function of surface tension of the suspending liquid (n-propanol/water) for conventional liposomes (■) and liposomes PEGylated by PEG 2000 (●) Minima are at 68.5 mN/m for conventional liposomes and 70.5 mN/m for PEG 2000 liposomes.

Fig 6.9 shows the sedimentation volume of conventional and PEG 2000 MLV following three weeks. From the figure it can be extrapolated that the surface tension of the conventional liposomes is 68.5 mN/m. This result compares well with previous experiments by Vargaha-Butler & Hurst 1995 in which the surface tension of empty phosphotidylcholine based liposomes having a cholesterol/ phosphotidylcholine ratio of 0.5 had a surface tension of approximately 68 mN/m. This work showed that increasing the cholesterol content of the liposome resulted in a fall in the surface tension of the liposome.

The surface tension of the PEG 2000 MLV can also be extrapolated from Figure 6.9. to be approximately 70.5 mN/m. The presence of the PEG therefore resulted in a shift in the surface tension of the liposomes towards that of water (72 mN/m), which might be expected, given that PEG is a highly hydrophilic polymer. Although the results for stability experiments have shown that under the conditions studied PEG results in complexes with improved stability behaviour this result suggests that under extended periods of storage that the PEGylated liposomes may in fact become increasingly likely to aggregate overtime if stored in buffers with surface tensions close to that of water.

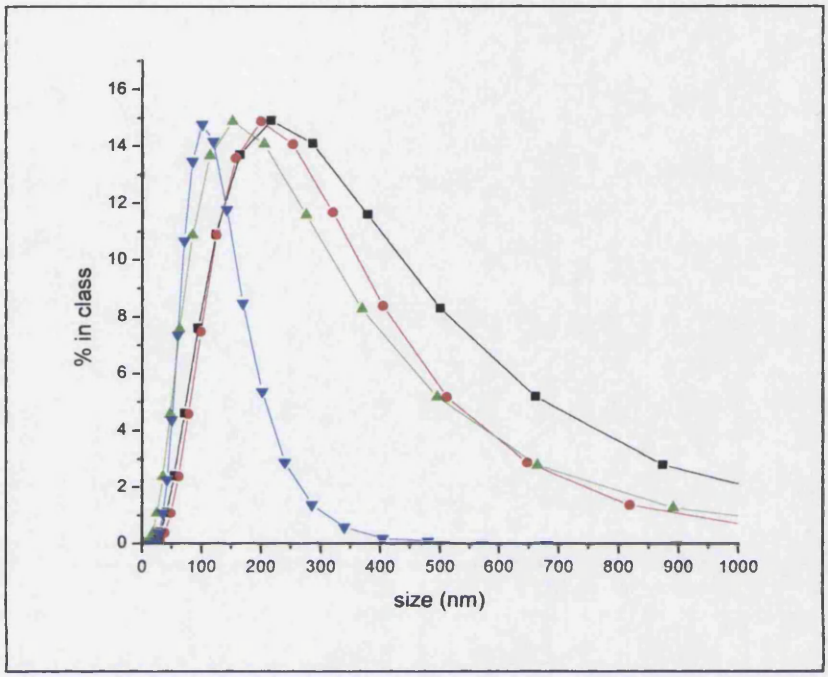
The surface tension of a liposome has several important consequences, which will affect the overall stability of the suspension. Because liposomes are composed of membranes similar to those of cells, fluids may diffuse in and out of them. They can therefore act as miniature osmometres. This was demonstrated in Fig 6.10 in which the size distribution of conventional and PEG 2000 liposomes is shown before and after incubation with 300

mM NaCl (twice the ionic strength of physiological fluids). The figure shows that the size of both suspensions decreases following addition of salt, a consequence of the osmosis of water from the low ionic strength conditions within the liposome to the high ionic strength conditions outside the liposomes. However this difference is less marked in the case of the PEGylated liposomes which may be an indication of the ability of these liposomes to tolerate the harsh conditions of the physiological environment better than conventional liposomes.

In studies with emulsions it has been shown that increased surface tension will result in an emulsion with increased droplet size (Garti, 2000). This has also been seen in this work. This may account for the increased size of the PEGylated unilamellar vesicles generated for a given pressure (using the jet homogeniser) than the corresponding conventional liposome (see fig 6.1.). This same work also shows that emulsions with increased surface tensions also exhibit reduced lifetime in suspension due to increased coalescence. This may therefore mean that although the PEG is improving some aspects of the stability of the particles other aspects will be adversely affected.

The knowledge of the surface tension of these particles may help in the design of long storage strategies. By selecting storage buffers with surface tensions significantly different from those of the suspended particles, settling can be reduced increasing stability.

Fig 6.10



Effect of the addition of 300mM NaCl to liposomes. Shown are typical size distributions plots for Conventional SUV prior to the addition of NaCl (▲), liposomes possessing PEG 2000 prior to NaCl addition (■) Conventional SUV following NaCl addition (▼) and PEG 2000 SUV following NaCl addition (●).

6.6. Summary.

The effects of the hydrophilic polymer Poly Ethylene Glycol upon the processing and biophysical characteristics of the liposomes and their complexes with PLL/DNA have been investigated. Several advantages and also some unexpected disadvantages of using PEG were uncovered. The advantages of using PEG included improved stability of the PLL/DNA/liposome complex under conditions which had in chapter 4 been shown to result in the rapid aggregation of the particles. Namely when lipid: DNA ratios are low which equates to a system at or close to its isoelectric point. The presence of the PEG was shown to effectively mask the charge of both the liposome and the PLL/DNA/liposome complexes which have been shown in previous work to improve circulation time within the body and therefore the likelihood of the transfection of target cells.

However, the presence of PEG was also shown to adversely effect the processing of the complexes by hindering the encapsulation of PLL/DNA within the liposome by as much as 20 %. In addition the presence of PEG resulted in an enlargement in the size of the liposomes. This was in part predicted from the increased surface/interfacial tension of the PEGylated liposomes compared to their conventional equivalents.

Similarities in the relationship between the biophysical characteristics and surface tension of liposomes and emulsions may suggest that some of the studies carried out on emulsion processing may have consequences upon liposomes.

7. Discussion and Conclusions.

Here the results introduced in chapters 3 to 6 will be brought together and discussed in the overall context of the processing of liposomes for use in the delivery of plasmid DNA for use in gene therapy and DNA vaccination. Each unit operation will be described and the implications of the experiences of this project and related literature will be discussed and future recommendations made. Points for consideration will primarily focus upon factors that are particularly important to the bioprocess industries such as cost and whether the process is scaleable.

7.1. Formation of MLV.

Multilamellar large vesicles (MLV) were prepared by a commonly used two step process in which the lipids were mixed together in an organic solvent mixture of chloroform and methanol (4/1 v/v) in a round bottom flask. The solvent was then removed by rotory evaporation and further dried in a vacuum oven both at a temperature of 60 °C. This process resulted in the formation of a thin lipid film on the bottom of the flask which, upon exposure to an aqueous medium (in this case 10 mM HEPES Buffer pH 8) and agitation will spontaneously form MLV due to the bi-polar nature of phospholipids.

Although this method is commonly used in the literature and is relatively simple and inexpensive to perform (especially at laboratory scale), it is difficult to see how it could be effectively scaled up. Even in the work carried out here when marginally larger

quantities of organic solvent were used for the preparation of quantities of liposomes greater than 50 ml at 3.6 μmol lipid, problems occurred due to condensation of methanol within the neck of the flask. This had the effect of causing some of the lipids to come out of solution resulting in the final liposome suspension being contaminated by insoluble lipid flakes which made accurate monitoring of lipid concentration difficult. This problem could be eliminated through the reduction of the percentage of methanol in the solvent buffer and the use of the minimum solvent volume possible. However this may not be feasible at industrial scale. Also at industrial scale it would be undesirable to handle large volumes of organic solvents.

Alternative methods for the production of liposomes which are suitable from an industrial perspective have not been investigated here but have been reviewed by Watwe & Bellare, (1998) and include Freon injection, detergent dialysis, microfluidisation and ethanol injection. Of these methods ethanol injection and modifications of this procedure for pilot and industrial scale are common in the literature. In a recent report Wagner *et al.*, (2002) have demonstrated a pilot scale modification to the ethanol injection method. In this process liposomes are prepared by way of a cross flow injection procedure.

One of the disadvantages of these procedures is there inability to form MLV. Although this is not an issue in this project, for many applications of liposomes MLV offer several advantages over small unilamellar vesicles (SUV) or large unilamellar vesicles (LUV) such as, increased reservoir volume in the case of drug loading. Endruschat & Henschke

(2000) discuss the use of a scaleable bench top method for the manufacture of multilamellar liposomes using a newly developed multistage pressure filtration device.

A one step method for the production of MLV has been put forward by Brandl *et al.*, (1990). This method involved the mixing of lyophilized lipids in aqueous media and homogenising the mixture to form liposomes. However in a recent study here at UCL (Tsai 1999) this method was found to be difficult to reproduce using the Lab 40 homogeniser. Due to the probability of blocking the nozzle of the jet homogeniser this method was not further investigated here.

7.2. Downsizing MLV.

Of the methods that have been discussed in this project for the downsizing of liposomes the best suited for the task at an industrial scale are homogenisation and extrusion. The results of chapter 3 clearly show the ability of the high pressure jet homogeniser for this task. Not only was it possible for efficient downsizing to occur after a single pass through the device but the resulting suspension had a size distribution suitable for filter sterilisation. The device also has the additional advantage of being able to control the size distribution of the suspension in a reproducible manner by altering the operational pressure or number of passes through the jet homogeniser.

Other homogenisers that have been discussed in the literature include the Microfluidiser (Talsma *et al.*, 1989); the Gaulin Micron Lab 40 (Brandl *et al* 1990) and the mini–lab 8.3

H high pressure homogeniser, from APV Rannie, Albertalund, Denmark (Bachmann *et al.*,1993). All of these methods share with the jet homogeniser the ability to alter the operating pressure and number of passes through them. The microfluidiser however requires multiple passes before a comparable size distribution can be obtained which is suitable for filter sterilisation (Talsma *et al.*, 1989; Sorgi & Huang 1996). These methods are reported to have the ability to manage high concentrations of lipids. The microfluidiser is able to effectively downsize liposomes at lipid concentrations of 400 μmol/ml, which is over 100x that used in this study. However, although it was not investigated here the similarity of the two devices would suggest that the jet homogeniser may also possess this ability. Tsai 1999 reported in an experiment using the Lab 40 for downsizing liposomes that upon overnight storage precipitates formed within the suspension. No such problem was observed with the jet homogeniser.

All the above studies also discuss the important role that lipid composition plays in the degree of downsizing that is likely to occur. Of the most widely used components of liposomes, cholesterol has been shown to have the greatest effect. Cholesterol containing liposome formulations will generally undergo downsizing up to between 5 and 10 passes through a device. Further passes will result in the gradual increase in the size of the particles produced (Bachmann *et al.*, 1993). For this reason the number of passes through the jet homogeniser in this study was maintained at five.

Extrusion through polycarbonate membranes is another technique for MLV disruption, which also has the ability to be scaled up to industrial level. By employing appropriate

pore sizes the final size of the liposome suspension can be controlled (Mayer *et al.*, 1986). The liposomes produced by passing through membranes with pores sizes between 200 and 400 nm are generally smaller than the diameter of the pores however those produced by passing through membranes of smaller pore sizes, between 30 and 50 nm produce liposome suspensions which are larger than the pore size.

The pore size was also shown to affect the lamellarity of the liposome suspension. 8 Passes through a membrane with pores of 100 nm was shown to be sufficient to produce a suspension of unilamellar vesicles. However, MLV remained in the suspension when passed through membranes with pore sizes greater than 200 nm. This however was improved by freeze thawing the suspension prior to extrusion (Mayer *et al.*, 1986). Extrusion pressure has also been shown to be an important factor in the efficient disruption of liposomes by extrusion. It is not possible to downsize a liposome suspension by extrusion below a lipid dependent minimum pressure (Hunter & Frisken, 1998).

One of the major disadvantages of extrusion from an industrial scale perspective is that the majority of the devices available for the extrusion of liposomes are operated discontinuously. However an extruder is now available which can operate continuously and at high pressures, Dispex Maximator®, (Schneider *et al.*, 1994). This device has been compared with a discontinuously operated extruder, the Avestin Extruder, which showed that it could operate at higher flow rates resulting in smaller liposomes and reduced processing times (Berger *et al.*, 2001). Although the jet homogeniser was used in a

discontinuous mode in this study it would be a simple procedure to operate it in a continuous mode.

An additional industrial related disadvantage of extrusion through polycarbonate membranes is the clogging of the filters, which is particularly a problem at high lipid concentrations, when there is a high cholesterol component to the formulation or when the size of the liposomes to be extruded is greater than that of the pores. This is also a problem since it will result in a loss of lipid from the suspension. It is difficult to clear most devices because they do not allow back flushing. The only solution for this problem is to remove and replace the membranes, however this can be expensive and may compromise the sterility of the procedure (Betageri & Kulkarni 1999).

7.3 Encapsulation of Plasmid DNA.

When liposomes are produced using the method employed in this study some of the hydrating solvent and solute molecules will be trapped within the liposome lumen. This is the traditional way of loading liposomes both for use in drug and gene delivery. Given that DNA is such a large molecule its encapsulation efficiency is much lower than for many other macromolecules such as proteins. This and the fact that liposomes generally do not encapsulate more than 50 % of the preparation buffer results in DNA encapsulation rarely exceeding 20 %.

In order to improve the encapsulation efficiency of liposomes one of two methods can be followed. Firstly, altering the method of liposome formation to maximise lipid concentration and vesicle size and secondly changing the physical nature of the DNA in solution in order to facilitate the entrapment of the DNA within the liposomes (Hug & Sleight, 1991). In this project the latter has been adopted.

The methods of liposome processing that have been developed for improved entrapment of DNA include reverse-phase encapsulation, dehydration-rehydration and detergent dialysis. These have been reviewed elsewhere (see chapter 1 section 1.5). Despite the improved encapsulation efficiency it still remains lower than what would be desirable. For example, reverse phase evaporation manages to encapsulate approximately 40 % and dehydration –rehydration 60 % of plasmid DNA. In addition the size of the resulting complexes is larger than is desirable for *in vivo* delivery (Hug & Sleight 1991). This poor encapsulation efficiency would therefore necessitate additional processing steps in order to remove free DNA and empty liposomes from the complexes thereby adding to manufacturing costs.

The formation of cationic liposomes is another method that has been developed for the improvement of plasmid DNA encapsulation. These have also been extensively reviewed elsewhere (see chapter 1). Although they show high levels of DNA encapsulation and transfection *ex vivo, in vivo* they present several problems including, reduced circulation time, increased non specific binding, increased immunogenicicty and high levels of cytotoxicity (Li & Huang 2000).

Methods that involve altering the structure of the DNA in order to improve encapsulation efficiency of DNA into anionic or neutrally charged liposomes generally involve methods that result in the condensation of the polymer. This may be achieved through the formation of DNA/polymer complexes (often referred to as polyplexes in the literature) with cationic polymers such as polyethylimine (PEI) or poly-L-lysine (PLL) (Lee & Huang 1996), which has been the method employed in this project. Alternatively Bailey & Sullivan (2000) have developed a method that exploits the fact that divalent metal ions such as Mn^{2+}, Ni^{2+}, Co^{2+} Cu^{2+} and Ca^{2+}, are capable of condensing DNA through neutralisation of the phosphate backbone. Additionally Ca^{2+} ions also binds to nitrogen and oxygen of guanine further increasing the effect. The presence of ethanol reduces the concentration of ions required. The presence of both Ca^{2+} and ethanol also results in the destabilising of liposomes. By mixing plasmid DNA and liposomes in the presence of Ca^{2+} and ethanol followed by dialysis they were able to trigger the condensation of DNA, and aggregation of SUV resulting in the encapsulation of the DNA within liposomes (Bailey & Sullivan, 2000). This method resulted in the formation of complexes less than 200 nm in size and with an encapsulation efficiency of 80 %, which compares well with the results from this study.

The method of encapsulation of DNA into anionic liposomes used in this project is close to that first developed by Lee & Huang (1996). The results obtained in this study also compare well with those reported with high levels of DNA encapsulation (~90 %) and the generation of complexes that were of a size suitable for *in vivo* delivery. The fact that these two similar methods employ different formulations demonstrate that the

condensation of DNA by cationic polymers followed by the encapsulation of these complexes by anionic liposomes may present a method of encapsulation which will eliminate the potential problems associated with cationic liposomes (see above) and anionic liposomes such as low levels of encapsulation.

As problems such as poor levels of transfection or low circulation times are discovered during the development of gene delivery vectors the addition of components to the formulation, which are designed to rectify the problem, becomes increasingly likely to be included into the final complex. It is reasonable to predict that as the complexity of a formulation is increased its processing may be adversely effected. In order to investigate this problem two molecules which are often attached to the surface of liposomes (Fab' to improve targeting and PEG to increase stability and circulation time within the blood stream) were investigated.

PEG being a large polymer conveys steric stabilisation upon the particle. Steric stabilisation of a suspension of colloidal particles is via the addition to the surface of the particles of a polymer the effect of which will be to prevent the particles from interacting with each other and therefore aggregation (Napper, 1983). Given that PEG will prevent the interaction of particles in suspension it would therefore be expected that its presence will also reduce the interaction between PLL/DNA complexes and liposomes thereby having a negative effect upon encapsulation of the DNA. This was indeed shown to be the case with PEG reducing the encapsulation efficiency of the liposome by approximately 20 %.

However, Fab' being a relatively small protein would not be expected to hinder the encapsulation of the PLL/DNA in such a dramatic manner. This however was not the case with reduction in encapsulation efficiency also of approximately 20 %. Why this occurred is unclear at this time and has not been reported elsewhere in the literature. One possible explanation is that the Fab' is acting in a similar manner to the PEG in that its effect is via steric hindrance. However this is unlikely given the different structures of these two molecules.

An alternative explanation can be reached if the interaction between liposomes and PLL/DNA is analysed by DVLO theory using the method of Lee *et al.,* 2001 in the same manner as has been employed to predict stability of the complexes in chapter 5. Figure 7.1 shows a plot in which the amount of energy two particles (one being a liposome the other being a PLL/DNA particle) of equal size must overcome in order to fuse together. The plot shows that although energetically favourable the formation of PLL/DNA/immunoliposome complexes is not as likely to occur as the interaction between conventional liposomes with PLL/DNA. This is seen as a lowering of encapsulation efficiency. It can only be expected therefore that the addition of any targeting moiety, which carries a charge (such as a peptide) to the surface of a liposome will adversely effect encapsulation of DNA especially if its presence results in a decrease in the anionic charge of the particle.

These problems will result in the amount of DNA and liposomes that will be required to be increased which will have the additional effect of increasing costs of manufacture. It

would therefore be necessary to evaluate the increased dose size for conventional liposome compared to the increased cost of manufacture of modified liposomes.

7.4 Storage.

Most of the studies focusing on non viral methods of gene delivery for gene therapy or DNA vaccines have focused upon improving the efficiency of gene transfer. By comparison there has been little research upon improving the physical stability and prolonged storage of the vectors in order to achieve the levels that will be required to satisfy the demands of regulatory authorities of approximately 2 years. There are three states in which non viral vectors can be stored, namely as a liquid formulation, frozen formulation or a dehydrated formulation.

Liquid formulations of non viral vectors are generally unstable and have a tendency to aggregate over time. This has been shown in several studies (Tsai *et al.*, 1999; Lee *et al.*, 2001) and was also shown in the results of the work carried out in this study (see chapter 5). Although it was shown in chapter 4 that liposome encapsulated PLL/DNA possessed higher levels of stability than unencapsulated PLL/DNA complexes, aggregation still occurred following 14 days storage at 4 °C.

There are two techniques that can be employed in order to improve the physical stability of liquid formulations. It has been shown that suspensions that have low polydispersity, i.e. are highly monodisperse, have greater stability than heterogeneous suspensions. Methods that increase the homogenicity of the suspension will therefore improve stability. Gao & Huang 1996, used sucrose density gradients in order to isolate the stable fraction of cationic liposome/DNA complexes which maintained size and

transfection efficiency for three months. However it is doubtful that this separation technique could be easily scaled up to industrial levels of manufacturing (Anchordoquy *et al.*, 2001).

Steric stabilisation is an alternative method for improving the stability of liquid formulations by preventing the interaction of particles in the suspension (see chapter 1 section 10). The attachment of PEG (see chapter 6) is one of the most popular methods employed in the literature. Several groups have reported the use of PEG in a large variety of DNA complexes including liposomes and this has also been the method employed in this study.

The results of chapter 6 have shown that the incorporation of PEG into the formulation will improve stability of the complexes. Even complexes that before aggregated following mixing showed improved levels of stability. In the case of the most stable complexes made with conventional liposomes the addition of PEG resulted in complexes that were stable for over twice as long.

However there are problems associated with the incorporation of PEG such as hindering the encapsulation of DNA as is discussed above. PEG may also hinder transfection by curtailing the interaction between the complex and the cell. Kaasgaard *et al.*, (2001) have shown that the attachment of PEG to liposomes reduces the binding of avidin to biotin attached to the liposome surface with both increased PEG chain length and surface density resulting in decreased binding.

An alternative way to improve stability in storage is to freeze the suspension. However, excipients are often required to prevent damage of the DNA or to aggregation during the freezing process. Even so this method is not a suitable method for storage over long periods of time. The stability of the formulation in the frozen state often requires strict maintenance of storage temperatures to prevent thawing or the crystallisation of excipients. Additionally transportation is difficult and costly even within a single country (Anchordoquy *et al.*, 2001).

The problems with storage at low temperatures has generated interest into the development of dehydrated formulations (lyophylisation) which can be stored at room temperatures and are ready to use after a simple rehydration step. Lipid-DNA complexes have been shown to loose transfection activity following lyophilisation. The addition of disaccharides to the complexes (up to 1 % w/v before lyophilisation) decreases the aggregation that may occur and preserves transfection (Allison *et al.*, 1999). The disaccharides replace the water and hydrogen bonds during the dehydration process and in so doing help to maintain the integrity of the bilayer. Polysaccharides do not hydrogen bond due to steric hindrance and therefore do not prevent the loss of transfection efficiency that occurs after lyophilisation (Allison *et al.*, 1999). The sugars are also believed to help to isolate particles in the unfrozen fraction of the suspension during the freezing step thus preventing aggregation. This is known as the particle isolation theory (Allison *et al.*, 2000). It should however be noted that the high excipient to DNA ratio that is required would make it difficult to achieve the DNA concentrations required for

clinical trials. No comparable studies have been carried out upon the effects of lyophilisation upon complexes resembling those used in this study.

7.5 Gene transfer.

Due to lack of available time it was not possible to carry out transfection studies upon the formulations used in this work. However as gene delivery will be the final use of these complexes it is worth discussing recent studies with non viral vectors that may shed some light on possible future directions for this work.

Cationic liposome/DNA complexes are the most commonly discussed non viral gene delivery vectors in the literature. There are currently over 50 different gene delivery formulations involving cationic lipid on the market word wide. Recently a number of these vectors have been compared together in their ability to transfect NIH 3T3 fibroblasts with a green fluorescent protein carrying plasmid (pQBI 25) (Simberg *et al.*, 2000). This study showed that the majority of the formulations transfect in a similar manner. It may be therefore that there is a rate limiting step in the transfection process that occurs after the lipid and DNA are no longer together which universally hinders transfection, such as cellular transport to the nucleus. The identification of this step would go a long way to improving non viral gene delivery.

The charge of the liposome has been shown to be an important factor in cell binding. In a resent study cationic liposomes with a zeta potential of +50 mV were shown to have

binding constants of 5 x10^{10} M^{-1} (Chenevier *et al.*, 2000). Although this is advantageous for *in vitro* gene delivery it will lead to non specific binding *in* vivo. Liposomes with a zeta potential of –20 mV (which is comparable to the complexes made in this study) were shown to have no detectable interaction with cells *in vitro* (Chenevier *et al.*, 2000). The addition of targeting moieties to the surface of the liposome can circumvent this for example; immunoliposomes with 0.5 % anti HeR2 Fab fragments similar to those used in this study have been shown to have binding constants of 7 x 10^9 M^{-1} to specific receptor bearing cells. (Kirpotin *et al.*, 1997).

Dioleoyl Phosphatidylethyamine (DOPE) is often incorporated into cationic liposome formulations in order to help increase the efficiency of transfection. DOPE is unique among common phospholipids in that in isolation it prefers to adopt a non-bilayer configuration but will adopt a stable bilayer in the presence of co-lipids. In this state it can be induced under specific conditions to form non-bilayer structures such as micelles, which can facilitate fusion. It is believed that fusion of the cationic liposome containing DOPE with the endosome membrane is what promotes transfection. However what may be more important is the ability of DOPE to facilitate mixing of the lipids, which causes perturbation of the endosome allowing the DNA to gain access to the cytoplasm (Mui *et al.*, 2000).

The presence of oleic acid (OA) in the formulation used in this study helps to convey an anionic charge to the liposome. It has however been shown that the presence of OA and DOPE in a liposome formulation results in pH sensitive liposomes which undergo

membrane fusion at approximately pH 6 (Duzgunes *et al.*, 1985). The lowering of the pH results in a neutralisation of the surface charge, which allows the close approach of membranes. This would therefore also occur within the endosome during transfection allowing escape of the DNA into the cytoplasm.

7.6. Conclusion.

The work discussed in this project has focussed on two key issues. These have an important impact on the commercial scale development of liposomes for use in the delivery of plasmid DNA for gene therapy and DNA vaccination. The two issues have been the scaleable and reproducible production of SUV and the effects of attaching targeting moieties (Fab') or stabilising polymers (PEG) to the liposome surface on the processing of the DNA complex. This has been investigated by biophysical characterisation of the liposomes and their complexes with DNA during their processing, focussing on the size, zeta potential, encapsulation of PLL/DNA into liposomes and stability.

Liposomes have potential to be used for the delivery of DNA. A method for their production that could form SUV of a size suitable for use *in vivo* was investigated. This device was a high velocity jet homogeniser, which had first been developed for the disruption of microbial cells. It is a small relatively easy to operate machine, which can be scaled up to industrial level. Previous studies had suggested that the device had potential for downsizing MLV. These studies had looked at the operation of the jet

homogeniser in the formation of oil/water emulations (Soon *et al.*, 2000). The jet homogeniser was indeed found to be able to downsize MLV and could also form a SUV suspension which had a size distribution suitable for sterilisation by way of sterile filtration using a 200 nm sterile filter medium following only a single pass at a pressure as low as 103.4 mNm^{-2}. By altering either the operating pressure or the number of passes through the jet homogeniser it was shown that the distribution of the final SUV suspension could be controlled between approximately 140 and 80 nm. This offers great advantages over conventional laboratory techniques such as sonication.

The method that was used to encapsulate DNA within the liposomes was to firstly condense the DNA using PLL and finally mix the liposomes with the PLL/DNA complexes. This method was first proposed by Lee & Huang (1996), however in this project the process had been modified by the carrying out the mixing using a syringe pump which had several advantages over hand mixing. These include improved potential for scale up and the elimination of variation between both experiments and researchers. It was found that the key processing criteria of the formation of the complexes was charge ratio between the liposomes and the PLL/DNA complexes, with charge ratios close to unity resulting in the formation of complexes which would aggregate quickly following mixing.

Targeting moieties such as antibody fragments are often incorporated into a gene delivery vector formulation in order to convey upon the complex improved selective targeting of cells and reduced non specific binding. Although much has been published upon the

and DNA vaccination. The results discussed advocate that when carrying out studies at laboratory scale the use of scaleable processing equipment should where ever possible be used. This will aid in future process development of the vector. Such devices include the high velocity jet homogeniser and the syringe pump, which have been at the centre of this study.

The sometimes adverse consequences upon processing a vector by the inclusion of additional molecules has also been discussed. The results illustrate the need to examine the effects of the proposed solution upon the biophysical characteristics of the vector. These effects may have important consequences on the large scale processing of the complex. A vector which is able to transfect 100 % of its intended target cells will not help anyone unless it can be manufactured in sufficient quantities. Researchers must identify potential problems as soon as possible in the development of a vector so that these may be addressed. This is only possible if the overall process and scale up is always being considered.

8. Future Work.

It was not possible to carry out transfection studies during the course of this project but this is of great importance for the next phase. It is important to understand how the process parameters discussed effect the gene delivery. In this way it will be possible to develop a vector, which is the best possible compromise between the requirements of the process and the delivery of the DNA

As the work carried out in this project has involved the use of Fab' antibody fragments and the formation of immunoliposomes it will be important to select cell lines that over express the target antigen. In the case of this project the Fab' antibody fragment used was raised against the product of the HER2 proto-oncogene which is over expressed in 25-30% of primary breast cancers (see chapter4). Cell lines that over express the target antigen HER2 include SK-BR-3, SK-OV-3, or MKN7 (Lewis *et al.*, 1993).

As well as transfection, flow cytometry can also provide some useful information regarding ability of a vector to deliver its payload. For example, the technique can be used to determine the binding kinetics of the complexes to cells (Chenevier *et al.*, 2000). The device separates and counts cells on the bases of fluorescence. The fact that the formulation used in this study contains a fluorescent lipid for concentration analysis makes it ideal for such a technique.

Stability of complexes over extended periods has been a subject that has largely been ignored in the literature. It would be useful to carry out extended stability for 2 years or more upon the complexes that have been produced here. Due to lack of available sterile storage facilities it was only possible to carry out short term stability studies during this project however, a longer term or accelerated stability study should form the bases of any future work.

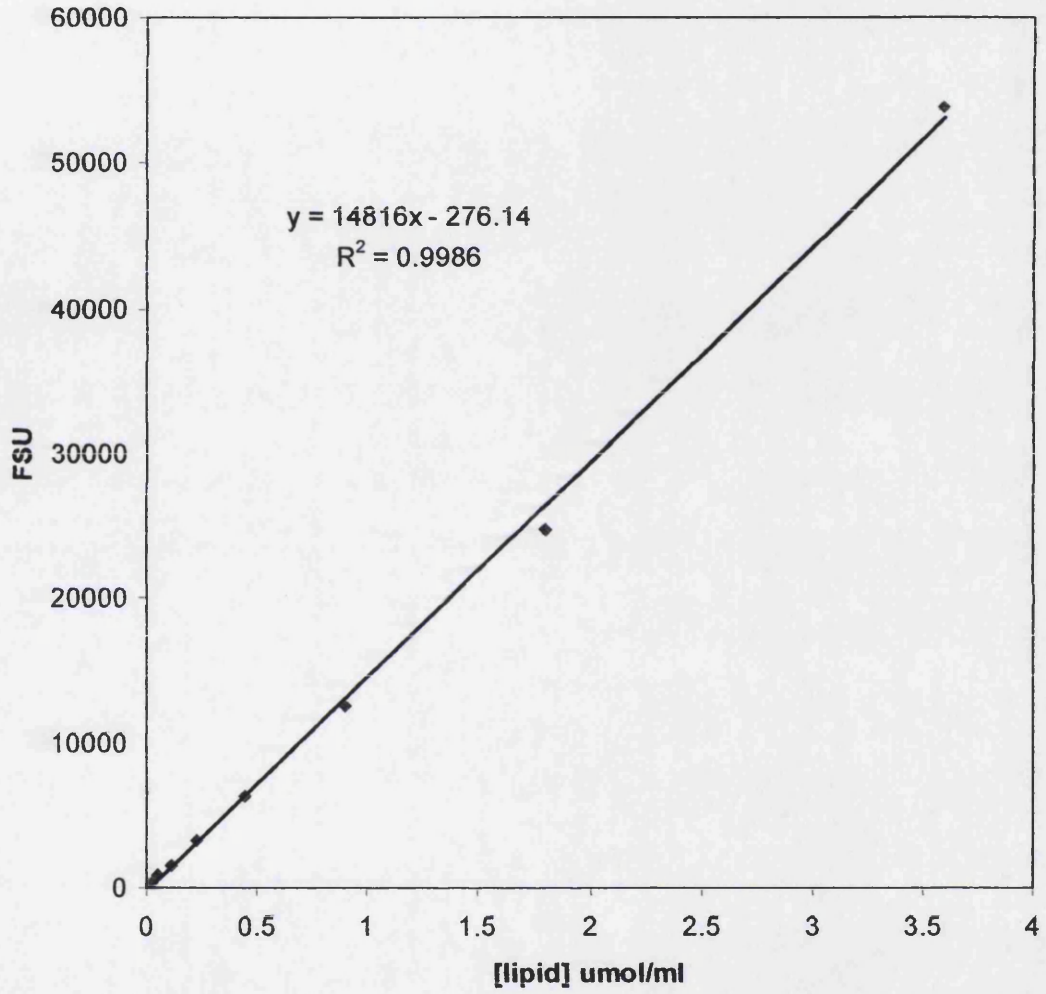
Important issues which need to be addressed insofar as stability is concerned include; optimum storage concentration; temperature; effect of freezing; lyophilisation; excipients; effect of PEG on shelf life; maintenance of gene delivery efficiency.

The use of large plasmids possibly in the order of 200Kbp, in gene therapy and DNA vaccination are likely to gain popularity in the future. Possible advantages of large plasmids include the ability to deliver multiple genes and thereby the prospect of transfection of whole pathways to cells. This would be useful for example in the treatment of cancer where the entire pathway leading to apoptosis could be delivered. Additionally in DNA vaccination one vaccination could be used to inoculate against several diseases at once.

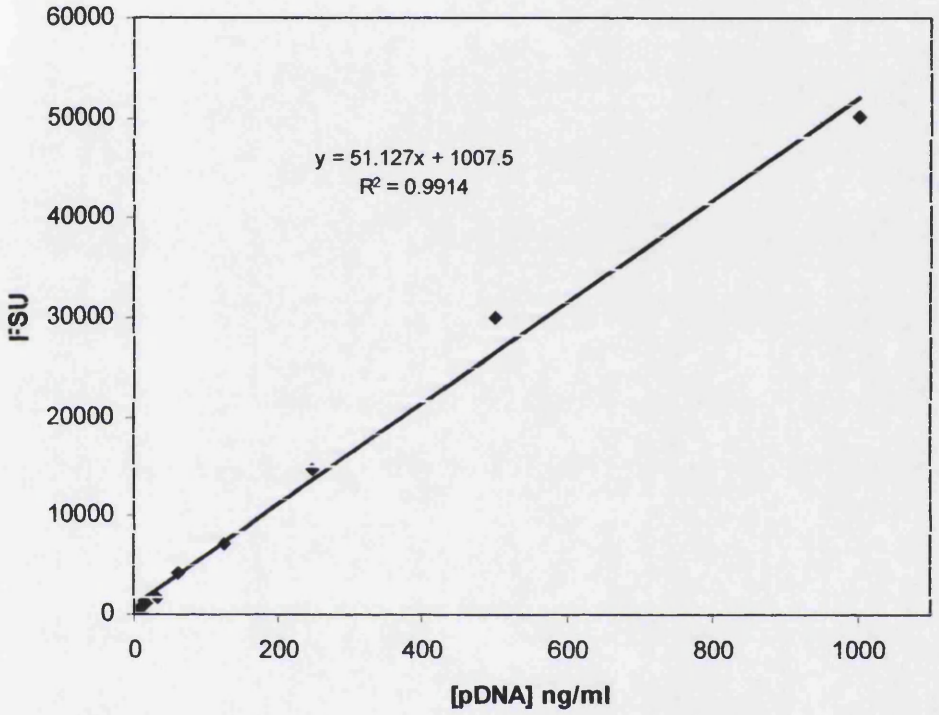
So far little has been published in the literature regarding the processing of large plasmids. One study in which plasmid size has been addressed with plasmids of 6 and 29 Kbp concluded that there were no formulation difficulties (Tsai *et al.,* 1999). However this is only a 5 fold difference in plasmid size and it is vital that if plasmids as much as 20

Appendix

Fig A1 Standard curve for lipid concentration monitoring using the fluorescent lipid marker.



A2. Standard curve for pico green dsDNA assay.



Calculation of Charge Ratios.

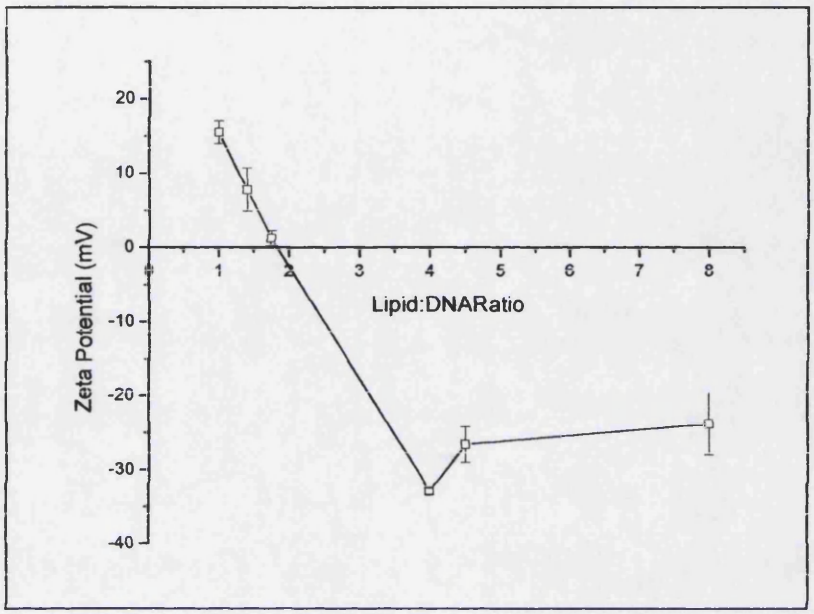
Table A1, Charges and molecular weights of molecules used in this project.

Molecule	**Molecular Weight**	**Charge /Mol.**
DOPC	786.2	0
DOPE	744.44	0
DOPE-MPB	1007.27	-1
Chol	386.7	-1
OA	282.5	-1
DNA (per nucleotide)	330	1
PLL (per residue)	209	+1

Example of calulation of charge ration in the formation of PLL/DNA complexes

Charge ratio = $\frac{\text{Mass of PLL}}{\text{Mass of DNA}} \times \frac{\text{Molecular weight of nuclotide}}{\text{Molecular weight of lysine residue}}$

Fig A4



Zeta Potentail of PLL/DNA/Liposomes prepared by mixing at various ratios of Lipid to DNA. The zeta potential of unencapsulated PLL/DNA is +20 mV while that of empty liposomes is approximately –45 mV.

n-prpanol/water V/V %	µl n-propanol/ 50ml water	γLv mN/m
0	0	71
0.1	50	70.5
0.25	125	69.5
0.4	200	68.5
0.6	300	67.5
0.8	400	66
0.9	450	65
1	500	64.5
1.5	750	62
2	1	60

Table A2. Surface tension of Biopolor buffers used for the preparation of Fig 6.9.

Publications

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Preparation of small unilamellar vesicles (SUV) and biophysical characterization of their complexes with poly-L-lysine-condensed plasmid DNA

Leigh A. Maguire, Hu Zhang and P. Ayazi Shamlou[1]

Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, U.K.

Liposomes have numerous applications in the (bio) pharmaceutical industries as agents in the synthesis of new biomaterials for use in areas including gene delivery. There is currently a need to establish efficient scaleable methods for the manufacture of liposomes, and in the present paper we describe the operation of a new high-velocity jet homogenizer for downsizing of multilamellar large vesicles to produce small unilamellar vesicles (SUV). Measurements of size distribution of SUVs are presented and compared with mathematical simulations based on the solution of a population balance equation combined with computational-fluid-dynamics analysis of flow in the homogenizer. Anionic SUVs are produced by the new method and incubated with poly-L-lysine (PLL)-condensed plasmid DNA (pDNA) to generate complexes under different physico-chemical conditions. The colloidal properties of the resulting complexes, including their size and charge, are measured using a Zetasizer and the encapsulation efficiency is obtained experimentally using a Pico Green assay. The results show that between 85 and 95% of the PLL-pDNA condensed plasmids were encapsulated by the liposomes, the smaller liposomes being more effective in encapsulating the complexes.

Introduction

The ability of liposomes to encapsulate chemical and bioactive reagents has many applications in the (bio)pharmaceutical sector, where they have been exploited for their potential as drug carriers [1–3]. Recent advances in lipid chemistry have led to new types of physiochemically sensitive liposomes with the ability to respond to an applied stimulus, such as a change in temperature or pH. These liposomes are being assessed as agents in the synthesis of new biomaterials for use in areas including tissue engineering, controlled drug delivery and gene therapy [4–7].

pH-sensitive anionic liposomes, which are the subject of interest in the present study, have the potential to mediate gene transfer. Huang and co-workers [5,6] and Düzgünes and co-workers [7] have demonstrated the encapsulation of plasmid DNA (pDNA) by pH-sensitive liposomes. These liposomes become unstable under acid conditions and do not require the presence of proteins or other macromolecules for fusion to other membranes. The fusion of the liposome and endosomal membrane is triggered in the endosome at about pH 5. In cell transfection, these liposomes were able to bind to, and disrupt, the endosomal membrane, allowing the release of the liposome contents into the cytoplasm. However, encapsulation of pDNA into liposomes is poor [6], and despite methods to improve it [8], encapsulation efficiency rarely exceeds 20%. Recently, anionic liposomes have been shown by electron microscopy to entrap positively charge DNA–polycation complexes via charge interactions with high levels of DNA encapsulation [9]. The present study focuses on this approach.

The ability of liposomes to interact with pDNA is determined largely by the physicochemical properties of the liposomes, including their size, size distribution, lamellarity and charge. The spherical shell of a small unilamellar vesicle (SUV), consisting of a single continuous lipid bilayer, has a size in the order of 100 nm or less. SUVs are more desirable than multilamellar large vesicles (MLVs), which consist of several bilayers and have diameters that can be up to several micrometres. Large vesicles have short half-lives in the blood circulation, and their unfavourable pharmacokinetic behaviour *in vivo* following injection makes them unsuitable for use in ligand-mediated and other forms of cell targeting [10].

Various methods are available for the preparation of SUVs, but, from a process perspective, the formation of stable SUVs with a narrow and predictable size distribution remains a challenge. For example, sonication, although a convenient method at the laboratory scale, does not produce liposomes of uniform distribution and the results are poorly reproducible [11]. Bath sonication cannot

Key words: DNA vaccines, gene therapy, homogenization, liposomes, poly-L-lysine–DNA complexes.

Abbreviations used: MLV, multilamellar large vesicles; SUV, small unilamellar vesicles; PLL, poly-L-lysine; pDNA, plasmid DNA; PDL, PLL-condensed pDNA (PLL-DNA)–liposome complex; CFD, computational fluid dynamics.

[1] To whom correspondence should be addressed (e-mail p.shamlou@ucl.ac.uk).

be scaled up, while probe sonication, although potentially scaleable, is problematic, owing to metal contamination, lipid degradation and generation of heat and aerosols [12]. Extrusion/homogenization [13,14] and microfluidization [15,16] have been shown to be scaleable, and we recently described the operation of a new high-velocity jet homogenizer, which was used to create fine emulsions [17]. Here we present new experimental data to illustrate the capacity of the jet homogenizer to form SUVs with predictable size distribution for use potentially in gene transfer. We used the SUVs to encapsulate poly-L-lysine (PLL)-condensed pDNA. The results provide insight into the impact of the size of SUVs on the efficiency of encapsulation.

Materials and methods

Liposome preparation

Dioleoylphosphatidylcholine, dioleoylphophatidylethanolamine, oleic acid and cholesterol were mixed together at a molar ratio of 2:0.05:1:1.95 in 5 ml of chloroform/methanol (4:1, vv) (Sigma–Aldrich Ltd, Poole, Dorset, U.K.) in a round-bottomed flask. When necessary 10 μg of cholesterol containing a fluorescent marker [D-(+)-*sn*-1-*O*-{1-[6-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacenepentan-3-oyl)amino]hexanoyl}-2-hexanoylglyceryl-(BODIPY®FL; Molecular Probes Inc., Eugene, OR, U.S.A.)] was added to provide a rapid quantitative method of monitoring lipid concentration. The organic solvent was removed by placing the flask on a rotary evaporator (Buchi rotavapor R-114; Buchi Labortechnik, Flawil, Switzerland) operating at 80 rev./min under a low vacuum at 60 °C, until a thin lipid film was produced on the bottom of the flask. The film was further dried by placing the flask in a Sanyo Gallenkamp (Loughborough, Leics., U.K.) vacuum oven at 60 °C. The dried lipid film was hydrated in an appropriate quantity of 10 mM Hepes buffer, pH 8 (Sigma–Aldrich). To facilitate the hydration process, a small amount of 2 mm-diameter glass beads were added. The flask was placed on the rotary evaporator at 60 °C without the vacuum, and was operated at 240 rev./min until all of the film was removed from the bottom of the flask.

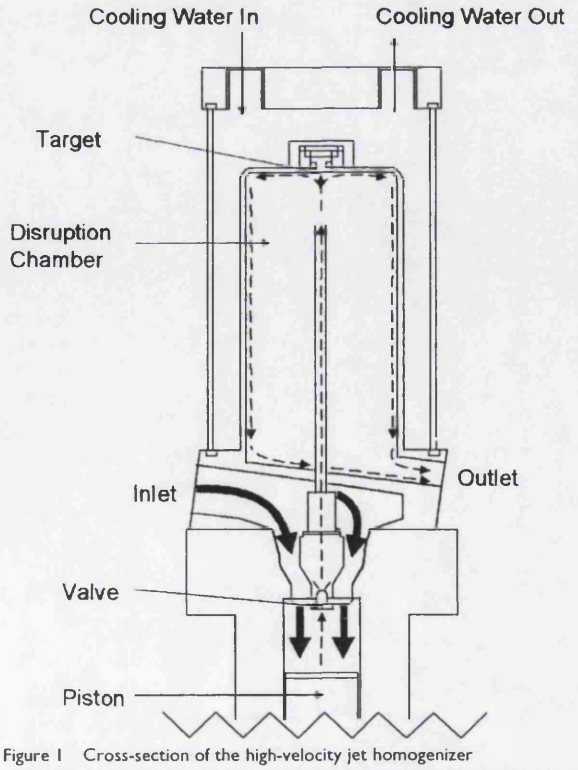

Figure 1 Cross-section of the high-velocity jet homogenizer

Downsizing of MLVs was achieved in a high-velocity jet homogenizer developed initially for cell disruption [18] and more recently used in our laboratory for preparation of fine emulsions. The main parts of the homogenizer are shown in Figure 1, and its method of operation is described elsewhere [17]. Briefly, a typical run starts with a downward stroke during which a crude dispersion of the MLVs is drawn into the piston chamber of the homogenizer. In the upward stroke, the dispersion is forced through a small orifice at high velocity, producing a jet. The jet travels through a pipe of variable cross-sectional area before impinging on the target. The disrupted solution containing the SUVs is cooled by contact with the walls of the disruption chamber, which are held at a low temperature by a re-circulating flow of coolant. The operating pressure of the homogenizer is in the range of 35–207 MPa (1 Pa = 1 N · m $^{-2}$) and orifice diameters in the range 0.1–0.18 mm are available. In the present study, data are provided for a single orifice of 0.18 mm diameter and an operating pressure in the range 103.4–172.3 MPa. The resulting SUVs were filtered using a 200-nm-mesh-size sterile disposable syringe filter.

The concentration of lipid was monitored by recording the intensity of the fluorescent marker using a 96-well plate fluorimeter (Fluorocount; Packard Bioscience, Pangbourne, Berks., U.K.) controlled by plate-reader software (Packard Bell; NEC Computers (UK) Ltd, White Waltham, Maidenhead, Berks., U.K.) using an excitation and emission filters of 485 and 530 nm respectively.

Preparation of PLL-condensed DNA (PLL-DNA) and PLL-DNA–liposome (PDL) complexes

pDNA (pSVβ) was produced in *Escherichia coli* DH5α, extracted from the host cells and purified using the method described previously by Levy et al. [19]. pDNA was condensed with PLL (Sigma–Aldrich) of molecular mass 29 kDa at a charge ratio of 2 by the method of Tsai et al. [20] using an infuse/withdraw syringe pump (PDH 2000; Harvard Apparatus, Holliston, MA, U.S.A.) equipped with a T mixing

head. The PDL complexes were formed by mixing PLL-DNA and liposomes vesicles (SUV) at the desired charge ratio using the same mixing apparatus. In the experiments described here, the pump was operated at an infusion rate of 6 ml/min. All concentrations were adjusted using 10 mM Hepes buffer, pH 8.

Zeta-potential and particle-size measurements

Zeta potential and particle-size measurements were made by laser doppler spectrometry using the Malvern Zetasizer 3000 (Malvern Instruments, Malvern, Worcs., U.K.). For zeta-size measurements we used the method of Tsai et al. [20]. Samples were injected into the *in situ* cuvette using 5 ml disposable syringes. Prior to each measurement the *in situ* cuvette was flushed with deionized water until the count rate of the instrument was less than 10. The calibration of the instrument was checked before each series of runs by using polystyrene standards supplied by the equipment manufacturer. Ten replicate runs were performed for each sample and the data averaged.

Particle-size measurements were carried out by the method of Lee et al. [21]. Disposable cuvettes (volume 4 ml) were filled with 2 ml of sample and placed in the instrument. The instrument was set to record five runs of 120 s duration per measurement. Size-distribution plots were obtained using the monomodel method of data analysis [21].

Surface-tension/interfacial-tension measurements

The surface tension of the liposomes was determined by the sedimentation technique [22]. A 1 ml portion of MLVs was centrifuged and the pellet was divided into equal amounts. These samples were resuspended into binary liquid mixtures of n-propanol/water. The n-propanol/water concentration ratios were selected to cover the range of surface tensions that included the surface tension of the liposome. Previous research [22,23] has shown that the surface tension of the binary mixture at which a maximum or minimum sedimentation volume is measured at the end of the test is equal to surface tension of the liposome.

The n-propanol/water binary mixtures were prepared by mixing various volume ratios of water and n-propanol between 0 and 50% n-propanol. This gave solutions with a range of surface tensions between 72 mN · m^{-1} (= 100% water) and 27 mN · m^{-1} (50% water). The surface tension of each binary mixture was measured using a torsion balance (Torsion Balance Supplies, Malvern Wells, Worcs., U.K.). These measurements gave a value of 68.2 mN · m^{-1} for the liposomes, which was used in population-balance simulations (see below).

Encapsulation efficiency

A 500 μl portion of sample complexes were incubated for 15 min at 37 °C with trypsin (Sigma–Aldrich) at a final concentration of 0.1 mg/ml. Any DNA released was separated from the system by extraction with phenol/chloroform/3-methylbutan-1-ol (Amersham Pharmacia Biotech, Rainham, Essex, U.K.), followed by ethanol precipitation and resuspension in 1 ml of TE buffer (10 mM Tris/HCl/1 mM EDTA, pH 8). DNA released was assayed using Pico Green (Molecular Probes Inc.) [20]. The percentages of free and encapsulated PLL-DNA were calculated in terms of the total DNA present in the complex, which was maintained constant at 2 μg in all of the experiments.

Symbols used

The following symbols are used in the equations in the Results and discussion section below:

$a(v,t)$	Probability density of droplet size in the feed
$A(v,t)$	Probability density of droplet size in vessel
C_1, C_2	Breakage constants
C_3, C_4	Coalescence constants
d	Drop diameter (m)
$f(v)$	Fraction of drops with volume between v and v+dv flowing out of the system per unit time
$g(v)$	Breakage frequency of the drops of volume v (s^{-1})
n_0	Number feed rate of drops (s^{-1})
N · t	Total number of drops · time (s)
v,v'	Drop volume (m^3)
ε	Local energy dissipation per unit mass W · kg^{-1}
μ	Viscosity (kg · m^{-1} · s^{-1})
ρ	Density of drops (kg · m^{-3})
σ	Interfacial tension (N · m^{-1})
$\beta(v,v')$dv	Number fraction of drops with volume v to v+dv formed by breakage of a drop of volume v'
$\Omega(v,v')$dvdv'	Number coalescence rate of drops of volume v with drops of volume v' (s^{-1})
ϕ	Volume fraction of drops

Results and discussion

Liposomes downsizing

The flow field in the jet homogenizer was simulated using computational fluid dynamics (CFD) in order to obtain the relevant hydrodynamic information, including jet-velocity and energy-dissipation-rate profiles. Using the method of Soon et al. [17] the transient flow of the jet emerging from the orifice was simulated for different operating pressures.

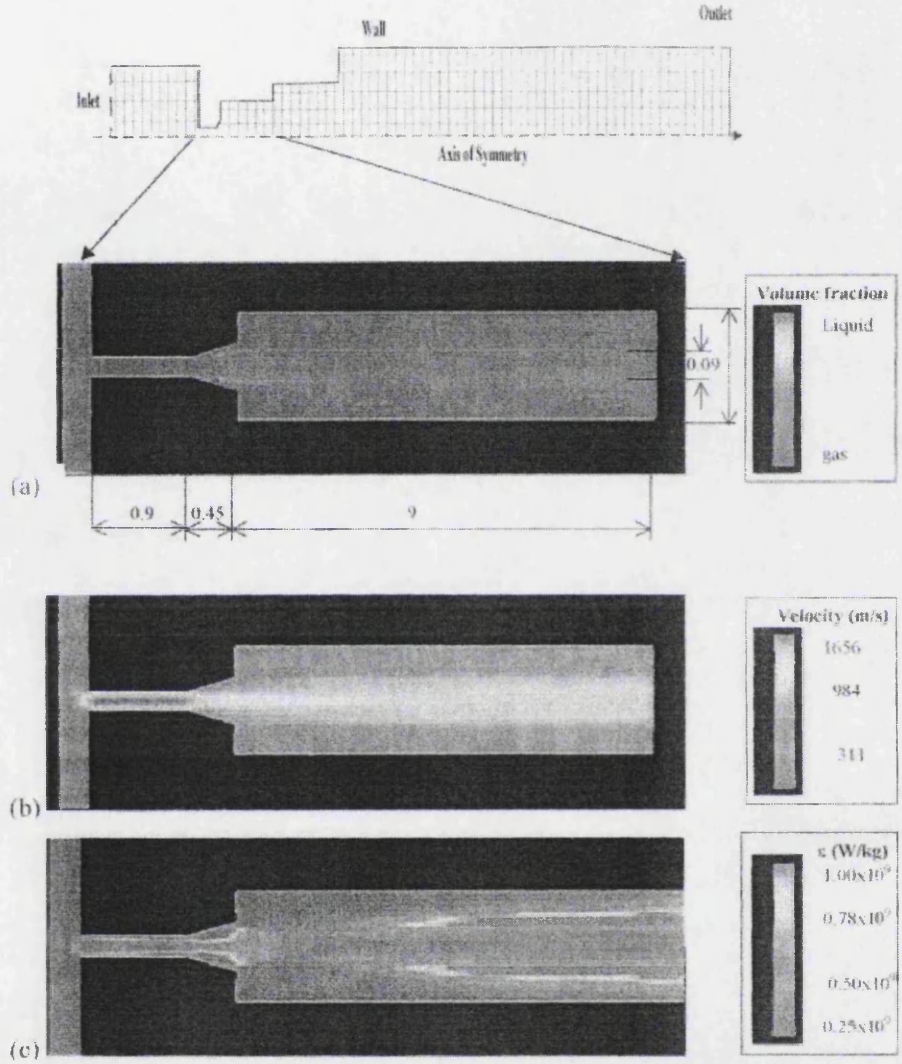

Figure 2 CFD simulations

(a) Volume fraction, (b) velocity and (c) energy-dissipation rates for flow through the homogenizer at an operating pressure of 137.9 MPa. Predictions of the energy dissipation rates plotted as a function of axial position for the region close to the orifice.

Figure 2 shows a snapshot of the volume fraction, velocity and energy-dissipation profiles for an operating pressure of 137.9 MPa. These simulations refer to conditions exactly 1.06×10^{-3} s after the jet emerged from the orifice, this being the predicted time for the jet to reach the target plate positioned 0.17 m away, directly opposite the orifice. We have previously verified these predictions by comparing them with high-speed video films of the jet as it emerged from the orifice during homogenizer operation [17]. Simulations shown in Figure 2 are confined to the region close to the orifice where most of the energy is dissipated. These simulations provide the basis for scaling-up the homogenizer and permit one to relate the physical properties of the vesicles to operational parameters, including orifice diameter and homogenizer pressure (see discussion below).

Figure 3 shows the impact of homogenization on the size distribution of the vesicles for an operating pressure of 103.3 MPa. The plots indicate that one pass through the homogenizer is sufficient to generate SUVs with a size distribution having a mode of 131±0.78 nm and a polydispersity of 0.4, indicative of a narrow distribution. Sterility and control of pyrogens are important considerations in the preparation of vesicles for human use. γ-Ray and heat sterilization have been shown to damage liposomes [24]. Conventional downsizing methods, for example sonication, require several passes to achieve adequate size reduction,

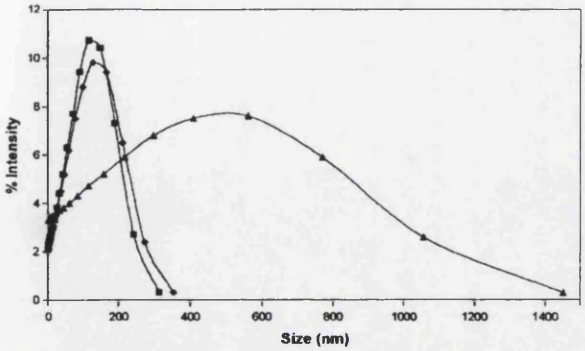

Figure 3 Comparison of the size distributions of MLVs (▲) and liposomes after a single pass through the high-velocity jet homogenizer at 103.3 MPa before (◆) and after (■) passage through the 200 nm filter

and, even then, often a centrifugation step needs to be included before the size distribution of the SUVs is reduced sufficiently for filter sterilization. Also shown in Figure 3 is the change in size distribution caused as a consequence of filtering the SUVs through a 200 nm-mesh-size filter medium. The average size of the liposomes shifts slightly to smaller sizes and the distribution narrows, as shown by a decrease in polydispersity from 0.4 to 0.2. This is consistent with the retention by the filter of the largest SUVs in the distribution. All subsequent experiments were carried out with the filtration step included in the vesicle-preparation protocol.

Figures 4(a) and 4(b) show the impact of the number of passes through the homogenizer and its operating pressure on the size distribution of the SUVs produced. The plots demonstrate that by increasing the operating pressure and with sufficient number of passes it is possible to prepare SUVs with a mean size of less than 100 nm. It has been reported that downsizing of liposomes by repeated passes using other types of homogenization causes the size distribution of the vesicles to decrease with the minimum size, depending on vesicle composition. According to these studies, as the number of passes through the homogenizer increases, a critical condition is reached beyond which further passes result in a broadening of the distribution [14]. Cholesterol-containing formulations have been shown to increase in size after five to ten passes [13]. The data shown in Figures 4(a) and 4(b) did not show such trends, but it is noted that our experiments were limited to a maximum of five passes. Lipid concentration, monitored by recording the intensity of the fluorescent labelled cholesterol in the sample, was found to decrease with each pass (results not shown). The reason for this loss has not been ascertained, but may be attributed to problems associated with handling very small quantities of test materials. Sorgi and Huang [15], who performed similar experiments with a microfluidizer, showed that loss of lipid was proportional for all of the lipids in the formulation. In our experiments the zeta potential

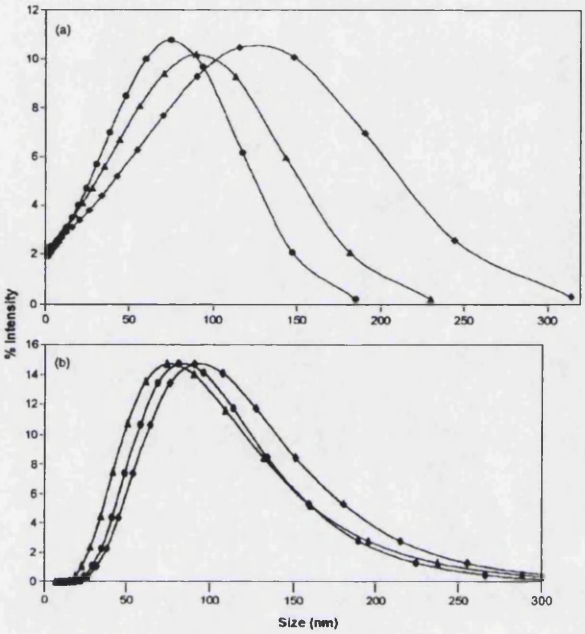

Figure 4 Effect on size distribution plots of increasing the number of passes through the homogenizer

(a) Data represent one (◆), three (▲) and five (●) passes through the homogenizer at an operating pressure of 172.3 MPa or (b) the operating pressure of the homogenizer [103.3 (◆), 137.9 (●) and 172.3 MPa (▲) and five passes].

of all samples remained constant (−45 mV), independent of number of passes, indicating that there was no preferential loss of any of the components in the formulation.

The impact of operational parameters (operating pressure and jet geometry) and physical properties of the dispersion (viscosity, density and interfacial tension) on size distribution of the liposome may be obtained by solving the following population balance equation [25]:

$$\frac{d[N(t)A(v,t)]}{dt} = n_o a(v,t)f(v) - N(t)A(v,t)f(v) + \int g(v')\beta(v,v')v(v')N(t)A(v',t)dv' - g(v)N(t)A(v,t) + \int \Omega(v-v',v')N(t)A(v-v',t)N(t)A(v',t)dv' - N(t)A(v,t)\int \Omega(v,v')N(t)A(v',t)dv' \quad (1)$$

In eqn (1), the first two terms on the right-hand side represent flow of liposomes of size v into and out of the system. The next two terms account for the rate of generation and loss of liposomes of size v respectively due to breakage. The final two terms represents the rate of generation and loss of liposomes of size v due to coalescence.

The solution of eqn (1) requires models of breakage and coalescence. Many such models are available for two-liquid phase dispersions and suspensions including oil drops in an aqueous phase. However, to our knowledge such models have not been extended to lipids in the past. We tested a number of breakage and coalescence equations and found the following to provide a good basis for analysis of data.

The two breakage functions in eqn (3), namely $g(v)$ and $\beta(v',v)$, were defined respectively as in [25]:

$$g(v) = C_1 v^{2/9} \varepsilon^{1/3} \exp\left[-\frac{C_2 \sigma}{\rho_d \varepsilon^{2/3} v^{5/9}}\right] \quad (2)$$

and:

$$\beta(v',v) = \frac{2.4}{v'} \exp\left[-4.5\frac{(2v-v')^2}{v'^2}\right] \quad (3)$$

The coefficient of coalescence rate is defined by the following equation [26]:

$$\Omega(v,v') = C_3 \frac{\varepsilon^{1/3}}{1+\phi}(v^{1/3}+v'^{1/3})^2(v^{2/9}+v'^{2/9})^{1/2} \times \exp\left[-\frac{C_4 \mu \rho \varepsilon}{\sigma^2(1+\phi)^3}\left(\frac{v^{1/3}v'^{1/3}}{v^{1/3}+v'^{1/3}}\right)^4\right] \quad (4)$$

The solution of the population balance equation requires a number of input parameters, including an initial size distribution of liposomes and the local energy dissipation rate, ε. The size distribution of the feed was used for the first pass. The predicted size distribution at the end of each pass was used as the input for the subsequent pass. The turbulent flow in the jet was assumed to be isotropic, and homogeneous and breakage and coalescence were considered to occur by the local turbulent fluid energy dissipation, ε, as predicted by CFD (Figure 2c). To reduce the number of calculations, the region between the orifice and outlet of the jet (Figure 2) was divided into six segments based on the velocity and energy distributions. A lumped-average value of energy dissipation was calculated for each segment from the distributions of energy dissipation rate in all the CFD cells in that segment. The calculation procedure was started by inputting the lumped-average value of the energy dissipation rate for the first segment in eqns (2)–(4). The results were substituted into eqn (1), which was solved numerically, together with the feed (initial) size distribution of liposomes using an in-house code. The output in the form of the predicted size distribution of the liposomes from the first segment was used together with the lumped-average energy dissipation rate for the second segment as input parameters to simulate the size distribution at the exit of the second segment, and the calculation procedure was continued till the final size distribution of liposomes were obtained at the outlet of the jet. The values of the four constants C_1, C_2, C_3, C_4 in eqns (2)–(4) which best fitted the data were found to be 0.03, 1.25, 10^{20} and 10^{-4} respectively. These values were obtained using one set of experimental data and applied to all segments.

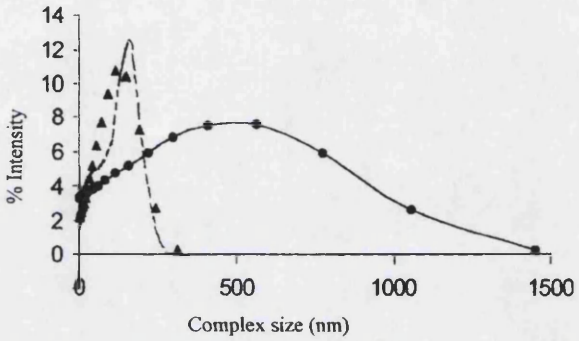

Figure 5 Predicted size distribution of liposomes for an operating pressure of 137.9 MPa (---●---●, initial-feed-size distribution) and after the third pass through the homogenizer (-▲-▲), based on CFD simulations of the local energy dissipation and solution of the population balance equation (eqn 1)

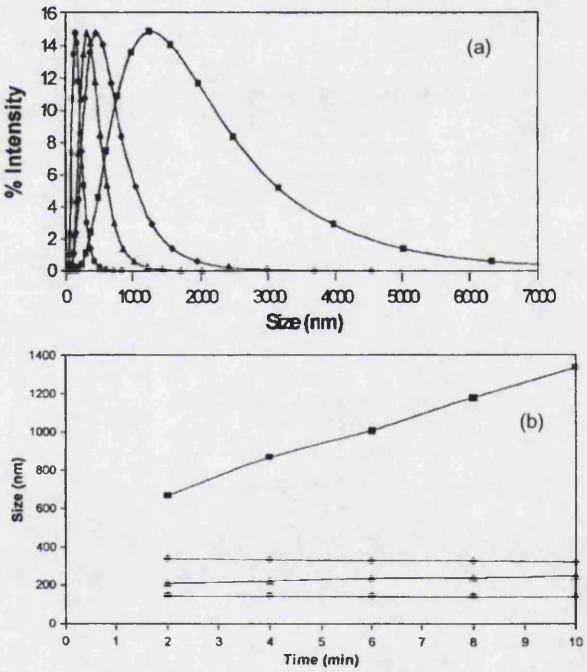

Figure 6 Effect of liposome/PLL-DNA charge ratio on the complex size

(a) Data refer to size distributions obtained at charge ratios of 0.4 (◆), 1 (■), 2.4 (▲) and 4 (●). All experiments were carried out 10 min after mixing of the components. (b) Shows the mean size of complexes as a function of time. Additional details are given in Figure 5.

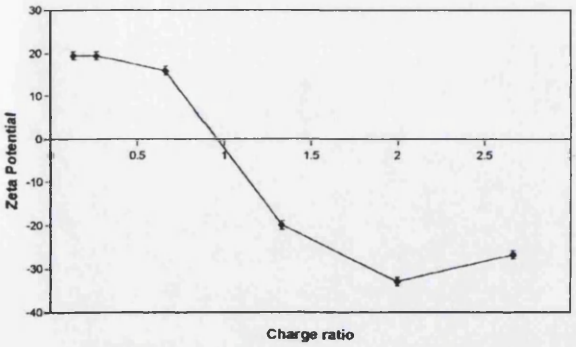

Figure 7 Effect of liposome/PLL-DNA charge ratio on the Zeta potential of the resulting complexes

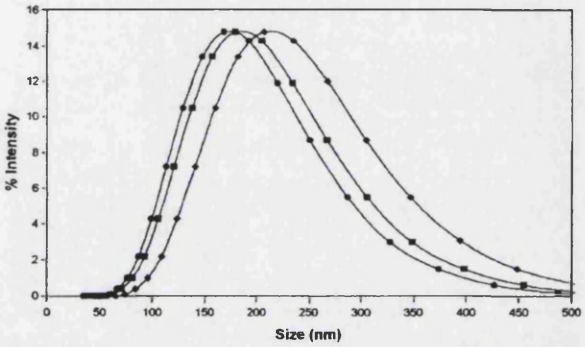

Figure 8 Comparison of the size distributions of PDLs formed with SUVs having an initial mean size of approx. 130 (◆), 100 (■) or 80 nm (●)

Data refer to complexes prepared at a charge ratio of 5. The distribution of the liposomes before complexing can be seen in Figure 4(a).

In Figure 5 the simulations obtained from the population balance equation are compared with experimental data obtained after the third pass through the homogenizer. Experimental size distributions are slightly smaller than the predicted distributions. One reason for this may be that in carrying out the simulations we assumed that the region of breakage was confined to the high-turbulent-energy zone near the orifice. It is possible that additional downsizing of the liposome particles occurs further downstream of the orifice region, particularly at the impact ring, where we have previously shown the energy level to be high [17]. Sensitivity analysis (simulations not shown) indicated that liposome size distribution was dominated by the breakage terms (eqns 2 and 3) in the population balance equation. Coalescence (eqn 4) had negligible effect on simulations.

Complex formation

pDNA was condensed with PLL (29 kDa) at a charge ratio of 2.0 and were complexed with the SUVs by using the twin-head pump and the T-mixer operating at a fixed perfusion rate of 6 ml/min [20,27]. Figure 6 shows the size distribution of the resulting PDL complexes as a function of charge ratio with the corresponding zeta potential data shown in Figure 7. Charge interactions between the cationic PLL-DNA complexes and the anionic liposomes play an important role in the formation of the complexes [9,11]. At a charge ratio of approx. 1.0, the complexes were close to their isoelectric point, and the vesicles showed a strong tendency for aggregation. This can be seen in Figure 7, where a switch in zeta potential from positive to negative is observed at a charge ratio of about 1.0. At both higher and lower charge ratios the complexes carried either a net positive or negative charge. The resulting charge repulsion between the complexes reduced the probability of aggregation. The data plotted in Figure 6 indicate that excess lipid is required to produce complexes of small size and prevent aggregation from occurring. The results of our experiments show that a charge ratio of at least 4, equivalent to a lipid/DNA ratio of 8:1, is required to produce complexes less than 200 nm in size. These complexes were shown to maintain their size for a period of 7–10 days before they start to aggregate. We have carried out tests aimed at improving the stability of these complexes through the attachment of poly(ethylene glycol) to the surface of the liposomes. The results will form the basis of a future study. Using a high charge ratio, however, may be unacceptable for gene transfer, for reasons of cost and possible lipid-induced toxicity. Lee and Huang [9] used anionic liposomes entrapped PLL-DNA at lipid/DNA ratios in excess of 15:1 to produce small, stable, complexes, which gave good encapsulation and low levels of empty liposomes.

Complex size also plays an important role in *in vivo* delivery of DNA to target cells. Large complexes (> 200 nm) are more likely to get trapped in the small capillaries of organs such as the lungs and liver, resulting in their accumulation within the organs. Large complexes are likely to be taken up by the cells of the reticuloendothelial system rapidly following intravenous administration [10]. For vectors taken up into target cells by endocytosis, it is thought that complex size should not exceed the size of the endosome. The endosome of lymphocytes is approx. 100 nm. However, large size may be an advantage when targeting solid tumours, which possess leaky blood vessels and have a tendency to accumulate large positively charge macromolecules [28].

Encapsulation efficiency

PLL-DNA was complexed with SUVs having different initial size distributions at a liposome/PLL-DNA charge ratio of 4. The results shown in Figure 8 demonstrate that, for otherwise similar conditions, decreasing the size of the SUVs

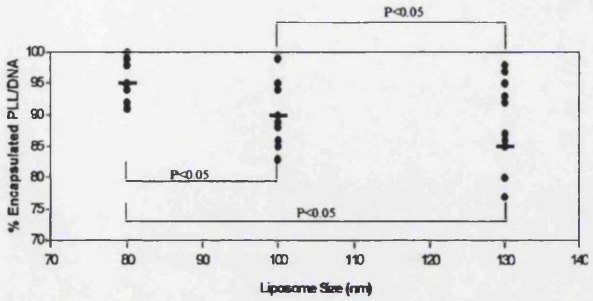

Figure 9 Efficiency of SUVs to encapsulate PLL-DNA plotted as a function of the mean size of SUVs

The size distribution of the SUVs used is shown in Figure 4(a). In control experiments in which PLL-DNA was treated with trypsin, near 100% release of the pDNA was recorded. pDNA released from PLL-DNA or PDL, which had not been treated with trypsin was below detectable levels by Pico Green assay. The statistical *P* values were calculated by using the standard Student t test. The bars represent the mean values.

produces complexes with smaller mean size. Figure 9 shows the impact of the size of the SUVs on the percentage of PLL-DNA encapsulated. There is considerable scatter in data, but, on the basis of a statistical analysis of the results, we assert that the encapsulation performance improves as the size of the SUVs decreases. No comparable data are available in the literature. However, experiments reported for small-molecule drugs suggest that encapsulation should improve with an increase in liposomes size [29]. Identification of the mechanisms by which PLL-DNA is encapsulated into the liposomes may provide further insight. The impact of the size of the liposomes vesicles on encapsulation efficiency must also consider the recent evidence [30] suggesting that the dissociation of the pDNA from the complexing agents in the cell may be influenced by the size of complexes.

The anionic liposome-entrapped PLL-DNA complexes described here are similar to those first demonstrated by Lee and Huang [9]. They offer advantages over DNA cationic liposome formulations, while also increasing the encapsulation efficiency of the liposome. One of the main challenges in using cationic complexes is the high level of binding to non-target cells, due to charge interactions. Through the binding of, for example, antibody molecules to anionic liposomes, complexes capable of targeting specific cells may be produced. The formulation used here allows this to be achieved through the modification of the dioleoylphosphatidylethanolamine component in the formulation [31].

Conclusions

We describe a new scaleable homogenization method for the rapid disruption of MLVs. The resulting SUVs had a narrow size distribution with a mean size below 100 nm, depending on the operating pressure and number of passes through the disruption equipment. Generally, however, a single pass at a moderate pressure was sufficient to produce SUVs for filtering sterilisation. CFD simulation of flow in the disruption equipment was carried out to define the critical region in the equipment where downsizing of MLVs occurred. SUVs produced by the new method were used to encapsulate PLL-pDNA at different charge ratios. The resulting anionic complexes were stable and had a mean size of below 200 nm, depending on the initial size of the SUVs. The results demonstrated that between 85 and 95% of the complexes were encapsulated by the liposomes depending on the initial size of the SUVs, the smaller liposomes being more effective in encapsulating the complexes.

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